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Genetic expansion of Chaperonin-containing TCP-1 (CCT/TRiC) complex subunits yields testis-specific isoforms required for spermatogenesis in planarian flatworms

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Abstract

Chaperonin-containing Tail-less complex polypeptide 1 (CCT) is a highly conserved, hetero-oligomeric complex that ensures proper folding of actin, tubulin, and regulators of mitosis. Eight subunits (CCT1-8) make up this complex, and every subunit has a homolog expressed in the testes and somatic tissue of the planarian flatworm *Schmidtea mediterranea*. Gene duplications of four subunits in the genomes of *S. mediterranea* and other planarian flatworms created paralogs to CCT1, CCT3, CCT4, and CCT8 that are expressed exclusively in the testes. Functional analyses revealed that each CCT subunit expressed in the *S. mediterranea* soma is essential for homeostatic integrity and survival, whereas sperm elongation defects were observed upon knockdown of each individual testis-specific paralog (*Smed-cct1B*; *Smed-cct3B*; *Smed-cct4A*; and *Smed-cct8B*), regardless of potential redundancy with paralogs expressed in both testes and soma (*Smed-cct1A*; *Smed-cct3A*; *Smed-cct4B*; and *Smed-cct8A*). Yet, no detriment was observed in the number of adult somatic stem cells (neoblasts) that maintain differentiated tissue in planarians. Thus, expression of all eight CCT subunits is required to execute the essential functions of the CCT complex. Furthermore, expression of the somatic paralogs in planarian testes is not sufficient to complete spermatogenesis when testis-specific paralogs are knocked down, suggesting that the evolution of chaperonin subunits may drive changes in the development of sperm structure and that correct CCT subunit stoichiometry is crucial for spermiogenesis.

Keywords

Chaperonin-containing TCP-1 (CCT) complex; TCP-1 ring complex (TRiC); Spermatogenesis; Spermiogenesis; Platyhelminthes

1 INTRODUCTION

Cytoskeletal polymers provide the cellular structure for shape, motility, and intracellular transport (Fletcher and Mullins, 2010; Pollard, 2003). One example of extreme cytoskeletal

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CONFLICT OF INTEREST STATEMENT

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rearrangement is observed during sperm development, during which changes in cell shape and size ensure the delivery of genetic material to the egg (Breitbart et al., 2005; Dvorakova et al., 2005; Toshimori and Ito, 2003). Actin is a fundamental component of the sperm acrosome, and microtubules assemble into flagella that make male gametes motile (Fouquet and Kann, 1994). Germ line-specific paralogs of both actin and tubulin exhibit intrinsically different characteristics from the ubiquitous versions that are essential for general cell viability (Fackenthal et al., 1993; Hoyle and Raff, 1990; Tanaka et al., 2003) – indeed, these specialized isoforms possess different primary sequences and receive distinct post-translational chemical modifications via testis-specific cofactors during sperm development (Breitbart et al., 2005; Fouquet et al., 1994; Kierszenbaum, 2002).

Folding of primary actin and tubulin polypeptides in eukaryotes is assisted by a highly conserved, multi-protein chaperone known as Tail-less complex polypeptide 1 (TCP-1) ring complex (TRiC), which is also called Chaperonin-containing TCP-1 (CCT) complex (Dunn et al., 2001; Lopez et al., 2015; Valpuesta et al., 2002). Each functional complex consists of two rings (Leitner et al., 2012; Yebenes et al., 2011). *TCP-1* (also known as *CCT1*), the founding member the CCT complex, was identified in *t* chromosome-mutant mice because of its abundant expression during spermatogenesis (Silver, 1981; Silver et al., 1979; Silver et al., 1987; Willison et al., 1986). CCT-1 is one of eight subunits that make up a single CCT-complex ring (Leitner et al., 2012; Yebenes et al., 2011). The other subunits in the complex (CCT2-8) share moderate sequence identity with each other (~30%), but high sequence identity between orthologs across species (~60% identity between yeast and mammals) (Kubota et al., 1994; Kubota et al., 1995). The sequence diversity among each subunit is thought to provide specificity for different substrates through interactions with a range of domains. CCT is estimated to participate in folding up to 15% of the human proteome (Thulasiraman et al., 1999; Yam et al., 2008), including cell-cycle regulators and polyglutamate repeat proteins (Boudiaf-Benmammar et al., 2013; Camasses et al., 2003; Kaisari et al., 2017; Nadler-Holly et al., 2012; Tam et al., 2006) – although actin and tubulin appear to be the major substrates of this complex, according to studies in yeast (Chen et al., 1994; Ursic et al., 1994; Vinh and Drubin, 1994), nematodes (Lundin et al., 2008), and mammalian experimental systems (Gao et al., 1992; Gao et al., 1993; Sternlicht et al., 1993; Yaffe et al., 1992).

Current efforts in the study of chaperonin function aim to identify the extent of CCT complex substrates in vivo and during specific cellular processes, as well as possible functions of individual subunits in the complex. Individual CCT complex subunits are hypothesized to bring specific attributes to the complex, as well as to perform activities apart from the chaperonin. For example, mutation of equivalent positions in individual CCT subunits in yeast result in different phenotypes (Amit et al., 2010). In human cell lines, studies of protein localization and function specifically implicate CCT5 as a regulator of cell shape (Brackley and Grantham, 2010). Mouse and human genomes contain two *CCT6* paralogs, one that is expressed ubiquitously (*CCT6A*) and one specifically expressed in testes (*CCT6B*) (Kubota et al., 1997). The restricted expression of the *CCT6B* paralog suggests that it may provide a particular function in spermatogenesis that is missing in the ubiquitous *CCT6A* paralog, but functional evidence for this hypothesis has not come to light.

