PPP1CC2 can form a kinase/phosphatase complex with the testis-specific proteins TSSK1 and TSKS in the mouse testis

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Abstract

The mouse protein phosphatase gene *Ppp1cc* is essential for male fertility, with mutants displaying a failure in spermatogenesis including a widespread loss of post-meiotic germ cells and abnormalities in the mitochondrial sheath. This phenotype is hypothesized to be responsible for the loss of the testis-specific isoform PPP1CC2. To identify PPP1CC2-interacting proteins with a function in spermatogenesis, we carried out GST pull-down assays in mouse testis lysates. Amongst the identified candidate interactors was the testis-specific protein kinase TSSK1, which is also essential for male fertility. Subsequent interaction experiments confirmed the capability of PPP1CC2 to form a complex with TSSK1 mediated by the direct interaction of each with the kinase substrate protein TSKS. Interaction between PPP1CC2 and TSKS is mediated through an RVxF docking motif on the TSKS surface. Phosphoproteomic analysis of the mouse testis identified a novel serine phosphorylation site within the TSKS RVxF motif that appears to negatively regulate binding to PPP1CC2. Immunohistochemical analysis of TSSK1 and TSKS in the *Ppp1cc* mutant testis showed reduced accumulation to distinct cytoplasmic foci and other abnormalities in their distribution consistent with the loss of germ cells and seminiferous tubule disorganization observed in the *Ppp1cc* mutant phenotype. A comparison of *Ppp1cc* and *Tssk1/2* knockout phenotypes via electron microscopy revealed similar abnormalities in the morphology of the mitochondrial sheath. These data demonstrate a novel kinase/phosphatase complex in the testis that could play a critical role in the completion of spermatogenesis.

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Introduction

Protein phosphorylation is a key post-translational regulatory mechanism that plays a role in countless cellular processes. Precise regulation of protein phosphorylation is carried out by the opposing activities of protein kinases and protein phosphatases. While the mammalian genome encodes ~400 Ser/Thr kinases, it encodes only ~40 Ser/Thr phosphatases (Moorhead *et al.* 2007, Bollen *et al.* 2010). Thus, many Ser/Thr phosphatases, including PP1s, obtain substrate specificity by functioning as holoenzymes via interactions with a large and diverse array of regulatory subunits (Hubbard & Cohen 1993). To date, almost 200 distinct PP1-interacting proteins have been identified (Bollen *et al.* 2010), and it is hypothesized that many more exist.

Spermatogenesis is no exception to the importance of protein phosphorylation-based regulatory processes. On searching the Gene Ontology database, it can be observed that 14 genes are linked to both the Ser/Thr protein

kinase molecular function (GO:0004674) and the spermatogenesis biological process (GO:0007283), including all six members of the testis-specific Ser/Thr kinase (TSSK) family. Using a similar database search for the Ser/Thr protein phosphatase molecular function (GO:0004722), it can be observed that no genes overlap with the spermatogenesis biological process; however, at least two Ser/Thr protein phosphatase genes, *Ppp1cc* (Varmuza *et al.* 1999) and *Ppm1d* (Choi *et al.* 2002), produce male infertility phenotypes when deleted, indicating that functional annotation in this database is not complete.

Ppp1cc is a member of the PP1 family of protein phosphatases that encodes two splice isoforms: the ubiquitous *Ppp1cc1* and the testis-specific *Ppp1cc2* (Okano *et al.* 1997). When *Ppp1cc* is deleted by targeted mutagenesis, the only observable phenotypic consequence is homozygous male infertility, due to a failure of spermatogenesis, reminiscent of the common human condition non-obstructive azoospermia (Varmuza *et al.* 1999). Inside the seminiferous tubules, a widespread loss

of germ cells is evident, most prominently in post-meiotic spermatids, leading to a breakdown of the spermatogenic cycle (Varmuza *et al.* 1999, Forgione *et al.* 2010). The few surviving germ cells feature a range of morphological abnormalities, including those involving meiosis, chromatin condensation, acrosome formation and mitochondrial organization (Varmuza *et al.* 1999, Chakrabarti *et al.* 2007, Forgione *et al.* 2010). In the mouse testis, a number of different proteins have been shown to interact with PPP1CC2. These include both proteins involved in isoform-specific interactions such as SPZ1 and Endophilin B1t (SH3GLB1, testis-specific isoform) (Hrabchak & Varmuza 2004, Hrabchak *et al.* 2007), and, more numerous, proteins that have the ability to interact with multiple PP1 isoforms, such as PPP1R11 (Cheng *et al.* 2009). In addition, another testis-specific protein, TSSK substrate (TSKS), was bioinformatically predicted to interact with PP1 based on the presence of a high-affinity PP1 docking motif, and *in vitro* experiments have confirmed that a TSKS fragment is capable of interacting with PPP1CA (Hendrickx *et al.* 2009). The ability of full-length TSKS to bind to other PP1 isoforms such as PPP1CC2 has not been tested.

In a search for additional PPP1CC2-interacting proteins, we carried out pull-down assays in mouse testis protein extracts using GST-PPP1CC1 and GST-PPP1CC2 as bait. Amongst the identified proteins was the testis-specific Ser/Thr kinase TSSK1, which is essential for spermatogenesis in the mouse (Xu *et al.* 2008, Shang *et al.* 2010). Herein, we describe experiments that demonstrate a clear biochemical link between TSSK1, TSKS and PPP1CC2 in the mouse testis.

Materials and methods

Mouse testis protein extract preparation

Whole mouse testes were homogenized in cold protein extraction buffer (10% (v/v) glycerol, 50 mM HEPES–NaOH, pH 8.0, 150 mM NaCl, 2 mM EDTA, 0.1% (v/v) NP-40, 1 mM dithiothreitol, 10 mM NaF, 0.25 mM sodium orthovanadate and 50 mM β -glycerol phosphate) supplemented with Sigma protease inhibitor cocktail using a Dounce homogenizer. After homogenization, samples were incubated on ice for 10 min, followed by centrifugation at a speed of 10 000 *g* for 10 min at 4 °C to remove non-soluble material. All animal protocols were approved by the Canadian Council on Animal Care.

