Control of a Novel Spermatocyte-Promoting Factor by the Male Germline Sex Determination Factor PHF7 of *Drosophila melanogaster*

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ABSTRACT A key aspect of germ cell development is to establish germline sexual identity and initiate a sex-specific developmental program to promote spermatogenesis or oogenesis. Previously, we have identified the histone reader Plant Homeodomain Finger 7 (PHF7) as an important regulator of male germline identity. To understand how PHF7 directs sexual differentiation of the male germline, we investigated the downstream targets of PHF7 by combining transcriptome analyses, which reveal genes regulated by *Phf7*, with genomic profiling of histone H3K4me2, the chromatin mark that is bound by PHF7. Through these genomic experiments, we identify a novel spermatocyte factor Receptor Accessory Protein Like 1 (REEPL1) that can promote spermatogenesis and whose expression is kept off by PHF7 in the spermatogonial stage. Loss of *Reep1* significantly rescues the spermatogenesis defects in *Phf7* mutants, indicating that regulation of *Reep1* is an essential aspect of PHF7 function. Further, increasing REEPL1 expression facilitates spermatogenic differentiation. These results indicate that PHF7 controls spermatogenesis by regulating the expression patterns of important male germline genes.

KEYWORDS spermatogenesis; germline sex determination; *Phf7*; REEP family; Genetics of Sex

GONADS are efficient factories for producing gametes and are comprised of germ cells in progressive stages of differentiation and maturation. In the testis, germline stem cells (GSC) provide a constant source of new germ cells committed to undergoing spermatogenesis, a process that involves multiple rounds of mitotic division followed by meiosis and specialization to give rise to sperm. This is a well-orchestrated developmental sequence that requires many genes to function at the appropriate times and locations. These include expression of stage-specific transcription and translation factors as well as activation of appropriate signaling

pathways based on interactions with the somatic niche. As many species have germline stem cell populations in both the testis and the ovary, one key question is how sex determination in the germline influences the GSC and their ability to differentiate into either sperm or eggs.

In the *Drosophila* testis, multiple signaling pathways, including the JAK/STAT and BMP pathways, are known to mediate the communication between the somatic niche and the GSCs to ensure both GSC self-renewal and the production of progeny committed to differentiation (Kiger *et al.* 2001; Tulina and Matunis 2001; Kawase *et al.* 2004; Wawersik *et al.* 2005; Leatherman and Dinardo 2008, 2010). GSCs divide asymmetrically along a specific axis so that one of the daughter cells leaves the GSC niche after mitosis (Yamashita *et al.* 2003). These cells initiate spermatogenesis and turn on the prodifferentiation gene *bag-of-marbles* (*bam*) at the four-cell spermatogonia stage (Gönczy *et al.* 1997). Subsequently, members of the testis-specific TBP-associated factors (tTAFs) and meiotic arrest complex (tMAC) work together to turn on genes important for spermatocyte development and

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spermatid differentiation (Hiller *et al.* 2004; Chen *et al.* 2005; Beall *et al.* 2007).

Recently, we identified a novel histone reader named *Plant Homeodomain Finger 7* (*Phf7*) as a factor that determines male identity in the germline (Yang *et al.* 2012). It is expressed specifically in male germ cells and is required for normal spermatogenesis. Further, *Phf7* is sufficient to induce spermatogenesis in XX germ cells, provided they are localized in a male soma. The best known function of PHD domains is that they can associate with specific modified histone residues (Bienz 2006). A wide variety of proteins that associate with chromatin contain PHD domains, including histone modifiers, transcription factors, and DNA-modifying enzymes, and the most common mark that PHD domains bind to is H3K4me3, though other specificities have also been reported (Peña *et al.* 2006; Shi *et al.* 2006; Matthews *et al.* 2007; Hu *et al.* 2011). PHF7 appears to have an uncommon preference for binding to H3K4me2 (Yang *et al.* 2012), a modification that is associated with active transcription and has been linked to epigenetic memory and repression of cryptic promoters (Santos-Rosa *et al.* 2002; Kim and Buratowski 2009; Light *et al.* 2013). It is not yet determined whether the ability to associate with modified histones is essential for the functions of PHF7 in the male germline, nor is the molecular mechanism of how PHF7 works known. Interestingly, *Phf7* is also expressed specifically in the testis in mammals, and human *Phf7* is able to rescue defects observed in *Phf7*-mutant flies (Yang *et al.* 2012). Therefore, there are likely many parallels between how fly and human PHF7 function.

PHF7 expression turns on in male germ cells during embryonic stages and persists until adulthood (Yang *et al.* 2012). We show here that PHF7 is in the nuclei of GSCs and early spermatogonia, consistent with this factor being a germline chromatin reader. This prompted us to look for downstream targets of PHF7 using a whole-genome approach, and we find that PHF7 regulates sex-biased gene expression during adult spermatogenesis. Further, we identify one such repressed gene as *Receptor Accessory Protein Like 1* (*Reep1*), which is a testis-enriched gene that facilitates spermatogenesis. These results indicate that PHF7 is important for the maintenance of proper spermatogonial identity and the balance between different stages of male germline development.

