A Testis Specific Isoform of Endophilin B1, Endophilin B1t, Interacts Specifically with Protein Phosphatase-1cγ2 in Mouse Testis and Is Abnormally Expressed in PP1cγ Null Mice

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ABSTRACT: Male mice homozygous for a null mutation in the protein phosphatase-1cγ (PP1cγ) gene are infertile, displaying a severe impairment in spermatogenesis that is not compensated by the presence of PP1cα and PP1cβ in mutant testes. A lack of the PP1cγ2 splice variant seems the most likely cause of the mutant phenotype, as it is the most heavily expressed PP1cγ isoform in wild type testes. Yeast two-hybrid screening using PP1cγ2 has identified several new binding partners, including endophilin B1t, a testis enriched isoform of endophilin B1a which differs from the somatic form by virtue of a carboxy terminal deletion spanning the last 10 amino acids. The testis isoform did not show an interaction with PP1cα, or with a truncated PP1cγ2 mutant lacking the unique carboxy terminus. In contrast, somatic endophilin B1a did not interact with any of the PP1c isoforms. Sedimentation and co-immunoprecipitation experiments using native testis proteins verified binding of endophilin B1t to PP1cγ2. Immunohistochemistry on wild type testis sections revealed a stage specific expression pattern for endophilin that appeared concentrated at discrete puncta throughout the seminiferous epithelium. Punctate endophilin expression in cells adjacent to the lumen was absent in PP1cγ null mice. Phosphatase assays indicate that chimeric endophilin B1t is able to inhibit recombinant PP1cγ2 activity toward phosphorylase *a* while having little effect on the activity of PP1cα. A potential role for endophilin B1t in mammalian spermatogenesis is discussed within the context of the PP1cγ knockout testis phenotype.

Protein phosphatase 1 (PP1[1]) is a major eukaryotic serine/threonine phosphatase composed of a single catalytic subunit (PP1c) in complex with one or more regulatory subunits. Catalytic subunits are highly conserved across phyla, while regulatory subunits are varied and thought to confer subcellular localization and substrate specificity (*1*). Higher eukaryotes typically contain multiple PP1c isoforms showing an overall sequence similarity of ~90% (*2*). Mutations in PP1c genes from different organisms have resulted in distinct physiological defects, suggesting that different isoforms have the potential for unique functions despite close sequence similarity (*3*−*6*). One way in which functional independence can be achieved is through preferential interaction with particular regulatory subunits. To date more than 50 regulatory subunits have been identified which target PP1 to diverse cellular processes including glycogen metabolism, protein synthesis, mitosis, synaptic depression, and smooth muscle contraction (*7*). Evidence suggests that regulatory subunits bind PP1c through multiple points of interaction, with many containing a degenerate "RVxF" motif (the more general consensus sequence is (R/K)X_{0-1}(V/I){P}(F/W), where X_{0-1} represents the possible presence of an amino acid, whose identity varies, while {P} is any amino acid except proline) (*8*). This motif has been implicated in an interaction with a conserved hydrophobic channel (*9*). Isolated PP1 holoenzymes typically contain only one inhibitor or targeting subunit, indicating that interaction of different regulatory subunits is often mutually exclusive (*10*). Of the known PP1 regulatory proteins only a small subset, such as the neurabins, actin-binding proteins that are thought to regulate synaptic depression in brain neurons, are known to preferentially interact with specific PP1c isoforms (*11*, *12*).

In mammalian systems, four different PP1c isoforms have been identified: PP1cα, PP1cβ, PP1cγ1, and PP1cγ2, where the latter two variants are derived through alternate splicing of the PP1cγ gene (*13*). The isoforms are over 98% identical excluding the extreme carboxy termini where most of the divergence in sequence occurs (*14*). Male mice homozygous for a null mutation in the PP1cγ gene are infertile, displaying a severe impairment in spermatogenesis despite the continued presence of PP1cα and PP1cβ (*3*). The phenotype is completely penetrant, with 100% of mutant males being sterile despite the occasional presence of mature, if grossly abnormal, sperm. Heterozygotes show no difference from wild type, suggesting that defects in homozygous mutants are not the result of a general decrease in PP1c gene dosage. Histochemical analysis of PP1cγ mutant testicular sections reveals gross reductions in numbers of condensing and

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[1] Abbreviations: PP1, protein phosphatase 1; PP1c, protein phosphatase-1 catalytic subunit; GST, glutathione *S*-transferase; PMSF, phenylmethylsulfonyl fluoride; HA, hemagglutinin; EST, expressed sequence tag; 3-AT, 3-aminotriazole.

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Table 1: Primers Used for Cloning and Expression Studies

	A. Cloning into pGAD7	
PP1cγ2	primer 1 (F)	5′-GGACGGCCATGGCGGATAT-3′
	primer 2 (R)	5′-ATGTTAGAATTCCCAACCAGG-3′
PP1cγ1	primer 3 (R)	5′-GTCACCGCAGAATTCAGAATGT-3′
PP1cα	primer 4 (F)	5′-GACGGCGCCATGGCCGACAGC-3′
	primer 5 (R)	5′-ACAGGGTGGAATTCGGGGGCT-3′
PP1cγ2 truncated	primer 6 (R)	5′-CTAATGGACGAAATTCAGGCCTG-3′
endophilin B1t	primer 7 (F)	5′-GCTGCCGCCCGGGATGAACATC-3′
	primer 8 (R)	5′-GGATGCTTCACTCGAGGGAGGCC-3′
endophilin B1a	primer 9 (R)	5′-ATGGGTAACCTCGAGTGGTCTCCC-3′
	B. RT-PCR	
endophilin B1t	primer 10 (F)	5′-AGCTGACCGAGAGCTGATTC-3′
	primer 11 (R)	5′-ACTACCGGATGCTTCACACC-3′
β-actin	primer 12 (F)	5′-AGCTGAGAGGGAAATCGTGC-3′
	primer 13 (R)	5′-TCAGCAATGCCTGGGTACAT −3′
	C. Cloning into pcDNA3.1	
PP1cγ2	primer 14 (F)	5′-GGGACGGCGCTAGCGGATATC-3′
endophilin B1t	primer 15 (F)	5′-CCCGGCTGCTAGCCGGCATGAAC-3′

elongate spermatids, as well as increased levels of apoptosis (*15*) and aneuploidy (*16*) in developing germ cells. In addition to the observed germ cell defects large "holes" may also be observed in the seminiferous epithelium that may be either vacuoles in the Sertoli cells, indicative of Sertoli cell dysfunction (*17*), or gaps left by dead germ cells. A lack of the PP1cγ2 splice variant seems the most likely cause of the mutant phenotype, as it is the most abundant PP1cγ isoform in the testis (*18*). To date, known physiological binding partners of PP1cγ2, including the glutamate receptor mGluR7b (*19*), the 78 kDa glucose-regulated protein (*20*) and a mammalian homologue of yeast Sds22p (*21*) are also known to bind other PP1c isoforms, or have not been characterized in terms of their specificity.