GST and His-tag pull-down assays

The PPP1CC1-coding sequence was PCR-amplified from the pGEM-7zf plasmid using the forward primer 5'-GGCGGATCCGCGATGGC-3' and the reverse primer 5'-GCTATGTTAGAATCCCAACCAGGC-3' and ligated into the BamHI and EcoRI sites of the pGEX-6P-2 plasmid (Amersham). GST-PPP1CC2- and GST-containing plasmids have been cloned previously and fusion proteins produced as described previously (Hrabchak & Varmuza 2004, Hrabchak

et al. 2007). Mouse coding sequences for *Tssk1* and *Tsk*s were cloned into the BamHI and NotI sites of the pET28a plasmid (Invitrogen) and transformed into BL21 cells. His-TSSK1 and His-TSKS were produced by inducing BL21 cells with 0.4 mM isopropyl β -D-1-thiogalactopyranoside (IPTG) for 4 h at 37 °C, and recombinant proteins were purified from the cleared lysate using Ni-NTA resin. Mouse testis lysates (500 μ g) were incubated with $\sim$ 2 μ g of GST-fusion proteins for 2–4 h at 4 °C with gentle rocking. Samples were centrifuged at 1500 *g* for 2 min at 4 °C, and GST-fusion protein beads were washed three times with 500 μ l of lysis buffer, after centrifugation steps. For pull-down experiments with LC–MS/MS analysis, testis lysates were initially pre-cleared via incubation with glutathione agarose beads, and recombinant proteins were subjected to additional washes, both before and after incubation with testis lysates. Recombinant human His-TSSK1 (Millipore cat. number 14-670) was bound to Ni-NTA resin and incubated with mouse testis lysates using the protocol used for GST fusions. *In vitro*, lysate-free pull-down assays were based on a previously described protocol (De Wever *et al.* 2012), where 500 ng of His-TSSK1 and/or His-TSKS were incubated with GST or GST-PPP1CC2 bound to glutathione agarose beads in 250 μ l of binding buffer (25 mM Tris, pH 7.5, 5% glycerol (v/v), 150 mM NaCl, 0.5% NP-40 (v/v) and 10 mM imidazole) for 2 h at 4 °C with rocking. Beads were then spun down at 1500 *g* for 1 min at 4 °C and washed three times with 1 ml of binding buffer. For all the experiments, bound proteins were eluted by boiling in SDS–PAGE sample buffer (50 mM Tris, 2% (w/v) SDS, 0.1% (w/v) bromophenol blue, 10% (v/v) glycerol and 25 mM β -mercaptoethanol) and analysed by SDS–PAGE followed by silver staining (FOCUS-FASTsilver, G Biosciences, St Louis, MO, USA) or western blotting using standard protocols.

In-gel digestion of silver-stained gel slices

Slices were excised from silver-stained polyacrylamide gel and washed with HPLC-grade water. To each gel slice, 200 μ l of acetonitrile were added, followed by incubation at room temperature for 15 min with mixing. Slices were then reduced with 10 mM dithiothreitol in 100 mM ammonium bicarbonate for 30 min at 50 °C, followed by removal of the reduction solution and wash with acetonitrile. Alkylation was carried out using 55 mM iodoacetic acid in 100 mM ammonium bicarbonate for 20 min in the dark at room temperature. Alkylation solution was removed and gel slices were washed with ammonium bicarbonate and dried. Gel slices were then incubated with proteomics-grade trypsin (Sigma–Aldrich, T6567) in 50 mM ammonium bicarbonate and 5 mM CaCl_2 on ice for 45 min and then overnight at 37 °C. After deactivating trypsin with trifluoroacetic acid, the supernatant was collected and 100 μ l of 60% (v/v) acetonitrile were added to the gel slices and incubated with rocking for 10 min. The supernatant was then collected and combined with that of the previous step and dried in a speed vac.

LC–MS/MS analysis and protein/peptide identification

LC–MS/MS analysis was carried out by the Advanced Protein Technology Centre (Toronto, ON, Canada <http://www.sickkids.ca/Research/APTC/index.html>). Peptides were loaded onto a

150 µm ID pre-column (Magic C18, Michrom Biosciences, Aburn, CA, USA) at 4 µl/min and separated over a 75 µm ID analytical column packed into an emitter tip containing the same packing material. The peptides were eluted over 60 min at 300 nl/min using a 0–40% (v/v) acetonitrile gradient in 0.1% (v/v) formic acid using an EASY n-LC nano-chromatography pump (Proxeon Biosystems, Odense, Denmark). The peptides were eluted into a LTQ-Orbitrap hybrid mass spectrometer (Thermo-Fisher, Bremen, Germany) operated in a data-dependent mode. Mass spectra were acquired at 60 000 FWHM resolution in the FTMS, and MS/MS was carried out in the linear ion trap. Six MS/MS scans were obtained per MS cycle. The raw data were searched using Mascot (Matrix Sciences, London, UK). Tandem mass spectra were extracted, charge state deconvoluted and deisotoped using BioWorks version 3.3. All MS/MS samples were analysed using Mascot (Matrix Science; version Mascot). Mascot was set up to search the NCBI nr_20110515 database for gel slice peptide samples and NCBI nr_20110813 database for phosphopeptide samples (both selected for *Mus musculus*) for trypsin digestion. Mascot was searched with a fragment ion mass tolerance of 0.40 Da and a parent ion tolerance of 20 ppm for phosphopeptide samples and a fragment ion mass tolerance of 0.50 Da and a peptide tolerance of 3.0 Da for gel slice peptide samples. Iodoacetamide derivative of cysteine was specified as a fixed modification, with the following variable modifications: Pyro-glu from E of the N-terminus, s-carbamoylmethylcysteine cyclization of the N-terminus, deamidation of asparagine and glutamine, oxidation of methionine, acetylation of the N-terminus for gel slice peptide samples with phosphorylation of serine, threonine and tyrosine as an additional variable modification for phosphopeptide samples. Scaffold (version Scaffold_3.1.2, Proteome Software, Inc., Portland, OR, USA) was used to validate MS/MS-based peptide and protein identifications. Peptide identifications were accepted if they could be established at >95.0% probability as specified by the Peptide Prophet algorithm (Keller *et al.* 2002). Protein identifications were accepted if they could be established at >95.0% probability and contained at least two identified peptides (or one for phosphopeptide samples). Protein probabilities were assigned by the Protein Prophet algorithm (Nesvizhskii *et al.* 2003). Proteins that contained similar peptides and could not be differentiated based on MS/MS analysis alone were grouped to satisfy the principles of parsimony.

