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The Role of Y Chromosome Genes in Male Fertility in *Drosophila melanogaster*

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ABSTRACT The Y chromosome of *Drosophila melanogaster* is pivotal for male fertility. Yet, only 16 protein-coding genes reside on this chromosome. The Y chromosome is comprised primarily of heterochromatic sequences, including DNA repeats and satellite DNA, and most of the Y chromosome is still missing from the genome sequence. Furthermore, the functions of the majority of genes on the Y chromosome remain elusive. Through multiple genetic strategies, six distinct segments on the Y chromosome have been identified as "male fertility factors," and candidate gene sequences corresponding to each of these loci have been ascribed. In one case, *kl-3*, a specific protein coding sequence for a fertility factor has been confirmed molecularly. Here, we employed CRISPR/Cas9 to generate mutations, and RNAi, to interrogate the requirements of protein coding sequences on the Y chromosome for male fertility. We show that CRISPR/Cas9-mediated editing of *kl-2* and *kl-5* causes male sterility, supporting the model that these gene sequences correspond to the cognate fertility factors. We show that another gene, *CCY*, also functions in male fertility and may be the *ks-2* fertility factor. We demonstrate that editing of *kl-2*, *kl-3*, and *kl-5*, and RNAi knockdown of *CCY*, disrupts nuclear elongation, and leads to defects in sperm individualization, including impairments in the individualization complex (IC) and synchronization. However, CRISPR/Cas9 mediated knockout of some genes on the Y chromosome, such as *FDY*, *Ppr-Y*, and *Pp1-Y2* do not cause sterility, indicating that not all Y chromosome genes are essential for male fertility.

KEYWORDS *Drosophila melanogaster*; CRISPR/Cas9; Y chromosome; male fertility; individualization complex

THE *Drosophila* Y chromosome contains ~40 Mb of DNA accounting for ~13% of the male genome, and is nearly all heterochromatic (Heitz 1933; Brosseau 1960; Kennison 1981; Hazelrigg *et al.* 1982; Gatti and Pimpinelli 1983). More than 70% of the Y chromosome is highly repetitive and consists of satellite DNA (Peacock *et al.* 1978). Despite the considerable size of the Y chromosome, the density of protein-coding sequences is extremely low (Piergentili 2010). Based on experimental and computational methods, including gene sequence scaffolds produced by the *Drosophila* Genome Project, it appears that there are 16 protein coding sequences: 12 single copy genes, and *Mst77Y*, which is multi-copy (Figure 1A) (Russell and Kaiser 1993; Carvalho *et al.* 2001; Piergentili and Mencarelli 2008; Vibranovski *et al.* 2008; Krsticevic *et al.* 2010; Piergentili 2010; Gunes and Kulac 2013; Carvalho *et al.* 2015; Hoskins *et al.* 2015; Krsticevic *et al.* 2015). Due to gaps in the Y chromosome sequence, and difficulties in performing fluorescent *in situ* hybridizations on heterochromatin, the map positions of many of these genes are not defined precisely (Gepner and Hays 1993; Carvalho *et al.* 2000; Carvalho *et al.* 2001; Koerich *et al.* 2008; Vibranovski *et al.* 2008; Hoskins *et al.* 2015).

The Y chromosome is critical for male reproduction, but is not necessary for viability, as *Drosophila* XO males are healthy, but infertile (Bridges 1916a,b; Stern 1929; Voelker and Kojima 1971). Using chromosomal deficiencies, translocations, temperature-sensitive mutations, and complementation tests, multiple groups have established that the Y chromosome includes six distinct intervals that are required for male fertility (male-fertility factors) (Stern 1929; Shen 1932; Brosseau 1960; Ayles *et al.* 1973; Hardy *et al.* 1981;

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Kennison 1981; Hazelrigg *et al.* 1982; Gatti and Pimpinelli 1992; Zhang and Stankiewicz 1998). Four of these male fertility factors (*kl-5*, *kl-3*, *kl-2*, and *kl-1*) are situated in *YL*, and the remaining two (*ks-1* and *ks-2*) are in YS (Figure 1A) (Stern 1929; Shen 1932; Brosseau 1960; Ayles *et al.* 1973; Hardy *et al.* 1981; Kennison 1981; Hazelrigg *et al.* 1982; Gatti and Pimpinelli 1992; Zhang and Stankiewicz 1998).