To explain the phenotype of PP1cγ null mice, we have hypothesized the presence of one or more regulatory subunits in the testis that interact specifically with PP1cγ2, but not PP1cα or PP1cβ, targeting the enzyme to a function required for spermatogenesis. Such a regulatory subunit would likely contain novel sequences that allowed for an interaction with the unique C-terminus of PP1cγ2. Our studies identified a small number of isoform specific interactors, including three unknown proteins, Spz1 (*22*) and a testis-enriched variant of endophilin designated as endophilin B1t. Here we characterize endophilin B1t expression in the testis and confirm the interaction between PP1cγ2 and endophilin B1t. We propose that endophilin, whose function in other cells is associated with endocytosis, may be involved in some of the dynamic tissue remodeling that occurs in the testis, and that the loss of PP1cγ may impair the activity of the testicular endophilin variant.

MATERIALS AND METHODS

Yeast Two-Hybrid Screening and Assays. To create two-hybrid vectors, coding sequences for mouse PP1cγ2, PP1cγ1, PP1cα, and a truncated PP1cγ2 lacking 18 amino acids from the C-terminus were amplified by PCR from pGEM7Z vectors containing full-length cDNAs (*23*). PCR was performed using the following primer sets (Table 1): for PP1cγ2, primers 1 and 2; for PP1cγ1, primers 1 and 3; for PP1cα, primers 4 and 5; for truncated PP1cγ2, primers 1 and 6 (primer sequences are summarized in Table 1). Resulting fragments were digested with *Nco*I and *Eco*RI and ligated separately into pGBKT7 (Clontech). Two versions of endophilin were cloned. Endophilin B1t was cloned by RT-PCR using primers 7 and 8 on RNA extracted from testis using Trizol (Invitrogen). The endophilin B1t fragment was digested with *Sma*I and *Xho*I and ligated into the multiple cloning site of pGADT7 (Clontech). Somatic endophilin B1a was cloned by two rounds of RT-PCR on testis RNA using primers 7 and 9. Two rounds of PCR were necessary to generate sufficient yield of the low abundance somatic splice variant from testis RNA. All two-hybrid vectors were sequenced to ensure coding sequences had been inserted in frame.

Bait vectors containing PP1c constructs were transformed separately into yeast *Saccharomyces cerevisiae* AH109 (*MATa, trp1-901, leu2*-3, *112, ura3-52, his3-200, gal4*Δ, *gal80*Δ, *LYS2::GAL1*$_{UAS}$-*GAL1*$_{TATA}$-*HIS3, GAL2*$_{UAS}$-*GAL2*$_{TATA}$-*ADE2, ura3::MEL1*$_{UAS}$-*MEL1*^{TATA}-*lacZ*) and maintained on synthetic dropout media without tryptophan. Screening was conducted using a mouse testis expression library constructed in pACT2 from pooled 8−12 week BALB/c males (Clontech). The library was amplified in *Escherichia coli* strain BNN132 as in Elledge et al. (*24*), and plasmid DNA was purified using a maxi-prep kit from Qiagen. Library plasmid DNA (500 μg) was transformed into yeast AH109 expressing PP1cγ2 by the LiAc method as described in the Clontech Yeast Protocols Handbook (2001). Transformed cells were selected after 5−7 days incubation at 30 °C on minimal media. Activation of the *lacZ* reporter gene was assessed via colony lift assay using X-gal as described in the Clontech manual. Plasmid DNA from positively interacting clones was extracted using a yeast plasmid purification kit (CPG Inc.) and electroporated (*25*) into previously prepared electrocompetent *E. coli* DH5α cells (*26*). Plasmids recovered from *E. coli* were retransformed into yeast expressing PP1cγ2 to confirm two-hybrid interactions, then separately into strains expressing bait constructs for PP1cα, PP1cγ1, lamin C, and truncated PP1cγ2.

Antibody Production and Western Blotting. Polyclonal antibodies recognizing both PP1cγ splice variants were generated in rabbits against the peptide "KPAEKKKP-NATRPVT". The peptide was synthesized commercially and coupled to keyhole limpet hemocyanin using an Imject sulfhydryl reactive antibody production kit (Pierce). Antibod-

ies were produced in rabbits, and sera were harvested using standard techniques (*27*).

Antibodies directed against endophilin (mouse anti-Bif-1 IgG) were obtained commercially (Imgenex). Antibodies directed against Bax (rabbit anti-Bax IgG) were obtained commercially (Santa Cruz Biotech). Antiserum directed against PP1cγ2 was kindly provided by S. Vijayaraghavan.

For Western blotting, testis protein extracts were prepared by homogenizing whole mouse testes with mortar and pestle in protein extraction buffer (100 mM NaCl, 1 mM EDTA pH 8.0, 10 mM Tris-HCl pH 7.5, 1 mM PMSF, with 1 μg/mL each of aprotinin, leupeptin, and pepstatin A) using 500 μL of buffer per 50 μg of tissue. Following centrifugation at 10500*g* for 10 min at 4 °C, the resulting supernatants were collected and 25 μg samples were boiled in SDS buffer prior to SDS−PAGE using 12.5% (w/v) acrylamide gels. Proteins were transferred to nitrocellulose with a Novex Western transfer apparatus and immunoblotted as described (*28*). PP1cγ, endophilin, and Bax antibodies were used at concentrations of 1:500. Goat anti-PP1cα, rat anti-GATA1, and HRP-coupled secondary antibodies (Santa Cruz Biotech) were used at 1:1000. Bound complexes were visualized using an enhanced chemiluminescence detection system (Amersham). For Western blotting with Bax antibodies, heart protein extracts were prepared as above due to the low concentration of Bax in testis and brain protein extracts.