Materials and Methods

Fly stocks and fecundity tests

The fly strains used are as follows: *w*¹¹¹⁸ (Bloomington Stock Center), *FM7a*, *Dfd-YFP* (Bloomington Stock Center), *nos-Gal4* (Van Doren *et al.* 1998), *bam*¹ (D. McKearin), *bam*¹¹⁴ (D. McKearin), *UAS-dPhf7* (Yang *et al.* 2012), *UAS-hPhf7* (Yang *et al.* 2012), and *Phf7*^{ΔN2} (Yang *et al.* 2012).

UAS-Reep1 was made by cloning a genomic fragment ranging from the start to the end of the longer *Reep1* isoform (isoform A) into pUASpB (Yang *et al.* 2012) and integrated into the fly genome via ΦC31-mediated site-specific integration.

Reep1^{CC4} was generated by CRISPR/Cas9 by expressing two short guide RNAs (5'-GATACACATACTGTCCCTTC-3' and 5'-GGAGCCTATTTCGAAGCCCA-3') in pBFv-U6.2 (Kondo and Ueda 2013). Successful deletion of the intervening region (11,572,836–14,573,431 bp on the X chromosome, Assembly R6.13) was confirmed by PCR and sequencing.

Fecundity of male flies was tested by mating single newly eclosed males to three virgin *w*¹¹¹⁸ females. The parents were removed on day 7 of the crosses, and the numbers of adult progeny were recorded on day 11.

Immunofluorescence staining, TUNEL labeling, and spermatocyte counts

Immunofluorescence staining on adult gonads was performed as previously described (Gönczy *et al.* 1997), and imaged on a confocal microscope (LSM780, Zeiss). Primary antibodies along with the concentrations used are as follows: rabbit-α-PHF7, 1:500; rabbit-α-VASA, 1:250 (d-260, Santa Cruz Biotechnology); goat-α-VASA, 1:250 (dC-13, Santa Cruz Biotechnology); rat-α-N-CADHERIN, 1:20 (EX-8, DSHB); mouse-α-α-SPECTRIN, 1:5 (3A9, DSHB); mouse-α-BAM, 1:25 (DSHB), rabbit-α-phospho-histone H3 (Ser10), 1:100 (Millipore). Embryos were sexed with a *Dfd-YFP* transgene on the X chromosome of the fathers. The antiserum for *Drosophila melanogaster* PHF7 was made in rabbits using as antigen a GST-tagged fragment containing amino acid residues 381–487 of PHF7 (LTK Biolaboratories). Labeling of dying spermatogonia with the TUNEL assay and determination of the numbers of spermatocytes within spermatocyte cysts were performed as previously described (Chang *et al.* 2013; Yacobi-Sharon *et al.* 2013).

In situ hybridization and RT-PCR

In situ hybridization in adult testes and embryos was carried out as previously described (Lehmann and Tautz 1994). A DIG-labeled (DIG RNA Labeling Mix, Thermo Fisher Scientific) antisense probe for *Reep1* was made using a complementary DNA (cDNA) fragment of the gene generated by PCR (forward primer: 5'-GAAT TAATACGACTCACTATAGGG GCTTTTTTTGTATAAACTTTATTGC-3'; reverse primer: 5'-GTAT CATACGATTTAGGTGACACTATAG TCAAACCAATGAACAAA-3'). Embryos were sexed with a *Dfd-YFP* transgene on the X chromosome of the fathers.

RT-PCR was done with cDNA samples (Fast RT Kit, Bio-Tools) converted from RNA extracted from different tissues (EasyPure Total RNA Reagent, Bioman). Primers for the PCR step are as follows: human REEP1: F-5'-GCACCTTTTACCCTGCGTAT-3', R-5'-TGCACAAGGGCATCGTAACT-3'; human REEP2: F-5'-TCCTGGACACCATCGAGGAC-3', R-5'-GGCCTCCTCCTCTTTTCCA-3'; human REEP3: F-5'-ACAGTTGCTTG GTTCCCCT-3', R-5'-AACAGCAGCAGTAGCTGCAA-3'; human REEP4: F-5'-TCCCAAAGGCCGGAAG-3', R-5'-CACCTGGTCC TCCAGGTAGA-3'; human REEP5: F-5'-GACAAGGCCAAAGAG ACTGC-3', R-5'-TGCAGGGAGAGCCAGTAAA-3'; human REEP 6: F-5'-CGGCCGGAATAACCAGGAAC-3', R-5'-GGATGCGCTG AAGACTCTGT-3'; *Reep1*: F-5'-TGCTCCGTTCTTAGCCATT-3',

R-5'-CTTTTGGCTATGGCATCGTT-3'; dREEP1: F-5'-TCCGAGG ATACTGACTCGGC-3', R-5'-AGGTGCTCTAAGCTGCCAAC-3'; CG4960: F-5'-GCGGCGTTACACAATTTTGATT-3, R-5'-CTGCAC CTGACTTACCTGGG-3'; CG5539: F-5'-GCCTGGGCCAAAT ACTGGAT-3', R-5'-ACTTGGTGGGCATGTAGAGC-3'; CG8331: F-5'-CTACAACAAGCTGGTGCGAC-3', R-5'-ACTCCAGCGGCT TTCTTCAT-3'.