PCR mutagenesis

For the generation of RVxF motif mutants, PCR mutagenesis was carried out using primers complementary to the relevant region of the TSKS-coding sequence with the exception of the required non-synonymous base pair substitutions. Primer sequences were as follows: for the KAASA mutation: forward primer 5'-CGAAAAAAGAAGGCTGCGTCCGCCCATGGGTGGA-GCCCCG-3' and reverse primer 5'-CGGGGCTCCACCCC-TAGGGCGGACGCAGCCTTCTTTTTTTCG-3'; for the KAVEF mutation: forward primer 5'-CGAAAAAAGAAGGCTGTG-GAGTTCCATGGGGTGGAGCCCCG-3' and reverse primer 5'-CGGGGCTCCACCCCATGGAAGTCCACAGCCTTCTTTTTTTCG-3'. Primers were used in PCR amplification of the pGEX-TSKS plasmid with PFU polymerase (BioBasic, Markham, Ontario, Canada), and the resulting reaction product was

purified and digested with DpnI restriction enzyme to digest the template plasmid. The digested DNA was transformed into DH5α for selection, and the presence of mutations was verified via plasmid sequencing. Plasmids containing TSKS-coding sequences with mutated RVxF motifs were then used to produce GST-fusion proteins as outlined above.

Co-immunoprecipitation

Aliquots of whole mouse testis protein lysates (500 µl; prepared as outlined above) were first pre-cleared via incubation with 50 µl of rProtein-G Agarose (Invitrogen) for 30 min at 4 °C. The cleared lysates were then incubated with either 5 µg of anti-PPP1CC or irrelevant antibody for 2 h at 4 °C, followed by isolation of antibody–antigen complexes via incubation with 50 µl of rProtein-G Agarose for a further 2 h at 4 °C. Agarose beads were then washed four times with lysis buffer, boiled in SDS–PAGE sample buffer, and subjected to SDS–PAGE and western blotting using rabbit anti-TSKS at a dilution of 1:500.

Antibodies

Goat anti-PPP1CC (N-19, Santa Cruz Biotechnology), which recognizes both PPP1CC1 and PPP1CC2, was used at a dilution of 1:500 for western blotting, with donkey anti-goat HRP secondary antibody (Santa Cruz Biotechnology) at a dilution of 1:5000. Guinea pig anti-TSKS and TSSK1 antibodies (Shang *et al.* 2010) were used at dilutions of 1:500 for western blotting in cell lysate samples and of 1:5000 using purified fusion proteins with goat anti-guinea pig HRP secondary antibody (Jackson ImmunoResearch, West Grove, PA, USA) at a dilution of 1:5000. For immunohistochemistry, the same anti-TSKS and TSSK1 primary antibodies were used at a dilution of 1:500, with Cy3-conjugated AffiniPure goat anti-guinea pig IgG (Jackson ImmunoResearch) at a dilution of 1:5000. Guinea pig anti-TSSK2 (Shang *et al.* 2010) was used at a dilution of 1:2000 for western blotting under the secondary antibody conditions used for other guinea pig antibodies listed above. Rabbit anti-TSKS (Shang *et al.* 2010) was used for western blotting at a dilution of 1:500 with anti-rabbit IgG HRP-linked antibody (Cell Signalling Technology, Beverly, MA, USA) at a dilution of 1:5000.

Phosphopeptide enrichment

Adult mouse testes were decapsulated and germ cell suspensions were produced as described previously (Henderson *et al.* 2011, MacLeod & Varmuza 2012). Germ cells were lysed in 400 µl of 7 M urea, 2 M thiourea, 4% CHAPs (w/v), and 40 mM Tris, reduced with 20 mM dithiothreitol for 1 h at room temperature and alkylated with 40 mM iodoacetic acid for 35 min at room temperature in the dark. Germ cell proteins were precipitated with acetone and dried using a speed vac, followed by resuspension in a 1 M urea, 50 mM ammonium bicarbonate solution containing 10 µg of proteomics-grade trypsin. Digestion was carried out overnight at 37 °C, and at the completion of digestion, the solution was acidified with 1% (v/v) formic acid and centrifuged to remove insoluble material. One quarter of the sample was used for phosphopeptide enrichment via sequential elution from IMAC (SIMAC) using the protocol of Thingholm

et al. (2009), but with 50 µl of TiO₂ Mag Sepharose (GE Healthcare, Milwaukee, WI, USA) substituted for TiO₂ phosphopeptide enrichment steps. Phosphopeptide-enriched samples were then analysed by LC–MS/MS (described earlier).

Immunohistochemistry

After removal of the tunica albuginea, WT and *Ppp1cc* mutant testes were fixed in 4% (w/v) paraformaldehyde overnight at 4 °C and dehydrated using a graded series of ethanol solutions and embedded in paraffin. Sections (7 µm) were dewaxed, hydrated and subjected to antigen retrieval by heating in 10 mM sodium citrate, 0.05% (v/v) Tween 20 and 1× PBS. Sections were permeabilized with 0.01% (v/v) Triton-X in PBS and blocked in 10% (w/v) goat serum, 1% BSA (w/v), 0.01% (v/v) Tween 20 and PBS solution. Primary antibody incubations were carried out in antibody dilution buffer (5% (w/v) goat serum, 1% (w/v) BSA, 0.01% (v/v) Tween 20 and PBS) overnight under the conditions described above. Secondary antibody incubations were carried out in antibody dilution buffer for 2 h in the dark. Nuclei were then stained with DAPI, and sections were mounted in 50% (v/v) glycerol for viewing with an Olympus BX60 microscope. Images were captured using the Cool Snap software and a CCD camera (RSPhotometrics, Tucson, AZ, USA). Images were adjusted for brightness and contrast using Photoshop 6.0 (Adobe) and then merged using ImagePro 4.1.

Electron microscopy

WT and *Ppp1cc* mutant testes were processed and analysed by electron microscopy (EM) as described previously (Forgione *et al.* 2010). *Tssk1/2* mutant testes were processed and analysed by EM as described previously (Shang *et al.* 2010).