Planarian flatworms (Platyhelminthes) hold a unique position in metazoan evolution compared to ecdysozoans (nematodes, flies) or deuterostomes (mice, rats). Planarian hatchlings lack a reproductive system at birth; instead, this system develops post-embryonically through differentiation of pluripotent somatic stem cells called neoblasts (Newmark et al., 2008). Neoblasts are the only mitotic cells in the planarian soma, and serve as the cellular source for growth, homeostatic integrity, as well as regeneration (Newmark and Sanchez Alvarado, 2002; Shibata et al., 2010). The development of planarian germ-line and somatic reproductive tissue after birth, as well as following experimental post-amputation, has facilitated the analysis of gene function during progression of gametogenesis: from pluripotent stem cell to germ-line stem cell, and through oogenesis or spermatogenesis (Chong et al., 2011; Hoshi et al., 2003; Ishizuka et al., 2007; Kobayashi et al., 2017; Rouhana et al., 2017; Wang et al., 2010; Wang et al., 2007; Zayas et al., 2005).

In this study, we characterized a dozen CCT complex subunit homologs encoded in the genome of the planarian *Schmidtea mediterranea*. Four subunits (CCT2, CCT5, CCT6, and CCT7) are represented by single orthologs expressed in the soma and testes, whose function is essential for survival. The other four subunits (CCT1, CCT3, CCT4, and CCT8) are each encoded by duplicated genes, which include an essential paralog and a paralog with testis-specific functions. Sperm-elongation defects were observed upon RNA-interference (RNAi)-mediated knockdown of any testis-specific CCT subunit, suggesting that spermatogenesis requires specialized functions of testis-specific subunits or robust CCT complex stoichiometry.

2 RESULTS

2.1 Characterization of CCT complex subunits in *S. mediterranea* reveals gene duplications with testis-specific expression for four subunits

Homologs of each human CCT complex subunit (Figure 1a–b) were identified among sexual *S. mediterranea* transcriptome sequences (Rouhana et al., 2012) using the protein-to-nucleotide basic local alignment search tool (TBLASTN) (Figure 1c; Table S1). These planarian CCT homologs were then verified in the *S. mediterranea* genome (Robb et al., 2015) and PlanMine (Brandl et al., 2016) databases (Table S1). Planarian CCT complex subunits shared 48% to 72% protein sequence identity with human orthologs, and 21% to 37% identity with other subunits of the planarian CCT complex (Figure 1d; Table S1). Single orthologs were identified for CCT complex subunits CCT2, CCT5, CCT6, and CCT7, whereas subunits CCT1, CCT3, CCT4, and CCT8 were each represented by two potential orthologs (Figure 1c–d; Table S1).

CCT subunits represented by an individual gene in *S. mediterranea* were named after their closest human ortholog (i.e., *Smed-cct2*, *Smed-cct5*, *Smed-cct6*, and *Smed-cct7*; addressed to as *cct2*, *cct5*, *cct6*, and *cct7* for brevity). Subunits represented by two paralogs in *S. mediterranea* were designated with the suffix “A” (based on higher protein sequence identity) or “B” (i.e., *Smed-cct1A* and *Smed-cct1B*, *Smed-cct3A* and *Smed-cct3B*, *Smed-cct4A* and *Smed-cct4B*, *Smed-cct8A* and *Smed-cct8B*; addressed as *cct1A* and *cct1B*, *cct3A* and *cct3B*, *cct4A* and *cct4B*, *cct8A*, and *cct8B* for brevity) (Figure 1d; Table S1). Both *cct1B* and *cct4A* (NCBI Accession KY847531 and KY847532, respectively) were recently

characterized as genes required for spermatogenesis in *S. mediterranea* (Rouhana et al., 2017), and were included for further analysis in this study for sake of comparison and completeness.

Nucleotide conservation between duplications of genes encoding CCT subunits is not commonly reported, with the exception of mouse and human CCT6 (Kubota et al., 1997), so we looked for evidence of gene duplications in other flatworm species (Tables S2–S6). Five CCT subunits (CCT 1, CCT3, CCT4, CCT5 and CCT8) were encoded by duplicated genes from the planarian flatworm *Polycelis nigra*, according to sequences deposited in PlanMine (Table S2), while duplicated genes for CCT1, CCT3, CCT4, and CCT8 were identified in the planarian *Dendrocoelum lacteum* (Table S3). We also searched for evidence of CCT subunit gene duplications in the parasitic flatworms *Schistosoma mansoni* (Trematoda) and *Echinococcus multilocularis* (Neodermata), using resources available for helminth genomic research through WormBase ParaSite (Howe et al., 2016; Howe et al., 2017). Interestingly, we were only able to identify a single ortholog for each CCT subunit in the genomes of *S. mansoni* (Table S4) and *E. multilocularis* (Table S5), suggesting that genetic expansion of CCT subunits is not present in the clade of parasitic flatworms. Genetic expansion of CCT subunits was also not evident in free-living flatworms that are not planarians, based on our search for CCT subunit homologs in sequences of *Macrostomum lignano* (Table S6), suggesting that genetic expansion of CCT subunits may be ancestral in planarians, but not in flatworms in general.