RNA-seq and ChIP-seq

For RNA-seq, 50 ng of oligo-dT-selected mRNA (Dynabeads Oligo dT, Thermo Fisher Scientific) isolated from total testis RNA (RNA-BEE, Tel-Test) was converted into barcoded cDNA libraries following the manufacturer's directions (NEXTflex Directional RNA-Seq Kit, Bioo Scientific) and sent for multiplexed high-throughput single-end sequencing at the IIGB Genomics Core Facility at the University of California (UC), Riverside. Using Tophat2, >90% of all reads were mapped to the *Drosophila* genome (BDGP R5/dm3), and ~60% of reads were unique matches. The uniquely mapped reads (14–23 million per library) were used for transcriptome analyses. Assembly of the transcriptome data and comparisons between datasets were carried out using Cufflinks and Cuffdiff through the Galaxy platform. The false discovery rate (FDR) for genes with significant expression changes between the samples was 0.05.

To perform ChIP, testes were fixed in 1% formaldehyde for 1 hr at 37°, washed in PBS, and resuspended in SDS lysis buffer (50 mM Tris-HCl pH 8, 1% SDS, 10 mM EDTA) to be homogenized using motorized plastic pestles. The homogenates were diluted to 250 μ l with ChIP dilution buffer (16.7 mM Tris-HCl pH 8, 167 mM NaCl, 1.2 mM EDTA, 1.1% Triton X-100) for sonication. After removal of cellular debris, the supernatants were diluted to 10-fold the volume of the SDS lysis buffer added with ChIP dilution buffer. 1/10 volumes were saved as input, and antibodies were added to the remaining samples for pull-down overnight at 4°. All complexes were immunoprecipitated by the addition of protein G-sepharose beads (GE Healthcare Life Sciences), and washed with three wash buffers (wash buffer #1: 20 mM Tris-HCl pH 8, 150 mM NaCl, 2 mM EDTA, 1% Triton X-100, 0.1% SDS; wash buffer #2: 20 mM Tris-HCl pH 8, 500 mM NaCl, 2 mM EDTA, 1% Triton X-100, 0.1% SDS; wash buffer #3: 10 mM Tris-HCl pH 8, 0.25 M LiCl, 1 mM EDTA, 1% NP40, 1% sodium deoxycholate) and twice more with TE pH 8. Inputs were diluted to 250 μ l, IP samples were resuspended in 250 μ l, and all underwent reverse cross-linking using proteinase K (1 μ g/ml) in 0.3% SDS at 65° overnight. DNA was recovered by phenol-chloroform extraction and resuspended in water. Antibodies used for IP were α -H3K4me2 (1:120, Y47, Abcam) and α -H3K4me3 (1:300, ab8580, Abcam).

For ChIP-seq, 10 ng of input or ChIP DNA samples were converted into barcoded ChIP-seq libraries according to manufacturer's instructions (NEXTflex ChIP-seq Kit, Bioo Scientific) and sent for multiplexed high-throughput single-end sequencing at the IIGB Genomics Core Facility at UC

Riverside. At least 50% of reads in each library were uniquely mapped to the *Drosophila* genome (BDGP R5/dm3) using Bowtie. Peaks of H3K4me2 and H3K4me3 were called by comparing IP results to the respective input samples using MACS at MFOLD (3, 30) and $P = 1 \times 10^{-3}$ (Zhang *et al.* 2008).

Data availability

Strains used are available upon request. The RNA-seq and ChIP-seq datasets are available at the National Center for Biotechnology Information (NCBI) Gene Expression Omnibus (GSE98968).

Results

PHF7 is a male germline-specific nuclear protein

Phf7 encodes a histone reader that associates with H3K4me2 and drives the male sexual program in the germline. Previously we generated N- and C-terminally HA-tagged PHF7 in the context of genomic rescue constructs, but the two tagged proteins exhibited different subcellular localization patterns, with one primarily nuclear and the other cytoplasmic (Yang *et al.* 2012). To resolve this discrepancy, we generated an antiserum against a protein fragment in the C-terminus of PHF7, and this allowed us to determine the expression pattern of the endogenous protein. Immunofluorescence staining of the adult gonads showed that PHF7 is clearly present in the nuclei of GSCs and spermatogonia up to the early 16-cell stage in the testis (Figure 1, A and A'). There are also low levels of signal in the nuclei of a few early spermatocytes, but the levels are much lower than those in earlier germline. The antibody is specific for PHF7 as staining in *Phf7*-mutant testes was minimal (Figure 1, B and B'). Consistent with PHF7 being expressed specifically in the male germline, we also observed only background PHF7 staining in the ovary (Figure 1, C and C'). Previously, we observed that *Phf7* RNA is expressed in a much larger domain of the testis than the HA-tagged proteins (Yang *et al.* 2012). Immunostaining with the PHF7 antibody confirms that, while RNA expression is in a broader range of germline in the testis, protein expression is restricted to spermatogonia, and its levels are reduced abruptly after the early 16-cell stage coincident with the BAM expression domain (Figure 1, E–E'''). These results demonstrate that PHF7 is a male germline-specific nuclear protein, consistent with a role as a chromatin factor. The different subcellular localization observed with the HA-tagged PHF7 is likely due to the balance of protein shuttling between nucleus and cytoplasm being altered by the tag.