Ten protein-coding genes on the Y chromosome map to the general regions assigned as six male fertility factors (Figure 1A), and are therefore candidate sequences encoding male fertility genes. Three genes, *kl-3*, *kl-2*, and *kl-5* (same names as the corresponding male fertility factors), encode dyneins (Gepner and Hays 1993; Carvalho *et al.* 2000), which function as microtubule motors to power flagella movements (Johnson *et al.* 1984). Disruption of either *kl-3* or *kl-5* with large deficiencies causes loss of the outer dynein arm of the axoneme in the sperm tail (Goldstein *et al.* 1982). While there are one or more candidate gene sequences associated with each male fertility factor, only in the case of *kl-3* has a protein coding region on the Y chromosome been specifically targeted through site-directed mutagenesis, and this was accomplished through CRISPR/Cas9-mediated editing (Yu *et al.* 2013).

The Y chromosome in flies shares some features with the human Y chromosome, which also has a relatively large proportion of heterochromatin, and encodes a small number of genes (54 in total) (Krausz and Casamonti 2017; Colaco and Modi 2019). As in flies, it was originally thought that the human Y chromosome functions exclusively in sex determination and fertility. However, some of these genes may have other roles, ranging from immune function to neurological roles (Colaco and Modi 2019).

In this work, we employed CRISPR/Cas9 to target genes on the Y chromosome, and were able to mutate seven genes. We also employed RNAi to suppress the expression of genes that we could not mutate by CRISPR/Cas9. We found that editing of the *kl-2* and *kl-5* genes by CRISPR/Cas9 resulted in male sterility, supporting the model that these are the coding sequences corresponding to the *kl-2* and *kl-5* male fertility factors (Stern 1929; Kennison 1981; Gepner and Hays 1993; Carvalho *et al.* 2000). We also demonstrate that knockdown of *CCY* causes male sterility, and propose that this gene is the *ks-2* male fertility factor. We also show that knockout or knockdown of four male fertility genes impairs spermatogenesis, including nuclear elongation, and individualization. In contrast to these effects, our data indicate that some Y chromosome genes are not essential for male fertility.

Materials and Methods

Drosophila culture and fly lines

Flies were cultured on standard fly food consisting of cornmeal, flour, agar, and yeast medium at 25°. The *tubulin-Gal4 > RNAi* flies were cultured at 29° to enhance Gal4 activity. w^{1118} was used as the control wild-type strain, and for embryo injections for targeted gene disruption using CRISRP/Cas9. The following fly strains were used in this study: w^{1118} (#5905; Bloomington Drosophila Stock Center); *tubulin-Gal4* (#5138; Bloomington Drosophila Stock Center); *ARY-RNAi* [#330637; Vienna Drosophila Resource Center (VDRC)], *ORY-RNAi* (#110132; VDRC), *Pp1-Y1-RNAi* (#110123; VDRC), *PRY-RNAi* (#58235; Bloomington Drosophila Stock Center) and *CCY-RNAi* (#61959; Bloomington Stock).

Male fertility tests

Fertility tests for males were performed over a 16-day period as described (Wen *et al.* 2016). To conduct the assays, we crossed one virgin male with one w^{1118} virgin female in a standard fly vial at 25°. Every 2 days over the course of 16 days, we transferred the parent flies to a new vial. To determine the number of progeny, we counted the numbers of adults that eclosed, and calculated the average number of progeny for each genotype ($n = 20$ vials/genotype).

RNA extraction and RT- PCR

Total RNAs were extracted from w^{1118} testes, male thoraxes, whole males and females and *tubulin-Gal4 > RNAi* male testes using TRIzol (Invitrogen), and treated with 2 μl DNase I (#M0303L; NEB) to remove residual DNA. The concentrations of the total RNAs were measured using a Nanodrop (Thermo 2000c; Thermo Fisher Scientific); 2 μg total RNA was used for cDNA synthesis (TIANGEN). All reactions were performed in triplicate, and *ribosomal protein 49* (*rp49*) was used as the internal control (Gupta *et al.* 2013).