Whole-mount in situ hybridization was performed using adult individuals from a sexual laboratory line of *S. mediterranea* to assess the expression of CCT subunit homologs (Figure 2a–l; Figure S2). Transcription of the gene encoding each primary CCT complex subunit (*cct1A*, *cct2*, *cct3A*, *cct4B*, *cct5*, *cct6*, *cct7*, and *cct8A*) was detected in somatic tissue throughout the anterior-posterior axis, often enriched in the head region (Figure 2a–h). Expression of the paralogs was also detected in the testes, which are distributed dorsolaterally throughout the body, but not in the head region (Figure 2a–h; insets). The somatic expression of CCT complex subunits was further evaluated in a subset of *cct* genes in asexual planarians, which are smaller and more amenable to riboprobe penetration through the fixed tissue than samples from sexual planarians. For example, expression of *cct3B*, which was only detected in the testes in sexual planarians (Figure 2j), was undetectable in asexual planarians (Figure S3a). Conversely, expression of *cct5* was detected throughout the asexual samples (Figure S3b), particularly in subsets of cells in the brain, intestine, pharynx, and under the muscular matrix (Figure S3d–g). Expression of the neoblast marker *Smedwi-1*, which was used as a positive control for specificity and analysis of internal regions in our samples, was detected in cells with the expected distribution for mesenchymal stem cells (Reddien et al., 2005).

Together, the phylogeny and in situ hybridization results demonstrate that an ortholog of every CCT complex subunit is expressed in planarian somatic tissues. Furthermore, paralogous genes encoding subunits CCT1, CCT3, CCT4, and CCT8 are expressed in testes.

2.2 Expression of every CCT complex subunit is essential to homeostatic integrity in *S. mediterranea*

Some *S. mediterranea* CCT homologs (*cct1B* and *cct4A*) were previously shown to be essential for sperm elongation (Rouhana et al., 2017), but the function of CCT subunit homologs in somatic development and homeostasis remained unknown. We hypothesized that expression of *cct3B* and *cct8B*, which were only detected in the testes, are not required for growth or survival, whereas their paralogs expressed in the soma are essential to planarian homeostatic integrity. We tested this by individually disrupting the expression of each CCT subunit homolog in *S. mediterranea* using RNAi. Groups of clonal individuals from a laboratory line of sexual *S. mediterranea* (Zayas et al., 2005) were subjected to an RNAi regimen of double-stranded RNA (dsRNA) feedings twice per week (Figure 3a), using a protocol shown to robustly disrupt expression of targeted gene sequences throughout the planarian anatomy (Rouhana et al., 2013).

Given that *cct* paralogs share nucleotide sequence identity ranging from 71% to 65% across most of their exon sequences (Table S1), including regions of sequence targeted by dsRNA (Figure S1), we assessed the possibility of off-target knockdown in the closest paralogous pair (*cct1A* and *cct1B*). Total RNA was extracted from individual planarians one week after *cct1A* dsRNA or *cct1B* dsRNA feedings, and transcript levels were measured for both paralogs by quantitative reverse-transcription PCR using two primer sets for each gene (Figure S4). A reduction of approximately 50% transcript abundance was observed for *cct1B(RNAi)* in *cct1A(RNAi)* planarians, whereas the intended reduction of *cct1A* transcript levels was over 95% (Figure S4a–b). A similar outcome was observed in planarians subjected to *cct1B* dsRNA, in which levels of *cct1B* transcripts were reduced by more than 95%. Conversely, *cct1A* transcript abundance was approximately half of that in *control(RNAi)* animals (Figure S4c–d). Regardless of indirect or “off-target” reduction in somatic *cct* paralog transcript abundance, the anatomy of planarians subjected to RNAi targeting any of the four testis-specific *cct* subunit paralogs was indistinguishable from that of *control(RNAi)* (Figure 3b and 3c; data not shown). On the other hand, planarians fed dsRNAs targeting CCT subunits expressed in the planarian soma began to display an array of physical defects two weeks into RNAi treatment (Figures 3d–h; data not shown). Noticeable abnormalities ranged from irregular crumpling (Figure 3d), curling (Figure 3e), head regression (Figure 3f), and lysis (Figure 3g–h). The vast majority of somatic-subunit knockdowns ultimately died within the experimental period, whereas testis-specific-subunit dsRNA-fed animals survived throughout the experimental period (Figure 3i). Considering the robust knockdown of targeted genes (>95%) and lack of observable phenotypes in the soma of planarians subjected to dsRNA targeting testis-specific *cct* subunits, the essential CCT complex in *S. mediterranea* is likely composed of *cct1A*, *cct2*, *cct3A*, *cct4B*, *cct5*, *cct6*, *cct7*, and *cct8A* gene products (Figure 3j), whereas *cct1B*, *cct3B*, *cct4A*, and *cct8B* are not required for the homeostasis of somatic tissue.

Curling, head regression, and lysis are phenotypes observed upon loss of functional stem cells after irradiation or knockdown of genes essential to neoblast function (Guo et al., 2006; Reddien et al., 2005; Rouhana et al., 2010). We therefore assessed neoblast distribution to determine whether or not a decrease in stem cell abundance preceded the homeostatic

defects observed upon knockdown of somatic CCT subunits. Planarians subjected to two weeks of RNAi were processed during the third week, when advanced physical abnormalities were readily observable in a subset of animals, and analyzed by confocal microscopy following immunostaining for neoblasts using the Y12 monoclonal antibody (Lerner et al., 1981; Rouhana et al., 2012). No significant decrease in the quantity of neoblast populations was observed in planarians subjected RNAi targeting somatic CCT subunits (*cct1A*, *cct3A*, and *cct5*) as compared to controls or knockdown of testis-specific subunit *cct3B* (Figure 4a–c). Neoblasts were even detected in planarians that had undergone head regression during somatic subunit knockdown (data not shown), suggesting that loss of neoblasts is not the primary cause for the homeostatic defects observed upon knockdown of somatic CCT subunits.