Genomics approaches to identifying *Phf7* targets

As PHF7 binds modified histones, we hypothesized that it is a chromatin-associated factor that localizes to, and regulates expression of, specific loci in the male germline genome. To identify PHF7 target genes, we carried out transcriptome comparisons between adult testes that were wild-type or mutant for *Phf7*. In addition, to enrich for the PHF7-expressing cell population, these testes were also mutant for *bam* (*bam*¹/*bam*¹¹⁴),

Figure 1 Expression patterns of PHF7 in the adult testis and ovary. (A and A') Adult w^{118} testis; (B and B') adult $Phf7^{\Delta N2}$ testis; (C and C') adult w^{118} ovary; (D and D') adult bam^1/bam^{114} testis. The antibodies used in A–D recognize PHF7 (green), VASA (red), and N-CADHERIN (blue); A'–D' displays the PHF7 signal alone. (E–E''') Colocalization of BAM and PHF7 staining in early germ cells of adult w^{118} testis. The antibodies used stain BAM (green), PHF7 (red), and VASA (blue); E' and E'' display the PHF7 and BAM signal alone, respectively. E''' displays the DAPI signals.

which arrests spermatogenesis at a stage that still expresses PHF7, creating testes filled with younger stage, PHF7-expressing germline (Figure 1, D and D'). We carried out high-throughput, strand-specific RNA sequencing using cDNA libraries of testes from newly eclosed *bam*- and *Phf7*, *bam*-mutants. Two biological replicates for each genotype were carried out to increase robustness of our results. Fourteen to 23 million uniquely mapped reads were obtained for each sample for transcriptome analysis. There was good agreement between the biological replicates; correlation coefficients were 0.968 (between *bam*- replicates) and 0.960 (between *Phf7*-*bam*- replicates).

Surprisingly, only 45 genes exhibited significant expression changes in both sets of replicates (FDR = 0.05, Figure 2A and Supplemental Material, File S1); expression of *Phf7* was also significantly reduced in the *Phf7*-*bam*- sample compared to controls and was not counted in the 45 candidate targets (Figure S1A in File S1). A subset of the 45 genes was validated by RT-qPCR with cDNA made from independently purified *Phf7*-, *bam*-, and *bam*- testis RNA and were found to have the same expression changes as observed in our

RNA-seq results (Figure S1B in File S1). Among the 45 genes, ~75% (34/45) were upregulated in *Phf7*, *bam*-mutants compared to *bam*-mutants, whereas the rest showed lower expression in the double-mutant samples. We then asked whether the genes altered in *Phf7* mutants were biased toward genes that are normally expressed sex-specifically by examining their expression in normal testes and ovaries using the tissue transcriptome data from modENCODE as well as the transcriptome comparisons between *bam*- testis vs. ovary (Brown et al. 2014; S. Primus and M. Van Doren, unpublished data). Interestingly, the majority of genes regulated by *Phf7* exhibit male-biased expression (41/45, 91%, Figure 2B and Figure S2 in File S1). While these genes may be directly or indirectly regulated by PHF7, these results are consistent with *Phf7* being involved in male germline development by regulating a highly specific set of sex-biased genes.

To determine which of these genes may be directly regulated by PHF7, we first attempted to localize PHF7 binding in the genome using ChIP-seq but were not able to obtain robust signals using available reagents. Since PHF7 binds to

Figure 2 Genomics analysis of possible *Phf7* target genes. (A) Transcriptome comparisons of *Phf7*⁻, *bam*⁻, and *bam*⁻ testes revealed 45 candidate genes whose expression is significantly different between testes of the two genotypes. The plot shows expression in *Phf7*⁻/*bam*⁻/*bam*⁻ testes. The orange bars indicate genes that have the H3K4me2 modification and are regulated significantly by *Phf7*. (B) Expression of the

Table 1 Summary of ChIP-seq analyses for H3K4me2 and H3K4me3

Chromatin modification	Peaks in replicate 1	Peaks in replicate 2	Common peaks ^a	Unique peaks ^b	<i>Phf7</i> -regulated genes ^c
H3K4me2	3532	3200	3081	520	8
H3K4me3	4859	4963	4513	1047	4

^a This number indicates the common regions from the two biological replicates for the respective modifications.

^b This number indicates out of the common peaks those that carried only the designated modification and not the other.

^c This number indicates the number of *Phf7*-regulated genes as determined by the RNA-seq analysis that carries the respective modification. All genes with H3K4me3 also carried H3K4me2.

