**ABSTRACT** Cilia are microtubule-based structures that protrude from the apical surface of cells to mediate motility, transport, intracellular signaling, and environmental sensing. Tau tubulin kinases (TTBKs) destabilize microtubules by phosphorylating microtubule-associated proteins (MAPs) of the MAP2/Tau family, but also contribute to the assembly of primary cilia during embryogenesis. Expression of TTBKs is enriched in testicular tissue, but their relevance to reproductive processes is unknown. We identified six TTBK homologues in the genome of the planarian *Schmidtea mediterranea* (*Smed-TTBK-a, -b, -c, -d, -e, and -f*), all of which are preferentially expressed in testes. Inhibition of *TTBK* paralogues by RNA interference (RNAi) revealed a specific requirement for *Smed-TTBK-d* in postmeiotic regulation of spermatogenesis. Disrupting expression of *Smed-TTBK-d* results in loss of spermatozoa, but not spermatids. In the soma, *Smed-TTBK-d* RNAi impaired the function of multiciliated epidermal cells in propelling planarian movement, as well as the osmoregulatory function of protonephridia. Decreased density and structural defects of motile cilia were observed in the epidermis of *Smed-TTBK-d(RNAi)* by phase contrast, immunofluorescence, and transmission electron microscopy. Altogether, these results demonstrate that members of the TTBK family of proteins are postmeiotic regulators of sperm development and also contribute to the formation of motile cilia in the soma.

**INTRODUCTION** Ciliopathies are a group of health disorders associated with mutations of factors involved in cilia formation and function. Included among these are variable pathologies of the kidney, liver, and retina, as well as brain dysgenesis, neurocognitive impairments, and infertility (Badano et al., 2006; Lee and Gleeson, 2011; Waters and Beales, 2011; Reiter and Leroux, 2017). Members of the tau tubulin kinase (TTBK) subfamily of casein kinase proteins are associated with ciliopathies (Ikezu and Ikezu, 2014). As the name implies, TTBKs are able to directly phosphorylate tubulin (the main structural element of microtubules) and the microtubule-associated proteins (MAPs) MAP2 and Tau (Takahashi et al., 1995; Tomizawa et al., 2001; Sato et al., 2006). Two members of the TTBK subfamily of proteins are encoded in the human genome. TTBK1 is almost exclusively expressed in the CNS, and certain alleles are associated with late onset Alzheimer’s disease (Vazquez-Higuera et al., 2011; Yu et al., 2011; Fagerberg et al., 2014). TTBK2 expression is detected broadly at the transcript level, but the protein is particularly abundant in the brain and testis (Bouskila et al., 2011; Fagerberg et al., 2014). Indeed, mutations in TTBK2 are associated with spinocerebral ataxia type 11, which is characterized by progressive loss of coordination, difficulty walking, abnormal eye signs, peripheral neuropathy, and dystonia (Houlden et al., 2007; Bauer et al., 2010). The contributions of TTBK proteins in the development or function of the reproductive system remain unknown.

Given the shared components involved in the assembly of microtubules in somatic cell cilia and sperm flagella, it could be postulated that TTBK activity contributes to the process of...
spermatogenesis. Second to levels of expression in neurons, for which functional relevance has been extensively established, the activity of mammalian TTBK paralogues is highest in male gonads (Sato et al., 2006; Bouskila et al., 2011; Fagerberg et al., 2014; Papatheodorou et al., 2018). However, functional studies in mice have not revealed a role for TTBK homologues in spermatogenesis. Humanized TTBK1 transgenic mouse display phosphorylated neurofilament aggregation and age-dependent memory impairment (Sato et al., 2008), whereas TTBK2 mutant mice manifest defects in primary cilia formation, loss of Sonic hedgehog signaling activity, and ultimately die during embryogenesis (Bouskila et al., 2011; Goetz et al., 2012). MAP2 and Tau are also expressed in the testes (Loveland et al., 1996; Inoue et al., 2014; Sigala et al., 2014). However, Tau and MAP2 are detected before development of sperm flagella and with nuclear localization during meiosis (Loveland et al., 1996, 1999; Inoue et al., 2014). Additionally, phosphorylation of nuclear Tau at residues targeted by TTBKs is temporally observed during meiosis in the mouse male germline, suggesting that regulation of MAPs by TTBKs may contribute to early events during spermatogenesis (Inoue et al., 2014). Given the potential redundancy between paralogues of TTBK and MAP2/Tau family proteins, as well as difficulties in studying TTBK2 function in mice, uncovering the function of TTBK phosphorylation during spermatogenesis may require analyses in nonmammalian organisms.

Several studies have recently demonstrated the value of the planarian flatworm Schmidtea mediterranea for in vivo evaluation of genes involved in cilia formation and function (Rompolas et al., 2009, 2013; Basquin et al., 2015; Azimzadeh and Basquin, 2016; King and Patel-King, 2016). Planarians are free-living members of the phylum Platyhelminthes, best known for their remarkable regenerative capacity (Newmark and Sánchez Alvarado, 2002; Shibata et al., 2010; Elliott and Sánchez Alvarado, 2013; Rink, 2013). Generation in flatworms is fueled by an adult population of pluripotent stem cells known as neoblasts. These neoblasts are the only proliferating cells of the planarian soma and undergo terminal differentiation to replace lost tissue (Saló and Baguñà, 1985, 1989; Newmark and Sánchez Alvarado, 2000; Wagner et al., 2011). Not only do planarians belong to a separate phylum from organisms more commonly used in the genetic analysis of development, such as the nematode and fly (Caenorhabditis and Drosophila), but also several unique features make planarians particularly desirable in the study of cilia. For example, cellular proliferation of neoblasts occurs in the complete absence of centrosomes, and de novo development of centrioles is observed during terminal differentiation of ciliated cells (Azimzadeh et al., 2012). The planarian genome has been sequenced (Robb et al., 2008, 2015; Grohme et al., 2018), and the distribution of expression of individual genes can be analyzed throughout the entire organism by whole-mount in situ hybridization. Gene function can be assessed systemically and by simply supplementing planarian food with double-stranded RNA targeting a specific gene (Newmark et al., 2003; Reddien et al., 2005; Rouhana et al., 2013). Defects in the assembly or function of cilia in epidermal cells of planarians result in locomotion defects that are readily detectable and do not affect regeneration or cause immediate lethality (Reddien et al., 2005; Rink et al., 2009; Glazer et al., 2010; Rompolas et al., 2013). Last, sexual strains of S. mediterranea develop their entire hermaphroditic reproductive system postembryonically, offering the opportunity to analyze genetic contributions to the formation of gametes and ciliated structures of the reproductive system after embryonic development is completed (Zayas et al., 2005; Wang et al., 2007, 2010; Chong et al., 2011; Rouhana et al., 2017). In this study, we take advantage of the unique characteristics of planarian biology to uncover potentially conserved contributions of TTBK homologues during the development of sperm and somatic ciliary structures.