2.3 Testis-specific CCT subunit paralogs are required for sperm elongation

Sexually mature planarian hermaphrodites commit a large portion of their anatomy to the development of testis lobes, which are located dorsolaterally from the neck to the tail and are composed mostly of germ cells at different stages of spermatogenesis. Sperm development can be visualized in individual testis lobes by 4',6-diamidino-2-phenylindole (DAPI) staining of DNA and confocal microscopy (Wang et al., 2010). When fully developed, each testis lobe contains spermatogonia along the outer layer of the lobe, cells in intermediate stages of spermatogenesis in the internal region of the lobe, and spermatozoa that accumulate in the center of the lobe (Figure 5a).

We sought to better understand the expression and function of CCT during spermatogenesis, as well as the relationship between paralogous subunits of this complex, by analyzing the expression of *cct* genes during spermatogenesis using fluorescence *in situ* hybridization; a sense riboprobe of *cct3A* sequence reveal the level of background signal during this analysis (Figure 5a). Transcripts for all 12 *cct* genes were detected in the outer layer of the testes lobes, where spermatogonia and primary spermatocytes reside (Figure 5b–m). Transcripts from a subset of CCT subunit genes – including *cct1A*, *cct2*, *cct5*, *cct6*, and *cct7* – were also detected in secondary spermatocytes and spermatids (Figure 5b, 5d, and 5i–k). Conversely, the abundance of transcripts from genes such as *cct4B* and *cct8A* decreased more rapidly as spermatogenesis progressed (Figure 5h and 5l). Given that gene expression for all 12 CCT subunits is detected in *S. mediterranea* spermatogonia as well as their progeny, functional CCT complexes are likely present throughout most of spermatogenesis – even in cells where corresponding transcripts are no longer detected.

Testes of planarians subjected to RNAi targeting individual subunit homologs by DAPI staining and confocal microscopy were observed to evaluate the functional requirement of CCT components during sperm development (Figure 6). The control group for normal spermatogenesis was subjected to feedings of dsRNA containing bacterial *ccdB* sequence, which does not alter planarian physiology (Collins et al., 2010) (Figure 6a–b); the positive-control group was presented with dsRNA targeting *nanos*, which is required for germ-line stem cell development and maintenance (Wang et al., 2007) (Figure 6c). Groups subjected to dsRNA targeting essential CCT subunits were fixed when somatic defects first appeared in a subset of animals since compromised somatic homeostasis could indirectly affect the

integrity of the testes. Groups subjected to dsRNA targeting testis-specific paralogs or *nanos* were processed after five weeks of treatment, in an attempt to attain the full effect of RNAi. Control groups subjected to *ccdB* dsRNA feedings were fixed at early and late time-points, both of which displayed normal testes and sperm development (Figure 6b).

As expected, testes were not detected in planarians subjected to *nanos* RNAi (Wang et al., 2007) (Figure 6c), demonstrating robust disruption of gene expression under the utilized experimental conditions. Consistent with our previous analysis (Figure 3), no lethality or defects in somatic homeostatic integrity were observed in knockdowns of testis-specific paralogs. With the exception of *cct1A* (Figure 6d) and *cct3A* (Figure 6g), however, knockdown of every CCT subunit homolog resulted in sperm defects (Figure 6e–o). Knockdown of any of the four testis-specific subunit paralogs (*cct1B*, *cct3B*, *cct4A*, and *cct8B*) resulted in obvious sperm-elongation defects (Figure 6e, 6h, 6i, and 6o). Knockdown of *cct4B* and *cct8A*, which are expressed in both testes and somatic tissue (Figure 2d and 2h), also compromised sperm development (Figure 6j and 6n). This suggests that the function *cct4B* and *cct8A* is not redundant with that of testis-specific paralogs, although one must also consider the possible effects of a partial reduction in *cct4A* and *cct8B* expression due to off-target RNAi. The quantity of elongated sperm present in planarians subjected to *cct1A* or *cct3A* RNAi (Figure 6d and 6g) suggested that contributions from testis-specific paralogs (*cct1B* and *cct3B*) are sufficient to complete spermatogenesis. Conversely, RNAi targeting subunits represented by single genes lead to loss of spermatozoa (*cct2*) (Figure 6f) and partial regression of testes (*cct5*, *cct6* and *cct7*) (Figure 6k–m). Together, these results indicate that expression of each testis-specific CCT subunit is required for spermatogenesis, thus confirming previous reports regarding *cct1B* and *cct4A* as well as providing functional characterization for two additional testis-specific CCT subunits (*cct3B* and *cct8B*). Normal expression of genes encoding other CCT subunits (*cct2*, *cct5*, *cct6*, and *cct7*) also seems to be required for proper sperm development, although one must consider the caveat that spermatogenesis in planarians could be affected indirectly by somatic defects observed during their knockdown.