Results

Testis-specific Ser/Thr kinase TSSK1 interacts with GST-PPP1CC1 and GST-PPP1CC2 in mouse testis lysates

In exploratory assays designed to identify PPP1CC-interacting proteins in the mouse testis, bacterially expressed GST-PPP1CC1 and GST-PPP1CC2 were purified and used in sedimentation assays with mouse testis protein lysates. Proteins were then subjected to SDS–PAGE, followed by silver staining. Selected gel

slices were excised from both the GST-PPP1CC1 and GST-PPP1CC2 lanes. In one such experiment, a region of ~45 kDa containing several visible bands was excised. After trypsin digestion, proteins present in the gel slices were identified via LC–MS/MS with MASCOT database searching. Common LC–MS/MS contaminant proteins such as keratins, actins and tubulins were removed from the list of candidate interactors. The remaining proteins for which ≥2 unique peptides were identified are listed in Table 1 (detailed peptide data are given in Supplementary Table 1, see section on supplementary data given at the end of this article). All four of the remaining proteins, UQCRC2, FADS2, SCCPDH and TSSK1, have been shown to be expressed in the mouse testis (Kueng *et al.* 1997, Stoffel *et al.* 2008, Guo *et al.* 2011), and targeted mutations of *Fads2* and *Tssk1/Tssk2* have been shown to disrupt spermatogenesis (Stoffel *et al.* 2008, Xu *et al.* 2008, Shang *et al.* 2010). *Tssk1* is a testis-specific kinase gene that plays a role in spermatogenesis, and the encoded protein, therefore, stood out to us as a particularly interesting candidate PPP1CC2 interactor in the testis. To test whether TSSK1 actually bound specifically to GST-PPP1CC2, the sedimentation assay in testis lysates was repeated, this time followed by western blotting for TSSK1. The results of this experiment confirmed the initial observation, with the GST-PPP1CC2 bait, but not GST alone, being able to pull down TSSK1 (Fig. 1A). Reciprocal pull-down experiments were carried out using His-TSSK1 bait, which successfully precipitated PPP1CC2 from mouse testis lysates (Fig. 1B). Additionally, a known substrate of TSSK1, testis-specific kinase substrate (TSKS), was successfully precipitated by His-TSSK1, verifying the functionality of the fusion protein (Fig. 1C).

Testis-specific kinase substrate, TSKS, interacts with PPP1CC2 via an RVxF docking motif

In a previously published study, the TSSK1 substrate TSKS was bioinformatically predicted to be a PP1-interacting protein, and subsequent *in vitro* experiments showed that a TSKS fragment was capable of interacting with PPP1CA (Hendrickx *et al.* 2009), the only PP1 isoform assayed. This prediction was based on the presence of a PP1

Table 1 Testis proteins identified in a SDS–PAGE gel band after sedimentation by GST-PPP1CC1 and GST-PPP1CC2. Only proteins identified by at least two significant unique peptides are listed. Common contaminant proteins, i.e. keratin and tubulin, have been removed from the list. Protein IDs are from UniProtKB/Swiss-Prot. Sequence coverage combines all the unique peptides from GST-PPP1CC1 and GST-PPP1CC2 pull downs.

Gene symbol	Name	Protein ID	Unique peptides		Sequence coverage (%)
			GST-PPP1CC1	GST-PPP1CC2	
<i>Uqcrc2</i>	Cytochrome b–c1 complex subunit 2, mitochondrial	Q9DB77	12	10	35.30
<i>Tssk1</i>	Testis-specific serine/threonine protein kinase 1	Q61241	10	2	25
<i>Sccpdh</i>	Saccharopine dehydrogenase-like oxidoreductase	Q8R127	7	5	16
<i>Fads2</i>	Fatty acid desaturase 2	Q9Z0R9	0	2	5

Figure 1 PPP1CC2 interacts with both TSSK1 and TSKS in the testis. GST and His-tag pull-down assays followed by SDS-PAGE and western blotting using the indicated antibodies were carried out. (A) Bacterially expressed mouse GST-PPP1CC2 successfully precipitates TSSK1 from mouse testis protein extract, while GST alone does not. (B) Ni-NTA-bound 6His-tagged human TSSK1 successfully precipitates PPP1CC2 from mouse testis protein extract, while Ni-NTA resin alone does not. Uneven signal observed in input lane is a gel artefact. (C) Ni-NTA-bound 6His-tagged human TSSK1 precipitates its substrate TSKS from mouse testis protein extract, while Ni-NTA resin alone does not. (D) Bacterially expressed mouse GST-PPP1CC2 successfully precipitates full-length TSKS from mouse testis protein extract, while GST alone does not. (E) Bacterially expressed mouse GST-TSKS successfully precipitates PPP1CC2 from mouse testis protein extract, while GST alone does not.

docking motif, known as an RVxF motif, in the TSKS amino acid sequence. In the mouse, this motif has the sequence KAVSF in amino acid positions 51–55. To test whether the PPP1CC2 isoform could interact with the full-length native TSKS in the testis, proteins pulled down by GST-PPP1CC2 were subjected to western blotting for TSKS. The results of this experiment indicate that the PPP1CC2 isoform can bind to full-length TSKS in the testis (Fig. 1D). The anti-TSKS signal observed via western blotting showed a doublet, which is consistent with previously published data (Shang *et al.* 2010). Furthermore, a reciprocal pull-down assay showed that GST-TSKS was able to bind to native PPP1CC2 in mouse testis lysates (Fig. 1E), confirming the interaction between these two testis proteins. The GST tag on its own was unable to precipitate either PPP1CC2 or TSKS (Fig. 1D and E). To verify that interaction with PPP1CC2 was dependent on the RVxF motif on the TSKS surface, we produced fusion

proteins containing TSKS with a mutated RVxF motif, changing the sequence from KAVSF to KAASA (GST-KAASA). When GST-KAASA was incubated with mouse testis lysates, it was unable to precipitate a detectable quantity of PPP1CC2 when compared with unaltered GST-TSKS (Fig. 2). Furthermore, GST-KAASA was still able to precipitate the known TSKS interactor TSSK2, indicating that the general structure of the TSKS protein was not affected by the RVxF motif mutation (Fig. 2). A second mutation, KAVEF, gave similar results, which will be discussed later. This experiment confirmed that TSKS and PPP1CC2 interact through the RVxF docking motif, but that the interaction of TSKS with TSSK2, and presumably also with TSSK1, does not depend on the RVxF motif.