RESULTS

Six TTBK homologues are encoded in the genome of S. mediterranea and preferentially expressed in testes

A TBLASTN search revealed six sequences with partial identity to human TTBK1 and TTBK2 (E value ≤ 2.37e-2) among contigs from a reference transcriptome assembly of the sexual strain of S. mediterranea (Rouhana et al., 2012). All six sequences were cross-referenced to sequences in independent transcriptomes deposited in PlanMine (Brandl et al., 2016), validated as individual loci in S. mediterranea genome sequences (Robb et al., 2015; Grohme et al., 2018), and named Smed-TTBK-a, -b, -c, -d, -e, and -f (Supplemental Table S1). Orthology to TTBK family proteins was confirmed using reciprocal BLASTP searches between predicted planarian translation products and human RefSeq protein sequences deposited in the National Center for Biotechnology Information (NCBI) (Supplemental Table S2). Planarian TTBKs share 61–71% protein sequence identity with human orthologues within the conserved kinase domain, whereas 85% identity is shared between human paralogues in this region (Figure 1A). In addition, conserved TTBK1 active site residues (Kiefer et al., 2014), and residues that are fundamental to the structure of kinases in general (Hanks and Hunter, 1995), are present in each of the TTBK paralogues of S. mediterranea (Supplemental Figure 1). No conservation was detected outside of the kinase domain between planarian and human TTBK sequences. However, regions of less than 50% identity are shared between human paralogues outside of the kinase domain, and a stretch smaller than 50 amino acids with 46% identity is shared between Smed-TTBK-c and Smed-TTBK-d (Figure 1A). These analyses suggest that all six planarian TTBK homologues are active kinases that derived from an ancestral TTBK sequence, and that human paralogues are a result of a recent duplication not ancestrally present before divergence of lophotrochozoans and deuterostomes. Furthermore, the lack of sequence conservation outside of the kinase domain corroborates with observations made by Ikezu and Ikezu (2014), who proposed that current TTBK homologues arose through evolution from a shorter TTBK gene composed of the kinase domain. Further analysis revealed the presence of TTBK orthologues in genomes of sponges and cnidarians, indicating that this protein family is present throughout the animal kingdom (Figure 1B). Interestingly, TTBK orthologues were also found in slime molds (i.e., Dictyostelium discoideum, Ancylostelium subglobosum, and Cavendiera fasciculata) (Figure 1B). However, TTBKs were not found in yeast (i.e., Saccharomyces cerevisiae, Neurospora crassa), the alga Chlamydomonas reinhardtii, or Arabidopsis thaliana, where the closest homologue to human TTBKs were members of the Casein Kinase I family of proteins (Figure 1B). These results suggest that TTBK was present in the last common ancestor shared between animals and slime molds (Mycetozoa), but may have been lost during the evolution of fungi.

We performed whole-mount in situ hybridization (WMISH) to assess the distribution of expression of TTBK paralogues in planarian hermaphrodites. WMISH analyses were performed on samples from a clonal sexual strain of S. mediterranea (Zayas et al., 2005) grown to a size of ∼1.3 cm, for which postembryonic development of a functional reproductive system is expected (Figure 2A). Two different riboprobes were used to analyze the expression of each S. mediterranea TTBK parologue. One set corresponded to sequence complementary to the region encoding the conserved kinase domain, whereas the other set targeted transcript regions that corresponded
FIGURE 1: Six genes in *S. mediterranea* are homologues of TTBKs with sequence conservation limited to the kinase domain. (A) Schematic of conserved regions of predicted amino acid sequence between human and planarian TTBK homologues. Location of conserved kinase domain and percent identity relative to that of HsTTBK1 are highlighted in black. Regions of conserved sequence outside the kinase domain and corresponding percent identity are highlighted in gray. The presence of a polyglutamate domain is indicated by a dotted box. Numbering at the bottom of each diagram indicates amino acid positions. (B) Phylogenetic tree based on maximum-likelihood principle estimates depicting the recent amplification of TTBK genes in *S. mediterranea*, and the relationship between TTBK and Casein Kinase family members. Phylogenetic analysis was performed on kinase domain protein sequences using default settings in phylogeny.fr (Dereeper et al., 2008). Abbreviations and shading: slime molds (orange) *D. discoideum* (Dd) and *C. fasciculata* (Cf); sponge (yellow) *Amphimedon queenslandica* (Aq); the ecdysozoans (green) *Caenorhabditis elegans* (Ce) and *Drosophila melanogaster* (Dm); the platyhelminth *S. mediterranea* (Smed; red); and the deuterostomes (blue) *Homo sapiens* (Hs), *Mus musculus* (Mm), *Gallus gallus* (Gg), *Xenopus tropicalis* (Xt), *Danio rerio* (Dr), and *Strongylocentrotus purpuratus* (Sp). TTBK homologues were not found in *S. cerevisiae* (Sc), *N. crassa* (Nc), *A. thaliana* (At), or *C. reinhardtii* (Cr), but their genomes do contain at least one Casein Kinase family I member (not shaded; top right). Scale bar = 0.5 substitutions per amino acid position.
TTBK homologues are expressed in the testes of adult sexual planarians. (A) Schematic representation of the planarian body plan, including sensory organs, the pharynx, and hermaphroditic reproductive anatomy. (B–G) Distribution of expression of each of the six S. mediterranea TTBK paralogues is preferentially detected in the testes of sexual samples by whole-mount in situ hybridization. Abbreviations: c.a., copulatory apparatus; n.c., nerve cord. Scale bars = 1 mm.

Expression of each of the six TTBK paralogues was robustly detected in the testes, which are distributed dorsolaterally throughout most of the planarian body (Figure 2, B–G). Surprisingly, expression of TTBK paralogues was not conclusively detected in the brain or elsewhere in the planarian anatomy using this approach. These results demonstrate that expression of planarian TTBK paralogues is particularly abundant in the testes, which is a feature that is conserved with human TTBK homologues.

Expression of Smed-TTBK-d is essential for sperm development
To identify potential functions of TTBK paralogues in S. mediterranea, we disrupted expression of Smed-TTBK-a, -b, -c, -d, -e, and -f individually using RNA interference (RNAi). Groups of five individuals from the clonal line of S. mediterranea sexual strain were subjected to a diet composed of beef liver supplemented with double-stranded RNA (dsRNA) targeting specific TTBK paralogues. Planarians were fed twice per week for 3 wk and analyzed 7 d after the last feeding (Figure 3A). This RNAi regimen has been shown to sustain disrupted expression of target sequences systemically without having nonspecific side effects in the anatomy or physiology of planarians (Collins et al., 2010; Chong et al., 2013; Rouhana et al., 2013, 2017; Steiner et al., 2016). A negative control group was fed liver containing dsRNA corresponding to firefly luciferase sequence. As a positive control for RNAi penetrance, a group was fed dsRNA targeting Smed-prohormone convertase 2 (PC2), which is required for proper development of the reproductive system (Collins et al., 2010). At the end of the RNAi treatment, the overall appearance of planarians subjected to knockdown of TTBK paralogues was comparable to that of luciferase(RNAi) controls, with the exception of Smed-TTBK-d knockdown planarians (Smed-TTBK-d(RNAi)), which manifested abnormal motility (Supplemental Table S3). To analyze the planarian anatomy at the cellular level, RNAi
groups were fixed 1 wk after the last dsRNA feeding and stained with 4′,6-diamidino-2-phenylindole (DAPI), which labels DNA in the nucleus of cells throughout the planarian anatomy. Again, the overall anatomy, distribution of testis lobes, and the presence of a copulatory apparatus (an indicator of sexual maturity) were comparable in TTBK knockdowns and luciferase(RNAi) planarians (Figure 3, B, C, B′, and C′; Supplemental Table S3).