3 DISCUSSION

3.1 Essential functions of somatic chaperonin in planarians

Group II chaperonins are conserved between archaea and eukaryotes (Lopez et al., 2015). The eukaryotic CCT complex is estimated to assist in folding as much as 15% of the proteome (Thulasiraman et al., 1999; Yam et al., 2008), with more than half of the active chaperones occupied by folding of actin and tubulin (Siegers et al., 2003), while the remainder include proteins that regulate cellular proliferation – such as the von Hippel-Lindau (VHL) tumor suppressor protein, CDC20, CDH1, and telomerase cofactor TCAB1 (Camasses et al., 2003; Feldman et al., 1999; Freund et al., 2014; Kaisari et al., 2017; McClellan et al., 2005; Melville et al., 2003). Given this diversity of target substrates, the lethal phenotype following a reduction in somatic CCT function in planarians is not surprising (Figure 3). The presence of comparable populations of neoblasts (the only mitotic cells in the planarian soma) between *control* and *cct* RNAi groups (Figure 4), however, suggests that this lethality may be due to compromised maintenance or differentiation of

specific cell types rather than loss of stem cells. Also of note is that similar phenotypic progression was observed after knockdown of each of the somatic CCT subunit-encoding genes, suggesting that: (i) basic CCT complex function, and not specialized roles of specific subunits, is essential for planarian homeostatic maintenance and survival and (ii) functional redundancy between different subunits is not sufficient to overcome loss of expression of any individual subunit in vivo.

3.2 Compromised CCT complex activity leads to sperm development failure: possible causes and implications

Microtubule assemblies are central to the molecular mechanics of sperm motility and structure, and slight changes to these structures may severely impact fertilization potential. We showed that sperm production in planarians is severely affected by reduced expression of any of four testis-specific paralogs of CCT subunits (*cct1B*, *cct3B*, *cct4A*, and *cct8B*) (Figure 6), suggesting that compromised activity of any CCT complex subunit could negatively impact fertility in this or other organisms. Biochemical studies of chaperonin activity showed that complexes made up of as few as one subunit are sufficient for substrate folding in vitro (Sergeeva et al., 2013); conversely, our results suggest that the contribution of all eight CCT complex subunits is crucial for proper sperm development, and their relative abundances may also influence spermatogenesis. One reason for this disconnect could be that specific CCT subunits bind different substrates, recruiting different sperm components for proper folding. Our preferred hypothesis, however, is that spermiogenesis is impaired due to incorrect folding of actin and tubulin, considering that the major abnormalities observed following individual *cct* gene knockdowns are related to sperm elongation and known association of the CCT complex with microtubular structures during spermiogenesis (Soues et al., 2003). Once transgenesis techniques become readily available for planarian research, testing whether over-expression of somatic subunits transcribed from promoters of testis-specific paralogs rescues *cct1B*, *cct3B*, *cct4A*, or *cct8B* RNAi phenotypes will be possible. This approach could establish whether testis-specific subunits have exclusive substrate affinity or bestow shared substrates with particular folding structures.

3.3 Testis-specific expansion of an essential chaperone in planarian flatworms

Budding yeast possess a single representative gene encoding each CCT subunit (Stoldt et al., 1996). Conversely, mouse and human genomes contain a gene duplication for the CCT6 subunit, with ubiquitous expression of one paralog versus testes-restricted expression of the other (Kubota et al., 1997). Sequence-alignment analyses revealed that further expansion of CCT subunit-encoding genes is shared in distant planarian species (Tables S2 and S3). Interestingly, analysis of sequences from parasitic flatworms *S. mansoni* (blood fluke) and *E. multilocularis* (tapeworm), as well as the free-living non-planarian flatworm *M. lignano*, identified only one ortholog for each CCT subunit (Tables S4–S6). Thus, the extensive expansion of chaperonin gene paralogs within the Platyhelminthes may be limited to the planarian clade. Indeed, multiple paralogs for CCT1, CCT3, CCT4, and CCT8 were identified in species from three different planarian genera, suggesting that expansion of these CCT subunits is ancestral in planarians. Such expansion could be related to the peculiarities of planarian reproductive and structural biology, which may have required evolutionary

changes in chaperonin function. For example, planarian spermatozoa contain two flagella that originate from the anterior end of elongated heads, and are composed of 9+1 axonemal microtubule arrangements in their flagella – which is different from the 9+2 structures generally observed in sperm of other animals (Henley et al., 1969; Silveira and Porter, 1964). Additionally, planarian cells completely lack centrosomes (Azimzadeh et al., 2012), which may require different microtubular structural components for nucleation.

3.4 Hypothesized origin for the selection of CCT gene duplication in planarians

Planarians can switch between sexual and asexual reproductive modes (reviewed by Newmark et al., 2008). Both modes of reproduction rely on a population of adult somatic stem cells (neoblasts) that give rise to all differentiated cells in the soma, as well as to germ-line stem cells that ultimately differentiate into gametes (Newmark and Sanchez Alvarado, 2002; Newmark et al., 2008; Wang et al., 2007). Progeny from a single neoblast are capable of replenishing cells for an entire organism (Wagner et al., 2011), which may allow mutations to accumulate in other neoblasts. Indeed, genetic analysis of individuals in a clonal, asexual line of *Dugesia japonica* revealed accumulation of an “unusually large number of mutations” (Nishimura et al., 2015). Our sequence analysis of CCT subunits revealed that the primary sequences of planarian paralogs are as different from each other as they are to their human orthologs (Figure 1d; Table S1), suggesting that selection against mutations in germ line-specific paralogs of essential genes (such as CCT subunit paralogs) is relaxed during periods of asexual reproduction in planarians, but become active when gametes develop from neoblasts during periods of sexual reproduction. Testis-specific isoforms of α - and β -tubulin have also been identified in planarians (Hase et al., 2007; Simoncelli et al., 2003; Wang et al., 2010), suggesting that the chaperone and its target(s) coevolved, resulting in the unique morphology planarian of sperm.