Reverse Transcriptase-PCR and Northern Blotting. Total RNA from multiple mouse tissues was isolated using Trizol according to manufacturer's instructions. All tissues were dissected from a 15 week old wild type male CD-1 mouse except ovary, which was pooled from three female littermates. Reverse transcription was carried out on 3 μg RNA samples using Superscript II (Invitrogen). Resulting cDNA was diluted 1:10 and used in separate PCR reactions to amplify a 726 bp endophilin B1t fragment with primers 10 and 11 (Table 1). A β-actin fragment was amplified as a positive control using primers 12 and 13.

Co-Immunoprecipitation from Testis Lysates. Wild type testis protein lysates were prepared as described above. 500 μg of testis lysate at 1μg/1 μL was incubated with 5 μg of anti-PP1cγ or 5μg of anti-endophilin for 2 h at 4 °C with gentle rotation. Antibody and bound proteins were precipitated with 50 μL of Protein G: Sepharose 4B (Sigma) for 1 h at 4 °C. Protein complexes were washed 5 times with protein extraction buffer before the addition of SDS−PAGE sample buffer and Western blotting.

Co-Immunoprecipitation in COS-1 Cells. COS-1 sells were cultured on plates in Dulbecco's modified Eagle's medium with 10% fetal calf serum according to standard procedures (*29*). For expression constructs, PP1cγ2 cDNA was amplified by PCR using primers 14 and 2 from a pGEM7Z vector as before. Endophilin B1t cDNA was amplified from testis RNA using primers 8 and 15 (Table 1). The cDNA fragments were digested with *Nhe*I and *Eco*RI (for PP1cγ2) or *Nhe*I and *Xho*I (for endophilin B1t) and cloned separately into *myc*- and *HA*-pcDNA3.1 plasmid vectors (Invitrogen). The PP1cγ2 construct was introduced into COS-1 cells by electroporation by stable transfection using a Bio-Rad Gene-Pulser according to the method of Misumi et al. (*30*), and transformed cells were selected in 0.8 mg/mL G418 for one week. Two 60 mm culture dishes were grown to 90% confluency before transient transfection with *HA*-pcDNA3.1-endophilin as before. Resulting cells were lysed after 3 days in 1 mL of immunoprecipitation buffer (50 mM Tris-HCL, pH 8.0, 120 mM NaCl, 0.5% NP40, 1 mM PMSF, and 1 μg/mL each of leupeptin, aprotinin, and pepstatin A) for 15 min at 4 °C. Lysates were centrifuged at 10500*g* for 10 min at 4 °C, and the supernatant was split into 500 μL samples. PP1cγ2 and endophilin B1t were precipitated separately using 5 μg of chicken anti-*myc* or anti-*HA* antibodies (Aves Labs) at 4 °C with gentle rotation for 1.5 h. Antibody and bound proteins were precipitated with 50 μL of anti-chicken IgY immobilized on agarose beads (Promega). Protein complexes were washed extensively before addition of SDS−PAGE sample buffer and Western blotting. For control experiments, 5 μg of anti-GATA1 or anti-PP1cα was used with COS-1 lysates as before. Bound proteins were precipitated with 10 μL of 50% Protein A: Agarose beads (Transduction Labs) before Western analysis.

Sedimentation Assays. Wild type testis protein lysates were prepared as described above. Varying amounts of GST-endophilin B1t were bound to 40 μL samples of glutathione sepharose 4B beads (Amersham) according to manufacturer's instructions. Binding of GST-endophilin B1t was verified via Coomassie Blue staining following electrophoresis. Beads were washed 2 times with PBS before addition of 5 mg total testis protein and incubation for 2 h at 4 °C. Protein complexes were washed 3 times with extraction buffer (100 mM NaCl, 1 mM EDTA pH 8.0, 10 mM Tris-HCl pH 7.5, 1 mM PMSF) before elution in SDS sample buffer and Western analysis. Relative amounts of PP1cγ2 were quantified using Optiquant software (Packard Biosciences). Interaction between both GST-endophilin B1t and GST-endophilin B1a, and Bax was performed as described above, except that heart extract was used instead of testis extract due to low expression of Bax in testis.

Protein Phosphatase-1 Activity Assays. To produce expression vectors for recombinant PP1c isoforms, coding sequences for PP1cγ2 and PP1cα were first obtained through restriction digest of plasmid DNA from respective two-hybrid baits using *Nco*I and *Sal*I. Respective cDNA fragments were cloned into corresponding sites on an NpT7-5 plasmid vector (generously provided by Dr. S. Shenolikar). Recombinant plasmids were transformed into *E. coli* BL21, and lysates expressing PP1c were prepared as in Ludlow (*31*).

To produce radiolabeled substrate, phosphorylase *b* was phosphorylated using phosphorylase kinase (Sigma) and 6000 Ci/mmol [γ-^{32}P]ATP (Amersham) as described (*32*). Unincorporated ^{32}P was dialyzed over a period of 2.5 days at 4 °C in 50 mM Tris-HCl pH 7.5, 1 mM $MnCl_2$, and 0.1% β-mercaptoethanol. A full length GST-endophilin B1t construct was prepared through digestion of the respective two-hybrid plasmid to release a cDNA insert, followed by cloning into pGEX-6P-2. GST-endophilin B1t protein was prepared as described previously. Phosphatase assays using 1:100 of bacterial lysates were performed for 15 min at 37 °C in 50 mM Tris-HCl, pH 7.5, 1 mg/mL bovine serum albumin, 1 mM $MnCl_2$, and 0.1% β-mercaptoethanol using 20 μM radiolabeled phosphorylase *a* (*31*, *32*). Specifically, 20 μL of diluted PP1c source was premixed with 20 μL of chimeric PP1c-binding protein or buffer before addition of 20 μL of phosphorylase *a* to start the reaction. Reactions were restricted to a maximum of 20% release of labeled phosphate to ensure linearity of phosphate release over time. GST-

neurabin 1 containing amino acids 374−516 of neurabin I, and GST-G_M containing amino acids 1−240 of G_M, were generous gifts from Dr. S. Shenolikar.