To confirm that PPP1CC2 interacts with TSKS *in vivo*, we carried out a co-immunoprecipitation experiment using whole-testis proteins. Immunoprecipitation using anti-PPP1CC followed by western blotting using anti-TSKS confirmed that these two proteins exist in a complex in the mouse testis (Fig. 3). Furthermore, in an unpublished proteomic analysis of whole-testis proteins following immunoprecipitation with anti-TSKS, PPP1CC was detected with a relatively high Mascot score in testes from both WT and *Tssk1/2* knockout animals (Shang *et al.* 2010; P Shang and J A Grootegoed 2013, unpublished result). This indicates that TSSK1/2 activity is not essential for the interaction between PPP1CC and TSKS.

TSKS is phosphorylated on at least two different serine residues in the mouse testis including the PP1 docking motif

Previous experiments have shown TSKS to be phosphorylated on the serine 281 residue in the mouse testis (Xu *et al.* 2008), which is hypothesized to be the target site of TSSK1 phosphorylation. In an experiment aimed at identifying novel phosphorylation sites in the mouse testis, we carried out sequential elution from IMAC (SIMAC) (Thingholm *et al.* 2008) phosphopeptide

Figure 2 The TSKS RVxF motif is required for interaction with PPP1CC2. GST-fusion proteins corresponding to unaltered TSKS protein, RVxF mutant KAASA and RVxF phosphoserine-mimic KAVEF were incubated with mouse testis lysates and assayed via western blotting for interaction with PPP1CC2 (top) and TSSK2 (bottom).

Figure 3 PPP1CC2 and TSKS interact in the mouse testis. Western blotting demonstrates that TSKS is immunoprecipitated by anti-PPP1CC but not by an irrelevant antibody (IP:Neg) from whole mouse testis lysates.

enrichment followed by LC–MS/MS on proteins extracted from adult germ cell suspensions (further data on site assignment are presented in [Supplementary Table 2 and Figure 1](#), see section on [supplementary data](#) given at the end of this article). MASCOT database searching of the resultant tandem mass spectra was used to map phosphorylation sites. Amongst the identified phosphopeptides were two different phosphorylated peptides that mapped uniquely to TSKS with >95% confidence (as calculated by the Scaffold Software). The first peptide, which was identified in both the IMAC and TiO₂ fractions, was the previously known TSKS phosphopeptide HGLSPATPIQGcSGPPGS*PEEPPR, which was phosphorylated on the serine 281 residue (best Mascot delta score=26.6; Ascore=94.9). The second phosphopeptide mapped to TSKS was AVS*FHGVEPR, which represents phosphorylation on the serine 54 residue (Mascot delta score=30.9; Ascore=1000), and has not been reported previously in the testis. According to the PhosphoSitePlus database ([Hornbeck et al. 2012](#)), this phosphopeptide has been identified previously in human Jurkat cell, T-cell leukaemia model, but not in the mouse, or specifically in the testis. Interestingly, this phosphorylated serine residue is found within the PP1 docking motif KAVSF.

Phosphorylation of the PP1 docking motif in TSKS probably inhibits interaction with PPP1CC2

Previous research with other PP1-interacting proteins has indicated that phosphorylation within and next to the RVxF docking motif can inhibit interaction with the PP1 catalytic subunit ([Beullens et al. 1999](#), [McAvoy et al. 1999](#), [Liu & Brautigan 2000](#), [Bollen 2001](#), [Grallert et al. 2013](#)). To test whether this was the case for TSKS, we produced a GST-fusion protein in which the serine residue of the RVxF docking motif (KAVSF) was mutated to glutamate (KAVEF) to mimic serine phosphorylation. When this phospho-mimic fusion protein (GST-KAVEF) was incubated with mouse testis lysates, no pull down of PPP1CC2 was observed, although there was no reduction in binding to TSSK2, indicating preservation of protein structure ([Fig. 2](#)). This experiment indicates that the phosphorylation of the RVxF motif in TSKS probably inhibits interaction with PPP1CC2 in the testis.

Interaction between PPP1CC2 and TSSK1 is mediated by TSKS

In vitro experiments have previously demonstrated direct interactions between TSKS and PP1 fragments, as well as between TSKS and TSSK1 ([Kueng et al. 1997](#), [Hendrickx et al. 2009](#)). While sedimentation assays indicated a reciprocal interaction between PPP1CC2 and TSSK1 in testis lysates, we sought to determine whether there was also a direct interaction between PPP1CC2 and TSSK1 *in vitro*. GST-PPP1CC2 is able to precipitate a significant amount of His-TSKS *in vitro* in the absence of cell lysates ([Fig. 4](#)), but only a trace amount of His-TSSK1. To test whether TSKS can mediate the interaction between PPP1CC2 and TSSK1, we incubated GST-PPP1CC2 coupled to glutathione agarose with His-TSSK1 and His-TSKS. As shown in [Fig. 4](#), in the presence of His-TSKS, GST-PPP1CC2 precipitated a significant amount of His-TSSK1, considerably more than when His-TSKS is not present. These experiments conclusively demonstrate that TSKS mediates the interaction between PPP1CC2 and TSSK1 and that all the three proteins can simultaneously interact.

TSKS and TSSK1 localization is impaired in Ppp1cc mutant seminiferous tubules

In the seminiferous epithelium, both TSSK1 and TSKS are expressed in the cytoplasm of elongating spermatids, with prominent accumulation of both at distinct cytoplasmic foci ([Shang et al. 2010](#)). Such stage-specific distribution is commonly observed in the testis as different seminiferous tubule cross sections contain different complements of spermatogenic cells. In response to the targeted deletion of *Tssk1* and *Tssk2*, the level of TSKS expression remains similar in the

Figure 4 The interaction between PPP1CC2 and TSSK1 is mediated by TSKS. *In vitro* pull-down assays were carried out, wherein bacterially expressed GST and GST-PPP1CC2 bound to glutathione Sepharose were incubated in the presence (+) or absence (-) of purified His-TSSK1 and/or His-TSKS followed by sedimentation, SDS–PAGE and western blotting with polyclonal antibodies directed against TSSK1 or TSKS. In the presence of His-TSKS, GST-PPP1CC2 can precipitate a significant amount of His-TSSK1, while only trace amounts of TSSK1 are detected in the absence of His-TSKS. In the bottom panel (anti-TSKS western blot), the upper band is an artefact produced by anti-TSKS in the presence of certain bacterially expressed constructs. The band representing His-TSKS is indicated with an arrow.