4 MATERIALS AND METHODS

4.1 Planarian culture

A hermaphroditic laboratory line of *S. mediterranea* (Zayas et al., 2005) was used for all experiments, with exception of the analyses presented in Figure S3, for which an asexual clonal strain was used. Clonal lines of sexual *S. mediterranea* were expanded by amputation, and maintained at 18°C in 0.75X Montjuïc salts, as per see Wang et al. (2007). Animals were fed calf liver puree, and starved for a week prior to experimentation.

4.2 Identification and cloning of *S. mediterranea* CCT subunit homologs

CCT complex subunit homologs were identified from a transcriptome assembly of *S. mediterranea* hermaphroditic laboratory strain cDNA (Rouhana et al., 2012) using human and *Saccharomyces cerevisiae* sequences as input for the Basic Local Alignment Search Tool (BLASTN) in CLC Genomics Workbench (QIAGEN, Hilden, Germany). Orthologs were confirmed via reciprocal searches against human protein sequences, using *S. mediterranea* homologs as input during BLASTX analyses at the National Center for Biotechnology Information (NCBI) website (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>).

Partial cDNA sequences of each CCT subunit homolog, except *cct1B*, were amplified from *S. mediterranea* sexual strain cDNA using the primers listed in Table 1. Amplicons were ligated into Eam1105I-digested pJC53.2 vector (Collins et al., 2010); recombinant construct content was verified by Sanger sequencing, and sequence information was deposited in the NCBI GenBank database (Accession Identifiers: MF669561-MF669571). A previously characterized pBluescript SK(+) construct (Rouhana et al., 2017) obtained from a *S. mediterranea* sexual cDNA library (Zayas et al., 2005) was used for analysis of *cct1B*.

4.3 Whole-mount *in situ* hybridization

Sexual planarians of 1.0 to 1.5 cm in length were used for *in situ* hybridization analyses and fixed, as per Pearson et al. (2009), with modifications implemented by King and Newmark (2013) and as listed below. Briefly, samples were sacrificed for 10 min in a phosphate-buffer saline (PBS) solution containing 10% N-acetyl cysteine (NAC), fixed for 1.5 h at 4°C with PBS containing 0.3% Triton-X (PBSTx) and 4% formaldehyde, gradually dehydrated, and placed overnight at -20°C in methanol. Samples were then gradually rehydrated into PBSTx, and bleached in a formamide-based solution under fluorescent light for 2 h, permeabilized for 20 minutes by treatment with Proteinase K (10 µg/ml), and post-fixed for 10 minutes in PBSTx containing 4% formaldehyde. Asexual planarians of 0.3 to 0.6 mm in length were processed for *in situ* hybridization, as described by King and Newmark (2013). Planarian samples were incubated for at least 36 hours at 56°C in hybridization solution containing DIG-labeled riboprobes corresponding to the antisense and sense sequence of each of the 12 CCT subunit homologs. The sense riboprobes were used as a control to determine the non-specific background (Figure S2). Subsequent washing, anti-DIG alkaline phosphatase antibody binding, and nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl-phosphate-based signal development was performed, as previously described (Pearson et al., 2009), for colorimetric analysis. These samples were mounted on microscope slides in 4:1 glycerol:PBS (v/v), and imaged using a Zeiss V.16 SteREO microscope equipped with a Canon EOS Rebel T3 camera.

For fluorescence *in situ* hybridization analyses, samples were processed post-hybridization, as described by King and Newmark (2013), stained with anti-muscle 6G10-2C7 antibodies (Developmental Studies Hybridoma Bank, Iowa City, IA), as per Ross et al. (2015), and/or DAPI, mounted on microscope slides in 4:1 (v/v) glycerol:PBS, and imaged under 20X magnification objectives using a Nikon C2+ confocal microscope equipped with NIS Elements C software.

4.4 Whole-mount immunofluorescence and DAPI staining

Planarians prepared for whole-mount analysis by immunofluorescence and/or DAPI staining were treated with a PBS solution containing NAC, fixed with PBSTx containing 4% formaldehyde, and bleached as described for whole-mount *in situ* hybridization (Section 4.3); methanol dehydration and overnight storage at -20°C were *not* performed. Bleached samples were washed twice with PBSTx, incubated for 2 h or overnight in 0.45% fish gelatin/0.6% bovine serum albumin/PBSTx blocking solution (Forsthoefel et al., 2014), and incubated with Y12 antibody (1:500 dilution of item MS-450-P) (NeoMarkers, Fremont, CA) for stem cell-distribution analysis, as per Rouhana et al. (2012), or processed for

nuclear staining. Nuclei were stained by incubation for 30 minutes at room temperature or overnight at 4°C in PBSTx solution containing 5 µg/ml DAPI, followed by washes in PBSTx. Whole-mount samples were imaged using a 20X objective in a Nikon C2+ confocal microscope equipped with NIS Elements C software.