Immunohistochemistry. Immunohistochemistry was performed on testes from 14−16 week old wild type CD-1 and PP1cγ null mice. In each case testes were dissected and fixed for 24 h in 4% formalin. Resulting fixed tissue was dehydrated in a graded ethanol series and embedded in paraffin, dewaxed, and hydrated as described (*33*). In order to unmask potential antigenic sites, heat antigen retrieval was performed in a microwave with the slides immersed in 0.01 M Tris-HCl pH 9.0 buffer. Slides were microwaved for two separate 8 min periods on medium high heat, with a 3 min cooling down period between. Following microwave treatment the slides were washed 10 min in PBS with 0.4% Photoflo (Kodak), 10 min in PBS with 0.005% Triton X-100, and 10 min in PBS with 10% antibody dilution buffer (10% goat serum, 3% bovine serum albumin, 0.05% Triton X-100). Incubation with bif-1 (endophilin) antibody (1:500) was performed overnight at 4 °C, after which slides were washed and blocked in dilution buffer as before. Secondary antibody treatment was for 4 h at 4 °C using 1:4000 Cy3 goat anti-rabbit IgG (Jackson Labs). Secondary antibodies were removed for 20 min in PBS with 0.4% Photoflo, before counterstaining with DAPI at 0.1 μg/mL for 10 min. After two final washes for 10 min in 0.4% Photoflo, the slides were mounted in 50% glycerol for viewing. Sections were examined with an Olympus BX60 microscope equipped with rhodamine and DAPI filters. Slides were imaged using an Olympus UPlanFl 0.75 N.A. lens. Images were captured using Cool Snap software and a CCD camera (RS Photometrics). Images were adjusted for brightness and contrast in Photoshop 6.0 (Adobe).

Animal Protocols. Mice were bred using standard animal husbandry. To maintain the PP1cγ mutant colony, individual mice were genotyped from genomic DNA isolated from tails using a PCR-based method previously described (*3*). In our laboratory, the PP1cγ mutant allele has been propagated on a CD-1 background (Charles River Labs). Rabbits used for antibody production were also maintained using standard animal husbandry procedures. All procedures involving laboratory animals were approved by The Canadian Council on Animal Care.

Sequence Information. The accession number for the endophilin B1t sequence is ABA54268.

RESULTS

Isolation and Identification of Endophilin B1t. In order to identify putative regulatory subunits of PP1cγ2, we have performed a yeast two-hybrid screen using the PP1cγ2 isoform as bait to screen a mouse testis expression library from 8−12 week males. Two-hybrid screening identified seven different clones as endophilin, and were thought to correspond to the B1a isoform. Initial attempts to amplify endophilin B1a from testis RNA using published sequences from the 5′ and 3′ ends were unsuccessful. Closer comparison of the published sequence with sequence derived from the clones isolated during screening revealed the presence of a divergent 3′ end. Using primers corresponding to the novel sequence, we were able to amplify the variant endophilin cDNA from testis RNA. Sequence comparisons of testis

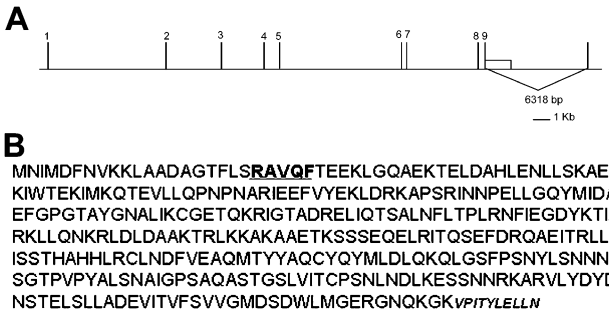

FIGURE 1: Endophilin B1t results from differential splicing that generates a slightly truncated protein. (A) The endophilin B1 gene encodes several different isoforms through differential splicing. Illustrated here is the variant splice that generates endophilin B1t, through the use of a cryptic splice donor site in the last exon of endophilin B1a (short open bar) and an exon approximately 6 kb downstream. (B) This splice event introduces a stop codon at the new splice junction, resulting in a protein that is missing the last 10 amino acids (small italics). The RVxF motif is underlined.

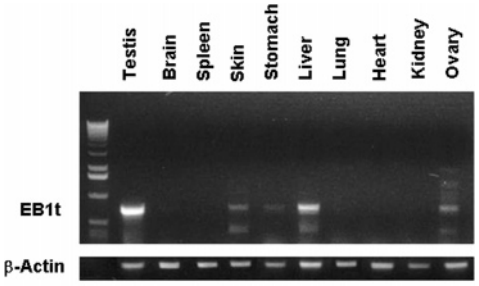

FIGURE 2: Endophilin B1t is expressed in a subset of tissues. RNA from indicated tissues was subjected to RT-PCR using endophilin B1t specific primers. Strong expression was observed in testis and liver, while weak but detectable expression could be seen in skin, stomach, and ovary. β-Actin was amplified from each sample as a control.

endophilin cDNA with genomic sequence revealed the presence of an additional exon approximately 6 kb downstream of the last exon in endophilin B1a. The splice donor site for this testis specific exon resides within the coding sequence of the last endophilin B1a exon, and results in a putative protein that is identical with endophilin B1a, with the exception that it is missing the last 10 amino acids (Figure 1; GenBank accession number DQ205686). All endophilin B1 isoforms possess a canonical RVxF motif (RAVQF) near the amino terminus. CrossMatch alignment reveals the presence of numerous mouse ESTs which have undergone this differential splice, and also the location of the most likely polyA addition signal. We have designated this new isoform endophilin B1t.

Endophilin B1t Is Predominantly Expressed in Testis. Endophilin B1 is a well-characterized protein, and is widely expressed (*34*). However, we had difficulty amplifying endophilin B1a from testis RNA, and were only successful using a 3′ primer from the newly discovered exon. We therefore assessed expression of this new isoform by RT-PCR on total RNA from a variety of mouse tissues using gene-specific primers (Figure 2). A DNA fragment corresponding to endophilin B1t was amplified robustly from testis and liver. Faint bands were also observed following RT-PCR on stomach, skin, and ovary. Brain, spleen, lung, heart, and kidney were completely negative for endophilin B1t in this assay.

Table 2: Yeast Two-Hybrid Interactions[a]

	PP1cγ2	*PP1cγ1*	*PP1cγ2Δc*	*PP1cα*	*lamin*
staufen	+++	+++	+++	+++	−
endophilin B1t	+++	−	−	−	−
endophilin B1a	−	−	−	−	−

[a] The various "bait" constructs are listed across the top, while the "prey" are listed in the leftmost column. "+++" represents strong interactions, while "−" represents no interaction, as judged by expression of the three target genes in this system.