Given the preferential expression of TTBK paralogues in the testis, as revealed by WMISH (Figure 2, B–G), we decided to analyze the cellular anatomy of this structure in more detail by confocal microscopy. Progression of spermatogenesis in planarian testis lobes shares structurally similarities with what is observed in human seminiferous tubules (reviewed by Newmark et al., 2008). Germline stem cells and spermatogonia populate and divide in the outer part of the lobe, meiosis and differentiation proceed inward, and spermatocytes open the possibility for spermatid stage of spermato genesis (Figure 3D). Normal distribution of developing sperm with the accumulation of elongated spermatid was observed in testes of luciferase(RNAi) (Figure 3D). By contrast, testes of Smed-TTBK-d(RNAi) displayed irregularities in sperm development that ranged from abnormal morphology and loss of spermatid (mild phenotype; Figure 3E) to the complete loss of elongating structures (severe phenotype; Figure 3F). Small testis lobes were observed in planarians subjected to PC2 RNAi (Figure 3G), which is known to block sexual maturation and progression of spermatogenesis from germ line stem cells (Collins et al., 2010). The applied RNAi protocol has been shown to decrease levels of target mRNAs by 90–95% (Counts et al., 2017). However, sperm development in testes of planarians subjected to RNAi targeting expression of Smed-TTBK-a, -b, -c, -e, or -f appeared to be normal, and no additive effects were observed from simultaneous knockdown of Smed-TTBK paralogues (Supplemental Table S3). Since we cannot rule out the possibility that residual gene expression during RNAi may be sufficient to mask potential phenotypes from complete loss-of-function of TTBK paralogues, and since RNAi efficiency is known to decrease when targeting multiple planarian genes simultaneously (Higuchi et al., 2008), we are unable to conclude whether other TTBK paralogues contribute to sperm development (or any other processes). However, our results demonstrate that Smed-TTBK-d is required for sperm development.

To determine the timing of Smed-TTBK-d expression during spermatogenesis more precisely, we analyzed expression of this gene by fluorescence in situ hybridization (FISH) and confocal microscopy. For reference, we included established markers of spermatogonia (germlinal histone H4 (gH4); Wang et al., 2007), spermatocytes (Smed-tkn-1; Chong et al., 2011), and spermatids (Smed-pka; Chong et al., 2011) for simultaneous analysis with Smed-TTBK-d (Figure 4). FISH analyses revealed that Smed-TTBK-d transcripts were present in spermatogonia (Figure 4, A and A′), spermatocytes (Figure 4, B and B′), and spermatids (Figure 4, C and C′). The distribution of Smed-TTBK-d transcript abundance more closely resembled that of Smed-pka, suggesting that the relative expression of Smed-TTBK-d peaks during the spermatid stage of spermatogenesis (Figure 4, C and C′). The peak pattern of Smed-TTBK-d expression corroborates with the loss of spermatid elongation observed in the testes of Smed-TTBK-d(RNAi) (Figure 3F). However, detection of Smed-TTBK-d expression in spermatogonia and spermatocytes opened the possibility that Smed-TTBK-d function may be required for earlier stages of spermatogenesis.

To pinpoint the earliest stage affected by disruption of Smed-TTBK-d expression during spermatogenesis, we analyzed the testes of luciferase(RNAi) and Smed-TTBK-d(RNAi) by whole-mount double-fluorescence in situ hybridization using genetic markers for specific stages of spermatogenesis (Figure 5). Spermatogonia (labeled by gH4) and spermatocytes (labeled by tkn-1) were observed in testes of every luciferase(RNAi) (Figure 5A; n = 7) and Smed-TTBK-d(RNAi) (Figure 5B; n = 11) sample. Analyses using the combination of spermatogonia (gH4) and spermatid (pka) markers also revealed the presence of both

**FIGURE 4:** Expression of Smed-TTBK-d in the planarian testes is detected early during spermatogenesis and peaks in spermatids. (A–C) Single plane confocal images of double-fluorescence in situ hybridization analysis using riboprobes for markers of different stages of spermatogenesis (A–C; green) and Smed-TTBK-d (A′–C′; magenta). Smed-TTBK-d expression is detected in a subset of cells that also express the spermatogonial marker germinal histone H4 (gH4; A–A′), the spermatocyte marker Smed-tkn-1 (B–B′), and the spermatid marker Smed-pka (C–C′). Nuclear morphology is visualized by DAPI staining in all panels (blue). The distribution of expression for specific spermatogenesis markers in delineated (dashed lines, A, A′ to C, C′). Merged views are shown (A″–C″). Scale bar = 50 µm.
of these cell types in testes of every luciferase(RNAi) (Figure SC; \( n = 5 \)) and Smed-TTBK-d(RNAi) (Figure SD; \( n = 9 \)) sample. As before, elongating spermatids and spermatogonia were observed by DAPI staining in the testes of most (83%) luciferase(RNAi) planarians (Figure 5, A, \( n = 5/7 \), and C and C'); \( n = 5/5 \)), but were absent in the testes of 75% of Smed-TTBK-d(RNAi) (Figure 5, B; \( n = 9/11 \), and D and D'); \( n = 6/9 \)). These results demonstrate that Smed-TTBK-d is required for spermatid elongation.

**Smed-TTBK-d is required for normal planarian behavior and protonephridia function**

In addition to defects in sperm development, planarians subjected to Smed-TTBK-d RNAi displayed behavioral and physiological irregularities not observed on knockdown of any other planarian TTBK parologue or in luciferase(RNAi) (Figure 6, A and B; Supplemental Videos S1 and S2; Supplemental Table S3). The motility of Smed-TTBK-d(RNAi) planarians transitioned from gliding around their body by each image is shown at the top right corner of each image. Scale bar = 50 µm.

Spermatozoa and elongating spermatids are absent in testes for Smed-TTBK-d(RNAi). (A–D) Single plane confocal images of double-fluorescence in situ hybridization analysis of luciferase(RNAi) (A, C) and Smed-TTBK-d(RNAi) (B, D) planarian testes. gH4 is used as a genetic marker of spermatogonia (A–D; green), tkn-1 as a marker for spermatocytes (A, B; magenta), and pka as a marker for spermatids (C, D; magenta). Nuclear morphology and elongated spermatid heads are visualized by DAPI staining (blue in A–D, gray in C and D), and threefold magnified views are shown in C’ and D’.

The fraction of individuals that displayed the phenotype represented by each image is shown at the top right corner of each image. Scale bar = 50 µm.

Based on those of Inoue et al. (2004, 2015). Groups of five planarians were placed in an illuminated end of a Petri dish and monitored for 3 min. Planarians subjected to luciferase RNAi readily moved away from the illuminated region and spent most of the time in the end of the Petri dish farthest from the light source (Figure 6, C and E), as was expected from previous observations of normal planarian behavior (Inoue et al., 2004, 2015). In contrast, Smed-TTBK-d(RNAi) planarians remained at the most illuminated end of the Petri dish for the majority of the time period of analysis (Figure 6, D and F). These results confirmed abnormal behavior in Smed-TTBK-d(RNAi) and suggested that Smed-TTBK-d is required for normal gliding motility of planarians.

Partial bloating (tissue edema) was observed in regions of planarians subjected to Smed-TTBK-d RNAi, which suggested that protonephridia may also require proper expression of Smed-TTBK-d. Protonephridia are ciliated structures of the planarian anatomy that regulate water and salt content (McKanna, 1968a,b; Ishii, 1980). Bloating is a phenotype caused by the defective function of protonephridia that has been extensively characterized in asexual planarians (Rink et al., 2009, 2011; Scimone et al., 2011; Thi-Kim Vu et al., 2015). Thus, we subjected asexual planarians to Smed-TTBK-d RNAi in order to verify whether expression of this gene is required for the function of protonephridia. Asexual planarians were fed twice per week for 4 wk with liver containing either luciferase dsRNA or Smed-TTBK-d dsRNA. All luciferase(RNAi) appeared normal a week following the last RNAi treatment (\( n = 17/17 \); Figure 7A). By contrast, the majority of Smed-TTBK-d(RNAi) planarians displayed either mild (\( n = 5/17 \); Figure 7B) or severe (\( n = 10/17 \); Figure 7C) bloated phenotypes. At this point, we amputated the heads and tails from luciferase(RNAi) and Smed-TTBK-d(RNAi) and waited a week to assess the regenerative capacity in these planarians. The development of photoreceptors was used as indicative of successful regeneration, which was observed in all but one luciferase(RNAi) trunk fragment (\( n = 14/15 \); Figure 7D). Most of the bloated planarians died after being amputated (\( n = 9/17 \)), but the majority of the fragments that did survive were able to regenerate (\( n = 6/8 \); Figure 7E), suggesting that Smed-TTBK-d is not directly required for stem cell-driven regeneration. Altogether, these results demonstrate that in addition to being essential for sperm development, Smed-TTBK-d is required in the soma for proper osmoregulation and motility.