4.5 Preparation of riboprobes and dsRNA by *in vitro* transcription

DNA templates for *in vitro* transcription containing cDNA sequences of *cct* genes (Figure S1) were synthesized by PCR using primers that anneal to flanking T7 promoter sequences in the parental vector pJC52.3 (5'-GGATCCTAATACGACTCACTATAGGG), or upstream of the T7 and T3 promoters in pBluescript SK(+) (5'-AAAGGGGGATGTGCTGCAAGGCGATTAAGTTGG and 5'-TAATACGACTCACTATAGGGAGACGCGCAATTAACCCTCACTA, respectively). The primer that annealed upstream of the T3 promoter in pBluescript SK(+) includes a T7 promoter sequence at its 5'-end, which facilitates dsRNA synthesis using T7 RNA Polymerase. Amplicons obtained after 38 cycles of PCR (denaturation set to 95°C for 30 seconds; annealing set to 59°C for 30 seconds; and extension at 72°C for 2 minutes) were purified using DNA Clean & Concentrator-5 columns (Zymo Research, Irvine, CA), and eluted in 20 µl of nuclease-free water. dsRNA was synthesized using T7 RNA Polymerase, as per Rouhana et al. (2013), and fed to planarians in liver puree at a concentration of approximately 100 ng/µl. Riboprobes labeled with digoxigenin (DIG)-UTP (Roche, Basel, Switzerland) containing antisense cDNA sequence were synthesized as per King and Newmark (2013) using SP6 RNA Polymerase (*cct1A*, *cct2*, *cct3B*, *cct4A*, *cct4B*, *cct5*, *cct6*, *cct7*, *cct8A*, and *cct8B*) or T3 RNA Polymerase (*cct1B* and *cct3A*), DNase treated, precipitated with lithium chloride, and resuspended in hybridization buffer for *in situ* hybridization. Control (sense) probes were synthesized using T3 RNA Polymerase (*cct1A*, *cct2*, *cct3B*, *cct4A*, *cct4B*, *cct5*, *cct6*, *cct7*, *cct8A*, and *cct8B*) or SP6 RNA Polymerase (*cct1B* and *cct3A*).

4.6 RNA interference

RNAi was performed as per Rouhana et al. (2013). Briefly, groups of six or seven planarians were fed to satiation with liver solution containing dsRNA [~100ng/µl] every 3–4 days for at least four weeks. DsRNA of the bacterial *ccdB* gene, which does not disturb planarian somatic or reproductive physiology (Collins et al., 2010), was used as a negative control. Planarians were subjected to dsRNA treatments for up to six weeks for survival studies, during which animals consumed liver containing dsRNA unless homeostatic defects or death occurred before the end of the treatment. For analysis of spermatogenesis defects, planarians were subjected to dsRNA treatments and fixed when somatic defects were observed in 40–60% of individuals in a group (somatic subunits), or after five weeks of dsRNA treatments (testis-specific subunits). Control *ccdB* RNAi samples were fixed at both time-points for comparative analysis.

4.7 Quantitative reverse-transcription PCR

Quantitation of mRNA levels for *cct1A* and *cct1B* relative to *beta-tubulin* were performed as per Miller and Newmark (2012) for three or four biological replicates of *control(RNAi)*, *cct1A(RNAi)*, and *cct1B(RNAi)*. Primers used for *cct* genes (two sets per paralog) are listed

in Figure S4, whereas primers for *beta-tubulin* were previously described by Miller and Newmark (2012).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

CCT	Chaperonin-containing tail-less complex polypeptide 1
DAPI	4',6-diamidine-2'-phenylindole dihydrochloride
dsRNA	double-stranded RNA
RNAi	RNA interference
TCP-1	Tail-less complex polypeptide 1

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Figure 1. Identification of four CCT subunit gene duplications in *S. mediterranea*
 (a-b) Structure of the CCT/TRiC hetero-oligomeric complex displaying two assembled rings from a side view (a) and the order of subunits from a top view (b). Images are reproduced from Leitner et al. (2012), under Copyright Clearance Center Agreement License Number 4082601509825. Each CCT subunit type is highlighted in a different color. (c) Neighbor-joining phylogenetic tree depicting the relationship between *S. mediterranea* (Sm) CCT subunit homologs and reference protein sequences from *Homo sapiens* (Hs) and *Saccharomyces cerevisiae* (Sc). Suffixes “A” and “B” are included for paralogs of HsCCT6

and *S. mediterranea* CCT1, CCT3, CCT4, and CCT8 subunits. Scale bar represents substitutions per position. **(d)** Possible subunits included in a chaperonin ring, given the identification of paralogs of four CCT complex subunits in *S. mediterranea*. Percent identity with human orthologs is shown for each subunit. Phylogenetic tree branches in (c) are shaded to match corresponding subunits in (a, b, and d).

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Figure 2. Expression of CCT complex subunits is enriched in the testes of *S. mediterranea*
 Whole-mount *in situ* hybridization analysis of one of each CCT subunit gene in anterior somatic cells and in testes of *S. mediterranea* (a-h), or of four duplicated subunit genes (*cct1B*, *cct3B*, *cct4A*, and *cct8B*) (i-l). The head region is indicated by a pound sign (#), and location of dorsolaterally distributed testes by an asterisk (*). Insets show magnified view of testis lobes. Scale bars, 1 mm. Results from analyses with sense control probes are included in Figure S2.