Endophilin B1t Interacts Specifically with PP1cγ2 in a Yeast Two-Hybrid Assay. We have hypothesized the presence of PP1 regulatory subunits that interact specifically with the γ2 catalytic subunit of PP1 to explain the phenotype of PP1cγ null mice. To determine if endophilin B1t was able to interact with multiple PP1c isoforms, two-hybrid assays were performed using full length endophilin B1t in combination with PP1cα, PP1cγ1, and a truncated version of PP1cγ2 lacking 18 amino acids from the C-terminus. A construct encoding a portion of human lamin C was used as a negative control. Expression of all fusion proteins in yeast was confirmed by Western blotting. A positive interaction was assessed through the ability of transformed cells to grow on minimal media, activating expression of *ADE2* and *HIS3* reporter genes. Expression of a lacZ reporter gene was also assessed after cell growth. Using these criteria endophilin B1t was found to interact solely with PP1cγ2 and not with PP1cα, PP1cγ1, or the truncated PP1γ2 (Table 2). Interaction with PP1cγ2 was strong as judged by the white color of resulting yeast colonies, indicating robust activation of the *Ade2* reporter in the *ade2-101* background. A bait construct expressing amino acids 148−486 of the mouse *staufen* gene was used as a control during testing, and found to interact strongly with all PP1c isoforms with no coloration due to *ade2-101*. Staufen is an RNA-binding protein and has recently been recognized as a regulatory subunit of PP1 in rat brain neurons (*35*). Staufen is also expressed in male germ cells during spermatogenesis in a stage-specific manner (*35*) and was isolated by us in the same two-hybrid screen as endophilin B1t. The strength of the interaction between endophilin B1t and PP1cγ2 was further demonstrated by the ability of cells containing both constructs to survive on media containing 10 mM 3-amino-1,2,4-triazole (3-AT), a competitive inhibitor of the *HIS3* gene product. In addition, lacZ colony lift assays demonstrated robust expression of the third reporter construct in the yeast two-hybrid system (data not shown).

Endophilin B1a did not interact with any of the PP1c isoforms in a yeast two-hybrid assay.

Endophilin B1t Associates with PP1cγ2 in Mammalian Cells. To confirm an interaction between endophilin B1t and PP1cγ2, co-immunoprecipitation on native testis proteins was performed. Antibodies to either PP1cγ or endophilin were able to pull out the opposite protein after purification and Western blotting (Figure 3A). Immunoprecipitation with preimmune serum failed to pull down PP1cγ. We also verified the interaction between endophilin B1t and PP1cγ2 in mammalian cells by cloning full-length coding sequences separately into pcDNA3.1 expression vectors to produce *myc*- and *HA*-epitope tagged fusion proteins. Expression vectors were then transformed sequentially into COS-1 cells, an immortalized kidney cell line derived from African green monkey. In co-immunoprecipitation experiments, antibodies to either of the *myc* or *HA* epitope tags were able to pull down the opposite protein following purification and Western blotting (Figure 3B). It should be noted that while the antibody used to recognize PP1cγ2 was capable of recognizing both of the PP1cγ splice variants, RT-PCR using COS-1 RNA showed that only PP1cα and not PP1cγ1 was naturally expressed (data not shown). PP1cα, which is naturally present in COS-1 cells (Figure 3B, far right), did not co-immunoprecipitate with endophilin B1t.

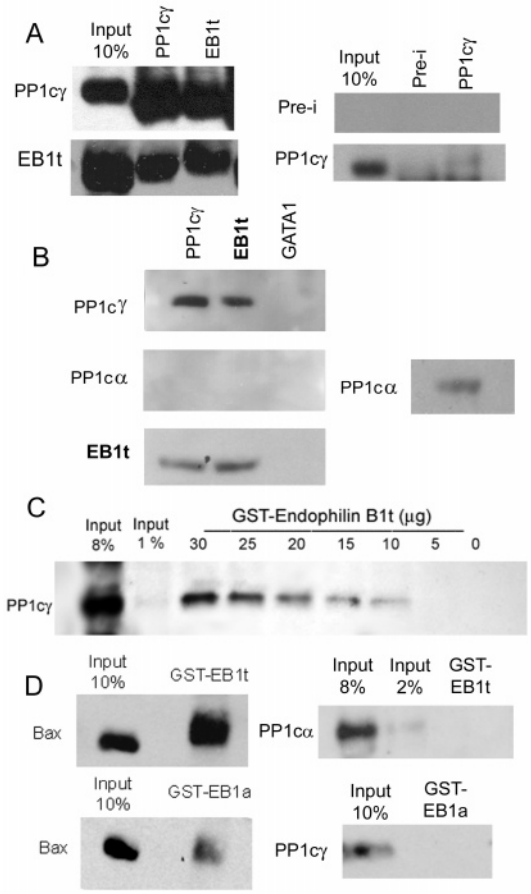

FIGURE 3: Co-immunoprecipitation and sedimentation of PP1cγ2 and endophilin B1t in mammalian cells. (A) Antibodies to PP1cγ2 and endophilin B1t (top) were used to precipitate associated proteins (left) visualized on Western blot from native testis protein extracts. Preimmune serum (pre-i) failed to immunoprecipitate PP1cγ2. (B) PP1cγ2 and endophilin B1t were artificially expressed in mammalian COS-1 cells with *myc* and *HA* epitope tags respectively. PP1cα was naturally present in COS-1 cells (far right blot). Antibodies to epitope-tagged PP1cγ2 and endophilin B1t (top) were used to precipitate associated proteins (left) visualized on Western blot. A negative control experiment was performed using a GATA-1 antibody. (C) Sedimentation assays were performed to isolate PP1cγ from crude testis lysates using endophilin B1t beads. Varying amounts of GST-endophilin B1t were immobilized on glutathione-Sepharose and incubated with testis lysates as described in Materials and Methods. The beads were washed and bound proteins eluted in SDS-sample buffer before separation by SDS−PAGE and Western blotting with antibodies to PP1cγ. (D) As a control, sedimentation assays were performed to isolate Bax from crude heart lysates with GST-endophilin B1a and GST-endophilin B1t beads. Negative control experiments were performed using GST-endophilin B1t and GST-endophilin B1a to attempt to isolate PP1cα or PP1cγ2 from crude testis lysates, respectively. For all controls 30 μg of either GST-endophilin B1t or GST-endophilin B1a was used.