H3K4me2, we therefore turned to profiling the H3K4me2 and H3K4me3 landscapes, again using *bam*-mutant testes for these experiments. It has not yet been shown that binding of PHF7 to H3K4me2 is required for the actions of PHF7, and we also do not expect that PHF7 would localize to all loci carrying the H3K4me2 modification. Nonetheless, we reasoned that obtaining the H3K4me2 profile may assist us in prioritizing which genes are more likely to be direct targets of PHF7.

Two biological replicates were performed for ChIP-seq of H3K4me2 and H3K4me3, and the results from the independent replicates were highly similar, with >90% of peaks called for each modification shared between the replicate samples (Table 1). H3K4me2 is known to be more highly enriched in the 5' portion of genes while H3K4me3 is localized to promoter areas, and our results are consistent with such a pattern (Figure 2D and Figure S3 in File S1). Of the 45 genes exhibiting significant expression changes in *Phf7* mutants compared to in control samples, eight of them also exhibited H3K4me2 peaks (orange bars, Figure 2A and Figure S3, A–H in File S1). Interestingly, *Phf7* itself is also enriched for H3K4me2 (Figure S3 in File S1). It is possible that the genes that are affected in *Phf7* mutants that do not bear H3K4me2 marks are indirect targets of PHF7; alternatively, PHF7 may be recruited to the chromatin through a mechanism independent of H3K4me2 binding.

Of all *Phf7*-regulated genes, *Reep1* (Receptor Accessory Protein Like 1, *CG11697*) was particularly interesting as it exhibits one of the largest expression fold changes upon loss of *Phf7* (Figure 2, A and C). Further, this gene carries H3K4me2 but not H3K4me3, which is consistent with the *in vitro* histone tail binding preferences of PHF7 (Figure 2D). For these reasons, our subsequent analyses focused on how *Reep1* may act downstream of PHF7.

***Reep1* expression is regulated by PHF7**

To investigate the link between *Phf7* and *Reep1*, we started by analyzing the expression of *Reep1*. RT-PCR of various fruit fly tissues revealed that *Reep1* is expressed exclusively in the

testis (Figure 3G). In addition, *in situ* hybridization of *Reep1* showed that, in the absence of *Phf7*, *Reep1* expression in the testis is elevated compared to wild-type controls (Figure 3, A and B). The *in situ* stains are specific as *Reep1*-mutant testes exhibit only background levels of signal (Figure 3C). These expression results are consistent with our transcriptome analysis. We further examined the expression of *Reep1* in the embryonic germline and find that, unlike *Phf7*, it is not detected in the embryonic gonad (Figure 3, D and E). In addition, in contrast to the adult testis, loss of *Phf7* in the embryonic testis does not activate *Reep1* expression (Figure 3F). Taken together, PHF7 acts as a repressor of *Reep1* in the undifferentiated adult male germline, though the directness of the relationship will have to be resolved in the future.

Based on protein sequence comparisons, REEP1 shares the closest homology to the Receptor Accessory Protein (REEP) genes. REEP genes are ER-shaping proteins whose mutations are associated with motor neuron diseases and retinitis pigmentosa (Züchner *et al.* 2006; Argasinska *et al.* 2009; Björk *et al.* 2013; Esteves *et al.* 2014; Arno *et al.* 2016). In *D. melanogaster*, there are four others that belong to this protein family whereas six REEPs are found in humans (Figure S4 in File S1). Phylogenetic analysis indicates that the evolution of the REEP family is complex and that REEP1 does not correspond to one specific mammalian counterpart. Of the human proteins, REEP1 is most similar to REEP5, but it is not the *Drosophila* REEP most similar to REEP5, and hence was given the name *REEP Like1* (*Reep1*).

We further investigated the expression patterns of all REEPs. Intriguingly, all REEP genes in *Drosophila*, with the exception of *CG8331*, exhibited a strong bias toward being expressed in the testis (Figure 3G). However, cross-examination with our own transcriptome database comparing *Phf7*-, *bam*-, and *bam*-mutant testes revealed that only *Reep1* expression is altered in *Phf7* mutants. These results suggest that multiple REEP genes in *Drosophila* may collectively participate in spermatogenesis, but only *Reep1* is regulated by *Phf7*. In humans, several REEPs have been reported to be expressed in the nervous system and other tissues including the testis

candidate genes from A in adult testes (blue bars) and ovaries (red bars) based on the modENCODE tissue expression database. The maximum value for the y-axis is set to 100 so that lower levels of expression can be visualized. The expression values for those higher than 100 are indicated on top of the respective bars. (C) RNA-seq profiles of both *Phf7-bam*- (blue) and *bam*- (red) testis samples in the genomic region around *Reep1* (*CG11697*). The green arrow underneath the *Reep1* gene name label indicates direction of transcription. (D) Profiles of H3K4me2 (top) and H3K4me3 (bottom) ChIP-seq in *bam*- testis samples in the genomic region around *Reep1*. The gene region for *Reep1* in both C and D is marked with green dotted lines.