cytoplasm of elongating spermatids, but its accumulation in distinct foci is lost (Shang *et al.* 2010), suggesting that regulation by TSSK1 (and/or TSSK2) is required for this punctate expression pattern. To test whether PPP1CC2 also plays a role in the regulation of TSKS localization, as well as TSSK1 localization, we carried out an immunohistochemical analysis on testis sections obtained from WT and *Ppp1cc* knockout mice. Mouse spermatogenesis can be divided into 12 different stages that arise in a cyclical fashion in the seminiferous tubules (Russell *et al.* 1990). It should be noted that the loss of *Ppp1cc* results in a bottleneck at stages VII/VIII of spermatogenesis, as well as a prominent loss of spermatids (Forgione *et al.* 2010). In WT seminiferous tubules, the previously described punctate expression pattern was observed for TSKS (Fig. 5A, B, C and D). This punctate expression pattern is stage specific, as the level of accumulation into distinct foci varies between seminiferous tubule cross sections. Strong TSKS expression throughout the cytoplasm begins at stage IX (Fig. 5D), with the emergence of foci during cytoplasmic staining by stage I (Fig. 5A). By stage IV, only distinct cytoplasmic foci are evident (Fig. 5B), which are no longer visible by stage VII (Fig. 5C). In *Ppp1cc* mutant seminiferous tubules, all these localization patterns can be found; however, there are statistically significant differences in their frequencies (Fig. 5E, F, G and H; Table 2). To demonstrate this, we classified 100 seminiferous tubules into one of four TSKS/TSSK1 staining patterns: absence of staining, cytoplasmic staining, cytoplasmic staining with visible puncta or punctate staining with limited cytoplasmic staining. In our analysis, 39% of the WT tubules exhibited punctate

staining with limited general cytoplasmic staining (Fig. 5B) and 24% of the WT tubules exhibited an absence of staining ($n=100$; Fig. 5C). By contrast, only 9% of *Ppp1cc* mutant tubule cross sections displayed the punctate staining pattern (Fig. 5F) and 51% displayed an absence of TSKS signal, which represent statistically significant differences from WT tubules ($P<0.001$; Fig. 5G). Therefore, we conclude that in *Ppp1cc* mutant seminiferous tubules the ability of TSKS to form its characteristic punctate staining pattern is significantly impaired. In addition to the quantitative differences, there were several qualitative changes in TSKS localization commonly observed in *Ppp1cc* mutant tubules. These defects included isolated elongating spermatids showing strong staining throughout the cytoplasm in tubules that otherwise exhibited a punctate expression pattern (Fig. 5F, inset), as well as tubules showing a large number of cytoplasmic foci instead of diffuse staining seen in WT counterparts (Fig. 5A and E, inset).

As has been reported previously, the expression pattern of TSSK1 in the WT testis is very similar to that of TSKS (Shang *et al.* 2010; Fig. 6A, B, C and D). We observed strong cytoplasmic staining beginning at stage VIII (step 8 spermatids; Fig. 6C), one stage before the onset of TSKS expression. Foci were evident during cytoplasmic staining by stage X (Fig. 6D), with only cytoplasmic foci being visible by stage II (Fig. 6A). TSSK1 staining was not visible in WT spermatids at stage VII (Fig. 6B). Again, similar to TSKS, we observed all of these staining patterns in *Ppp1cc* mutant seminiferous tubules (Fig. 6E, F, G and H; Table 2), but frequencies differed, with the fully developed punctate staining pattern being observed in only 11% of the mutant tubules (44% in WT

Figure 5 TSKS localization is impaired but not abolished in *Ppp1cc* mutant seminiferous tubules. Immunohistochemical analysis was carried out on WT (A, B, C and D) and *Ppp1cc* mutant (E, F, G and H) testes using a polyclonal antibody for TSKS (red) and nuclear staining using DAPI (blue). Representative seminiferous tubules at four different approximate time points of the spermatogenic cycle are shown. For WT tubules, approximate stages depicted are as follows: (A) stage I, (B) stage IV, (C) stage VII and (D) stage IX. Degeneration of seminiferous tubules in *Ppp1cc* mutant tubules makes staging challenging, but the depicted mutant tubules were matched as closely as possible to their WT counterparts. Numbers in the bottom right corner of each panel indicate the percentage of tubules that exhibit the depicted staining pattern. Asterisks indicate values significantly different from those of the WT counterparts ($P<0.05$). Arrow indicates paired TSKS puncta in WT spermatids. Scale bar represents 20 μm ; all the panels are equal in scale.

Table 2 Quantitative evaluation of TSKS and TSSK1 staining patterns in WT and *Ppp1cc* mutant seminiferous tubules.

Genotype	Staining pattern			
	Absence of staining	Cytoplasmic	Cytoplasmic + puncta	Puncta
TSKS				
WT	24	21	16	39
<i>Ppp1cc</i> KO	51*	18	22	9*
TSSK1				
WT	14	26	16	44
<i>Ppp1cc</i> KO	41*	15	33*	11*

*Indicates values significantly different ($P\leq0.05$) from those of the WT counterparts.

tubules, $P<0.001$; Fig. 6A and E) and 41% of the tubules lacked TSSK1 signal (14% in WT tubules, $P<0.001$; Fig. 6B and F). There was also a statistically significant increase in the proportion of tubules displaying TSSK1 foci with accompanying signal throughout the cytoplasm, with this pattern being visible in 33% of the *Ppp1cc* mutant tubules compared with 16% of the WT tubules ($P<0.01$; Fig. 6D and H). Qualitative abnormalities in TSSK1 staining patterns similar to those described for TSKS were also observed in *Ppp1cc* mutant seminiferous tubules. Clouds of cytoplasmic staining were evident alongside developed foci (Fig. 6E, inset), and aggregates of numerous foci (Fig. 6H, inset) as opposed to pairs of foci were observed in WT tubules (Fig. 6D, inset). The results of this experiment indicate that both TSKS and TSSK1 are able to achieve correct localization in the absence of PPP1CC isoforms; however, they do so at a lower frequency and with

several observable defects. As has been mentioned above, anti-PPP1CC staining in the testis is very strong and found throughout the cytoplasm (Hrabchak *et al.* 2007) to the extent that the examination of PPP1CC2 localization with TSSK1 and TSKS would not be informative.