**Smed-TTBK-d is required for the structural integrity of planarian motile cilia and protonephridial units**

Smed-TTBK-d expression was detected conclusively only in the testes from analyses by WMISH in sexual planarians (Figure 2E), but the behavioral phenotype of Smed-TTBK-d(RNAi) suggested that this gene is active in somatic cells responsible for motility and osmoregulation. To identify the somatic distribution of Smed-TTBK-d expression, we consulted the single-cell transcriptome analyses of asexual planarians available from Planarian SCS (https://radiant.wi.mit.edu; Wurtzel et al., 2015). Planarian digiworm (https://digiworm.wi.mit.edu; Fincher et al., 2018), and single-cell RNASeq data from Plass et al. (2018). According to the data deposited in Planarian SCS, expression of Smed-TTBK-d (dd_Smed_v4_12470_0_1) in asexual planarians is enriched in ciliated epithelia (epidermis II), as well as in cells of the protonephridia, and in ciliated neurons (Supplemental Figure S2). The records in Planarian digiworm also indicated enrichment of Smed-TTBK-d expression in neuronal and epidermal cell types (Supplemental Table S4), whereas the transcriptome studies of Plass et al. (2018) detected enriched expression for this gene in secretory cells (secretory 2 category), cells of the **Molecular Biology of the Cell**
We readily deposited in PlanMine (dd_v4; Liu et al., 2013; others). Three-dimensional analysis by digital stitch microscopy (as per Rompolas et al., 2011), and Ross et al. (2011), and Alvarado and Newmark (1999), Rink et al. (2011), and Ross et al. (2018). We readily detected ciliary projections in the dorsal, ventral, and peripheral epithelium of luciferase(RNAi) planarians (Figure 8, E, G, and I). Conversely, Smed-TTBK-d(RNAi) planarians appeared to have lost most of the cilia in the dorsal, ventral, and peripheral epithelium (Figure 8, F, H, and J). Detection of cilia in the auricles and the dorsal ciliated stripe, which are anatomical regions hypothesized to contain a high density of ciliated sensory neurons, was reduced in Smed-TTBK-d(RNAi) planarians as compared with luciferase(RNAi) controls (Figure 8, E and F). Finally, irregularities in the morphology of structures decorated by anti-AcTub in protonephridial units, which are largely composed of motile cilia, were also observed in Smed-TTBK-d(RNAi) (Figure 8, K and L). These results demonstrate that Smed-TTBK-d RNAi is required for the proper formation of ciliated structures involved in planarian motility, behavior, and fluid homeostasis.

To better characterize the defects in cilia formation observed on Smed-TTBK-d(RNAi), we analyzed multiciliated ventral epithelial cells by anti-α-tubulin immunofluorescence and high-magnification confocal microscopy (as per Rompolas et al., 2010, 2013; and Vij et al., 2012). Control luciferase(RNAi) planarians possessed filamentous cilia of homogeneous dimensions distributed throughout the regions of peripheral ventral epidermis (Figure 9A). By comparison, the structures illuminated by anti-α-tubulin immunofluorescence in analogous cells of Smed-TTBK-d(RNAi) planarians were less densely distributed and varied in thickness and architecture from normal epidermal cilia described in the literature (Figure 9B; Rompolas et al., 2009, 2010; Almuedo-Castillo et al., 2012; Rompolas et al., 2013). Three-dimensional analysis by digital stitching deconstruction of confocal images taken at different planes along the z-axis (i.e., z-stacks) revealed a gap of ~10 µm between the nuclei of these cells and the region of cilia illuminated by anti-α-tubulin in luciferase(RNAi) planarians (Figure 9A). However, the distance between the nucleus and the ciliary structures illuminated

![Image](image-url)
by anti-α-tubulin in cells of the ventral epidermis was not present in *S. mediterranea* MAP2/Tau-like-1 (Supplemental Table S5). These three genes, hereafter referred to as *S. mediterranea* MAP2/Tau-like-1 (*Smed-MAPT-L1*), MAP2/Tau-like-2 (*Smed-MAPT-L2*), and MAP2/Tau-like-3 (*Smed-MAPT-L3*), encode for proteins that do not group with specific vertebrate MAP2/Tau family proteins (i.e., MAP2, MAP4, or Tau) in phylogenetic analyses (Figure 10A), suggesting that vertebrate and planarian MAPs emerged from independent gene duplication events. Tubulin-binding domain repeats of *Smed-MAPT-L1* (Figure 10B; Supplemental Figure S3). The core tubulin-binding motif KGKS (Sundermann et al., 2016) is present in a subset of repeats in each planarian MAP-L parologue (Supplemental Figure S3). Additionally, the consensus phosphorylation site for CK1 kinase isoforms (S/T*-X-X-S/T, where the asterisk marks the target residue; Flotow and Roach, 1989; Flotow et al., 1990; Nakielny et al., 1991), as well as preferred substrate sites for mammalian TTBK2 (S/T*-X-pY, where a primed phosphotyrosine is found at the +2 position; Bouskila et al., 2011), are both present within the planarian MAP-L paralogues. The core tubulin-binding motif KGKS (Sundermann et al., 2016) is present in a subset of repeats in each planarian MAP-L parologue (Supplemental Figure S3). Additionally, the consensus phosphorylation site for CK1 kinase isoforms (S/T*-X-X-S/T, where the asterisk marks the target residue; Flotow and Roach, 1989; Flotow et al., 1990; Nakielny et al., 1991), as well as preferred substrate sites for mammalian TTBK2 (S/T*-X-pY, where a primed phosphotyrosine is found at the +2 position; Bouskila et al., 2011), are both present within the planarian MAP-L paralogues. *Expression of MAP2/TAU homologues in testes of S. mediterranea.* Our data suggest that *Smed-TTBK-d* contributes to the initiation and assembly of motile cilia/flagella through conserved mechanisms reported for mammalian TTBK2 during the development of primary cilia (Goetz et al., 2012; Bowie et al., 2018). Homologues of centriolar proteins that interact with mammalian TTBKs during the regulation of primary cilia formation (i.e., CP110 and Cep164; Goetz et al., 2012; Cajanek and Nigg, 2014; Oda et al., 2014) have been shown to be required for normal motility in *S. mediterranea* (Azimzadeh et al., 2012). To gain further insight into the potential roles of other well-known TTBK targets during spermatogenesis and motile cilia formation, we looked for *S. mediterranea* homologues of the MAP2/Tau microtubule-associated protein family. Neuronal members of the MAP2/Tau protein family are known TTBK substrates in mammals (Takahashi et al., 1995; Sato et al., 2006) and show dynamic phosphorylation at target residues during spermatogenesis (Inoue et al., 2014). Three MAP2/Tau homologues were identified in reference transcriptomes of sexual and asexual *S. mediterranea* (Supplemental Table S5). These three genes, hereafter referred to as *S. mediterranea* MAP2/Tau-like-1 (*Smed-MAPT-L1*), MAP2/Tau-like-2 (*Smed-MAPT-L2*), and MAP2/Tau-like-3 (*Smed-MAPT-L3*), encode for proteins that do not group with specific vertebrate MAP2/Tau family proteins (i.e., MAP2, MAP4, or Tau) in phylogenetic analyses (Figure 10A), suggesting that vertebrate and planarian MAPs emerged from independent gene duplication events. Tubulin-binding domain repeats can be identified in each of the proteins encoded by *Smed-MAPT-L* genes (three in *Smed-MAPT-L1*, four in *Smed-MAPT-L2*, and three in *Smed-MAPT-L3* (Figure 10B; Supplemental Figure S3). The core tubulin-binding motif KGKS (Sundermann et al., 2016) is present in a subset of repeats in each planarian MAP-L parologue (Supplemental Figure S3). Additionally, the consensus phosphorylation site for CK1 kinase isoforms (S/T*-X-X-S/T, where the asterisk marks the target residue; Flotow and Roach, 1989; Flotow et al., 1990; Nakielny et al., 1991), as well as preferred substrate sites for mammalian TTBK2 (S/T*-X-pY, where a primed phosphotyrosine is found at the +2 position; Bouskila et al., 2011), are both present within the tubulin-binding repeats of *Smed-MAPT-L3* and outside of the tubulin-binding repeats of *Smed-MAPT-L1* (Figure 10B; Supplemental Figure S3). These primary sequence analyses indicate that the regulatory circuitry among TTBKs, MAP2/Tau, and tubulin may participate in directing microtubule dynamics in *S. mediterranea.*