(Saito *et al.* 2004; Behrens *et al.* 2006; Hurt *et al.* 2014). Using RT-PCR with cDNA samples from various human tissues, we find that while human REEPs appear to be expressed much more broadly than in *Drosophila*, all are expressed in the testis, and in several cases their expression is enriched in the testis (Figure 3H). It is worth noting that a recent report showed that expression of REEP1 and REEP2 proteins is far less ubiquitous than suggested by RT-PCR analysis and the two proteins are found predominantly in the brain and the testis (Hurt *et al.* 2014). It is possible that other REEPs also exhibit a similar brain and testis bias at the protein expression level. These results are consistent with there being a common role of REEP genes in spermatogenesis.

REEP1 accelerates spermatogenesis

To gain insights into the function of *Reep1* in the testis, we first investigated whether overexpression of *Reep1* would have an impact on spermatogenesis. This was done by examining male flies carrying an upstream activating sequence (UAS)-driven *Reep1* construct (*UAS-Reep1*) and *nanos-Gal4* (*nos-Gal4*), which together express *Reep1* only in germ cells. This caused a twofold overexpression of *Reep1* RNA without dramatically changing its spatial pattern of expression (Figure 4, A–C). We further characterized different stages of germline development in testes that overexpress *Reep1*. Interestingly, we observed that, while all stages of germline development are present, there is clear evidence of increased spermatogenesis. The numbers of spermatogonial cysts are elevated when *Reep1* is overexpressed in the germline as documented by scoring the number of BAM+ 4- to 8-cell cysts (Figure 4D). Moreover, overexpression of *Reep1* in the germline also enhances fecundity of such male flies (Figure 4E). The increase in BAM+ spermatogonial cysts is not due to premature commitment of spermatogonia to spermatocytes as virtually all spermatocyte cysts (98.4%) in *Reep1*-overexpressing testes contain 16 spermatocytes, though we will note that there are occasional eight-cell spermatocyte cysts in *nos-Gal4/UAS-Reep1* testes (Figure 4F, Figure S5, A–C in File S1).

The increase in spermatogonial cysts upon *Reep1* overexpression could be caused by either an increase in GSC number or in their rate of division. However, no differences in GSC number were observed (Figure 4G). We did observe a small increase in GSC division as assayed by phospho-histone H3 (pH 3) staining, but this was not statistically significant (Figure 4H). We also analyzed whether the increase in spermatogenic cysts could be due to a decrease in germline cyst death. However, no significant changes in germline death were observed by TUNEL staining upon *Reep1* overexpression (Figure 4I).

Loss of *Reep1* in *Phf7* mutants restores normal spermatogonia development

To further investigate the possible roles that *Reep1* have in spermatogenesis, we generated a mutant of *Reep1* by CRISPR/Cas9 in which ~90% of the coding sequence is de-

Figure 3 Expression analyses of *Reep1* (CG11697) and related genes. (A–F) *In situ* hybridization of *Reep1* on a wild-type adult testis (A), a *Phf7^{ΔN2}* adult testis (B), a *Reep1^{CC4}* (CG11697-mutant) adult testis (C), a wild-type st. 17 female embryo (D), wild-type st. 17 male embryo (E), and *Phf7^{ΔN18}* st. 17 male embryo (F). Images are taken with a 10× objective. (G) RT-PCR analysis of *Reep1* and other REEP-related genes in gonads and other tissues of *Drosophila*. Ts, adult testis; MC, adult male carcass; Ov, adult ovary; FC, adult female carcass; L3Ts, 3rd-instar larval testis; L3MC, 3rd-instar male carcass; L3F, 3rd-instar female larva. RT indicates whether the lane included reverse transcriptase during reverse transcription; NTC is no template control. (H) RT-PCR analysis of human REEP homologs across different tissues. Co, colon; Th, thymus; Int, small intestine; Lk, leukocytes; Ov, ovary; Pr, prostate; Sp, spleen; Ts, testis. TUB is the internal control using tubulin primers; NTC is no template control.

leted. However, we found that male fecundity, development of various germline populations, and the size and shape of the testis are all similar to controls (Figure 5, A, B, and F). Given that all five REEP family genes are expressed in the testis, it is possible that they function redundantly.

Phf7 is required for normal spermatogenesis, and *Phf7* mutants exhibit decreased male fecundity and a reduction in the number of BAM-positive germline cysts (Yang *et al.* 2012). The reduction in BAM+ cysts is not due to their precocious commitment to spermatocytes as all spermatocyte cysts we analyzed contained 16 spermatocytes ($n = 215$, Figure S5D in File S1). As *Reep1* is strongly upregulated in *Phf7* mutants, we wanted to test whether the upregulation of *Reep1* contributes to the spermatogenesis defects in *Phf7* mutants. This was carried out by examining testes mutant for both *Phf7* and *Reep1* to see if the loss of *Reep1* would restore normal spermatogonial development in *Phf7*-mutant testes. We find that the loss of fecundity observed in *Phf7* mutants (*Phf7^{ΔN2}*) is rescued in males simultaneously mutant for *Reep1* (*Phf7^{ΔN2}, Reep1^{CC4}*) to levels similar to those observed when restoring *Phf7* expression (*Phf7^{ΔN2}, nos-Gal4/UAS-Phf7*, Figure 5A). Moreover,