To perform the RT-PCR, we used rTaq Enzyme (Lot# 9AF03; GenStar) and 2 μl of each cDNA in a 20 μl total volume. Both the forward and reverse primers were diluted to 10 μM (0.5 μl) in the 20 μl reaction system. The PCR program using a PCR e-1000 thermocycler (GENESD: http://www.genesd.net.cn/) was as follows: 94° for 5 min, 30 cycles of 94° for 30 sec, 55° for 30 sec, 72° for 30 sec, and 72° for 7 min. The temperature was then shifted to 4°. We loaded all PCR products onto 1.2% agarose gels containing 0.5 μg/ml ethidium bromide, performed electrophoresis, and used ImageJ software to quantify the intensities of the DNA bands.

The primers corresponding to each of the 12 known protein-coding genes on the Y chromosome are indicated in Supplemental Material, Table S4. The following primers were used in conjunction with following control genes: *rp49* (ubiquitously expressed), *Mst35ba* (also known as Protamine A, and is a testes-specific control), and *X8C* (negative control, which corresponds to sequences in an intergenic region on the X chromosome).

rp49 forward primer: 5′ CGGATCGATATGCTAAGCTGT 3′;
rp49 reverse primer: 5′ GCGCTTGTTCGATCCGTA 3′;
Mst35Ba forward primer: 5′ CATCCAATAAGGAGAGCACCTCA 3′;
Mst35Ba reverse primer: 5′ CTTTCTATTCTCCGAGAGCCTG 3′;
X8C forward primer: 5′ TTCACTTGTCCGACGTTGTT 3′;
X8C reverse primer: 5′ CGTTTAGATCTGCGGCAATT 3′.

Design and in vitro synthesis of gRNAs for gene targeting CRISPR/Cas9

All of the gRNAs (5′GGA/G-N17/18-NGG-3′) were designed as described (Yu *et al.* 2013) using the "CRISPR Optimal Target Finder" (http://targetfinder.flycrispr.neuro.brown.edu/), and are listed in Tables S1 and Table S2. We selected gRNAs that target the first third of the coding sequence and that have low off-target scores. Then, we used the gRNA efficiency prediction tool (https://www.flyrnai.org/evaluateCrispr/) to select the gRNAs with the highest efficiency scores.

To transcribe the gRNAs *in vitro*, we obtained the DNA template by PCR using the pMD19-T gRNA scaffold vector (provided by the laboratory of Guanjun Gao, Shanghai Tech University, China) (Yu *et al.* 2013). We generated the DNA sequence for the gRNAs by PCR-amplifying the gRNA scaffold using primers that included the gRNA target sequence. We used the RiboMAX Large Scale RNA Production System-T7 Kit (Promega) to transcribe the gRNAs, and performed phenol-chloroform-isopentanol extraction (Solarbio) to purify each gRNA sample. We used 50 ng/μl of each gRNA for micro-injections.

Embryo micro-injections

To target the Y chromosome genes, we micro-injected embryos with *Cas9* mRNA and gRNAs as described (Yu *et al.* 2013). We performed *in vitro* transcription of *Cas9* mRNA using the pSP6-2sNLS-spcas9 plasmid (Yu *et al.* 2013) and the mMESSAGE mMACHINE SP6 (Lot# 00401332; Invitrogen). Poly(A) tails were added to the 3′ end of the 5′ capped mRNAs with the *Escherichia coli* poly(A) polymerase (M0276S; New England BioLabs). We then combined the purified *Cas9* mRNA and the gRNAs at final concentrations of 1 μg/μl and 50 ng/μl, respectively, which we injected into 0- to 1-hr-old embryos (from 3- to 4-day-old flies maintained at 25°) using an automatic micro-injection system (FemtoJet 4i, Eppendorf). For each gene disruption, we injected ∼200 embryos.