**FIGURE 7:** Asexual planarians subjected to *Smed-TTBK-d* RNAi develop a bloating phenotype representative of defects in protonemaphridia function. (A–C) Asexual planarians imaged by bright-field microscopy exhibit normal anatomical features after a 3-wk treatment of luciferase dsRNA (A), whereas planarians subjected to dsRNA targeting *Smed-TTBK-d* developed mild (B) and severe (C) bloating phenotypes. (D, E) Trunk fragments with regenerated heads and tails imaged by dark-field microscopy 7 d after amputation. Representative images of luciferase(RNAi) (D) and *Smed-TTBK-d* RNAi (E) trunk fragments able to regenerate heads and tails (unpigmented tissue) 7 d postamputation. The fraction of planarians represented by the imaged phenotype is indicated the top right corner of each frame in A–E. Planarians that did not survive 48 h postamputation and were excluded from the denominator in D and E. Scale bar = 1 mm.
FIGURE 8: Smed-TTBK-d is required for normal development of ciliated epithelia and protonephridia. (A, B) Colorimetric in situ hybridization in asexual S. mediterranea reveals Smed-TTBK-d expression in the periphery of the planarian body, where ciliated epithelia reside (A). Expression of Smed-pka, a spermatid marker used as a negative control, is not detected in the planarian periphery (B). (C, D) Confocal plane showing detection of Smed-TTBK-d mRNA (magenta; C and C′) in peripheral epithelial cells (arrows in C′) of sexual planarians analyzed by fluorescence in situ hybridization. Magnified view of area marked by a dashed square is shown C′. Specificity of Smed-TTBK-d transcript detection in epithelial cells is verified by parallel analysis using luciferase riboprobes as a negative control (magenta; D). (E–L) Maximum projections of confocal z-sections reveal ciliary structures in luciferase(RNAi) (E, G, I, and K) that are lost in Smed-TTBK-d(RNAi) (F, H, J, and L). Cilia decorated with acetylated-tubulin antibodies (green) in the dorsal (E, F), ventral (G, H), and peripheral epithelium (I, J) of asexual planarians are more prevalent in luciferase(RNAi) (E, G, and I) than in Smed-TTBK-d(RNAi) (F, H, and J). Fivefold magnified view of area marked by a dashed square is shown in insets for I and J. Cilia in areas of high sensory neuron density along the dorsal midline (arrowhead) and auricles (arrows) are indicated in (E). Acetylated-tubulin staining also reveals defects in the anatomy of protonephridial units of Smed-TTBK-d(RNAi) (L) as compared with those in luciferase(RNAi) controls (K). Cell nuclei labeled by DAPI are shown in white (C, D) and blue (E–J). Scale bars = 1 mm (A, B); 100 μm (C, E–H); and 50 μm (D, I–L).

To determine whether MAP2/Tau homologues are coexpressed with Smed-TTBK-d, we consulted the above-mentioned sexual planarian transcriptome (Rouhana et al., 2017) and asexual planarian single-cell RNAseq analyses (Wurtzel et al., 2015; Fincher et al., 2018; Plass et al., 2018). Transcriptomic data for Smed-MAPT-L1 (Contigs 39776, 52868, and 33308) and Smed-MAPT-L3 (Supplemental Figures S4 and S5). The single-cell expression analysis from Plass et al. (2018) indicates enriched expression for Smed-MAPT-L1 in several neuronal precursors and some specified neuronal cell types (e.g., GABAergic neurons that also express Smed-TTBK-d), as well as in subtypes of parenchymal cells (Supplemental Figure S5). This same study detected modest, but enriched, expression of Smed-MAPT-L2 in precursors of both muscle and epidermal cells (but not in terminally differentiated cells), as well as enriched expression of Smed-MAPT-L3 in a specific subset of neurons (npp-18+ neurons) (Supplemental Figure S5). Altogether, these analyses suggest that overlapping expression of Smed-TTBK-d and Smed-MAPT-L genes is likely to occur in a subset of neurons, temporally during epidermal differentiation, and potentially in reproductive structures.

To directly test whether MAP2/Tau homologues are expressed in the planarian testes, we performed WMISH analyses of Smed-MAPT-L1, -L2, and -L3 expression in sexual planarians (Figure 10, C–E). Expression of Smed-MAPT-L1 was detected broadly in the head and throughout most of the body of sexual planarians (Figure 10C). No particular tissues were distinguishable from the distribution of Smed-MAPT-L1 detection by WMISH, but this may be reflective of a combination of expression in neurons, muscle, piwi+, parenchymal, and/or cathepsin+ cells, as observed in single-cell RNAseq analyses (Supplemental Figures S4 and S5; Supplemental Table S4). On the other hand, expression of Smed-MAPT-L2 and -L3 was clearly enriched in the testes of sexual planarians (Figure 10, D and E). In addition, Smed-MAPT-L3 expression was detected in foci distributed from the posterior region of the head to the tail of the planarian body, but not in the pharynx and copulatory apparatus (Figure 10, E and

MAPT-L2 and -L3 in the same sexual planarian reference transcriptome or analysis mentioned above (Supplemental Table S5). However, transcriptome comparisons between S. mediterranea sexual and asexual strains performed by Davies et al. (2017) indicate that expression of these paralogues is enriched in sexual planarians (3.4-fold enrichment for Smed-MAPT-L2, dd_Smed_v4_32444_0_2, and 3.2-fold enrichment for Smed-MAPT-L3, dd_Smed_v4_33588_0_2). Single-cell RNAseq data from asexual planarians from Wurtzel et al. (2015) and Fincher et al. (2018) indicate that Smed-MAPT-L1 is expressed at highest levels in neural and muscle cells (Supplemental Figure S4; Supplemental Table S4), as well as in some cathepsin+ cells (Supplemental Table S4). Records for Smed-MAPT-L2 and -L3 expression in these single-cell RNAseq analyses were also either unavailable (Planarian SCS) or not enriched at significant levels in any cell type (Planarian digiworm; Supplemental Table S4). The single-cell expression analysis from Plass et al. (2018) indicates enriched expression for Smed-MAPT-L1 in several neuronal precursors and some specified neuronal cell types (e.g., GABAergic neurons that also express Smed-TTBK-d), as well as in subtypes of parenchymal cells (Supplemental Figure S5). This same study detected modest, but enriched, expression of Smed-MAPT-L2 in precursors of both muscle and epidermal cells (but not in terminally differentiated cells), as well as enriched expression of Smed-MAPT-L3 in a specific subset of neurons (npp-18+ neurons) (Supplemental Figure S5). Altogether, these analyses suggest that overlapping expression of Smed-TTBK-d and Smed-MAPT-L genes is likely to occur in a subset of neurons, temporally during epidermal differentiation, and potentially in reproductive structures.