Figure 3. Expression of every somatic CCT subunit is required for survival and homeostatic integrity in *S. mediterranea*

(a) Experimental design for dsRNA feeding regime to knockdown of each of the 12 CCT subunit homologs in *S. mediterranea*. (b-h) Representative samples of planarians displaying normal anatomical appearance after 6-weeks of bacterial *ccdB* dsRNA (control RNAi) (b) or *cct8B* dsRNA feedings (c), compared to groups subjected to *cct8A* dsRNA feedings, which displayed dark, crumpling of the body (d), curling (e), head regression (f) and lysis (g, h) during the initial 3 weeks of RNAi treatment. (i) The percentage of individuals who survived

the RNAi treatment (ordinate) ($n = 20$ planarians per group, and at least 3 replicates) targeting expression of individual CCT subunits (abscissa). **(j)** Composition of the essential CCT complex in *S. mediterranea*. Subunit paralogs not necessary for survival are faded. Scale bar, 1 mm.

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Figure 4. Lethality caused by knockdown of essential CCT complex subunits is not due to loss of neoblasts

(a) Graph depicting the average quantity of neoblasts (ordinate) detected in single confocal planes of individuals from different RNAi groups (abscissa); no significant differences were observed between *control*, *cct1A*, *cct3A*, *cct3B*, and *cct5* knockdowns ($n = 4$ individuals per group). (b–c) Representative images of *control* (b) and *cct1A* (c) neoblast populations detected by Y12 immunofluorescence (magenta) in single confocal frames of anterior midline mesenchyme when essential gene RNAi groups reached the 50% lethal-dose RNAi. DAPI staining (cyan) identified the nuclei of cells.

Figure 5. Expression of CCT complex subunits is detected during early stages of spermatogenesis (a-m) Morphology of nuclei in cells undergoing spermatogenesis is revealed by DAPI staining (white) in single confocal images of testes from samples subjected to whole-mount fluorescence *in situ* hybridization (magenta). **(a)** Background signal detected from *cct3A* sense riboprobes. **(b-m)** Antisense riboprobes reveal expression of each *cct* gene in the outer layer of testes, where spermatogonia and primary spermatocytes reside. Scale bars, 50 μm.

Figure 6. Spermatogenesis defects resulting from knockdown CCT complex subunits
(a) Whole-mount sample of planarian hermaphrodites, imaged by fluorescence stereomicroscopy, revealed areas of high nuclear density stained by DAPI. Normal progression of spermatogenesis towards the inner region of testes lobes can be observed by confocal microscopy in these samples (inset). **(b-o)** Accumulation of spermatozoa was observed in testes of planarians subjected to dsRNA of bacterial *ccdB* sequence (control) (b), *cct1A* (d), and *cct3A* (g). Testes were not detected in planarians subjected to *nanos* dsRNA feedings (c), whereas sperm development defects were observed in the testes of

planarians subjected to dsRNA targeting *cct1B* (e), *cct2* (f), *cct3B* (h), *cct4A* (i), *cct4B* (j), *cct5* (k), *cct6* (l), *cct7* (m), *cct8A* (n), or *cct8B* (o). Twofold magnifications are shown in insets. Scale bar, 100 μ m.

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Table 1
Sequence of primers used for amplification and cloning of *S. mediterranea* CCT complex subunit cDNAs

The name assigned to each *S. mediterranea* CCT subunit homolog is indicated in the first column, along with corresponding reference contig number from the sexual strain transcriptome assembly published by Rouhana et al (2012) (<http://hdl.handle.net/2142/28689>).

<i>S. mediterranea</i> gene	Reference sequence	Forward primer	Reverse primer
<i>cct1A</i>	Contig49594	5'-TATGTGCATGAAGTATTTTCGTTGAAGC	5'-TTTGTACATAGAAGGTTTCGAAAATGCC
<i>cct1B</i>	Contig40409	Obtained from Zayas et al. (2005) cDNA library	
<i>cct2</i>	Contig49096	5'-TGCCAAGATTTTAATTGCCAATACTCC	5'-GAGCAGCTTTAATGATGTTATCGACAC
<i>cct3A</i>	Contig39123	5'-AAATTGCCAGAACACAAGATGAAGAAG	5'-TCATAACGTTTCTCGCTACAGACATAG
<i>cct3B</i>	Contig40653	5'-CTATGCTCAACATTTTCTGATCAAGGC	5'-TAGTTCCAGATACAATGTCGTCGATTC
<i>cct4A</i>	Contig40358	5'-GTTGTAGTGCTAGCTGGATCATTC	5'-TTTGCAAATAAACTCCACCTTCTCC
<i>cct4B</i>	Contig55656	5'-TGGTTCAATTATCTCAATCACAAGA	5'-CAGCAATTAGAGCAGGTTTCTTTAC
<i>cct5</i>	Contig55185	5'-TTGTATTAGCGGGATCGTTATTAGAGC	5'-TCTCTTAGCCTCATCAACAATCATACG
<i>cct6</i>	Contig49080	5'-ACAGTGAATTAGATTTGCATCGTGTTG	5'-TTCACCTGTCATTTTATTTCCATTTCGC
<i>cct7</i>	Contig53812	5'-TAGTTCCTTCAAAAATTGCCGATTGGTG	5'-GAATGTCGACTCCACACCAAATTTTAC
<i>cct8A</i>	Contig48870	5'-AGGCTTTGAAATAGCAGCAAAACATAG	5'-CAACTTCGTCCATTACATCATGCATAC
<i>cct8B</i>	Contig38023	5'-TTGTTATATCCGGTGGTAAAGTTGGAG	5'-CATCGAATTCTGTCTTGCTTGATTATGCC

The name assigned to each *S. mediterranea* CCT subunit homolog is indicated in the first column, along with corresponding reference contig number from the sexual strain transcriptome assembly published by Rouhana et al (2012) (<http://hdl.handle.net/2142/28689>).