Validity of the interaction between endophilin B1t and PP1cγ2 was illustrated using a GST-sedimentation assay on native testis proteins. Specifically, varying amounts of full-length GST-endophilin B1t were covalently linked to glutathione-Sepharose beads and used to precipitate total protein from wild type mouse testis lysates (Figure 3C). Under our conditions, 30 μg of GST-endophilin B1t was shown to precipitate approximately 6% of the input PP1cγ. By comparison, 20 μg of neurabin I was recently found to bind 9−20% of input PP1cα/γ1 from brain lysates under similar conditions (*11*). We cannot exclude the possibility that endophilin B1t binding to PP1cγ2 is stage specific, in which case only a subset of whole testis protein would be available for binding in our assay. GST-endophilin B1a was not able to precipitate PP1cγ from wild type testis lysates. Similarly, GST-endophilin B1t was unable to isolate PP1cα from wild type testis lysates (Figure 3D). These observations further confirmed the specificity of the endophilin B1t/PP1cγ2 interaction. However, both GST-endophilin B1t and GST-endophilin B1a were able to precipitate Bax, a binding partner of endophilin B1a (*36*), from heart extracts, where Bax is expressed abundantly, indicating that the GST fusion proteins have normal conformation.

Inhibition of PP1 Activity by Endophilin B1t. Previous studies (*10*, *37*) have shown that association of PP1c with targeting subunits often decreases activity of the enzyme *in vitro*, at least toward phosphorylase *a*, which is the classic substrate for dephosphorylation by PP1. To examine the possible effect of binding to endophilin B1t on PP1c activity, we performed a series of phosphatase assays using a recombinant PP1c source in the presence of increasing concentrations of full length GST-endophilin B1t. Since *E. coli* has no native phosphatase activity toward phosphorylase *a* (*31*), crude bacterial lysates in physiological salt concentrations including 2 mM $MnCl_2$ could be used rather than affinity-purified enzyme. Unfortunately, there is no reliable method of separating native PP1c isoforms directly from testis without contamination. Measured phosphatase activities were calculated as a percentage of control (Figure 4). Under our conditions chimeric endophilin B1t was able to decrease PP1cγ2 activity in a dose dependent manner with a 60% decrease in total at 10^{-6} M. The IC_{50} for the inhibition was calculated at ~0.5 μM. Endophilin B1t had relatively little effect on PP1cα activity, with only a 10% decrease from control levels at the highest concentration of 10^{-6} M. As a control, phosphatase activities were also measured in the presence of increasing amounts of neurabin I and the skeletal muscle glycogen-targeting subunit (G_M), both known regulatory subunits of PP1 that have been previously characterized in terms of their inhibitory effect on the α catalytic subunit (*37*). Neurabin I recruits PP1 to the actin cytoskeleton in brain neurons and was recently shown to interact preferentially with PP1cα and PP1cγ1 but not PP1cβ (*11*). Consistent with previous data, GST-neurabin I containing amino acids 374−516 inhibited PP1cα activity up to 90% of maximum at 10^{-6} M with an IC_{50} of 9 nM (*38*). PP1cγ2 was inhibited by up to 80% of control levels with a corresponding IC_{50} of 12 nM. The glycogen targeting subunit G_M was previously reported as decreasing PP1cα activity toward phosphorylase *a* up to 50% of maximum, compared to the 80−90% reduction observed for high levels of neurabin I (*37*). Under our conditions a GST construct containing amino acids 1−240 of G_M was found to inhibit PP1cα and PP1cγ2 at 40% and 49% respectively of maximum at 10^{-6} M. The inhibitions of PP1cγ2 by G_M and endophilin B1t were highly similar.

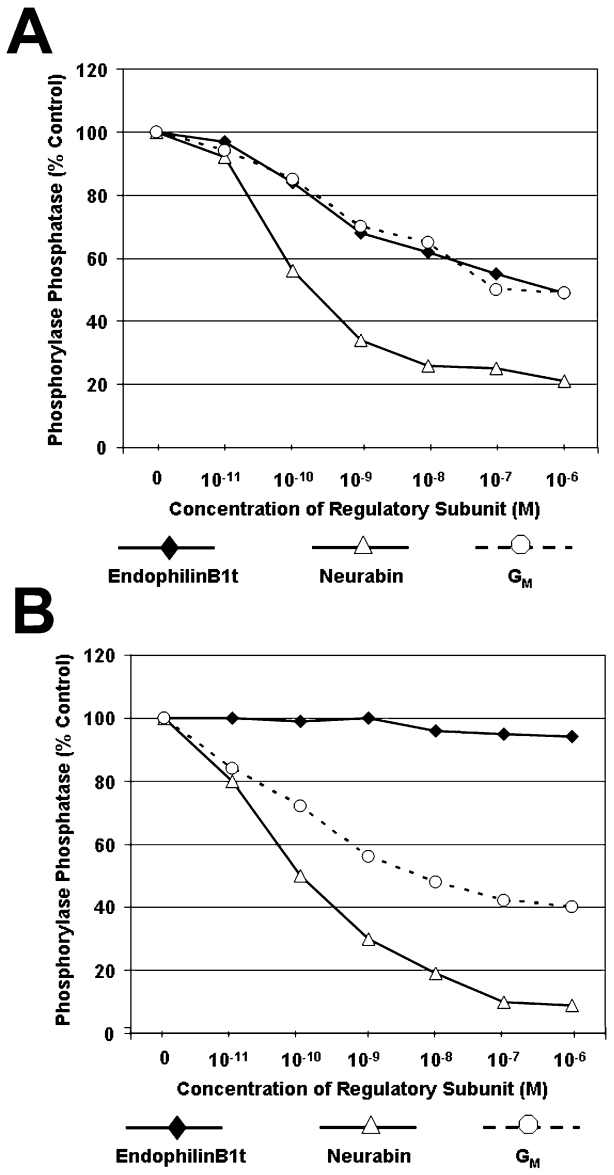

FIGURE 4: Inhibition of PP1cγ2 activity by chimeric endophilin B1t in a phosphatase assay. Diluted bacterial lysates expressing recombinant PP1cγ2 (A) and PP1cα (B) were used in phosphatase activity assays using radiolabeled phosphorylase *a* as described in Materials and Methods. Dephosphorylation of phosphorylase *a* was monitored in the presence of increasing concentration of GST-endophilin B1t (diamonds), GST-G_M (amino acids 1−240) (octagons), and GST-neurabin I (amino acids 374−516) (triangles). Phosphatase activity is expressed as a percentage of control. Data points represent an average of three independent experiments varying by less than 6%. Assays were restricted to 15 min time periods to ensure linearity of phosphate release.