Figure 4 Effects of *Reep1* overexpression in spermatogenesis. (A and B) *In situ* hybridization of *Reep1* in *nos-Gal4/+* (A) and *nos-Gal4/UAS-Reep1* (B) adult testes. (C) qRT-PCR analysis of *Reep1* in the *nos-Gal4/+* and *nos-Gal4/UAS-Reep1* adult testes normalized with *Actin5C* expression. (D) Numbers of all BAM+ spermatogonial cysts in either controls (*nos-Gal4/+*) or *Reep1* overexpressing flies (*nos-Gal4/UAS-Reep1*). BAM+ cyst counts were grouped into three intervals, 4–7 (dotted), 8–11 (gray), and 12–15 (black). The data are presented as the percentage of testes of each genotype that fall into each interval. (E) Average progeny produced by single male flies of either the controls (*nos-Gal4/+*) or *Reep1* overexpressing flies (*nos-Gal4/UAS-Reep1*). (F) Numbers of spermatocytes per cyst in either controls (*nos-Gal4/+*) or *Reep1* overexpressing flies (*nos-Gal4/UAS-Reep1*). (G) Average numbers of GSCs per testis of controls (*nos-Gal4/+*) or *Reep1* overexpressing flies (*nos-Gal4/UAS-Reep1*). (H) Average numbers of phosphorylated histone H3 + cells or cysts of various stages (GSCs, gonialblasts, 2-, 4-, and 8-cell cysts) in controls (*nos-Gal4/+*) or *Reep1* overexpressing flies (*nos-Gal4/UAS-Reep1*). (I) Numbers of TUNEL+ spermatogonial cysts in each testis of controls (*nos-Gal4/+*) or *Reep1* overexpressing flies (*nos-Gal4/UAS-Reep1*). In D–I, numbers in parentheses indicate sample sizes. For H, in each set of parentheses the first number is for *nos-Gal4* and the second is for *nos-Gal4/UAS-Reep1*. *Indicates statistical significance of $P < 0.05$. Numbers in parentheses indicate sample sizes.

the reduction in BAM-positive cysts observed in *Phf7* mutants is significantly rescued in *Phf7*, *Reep1* double mutants (Figure 5, B–F). It is seemingly contradictory that directed overexpression of *Reep1* accelerates spermatocyte development (Figure 4), yet loss of *Phf7*, which also causes increased expression of *Reep1*, is detrimental to spermatogenesis. This may be due to the fact that *Reep1* overexpression appears even higher in *Phf7* mutants (compare Figure 3, A and B to Figure 4, A and B) or to other differences caused by loss of *Phf7*. Regardless, the ability of *Reep1* loss to suppress the *Phf7* mutant phenotype indicates that *Reep1* mis-expression is an important component of the abnormal spermatogenic development caused by loss of *Phf7*. We conclude that PHF7 influences the spermatogenic program by regulating downstream factors including *Reep1*.

Discussion

Phf7 regulates both germline sex determination and spermatogenesis

In this work, we show that in addition to regulating male germline sex determination, PHF7 also controls spermatogenesis partly by regulating *Reep1*. One interesting question that remains unanswered is the molecular mechanisms by which PHF7 acts. PHF7 contains three PHD domains in its N-terminus that presumably mediate its interaction with H3K4me2-modified chromatin, whereas the C-terminus bears no resemblance to any known protein domains. Recently we found that the three PHD domains are sufficient to rescue the fecundity defects of *Phf7* mutant male flies (Wang *et al.* 2017). It is possible that upon association of

Figure 5 Analysis of *Reep1* function in spermatogenesis. (A) The effect of loss of *Phf7* or *Reep1* function on male fecundity indicated with boxplots and whiskers. The top, bottom, and lines in the middle of the boxplots indicate the 75th-percentile, 25th-percentile, and median of each genotype, respectively. The whiskers indicate the 90th- and 10th-percentile points. *Phf7^{ΔN2}; nos-Gal4/UAS-Phf7*, mutant of *Phf7* rescued with cDNA construct as positive control; *Phf7^{ΔN2}; nos-Gal4/+*, mutant of *Phf7* with a copy of *nos-Gal4*; *Phf7^{ΔN2}*, mutant of *Phf7*; *Phf7^{ΔN2}, Reep1^{CC4}, Phf7*, *Reep1* double mutant; *Reep1^{CC4}*, *Reep1* mutant. The numbers in parentheses indicate sample sizes. (B) BAM + spermatogonia cyst counts of control (*w¹¹¹⁸*), *Phf7*-mutant (*Phf7^{ΔN2}*), *Phf7*, *Reep1*-double mutant (*Phf7^{ΔN2}, Reep1^{CC4}*), and *Reep1*-mutant (*Reep1^{CC4}*) testes. BAM + cyst counts were grouped into three categories, ≤ 3 (cross hatched), 4–7 (light gray), and at least 8 (dark gray). The data are presented as the percentage of testes of each genotype that fall into each category. In A and B, $*P < 0.05$. (C–F) Representative images of wild-type (*w¹¹¹⁸*, C), *Phf7*-mutant (*Phf7^{ΔN2}*, D), *Phf7*, *Reep1*-double mutant (*Phf7^{ΔN2}, Reep1^{CC4}*, E), and *Reep1*-mutant (*Reep1^{CC4}*, F) testes stained with BAM (green), VASA (red), and N-CADHERIN (blue) antibodies. (C'–F') displays the BAM signal from C–E with the hubs outlined by white-dotted circles.