***Ppp1cc* and *Tssk1/2* knockout spermatids display similar defects in mitochondrial sheath morphology**

Defective mitochondrial sheath morphology has been observed previously in both the *Ppp1cc* and *Tssk1/2* knockout mouse models (Chakrabarti *et al.* 2007, Shang *et al.* 2010). Comparative analysis of these structures via EM revealed key similarities between the two mutants. It is important to note that only a small number of spermatids reach this stage of development in *Ppp1cc* mutant seminiferous tubules. In WT spermatid mid-pieces (Fig. 7A), the mitochondria (arrows) effectively migrate to the axoneme and form a compact mitochondrial sheath, while in *Ppp1cc* mutants, this process is impaired and mitochondria are often observed to cluster apart from the axoneme (Fig. 7B, arrowhead). Furthermore, when mitochondria do successfully migrate to the axoneme, the mitochondrial sheath is disorganized and not tightly associated with the axoneme (Fig. 7C). This loose arrangement of the mitochondria in *Ppp1cc* mutant spermatids bears a close similarity to what is observed in *Tssk1/2* mutants (Fig. 7D; see also Fig. 5 in Shang *et al.* (2010)). These data strongly suggest that the PPP1CC2–TSKS–TSSK1 complex plays a role in mitochondrial sheath morphogenesis.

Figure 6 TSSK1 localization is impaired but not abolished in *Ppp1cc* mutant seminiferous tubules. Immunohistochemical analysis was carried out on WT (A, B, C and D) and *Ppp1cc* mutant (E, F, G and H) testes using a polyclonal antibody for TSSK1 (red) and nuclear staining using DAPI (blue). Representative seminiferous tubules at four different approximate time points of the spermatogenic cycle are shown. For WT tubules, approximate stages depicted are as follows: (A) stage II, (B) stage VII, (C) stage VIII and (D) stage X. Degeneration of seminiferous tubules in *Ppp1cc* mutant tubules makes staging challenging, but the depicted mutant tubules were matched as closely as possible to their WT counterparts. Numbers in the bottom right corner of each panel indicate the percentage of tubules that exhibit the depicted staining pattern. Asterisks indicate values significantly different from those of the WT counterparts ($P<0.05$). Arrow indicates paired TSSK1 puncta in WT spermatids. Scale bar represents 20 μ m; all the panels are equal in scale.

Figure 7 *Ppp1cc* and *Tssk1/2* mutants display similar abnormalities in mitochondrial sheath morphology. (A) WT spermatids show organized mitochondrial sheaths (arrows) closely and uniformly associated with the axoneme. (B and C) The few remaining *Ppp1cc* mutant spermatids display abnormal mitochondrial sheaths with defects in the migration of the mitochondria (arrows) to the axoneme (B, arrowhead) and loosely packed sheaths (C). (D) *Tssk1/2* mutant spermatids have mitochondrial sheaths that also display loosely associated mitochondria.

Discussion

The necessity of the protein phosphatase gene *Ppp1cc* for the completion of spermatogenesis in mice is well established (Varmuza *et al.* 1999). However, the precise role in this process, particularly that of the testis-specific isoform PPP1CC2, remains unknown. The multitude of defects within the *Ppp1cc* mutant seminiferous epithelium suggests the possibility of pleiotropic functions, with defects in both meiotic and post-meiotic germ cells being evident (Varmuza *et al.* 1999, Forgione *et al.* 2010). In an effort to learn more about the function(s) of PPP1CC2 in spermatogenesis, we carried out GST pull-down assays to identify PPP1CC2-interacting proteins in the testis. It should also be noted that the results reported herein arise from the LC-MS/MS analysis of a single gel slice from GST-PPP1CC2 pull downs in the testis and are by no means an exhaustive survey of PPP1CC2 interactors in the testis. Analysis of additional bands can reasonably be expected to identify more PPP1CC2-interacting proteins in the testis, some of which may play a role in spermatogenesis. Amongst the identified proteins was the testis-specific kinase TSSK1, which is also required for male fertility, although with a phenotype quite different from that of the *Ppp1cc* deletion (Xu *et al.* 2008, Shang *et al.* 2010). While the

loss of *Ppp1cc* results in a severe impairment in spermatogenesis including the widespread loss of post-meiotic germ cells and a bottleneck in the spermatogenic cycle (Varmuza *et al.* 1999, Forgione *et al.* 2010), the loss of the closely linked *Tssk1* and *Tssk2* genes results in a severely reduced number of epididymal spermatozoa but no major loss of germ cells during spermatogenesis (Shang *et al.* 2010). This difference in phenotypic severity is consistent with a requirement for *Ppp1cc* earlier in spermatogenesis than *Tssk1/2*, which is reflected by the expression patterns of these genes in the developing spermatogenic cells. *Ppp1cc2* is expressed throughout spermatogenesis and at a high level from meiotic pachytene spermatocyte stage onwards (Hrabchak & Varmuza 2004). Conversely, *Tssk1* and *Tssk2* are only expressed in post-meiotic spermatids (Li *et al.* 2011). Despite these differences, the time point of peak PPP1CC2 protein levels in the testis overlaps with that of TSSK1/2, and there are several key similarities between the knockout phenotypes. Both mutants exhibit a significant reduction in the number and motility of epididymal spermatozoa as well as prominent defects in the organization of the mitochondrial sheaths (Varmuza *et al.* 1999, Chakrabarti *et al.* 2007, Soler *et al.* 2009, Shang *et al.* 2010). *Ppp1cc2* knock-in mice with low levels of transgene expression (>50% of heterozygous levels) are able to rescue the loss of post-meiotic germ cells found in *Ppp1cc* knockouts, but are still infertile and mitochondrial sheath abnormalities are still visible (Soler *et al.* 2009, Sinha *et al.* 2012). Taken together, all this evidence points to a functional link between PPP1CC2 and TSSK1 late in spermiogenesis, after the time point where PPP1CC2 is first required, and suggests that PPP1CC2 plays a role in multiple events in spermatogenesis, not unexpected for a member of the PP1 family of protein phosphatases.