Expression Pattern of Endophilin B1t in Mouse Testis. To examine the expression pattern of endophilin B1t, immunohistochemical analyses of wild type testis sections from 14 week old CD-1 mice were performed with the bif-1 antibody. Bif-1 was the original name for endophilin B1a. In wild type testis sections, endophilin B1t staining was stage specific and presented two kinds of protein distributions (Figure 5A). Most tubules displayed diffuse cytoplasmic staining, although there was clearly some stage specificity in the intensity, with

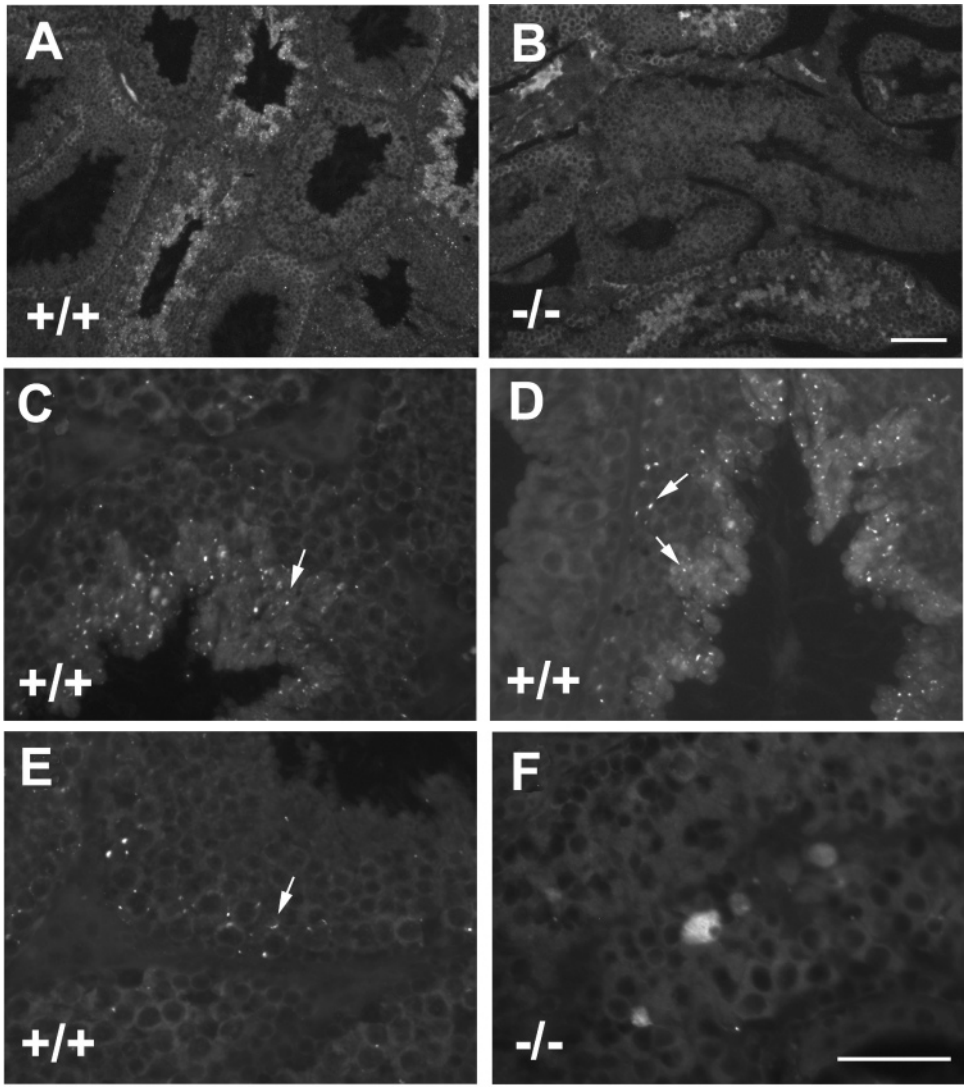

FIGURE 5: Expression pattern of endophilin B1t in wild type and PP1c$\gamma^{-/-}$ testes. (A, B) Immunohistochemical analyses were performed using bif-1 (endophilin) antibody (Cy-3 labeled) on adult wild type (A) and PP1c$\gamma^{-/-}$ (B) testes as described in Materials and Methods. This image was obtained at lower magnification to illustrate the stage variation in expression patterns. Bar represents 100 μm. (C−F) Immunohistochemistry was performed on adult wild type (C, D, E) and PP1c$\gamma^{-/-}$ (F) testes using antibody directed against bif-1 (endophilin). Higher magnification images better illustrate the puncta (arrows) observed in wild type, but not mutant seminiferous epithelia. Bar represents 50 μm.

those tubules containing maturing spermatids displaying concentrated accumulation in the shrinking cytoplasm. Additionally, strong punctate staining could be observed in a subset of tubules, with distinctive patterns of puncta in different tubules (Figure 5C, 5D, 5E). Some tubules contained large numbers of puncta in the adlumenal layers of maturing spermatids (Figure 5C, 5D), while others contained smaller numbers of puncta in the more basal layers (Figure 5E). A few tubules displayed both patterns of puncta staining (Figure 5D).

Tubules of PP1cγ null mice were almost devoid of punctate staining (Figure 5B, 5F). Where it was found, it was limited to the basal compartment pattern; the adlumenal pattern was completely missing. The variation in intensity of diffuse cytoplasmic staining could be observed, but the circumferential regularity observed in wild type testis sections was lost. This latter pattern likely reflects the general breakdown in seminiferous epithelial organization in mutant testes observed previously (*3*, *39*, *40*). It should be noted that the bif-1 antibody recognizes all of the endophilin B1 isoforms.

Immunohistochemistry with the PP1cγ antibody revealed a concentrated cytoplasmic distribution in both germ cells and Sertoli cells throughout the seminiferous epithelium (Figure 6). Although the intensity of the PP1cγ signal appeared to vary slightly between tubules, especially in regions abutting the lumen, the overall expression throughout spermatogenesis was uniformly high.