Preparation of genomic DNA and characterization of mutations

Drosophila adults were homogenized in 100 μl lysis buffer (10 mM Tris-HCl pH 8.0, 0.5 mM NaCl, 20 mM EDTA, 50 μg/ml RNase A, and 1% SDS) and incubated at 37° for 1 hr. We transferred the tubes to a 65° heating block for 30 min, added 100 μl phenol/chloroform/isoamyl alcohol and vortexed gently. We centrifuged the tubes at 15,000 rpm for 10 min and transferred the supernatants into new tubes. We precipitated the DNAs with equal volumes of isopropanol at −20° for ≥1 hr, centrifuged at 15,000 rpm at 4° for 10 min, discarded the supernatants, washed the DNAs with 500 μl 75% ethanol, and dried the DNAs under vacuum at room temperature for 5 min. We dissolved each genomic DNA in 10 μl ddH_2O and stored the samples at −20°.

To conduct the PCR, we used 200 ng of each genomic DNA in a 10 μl total volume. Both the forward and reverse primers were diluted to 0.1 μM. The PCR program was as follows using rTaq Enzyme (Lot# 9AF03; GenStar): 94° for 5 min, 30 cycles at 94° for 30 sec, 55° for 30 sec, 72° for 90 sec, and 72° for 7 min. The samples were then kept at 4°. To analyze the sizes of the PCR products, we combined 1 μl PCR products with 6× loading buffer, which we loaded onto 1.5% agarose gels containing 0.5 μg/ml ethidium bromide, and performed electrophoresis. We subjected the same PCR products to DNA sequencing. The PCR primers and sequence results are shown in Table S3 and Figure S2 and S3, respectively.

Imaging testis and sperm stained with DAPI and phalloidin

To examine the testes and mature sperm in seminal vesicles, testes were dissected out from 3- to 7-day-old adult males in 1× PBS buffer, and transferred onto slides with a small drop of 1× PBS buffer. We stained the testes with 4′,6-diamidino-2-phenylindole (DAPI, 1 μg/μl) in 1× PBS buffer for 5 min, mounted the tissue on slides with 1× PBS buffer, placed a cover slip on the sample, and flattened the tissue gently. We acquired the images using a Zeiss Imager Z2.

To stain individualization complexes with phalloidin, we followed a protocol described previously (Fabrizio *et al.* 1998) with modifications. Briefly, we dissected testes in 1× PBS buffer, transferred them into 0.5% sodium citrate for 5 min, and 4% paraformaldehyde (Sigma) for 20 min on ice. We flattened the testes using a siliconized cover slip, froze them in liquid nitrogen, removed the cover slip with a razor blade, and washed the slides with 1× PBST (1× PBS, 0.1% TritonX-100, 4°) for 15 min each. The tissues were blocked in PBSTA (PBST, 3% BSA) for 1 hr, and incubated for 2 hr at room temperature in the dark with 0.14 μM phalloidin (Yeasen, Shanghai, China 40735ES75). The tissues were washed in PBST, stained with 1 μg/μl DAPI for 1 min, mounted, and observed under a Zeiss Confocal LSM 780.

Statistics

All data were analyzed by performing unpaired Student's *t*-tests using GraphPad Prism version 6.00 for Mac (GraphPad Software, La Jolla, CA). Statistical results are presented as means ±SEMs. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

Data availability

Strains, plasmids and DNA sequences are available upon request. The authors state that all data necessary for confirming the conclusions of the article are represented fully within the article, figures, and tables. Supplemental material available at figshare: https://doi.org/10.25386/genetics.12284450.

Results

All Y chromosome genes are expressed in the testis

The transcriptional expression patterns of the genes encoded on the Y chromosome (Figure 1A) have not been analyzed extensively. Nevertheless, existing RNA-seq data (Flybase