While sedimentation assays revealed reciprocal interactions between PPP1CC2 and TSSK1 in testis lysates, *in vitro* binding was unsuccessful, suggesting indirect interactions *in vivo*. One potential link between PPP1CC2 and TSSK1 is the common interactor, TSKS, which we demonstrated to interact with both PPP1CC2 and TSSK1 *in vivo*. Further *in vitro* binding experiments demonstrated that the interaction between PPP1CC2 and TSSK1 is mediated by TSKS. This multiprotein complex thus contains both a protein kinase and a protein phosphatase, and all three proteins are known to be phosphorylated in the testis (Kueng *et al.* 1997, Huang & Vijayaraghavan 2004, Jaleel *et al.* 2005). We have shown conclusively that interaction with PPP1CC2 is dependent on the presence of a PP1 docking RVxF motif in the N-terminal region of TSKS (amino acids 51–55). Previous studies have demonstrated that the N-terminal region of TSKS is also required for interaction with TSSK proteins (Xu *et al.* 2008), indicating that both proteins are probably in close contact while bound to their common interactor. Future studies are needed to determine

enzyme–substrate relationships between any of these proteins aside from the known phosphorylation of TSKS by TSSK1.

The kinase activity of TSSK1 towards TSKS has been demonstrated previously, and it is thought to occur on the serine 281 residue (Kueng *et al.* 1997, Xu *et al.* 2008). During the course of this study, we identified a second phosphorylated residue, serine 54, which interestingly lies within the PP1 docking RVxF motif. Previous studies have shown phosphorylation in and around RVxF motifs to inhibit interaction with PP1 isoforms (Beullens *et al.* 1999, McAvoy *et al.* 1999, Liu & Brautigan 2000, Bollen 2001), which our experiments confirmed for TSKS. These data suggest that within the testis, there exists a pool of TSKS that is incapable of interacting with PPP1CC2 (phosphorylated S54), while another portion is permissive to interaction (unphosphorylated S54), demonstrated by the successful sedimentation assays that we carried out. Precisely how this is regulated remains an open question. PPP1CC2 itself is unlikely to dephosphorylate this residue, as the RVxF binding surface is distant from the active site on the surface of the phosphatase (Egloff *et al.* 1997). Moreover, the phosphorylation of the RVxF motif inhibits binding of PPP1CC2. This indicates the involvement of another protein phosphatase in the regulation of this interaction, the identity of which remains unknown, although a number of other non-type 1 protein phosphatases are known to be expressed in the testis (Fardilha *et al.* 2011). Similarly, the kinase responsible for this phosphorylation remains in question. The kinase prediction tools Scansite (Obenauer *et al.* 2003) and KinasePhos (Huang *et al.* 2005) suggest PKC kinases to be the top candidate based on the sequence surrounding the phosphorylation site; however, this would require further testing before any definitive conclusions are drawn.

Our immunohistochemical analysis of TSKS and TSSK1 expression in the *Ppp1cc* knockout testis indicated reduced localization of both proteins to distinct puncta as well as a number of qualitative defects. The puncta correspond to previously described structures proposed to originate in the chromatoid body: a ring-shaped structure and a satellite (Shang *et al.* 2010). These abnormalities are consistent with the phenotype of the *Ppp1cc* knockouts as the TSKS/TSSK1-expressing elongating spermatids are frequently missing in mutants, resulting in tubules lacking any observable staining for these proteins. The loss of *Ppp1cc* also causes a bottleneck at stages VII/VIII of the spermatogenic cycle and a disorganization of the seminiferous epithelium resulting in tubules displaying a seeming mixture of stages (Forgione *et al.* 2010). Taken together, these findings suggest that the abnormalities in TSKS/TSSK1 staining are secondary to the initial effects of the *Ppp1cc* mutation early in spermatogenesis, but place these proteins upstream of PPP1CC2 in a biochemical pathway later in spermatogenesis. A previous analysis of TSKS expression in *Tssk1/Tssk2* mutant tubules has shown

that TSSK1 or TSSK2 or both are required for the formation of the chromatoid body-derived ring and satellite structures as well as the correct localization of TSKS to these structures during spermiogenesis (Shang *et al.* 2010). Also, recent analysis of the evolutionary history of the *Tssk1* and *Tssk2* genes in rodents and primates suggests that the two genes may have not completely overlapping functions (Shang *et al.* 2013). One can thus envision a scenario where TSSK1 binds to and phosphorylates TSKS resulting in the localization of TSKS (and TSSK1) to specific puncta in the developing elongating spermatids, whereupon it binds to PPP1CC2, forming, at least transiently, a trimeric (or higher oligomer) complex. The biological consequences of this interaction are yet to be uncovered, but a critical role in spermiogenesis seems likely. TSSK1 and TSKS play a role in the formation and/or function of chromatoid body-derived structures in elongating spermatids (Shang *et al.* 2010). The chromatoid body is a centre of RNA processing in developing round spermatids, but this may not be the only role for this structure (Meikar *et al.* 2011). The role of the structures derived from the chromatoid body in further developed elongating spermatids is less clearly defined, but it has been hypothesized that they may have a role in the assembly of the mitochondrial sheath (Shang *et al.* 2010). The fact that the mitochondrial sheath is also abnormal in *Ppp1cc* mutant spermatids leaves open the possibility that PPP1CC2 is important for post-translational regulation of proteins during the development of this structure. Furthermore, as our analysis demonstrates, there is a clear similarity in mitochondrial defects between the *Ppp1cc* and *Tssk1/2* knockout models, strengthening the hypothesis of a functional link to mitochondrial sheath morphogenesis. Currently, there is no published account of a *Tsk*s knockout mouse, so the impact of the loss of this gene on the mitochondrial sheath is unknown.

In conclusion, the results of this study indicate an interaction between the testis-specific Ser/Thr phosphatase PPP1CC2 and two additional testis-specific proteins, the kinase substrate TSKS and the kinase TSSK1. Furthermore, the results indicate that the interaction between TSSK1 and PPP1CC2 is indirect and mediated by TSKS, which binds to PPP1CC2 via the classical PP1 docking RVxF motif. The RVxF motif on the TSKS surface can be phosphorylated in the testis, which is inhibitory to PPP1CC2 interaction.

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/REP-13-0224>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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