DISCUSSION

PP1cγ2 is strongly expressed in the testis, but it is not the only PP1c isoform present. PP1cα and PP1cβ are also expressed, both in germ cells and Sertoli cells, the latter being the somatic component of the seminiferous tubules (*3*). A small amount of PP1cγ1 is also present, mainly in Sertoli cells. This has led us to hypothesize that the male sterile phenotype observed in PP1cγ null mice is mediated by a PP1 holoenzyme composed of PP1cγ2 and a regulatory

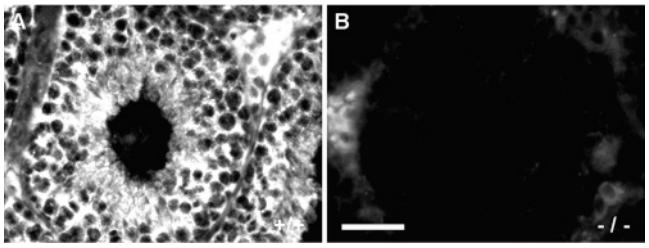

FIGURE 6: Expression of PP1cγ in wild type and PP1cγ mutant testes. Immunohistochemistry was performed on adult testes from the wild type (A) and PP1c$\gamma^{-/-}$ mice (B), using an antibody directed against a peptide specific to PP1cγ isoforms (see Materials and Methods). Consistent with previously published Western blot data (*3*), no signal is observed in mutant testes. PP1cγ protein is uniformly distributed in wild type testes.

subunit that binds specifically to this isoform. Our results from two-hybrid screening support this hypothesis. Several clones were isolated which interact specifically with the PP1cγ2 splice variant. One of these clones, endophilin B1t, was isolated seven times in the screen, which covered the cDNA library 3-fold.

A variety of *in vitro* assays verified the yeast two-hybrid results. Endophilin B1t co-immunoprecipitated with PP1cγ2 and was able to pull out PP1cγ2 from testis protein extracts. It can also be co-immunoprecipitated with PP1cγ2, but not PP1cα, following transient transfection of COS-1 cells. Furthermore, endophilin B1t inhibits PP1cγ2 phosphatase activity *in vitro* at least as well as a known PP1c regulatory subunit, G_M, while having no effect on PP1cα activity.

While endophilin B1 is widely expressed (*34*), the testis variant we identified in our screen is limited to the testis, liver, ovary, skin, and stomach, with expression in the last three tissues being very weak. This variant lacks 10 amino acids from the extreme carboxy terminus through differential splicing of a downstream exon to a cryptic splice donor in the last exon of endophilin B1a (Figure 1B). Full length endophilin B1a does not interact with any of the PP1 isoforms, in spite of the presence of a canonical RVxF motif near its amino terminus, indicating that the motif alone is insufficient for binding to PP1cγ2. Endophilin B1t acquires the ability to bind PP1cγ2, but not PP1cγ1 or PP1cα through loss of the last 10 amino acids on the COOH terminus. This suggests that the carboxy terminus of endophilin B1a may prevent the isoform specific binding to PP1cγ2. The expression of endophilin B1t in liver and ovary is interesting, given that there does not appear to be any defect in either tissue in mutants.

Endophilin B1 isoforms are expressed in seminiferous epithelial tubules in a stage specific manner. There are two kinds of protein distribution revealed by immunohistochemistry: diffuse and uniform cytoplasmic staining, and punctate accumulation at cell membranes. Both kinds of distribution display stage specific patterns. The diffuse staining is slightly intensified in basal spermatocytes in some tubules, and strongly intensified in elongating spermatids in other tubules. Abundant punctate distribution can be observed in the adlumenal compartment of tubules containing condensing spermatids, while sparse puncta are present in the basal compartment of some tubules. The diffuse staining pattern is retained in PP1cγ mutant testes, although underlying disorganization of the seminiferous epithelium contributes to disorganized distribution of the intense staining pattern. The most interesting observation is the almost complete disappearance of the punctate staining pattern. Even those tubules that retain the intense diffuse staining found in the maturing spermatid compartment are missing puncta in this region. There are very few puncta in the basal compartment in mutant testes.

Endophilin has been demonstrated to be involved in the endocytic pathway (*41*−*44*). This raises interesting possibilities for explaining the phenotype in the PP1cγ mutant mice. Light microscopic examination of developing testes led us to propose that PP1cγ mutant testes prematurely release germ cells from the seminiferous epithelium (*39*). Germ cell retention and release is thought to involve complicated recycling of cell−cell junctional complexes (*45*). Among these are ectoplasmic specializations (ES), adhesion junctions found between adjacent Sertoli cells near the basal compartment of the seminferous epithelium, and between germ cells and Sertoli cells closer to the adlumenal compartment. Located close to ES are structures called tubulobulbar complexes. Recent evidence suggests that a key function of tubulobulbar complexes is the recycling of junctional complexes such as ES through a complicated endocytic cycle (*46*). Tubulobulbar complexes bear superficial similarity to capitate projections, structures that involve interdigitations of glial cells and photoreceptors, and whose function is disrupted in endophilin mutants in *Drosophila melanogaster* (*44*). The phenotype of the PP1cγ mutant testes, and the strong association of endophilin B1t with PP1cγ2, suggest that disregulation of endophilin B1t function in the testis may be a proximate cause of the premature germ cell release we observe. This hypothesis is under further investigation in our lab.

Our studies provide additional evidence of a regulatory subunit that preferentially interacts with PP1cγ2 and not PP1cα or PP1cγ1, and has the potential to target the isoform to a function required for spermatogenesis. We have reported recently the isolation of another isoform specific regulatory subunit, Spz1 (*22*), identified in the same yeast two-hybrid screen as endophilin B1t. Together with three unknown interacting clones (Hrabchak and Varmuza, unpublished), endophilin B1t and Spz1 are part of a growing list of PP1c isoform specific regulatory subunits. They represent a small proportion of the PP1cγ2 interacting clones identified in the screen (see Table 1, Supporting Information). Examination of PP1cγ null mice has revealed multiple testis defects including increased aneuploidy (*16*) and apoptosis (*15*), secondary to the central defect(s) resulting in infertility. To explain this we have postulated that PP1cγ2 plays multiple roles in the testis governed by a varied subset of regulatory proteins, some of which are isoform specific. Combinatorial control of PP1cγ2 by a subset of regulatory proteins including endophilin B1t and Spz1 has the potential to best explain the phenotype of PP1cγ null mice.

SUPPORTING INFORMATION AVAILABLE

A table of all the isoform nonspecific interactors pulled out of the testis library in the yeast two-hybrid screen. This material is available free of charge via the Internet at http://pubs.acs.org.

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