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These results demonstrate that expression of MAP2/Tau homologues in the testes is a shared feature between planarians and mammals, which suggests the presence of conserved tissue-specific functions for these microtubule regulators outside of the brain. We considered the possibility that Smed-TTBK-d may work with Smed-MAPT-L2 and/or Smed-MAPT-L3 in the male germline of planarians. However, functional assessment of planarian MAP2/Tau homologues by RNAi failed to support the hypothesis that Smed-MAPT-L1, -L2, or -L3 is required for the development of spermatozoa, as testes morphology of individual, double, and triple knockdowns was comparable to that of luciferase(RNAi) (Supplemental Figure S6).

DISCUSSION

This study documents the characterization of six TTBK protein subfamily members expressed in the testes of S. mediterranea. Analysis of TTBK orthologues suggests that genetic expansion of TTBK genes is not ancestral among metazoans (Figure 1B). Pairwise comparison of planarian and human TTBKs found no evidence for selective pressure outside of the kinase domain (Figure 1A). These observations corroborate with the hypothesis proposed by Ikezu and Ikezu (2014) regarding the emergence of this subfamily from a protein composed mainly of the kinase domain. Taking into consideration that 1) defects in motile cilia formation are observed on knockdown of Smed-TTBK-d, 2) TTBK2 function is required during primary cilia formation in mice (Goetz et al., 2012; Bowie et al., 2018), and 3) primary sequence conservation between planarian and mammalian TTBKs is observed only in the kinase domain, we postulate that the kinase domain is sufficient for the function of TTBKs during cilia formation (be it primary or motile). Given the functional similarities and high degree of sequence conservation between the kinase domain of Smed-TTBK-d and human homologues (61% for TTBK1; 63% for TTBK2), it seems promising to consider the use of planarians in screens for potential therapeutic agents that can modulate the activity of TTBKs.

Interestingly, Smed-TTBK-d was the only gene of six TTBK paralogues that resulted in detectable phenotypes on RNAi. Differences in behavior, motility, osmoregulation, and sperm development were observed in Smed-TTBK-d(RNAi), but not on knockdown of Smed-TTBK-a, -b, -c, -e, or -f (Figures 3, 6, and 7; Supplemental Table S3). The possibility of functional redundancy was tested by

FIGURE 9: Smed-TTBK-d is required for assembly and normal architecture of ciliated structures. (A–D) Maximum projection of confocal z-sections shows that the architecture of epithelial ciliary structures decorated by alpha-tubulin antibody staining (magenta) in luciferase(RNAi) (A) is distorted in Smed-TTBK-d(RNAi) (B). Transverse views of z-stack confocal projections of samples (A, B) reveal differences in distance between the nucleus (white) and presumptive ciliary structures (magenta) in epithelial cells of luciferase(RNAi) (A') and Smed-TTBK-d(RNAi) (B'). (C, D) Concanavalin A staining (green) reveals that ciliary structures visualized using alpha-tubulin antibodies protrude from the cell membrane in control luciferase(RNAi) cells (C), but are proximally misplaced in Smed-TTBK-d(RNAi) (D). Cell nuclei are labeled by DAPI staining (grayscale in A and B and A' and B'; blue in C and D). (E, F) Video still captures of epithelial cilia (arrows) as seen in during live imaging analysis of luciferase(RNAi) (E) and Smed-TTBK-d(RNAi) (F) by phase contrast microscopy (shown in Supplemental Videos S3 and S4). (G, H) Transmission electron micrographs showing basal body of cilia (black arrows) anchored to the epithelial cell membrane and extended axonemes (black arrowheads) in a representative section of luciferase(RNAi) (G), whereas Smed-TTBK-d epithelial cells (H) are surrounded by aberrant axonemes (empty arrowheads) and contain presumptive uncommitted rootlets (white arrows) in their cytoplasm. Insets showing 200% magnified view of regions marked by dashed lines (G' and H'). Scale bars = 10 µm (A–D) and 500 nm (G and H).
performing simultaneous knockdown with some combinations of Smed-TTBK paralogues, but these did not yield evidence to support cooperative or redundant functions among the different planarian TTBKs (Supplemental Table S3). Although not all possible combinations were tested, aberrant phenotypes were only observed in RNAi treatments that included dsRNA targeting Smed-TTBK-d. Furthermore, the spermatogenesis and motility phenotypes observed on simultaneous knockdown of all six Smed-TTBK paralogues were indistinguishable from knockdown of Smed-TTBK-d alone. These observations suggest that there is something unique about Smed-TTBK-d expression or the specificity of interactions that makes it particularly important for the development of sperm and epidermal motile cilia. Single-cell transcriptome analyses suggest that specificity of cell-type expression may contribute to Smed-TTBK-d being required for osmoregulation and locomotion. The analyses from Wurtzel et al. (2015) suggest that Smed-TTBK-d is the only TTBK paralogue preferentially expressed in protonephridia, whereas Smed-TTBK-d and Smed-TTBK-c are preferentially expressed in ciliated epithelia (Supplemental Figure S2). On the other hand, the data from Fincher et al., (2018) indicate that Smed-TTBK-d is preferentially expressed in epidermal cells and the pharynx, whereas Smed-TTBK-c is expressed in the protonephridia (Supplemental Figure S2). Conversely, the question of possible redundancy among TTBK paralogues during spermatogenesis is less clear, since all six of these genes are preferentially expressed in the testes (Figure 2), but our functional analyses only supported the requirement of Smed-TTBK-d during sperm development. One possibility could be that Smed-TTBK-d expression during spermatogenesis is much higher than that of its paralogues, but average expression values from RNA-seq analyses show that Smed-TTBK-d transcript abundance is actually the lowest from the six paralogues in sexual planarians (Supplemental Table S5). Given that Smed-TTBK-d does not seem to be expressed differently from its paralogues during spermatogenesis (at least at the transcriptional level), we hypothesize that the primary structure of Smed-TTBK-d bestows it with unique biochemical characteristics that are particularly important during sperm development. Unfortunately, we are unable to test this hypothesis due to current technical limitations with transgenesis for the study of gene function in planarians.

Smed-TTBK-d RNAi resulted in the loss of elongating spermatids and spermatozoa in the testes of 58% of our Smed-TTBK-d(RNAi) samples (n = 28/48; Figures 3E and 5, B and D). Detailed analysis using stage-specific markers revealed that the disruption of...
spermatogenesis observed on Smed-TTBK-d RNAi occurs after meiosis (Figure 5D). We hypothesize that spermatid elongation in Smed-TTBK-d(RNAi) testes is halted due to a failure in flagellar formation under reduced Smed-TTBK-d activity. Although our current readout of spermatid elongation is based on the morphological transition of round nuclei into the thinly stretched structure seen in spermatooza (Figure 3D), defects in sperm tail formation have been shown to affect sperm head shape (Lehti et al., 2013). We further hypothesize that Smed-TTBK-d contributes to flagella formation through shared mechanisms active during the initial stages of cilia assembly in planarian epithelial cells (Figure 9, B, D, F, and H) and primary cilia in mammals (Goetz et al., 2012; Bowie et al., 2018).