PHF7 with H3K4me2, located at the 5' end of the target gene body, PHF7 inhibits transcription of the target genes by directly blocking access of chromatin remodelers and transcriptional coactivators, or by recruiting transcriptional corepressors. It is also important to note that H3K4me2 is most likely localized to many more gene loci than those that are targets of PHF7. Therefore, there are probably yet unidentified interacting partners of PHF7 that help determine the target selectivity of PHF7. These are interesting ideas that will be addressed in the future to understand the

molecular details of how PHF7 modulates male germline gene expression.

Reep1 is a critical target of PHF7 regulation

In this work we identify a novel spermatocyte factor *Reep1* downstream of PHF7. Loss of *Phf7* leads to derepression of *Reep1* expression in spermatogonia and spermatocytes (Figure 3B), and the fact that *Reep1* also bears the H3K4me2 mark suggests that it is a candidate for direct regulation by PHF7. Interestingly, the substantial increase in *Reep1* RNA

observed in *Phf7* mutants extends beyond the domain where PHF7 is expressed (compare Figure 3B to Figure 1A). It could be that PHF7 regulates *Reep1* expression at early stages, which then persists into later stages. Alternatively, this could be due to PHF7-dependent epigenetic changes that are established earlier and which repress *Reep1* in spermatocytes. In comparison, though PHF7 is also expressed in the embryonic testis (Yang *et al.* 2012), we do not detect *Reep1* expression in the gonads of either sex, or in *Phf7* mutant embryos. These observations suggest that *Reep1* likely regulates only later aspects of male germline identity and spermatogenesis. There may yet be other targets of *Phf7* during early germline development to be investigated in the future.

Reep1 is an important pro-spermatogenesis factor downstream of PHF7 as loss of *Reep1* function is able to significantly rescue the spermatogenesis defects observed in *Phf7* mutants (Figure 5). *Phf7*-mutant males produce fewer progeny and harbor fewer differentiating spermatogonial cysts in their testes, and both of these defects are greatly rescued when *Reep1* is also mutated. However, unlike *Phf7*, *Reep1* mutants alone do not cause any defects in spermatogenesis that we could observe. This may be due to functional redundancies of *Reep* genes as multiple family members show enriched expression in the testis (Figure 3G), though only *Reep1* is regulated by *Phf7*. Interestingly, moderate overexpression of *Reep1* enhances spermatogenesis while we observed retarded spermatogonial development and reduced fecundity in *Phf7* mutants in which greater derepression of *Reep1* expression occurs. The dose-dependent effects of *Reep1* highlight the importance of expressing genes at the right levels and at the right time for proper germline development. Moreover, our results show that PHF7 regulates key spermatogenesis genes to maintain the balance of different developing germline stages and achieve efficient sperm production.

Reep genes and spermatogenesis

There are five *Reep* family genes in *Drosophila*, and expression of four of them are specific in the testis (Figure 3G). In humans, *Reep* genes are more broadly expressed but also show some enrichment in the testis (Figure 3H), suggesting that REEP proteins may be generally important for spermatogenesis. REEP proteins were first described as molecules capable of enhancing expression of olfactory receptors in cultured cells (Saito *et al.* 2004; Behrens *et al.* 2006). Further studies indicated that REEPs are determinants of ER structure and can facilitate trafficking of transmembrane proteins, in particular G-protein-coupled receptors (GPCRs) such as olfactory receptors (Beetz *et al.* 2008; Schlang *et al.* 2008; Park *et al.* 2010; Björk *et al.* 2013). Mutations in *Reep* genes have been associated with motor neuron diseases and retinitis pigmentosa (Züchner *et al.* 2006; Argasinska *et al.* 2009; Esteves *et al.* 2014; Arno *et al.* 2016). Interestingly, olfactory receptors have long been known to be expressed in the male germline and have even been implicated in the chemotaxis of human sperm (Parmentier *et al.* 1992; Spehr 2003).

Given the general role of REEP proteins in ER structure and promoting proper expression of GPCRs, our current hypothesis is that REEP family members regulate the expression of GPCRs or other transmembrane proteins that are essential for the normal progression of spermatogenesis. These may represent key signaling molecules that control different stages of spermatogenesis and the transitions in between. Indeed, a number of GPCRs are expressed in a sex-biased manner in the germline (S. Primus and M. Van Doren, unpublished results) that are candidates for regulation by REEP1. It will be of interest in the future to examine whether REEP-dependent transmembrane proteins are important for male germline development and spermatogenesis.

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