Asator is an essential TTBK homologue in Drosophila that localizes to the mitotic spindle and interacts with spindle matrix proteins, presumably to regulate microtubule spindle dynamics (Qi et al., 2009). Both MAP2 and Tau can be phosphorylated by TTBKs (Takahashi et al., 1995; Tomizawa et al., 2001; Sato et al., 2006) and are expressed in germ cells of mammalian testes (Ashman et al., 1992; Gu et al., 1996; Loveland et al., 1996; Inoue et al., 2014). In rat testes, MAP2 is found in the nucleus of spermatocytes and round spermatids (Loveland et al., 1996), and changes in Tau nucleocytoplasmic localization correlates with dynamic phosphorylation during spermatogenesis in mice (Inoue et al., 2014). Phosphorylation of Tau during meiosis includes TTBK substrate residues associated with hyperphosphorylation in Alzheimer’s disease (Tomizawa et al., 2001; Inoue et al., 2014). Thus, we considered the possibility that Smed-TTBK-d may regulate microtubule spindle dynamics during meiosis, and that planarian homologues of MAP4 (which contributes to spindle stability; Samora et al., 2011) may be involved in this process. Two lines of evidence contradict this hypothesis. First, spermatid development is observed using stage-specific spermatogenesis markers in Smed-TTBK-d(RNAi) planarians (Figure 5D), indicating that completion of meiosis is not inhibited by the loss of Smed-TTBK-d function. Second, although testicular expression of MAP2/4/Tau homologues is conserved in planarians (Figure 10, D and E), knockdown of Smed-MAPT-L2 and Smed-MAPT-L3, individually or in combination, did not reveal any defects in spermatogenesis (Supplemental Figure S6). An alternative mechanism that has not been ruled out to explain the requirement of Smed-TTBK-d during spermatogenesis is the potential involvement of TTBK substrates that are not MAPs. These could include homologues of the transactive response DNA-binding protein 43 kD (TDP-43; Liachko et al., 2014; Taylor et al., 2018), which is known to function in sperm development in mice (Lalmansingh et al., 2011). Nevertheless, our preferred hypothesis is that Smed-TTBK-d is required for spermatid elongation through its role in flagellar assembly.

**MATERIALS AND METHODS**

**Planarian cultures**

A hermaphroditic clonal line of *S. mediterranea* (Zayas et al., 2005) was used for all experiments, except where the use of individuals from the assexual strain (Sánchez Alvarado et al., 2002) was specified. Laboratory populations of sexual *S. mediterranea* were expanded by amputation and maintained at 18°C in 0.75x Montjuïc salts as per Wang et al. (2007). Asexual animals were maintained at 21°C as described by Sánchez Alvarado et al. (2002) with modifications to the Montjuïc salts formula as per Pearson and Gurley (http://lab.research.sickkids.ca/pearson/wp-content/uploads/sites/54/2015/10/Planarian_careNFeedingNliverprep_Pearson.pdf). Both strains are maintained on a weekly to semiweekly diet of calf liver and starved for a week prior to experimentation.

**Identification and cloning of *S. mediterranea* TTBK homologues**

Planarian homologues of human TTBK1 and TTBK2 protein sequences were identified in a reference transcriptome assembly including cDNA and RNAseq reads from the sexual strain of *S. mediterranea* (Rouhana et al., 2012) using the TBLASTN function in BlastStation Local64 (TM Software, Arcadia, CA). Orthologous TTBK sequences were confirmed via reciprocal BLAST searches of predicted planarian protein products and human reference protein sequences deposited in the NCBI (http://blast.ncbi.nlm.nih.gov). The existence of six planarian TTBK orthologues in *S. mediterranea* was verified in genomic sequences from Robb et al. (2008) and Grohme et al. (2018) by BLASTN searches in SmedGD (Robb et al., 2015) and PlanMine (Brandl et al., 2016). Amplicons containing kinase domain sequence corresponding to each of the six TTBK paralogues as well as casein kinase homologues were amplified from sexual *S. mediterranea* total cDNA using the following primers: Smed-TTBK-a, 5′-ACAAGGAAAGAAGACATCTTTGAGA-3′ and 5′-ATCAGGGAATCAGATGTGGAGG-3′; Smed-TTBK-b, 5′-GGAATGACAGTGTGGAAAAGAAGTTA-3′ and 5′-CAACTCTGAATATTATGCTCCGCC-3′; Smed-TTBK-c, 5′-GCTAAGTGCTCAAAATCTGTTACAT-3′ and 5′-ACCTACTCGGAACCTATTCCTTGAGC-3′; Smed-TTBK-d, 5′-CTTCCACTCAAGAACCTGATTTAGC-3′ and 5′-TATCTAATCCTTTCCAGCAGAAACCAG-3′; Smed-TTBK-e, 5′-TATAAGAGCCAGGTACCTCAT-3′ and 5′-CATCTAATCGTTCGCAAGAACC-3′; Smed-TTBK-f, 5′-GTAATGTCTCTCAAGGAAACCT-3′ and 5′-CCAAGTATTGTGTTAACCCTGAGG-3′; Smed-CSNK1-a, 5′-GAATGGAATTTAAGCTCTGCTGT-3′ and 5′-CCAAAGATTACTTACGCTATGTC-3′; Smed-CSNK1-e, 5′-GAAGAAAAATGTGCTGACTTCTTTCC-3′ and 5′-ACTTGTGTTCCTGTGTTAAGTCTCTTG-3′. Amplicons were ligated into pGEM-T as per the manufacturer’s protocol (Promega, Madison, WI), verified by Sanger sequencing, and sequence records were deposited in GenBank (Accession No. MH367867-MH367874). GeneArt Strings DNA fragments (ThermoFisher, Waltham, MA) containing sequences that correspond to different regions of Smed-TTBK-a, b, c, d, e, and f cDNA (Supplemental Figure S7) than the ones cloned in pGEM-T were used to verify results obtained from RNAi and in situ hybridization. Sequences corresponding to *S. mediterranea* homologues of the MAP2/Tau family transcripts were also synthesized as GeneArt Strings DNA fragments (Supplemental Figure S7) and used as templates for downstream applications. Firefly luciferase and PC2 (Collins et al., 2010) partial cDNA sequences were included as negative and positive controls, respectively.

**Whole-mount in situ hybridization**

Sexual planarians of 1.0–1.5 cm length, or asexual planarians of 0.3–0.6 cm length, were processed for in situ hybridization as described by King and Newmark (2013), with modifications for sexual planarians as described below. Briefly, samples were rinsed with husbandry media and killed by incubating placed horizontally for 10 min on a rocking platform in a phosphate-buffered saline (PBS) solution containing 7.5% N-acetyl cysteine (NAC). The NAC solution was replaced with PBS containing 0.3% Triton-X (PBSTx) and 4% formaldehyde, and samples were incubated for 1 h for fixation. Then, samples were gradually dehydrated into xylenes, placed overnight at −20°C, gradually
rehydrated into PBSTx, bleached in a formamide-based hydrogen peroxide solution under fluorescent light for 1.5–2 h, rinsed in PBSTx, and treated for 12 min with 10 µg/ml Proteinase K (Roche, Mannheim, Germany) in a PBSTx solution containing 0.1% SDS and postfixed for 10 min in PBSTx containing 4% formaldehyde. Subsequent steps for incubation with digoxigenin-11-UTP (DIG; Roche, Mannheim, Germany)-labeled riboprobes, posthybridization washes, incubation with anti-DIG antibodies, and washes in TNTx (100 mM Tris, pH 7.5, 150 mM NaCl, 0.3% Triton-X) after the antibody-binding step were performed as per King and Newmark (2013). Samples subjected to colormetric detection of riboprobe hybridization were incubated with anti–DIG-AP (1:4000 dilution; Roche, Mannheim, Germany, Catalog No. 11093274910), developed as per Pearson et al. (2009), mounted on microscope slides in 4:1 glycerol:PBS (vol/vol), and imaged using a Zeiss V.16 SteREO microscope equipped with a Canon EOS Rebel T3 camera. For FISH analyses, samples were incubated overnight in anti–DIG-POD (1:1000 dilution; Roche, Mannheim, Germany, Catalog No. 1120773910), developed using FAM tyramide solution as per (King and Newmark, 2013), cleared and mounted in 4:1 glycerol/PBSTx, and imaged using a Nikon C2+ confocal microscope equipped with NIS Elements C software.

Whole-mount immunofluorescence, lectin, and DAPI staining

Analyses using anti-AcTub (1:500 dilution; clone 6-11B-1, Sigma Aldrich, St. Louis, MO) were performed on whole-mount asexual planarians fixed by HCl and paraformaldehyde treatment as per Ross et al. (2015). For whole-mount immunofluorescence using anti-α-tubulin (1:1000 dilution; clone B-5-1-2, Sigma Aldrich, St. Louis, MO), ConA (1:1000 dilution; Vector Laboratories, Burlingame, CA, Catalog No. FL-1001), and/or DAPI (5µg/ml; Acros Organics, Morris, NJ), planarian samples were prepared through NAC, formaldehyde, and bleaching treatments as described for whole-mount in situ hybridization (above). Steps for methanol dehydration and overnight storage at −20°C usually performed for in situ hybridization were omitted. For DAPI staining alone, bleached samples were washed twice with PBSTx, incubated overnight in a PBSTx solution containing DAPI, washed six times with PBSTx, mounted on slides, and imaged under UV light using a Zeiss V.16 SteREO microscope equipped with a Canon EOS Rebel T3 camera (low magnification) or a Nikon C2+ confocal microscope equipped with NIS Elements C software (20× objective or 60× oil immersion objective). For visualization of the cilia of the planarian epidermis, bleached samples were incubated for 2 h in Blocking solution (0.5% fish gelatin; 1% bovine serum albumin in PBSTx; Forsthoefel et al., 2014), followed by overnight incubation at 4°C in blocking solution supplemented with monoclonal anti-α-tubulin antibody or anti-AcTub. After overnight incubation, the samples were rinsed twice with PBSTx and washed six additional times for 15 min each with PBSTx, incubated for 3 h at room temperature or overnight at 4°C with secondary antibody in blocking solution (1:400 dilution of goat-anti-mouse immunoglobulin G (IgG) Alexa Fluor 488-conjugated [Cat. No. A11029, Thermo-Fisher, Waltham, MA] or goat-anti-mouse IgG Alexa Fluor 633 [Cat. No. A21050, ThermoFisher, Waltham, MA]), washed with PBSTx, stained with DAPI (as above), washed, and mounted on slides in 4:1 glycerol/PBS (vol/vol). Epithelial cells visualized by ConA were stained by extending the incubation with DAPI overnight at 4°C and including 5µg/ml Fluorescein–conjugated ConA lectin during the incubation. Samples were mounted with their dorsal end adjacent to the slide cover for analysis of testes, or with their ventral side facing the slide cover for analysis of ventral epithelial cells, with NIS Elements C software (60× oil immersion).

Preparation of riboprobes and dsRNA by in vitro transcription

Amplicons containing Smed-TTBK parologue, PC2, or luciferase partial cDNA sequence, upstream of an antisense SP6 promoter sequence, and flanked by T7 promoters were synthesized from pGEM-T constructs using the following primers: 5′-GGCGGCAATTAACCTC ACTAAAGTAATACGACTCATAAGGGCGAATTGG-3′; 5′-GGCGCGCTATAATCGACTCATAAGGGCGAATTGG-3′. Amplicons containing an antisense T3 promoter, flanking T7 promoters, and partial cDNA sequence corresponding to Smed-TTBK cDNAs (outside of the kinase domain), Smed-MAPT-L paralogues, PC2, or luciferase were generated by PCR from GeneArt Strings DNA templates (ThermoFisher, Waltham, MA; Supplemental Figure S7) using the following primers: 5′-GAATTTAATACGACCTCACTATAAGGGCGAATTGG-3′; 5′-GAATTTAACATCGACTCATAAGGGCGAATTGG-3′ as in Counts et al. (2017). Amplicons were purified using DNA Clean & Concentrator-5 columns (Zymo Research, Irvine, CA), eluted in 20 µl of nuclease-free water, and used as templates for in vitro transcription. For RNAi, dsRNA was synthesized using T7 RNA Polymerase as per Rouhana et al. (2013) from the same templates generated by PCR from pGEM-T constructs and GeneArt Strings as described above. DIG-11-UTP and Fluorescin-12-UTP (Sigma Aldrich, St. Louis, MO)-labeled riboprobes were synthesized as per King and Newmark (2013) using either SP6 RNA Polymerase (amplicons generated from pGEM-T cDNA constructs) or T3 RNA Polymerase (GeneArt Strings amplicons). DIG-labeled riboprobes were precipitated using lithium chloride after DNase treatment and resuspended in hybridization buffer.

RNAi

RNAi was performed as per Rouhana et al. (2013). Briefly, groups of five planarians were fed to satiation with liver solution containing gene-specific dsRNA at a concentration of ∼100 ng/µl every 3–4 d for 3 or 4 wk. dsRNA with sequence corresponding to firefly luciferase, which does not disturb planarian somatic or reproductive physiology, was used as a negative control. One week after their final dsRNA feeding, planarians were subjected to behavioral assays, or fixed for anatomical analysis. For combinatorial gene knockdown, liver was supplemented with 125–150 ng/µl of dsRNA composed of equal amounts of all six Smed-TTBK paralogues. For mixtures of dsRNA targeting less than six paralogues, the amount of dsRNA for each gene was kept constant (one-sixth) and brought up to a total dsRNA concentration of 125–150 ng/µl with luciferase dsRNA.

Light-response assay

On completion of RNAi treatment groups of five planarians subjected to either luciferase or Smed-TTBK-d knockdown were collected in a 50-ml conical tube, washed with 5 ml of 0.75X Montjuic salts, and decanted into a 150 × 15-mm Petri dish filled with 25 ml 0.75X Montjuic salts. White light from a Fiber-Lite high intensity illuminator (Series 180, Dolon-Jenner Industries, Boxborough, MA) at the maximum strength was directed to the most proximal region of the plate (see Figure 6, C and D) from a vertical distance of 20 cm. Planarians were decanted in the illuminated region and migration to less illuminated regions of the Petri dish were recorded for 3 min for...
both luciferase(RNAi) and Smed-TTBK-d(RNAi) groups. At the end of 2 min, the experiment was conducted again with reciprocal placement of planarian groups in Petri dishes. Time spent by each planarian in each of the five positions of the plate was averaged for four runs using two groups of biological replicates. Standard deviation from the mean and two-tailed Student's t test were calculated for statistical analysis.

Live imaging of motile cilia and TEM
Asexual planarians of 1–3 mm length were subjected to 3 wk of luciferase or Smed-TTBK-d RNAi and either mounted on glass slides for live imaging of cilia or fixed overnight at room temperature in a PBS solution supplemented with glutaraldehyde. Fixed samples were submitted to the Campus Microscopy and Imaging Facility at The Ohio State University for TEM processing and analysis. For live imaging of cilia, planarians were mounted in modified Montjuïc salts (see Planarian cultures above) on a glass microscope slide adhered to a perforated sheet of Parafilm and capped with a slide cover as per Rompolas et al. (2013). Movement of cilia in the peripheral epithelium of luciferase(RNAi) and Smed-TTBK-d(RNAi) were imaged by phase contrast under oil immersion using a 100× objective on a Nikon Eclipse E200 microscope and recorded with an Apple iPhone X mounted on a universal cell phone photography adapter (Amazon Standard Identification Number B07BC85SR7; Gosky Optics, China). Videos were cropped and converted to .mov files using Apple iMovie software.

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