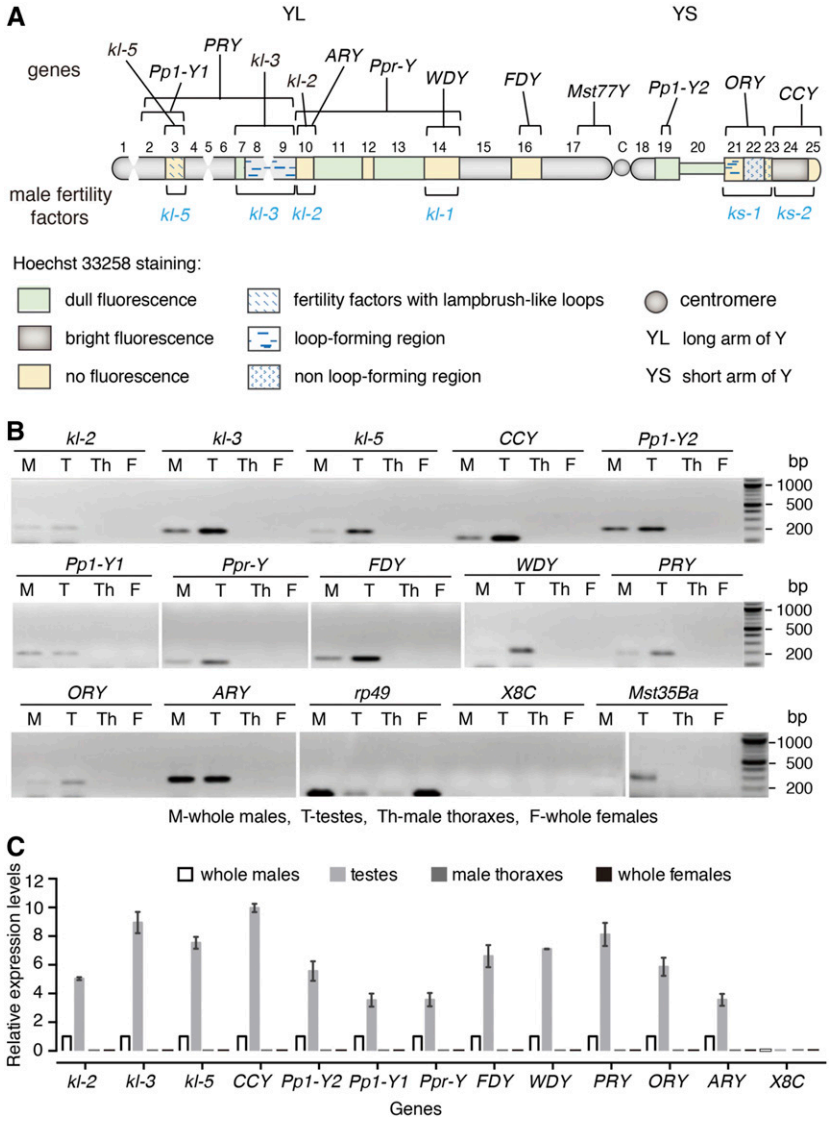

Figure 1 Location and expression of Y chromosome genes. (A) Distribution of genes on the Y chromosome. The Y chromosome is divided into 25 heterochromatic regions, from h1 to h25 (1–25). The colors and patterns indicate the different levels of staining with Hoechst 33258. The 13 defined genes are indicated above the Y chromosome. There are multiple copies of *Mst77Y* (Krsticevic *et al.* 2010, 2015). The bracket associated with *Mst77Y* refers to the small gene cluster in that interval. The identities and locations of the genes corresponding to the three male fertility factor (*kl-1*, *ks-1*, and *ks-2*) have not been clarified. The brackets indicate the uncertainties of their chromosomal locations. The regions corresponding to the six fertility factors (Brosseau 1960) are indicated in blue below the Y chromosome. The cartoon is adapted from previous publications (Piergentili and Mencarelli 2008; Hoskins *et al.* 2015). (B) Expression of Y chromosome genes (analyzed by semiquantitative RT-PCR) in: whole males, testes, thoraxes, and whole females. The PCR products were fractionated on 1.2% agarose gels. DNA size markers are indicated. *rp49* was used as an internal control, *X8C* is an X-linked intergenic region that is transcriptionally silent (Wen *et al.* 2016) and served as a negative control. *Mst35Ba* is a testis-specific control. (C) Relative expression levels of RT-PCR products in whole males, testes, male thoraxes, and whole females; 1 is defined as the level in whole males. We used ImageJ software to quantify the PCR products. Means ± SEMs. $n = 3$ replicates.

website: http://flybase.org/) indicate that most Y genes are expressed, beginning during the third-instar larval stage, and continue to be expressed throughout the pupal stage (Figure S1A). An exception is *FDY*, which is expressed during all developmental stages (Figure S1A). Multiple Y chromosome genes are expressed in imaginal discs, fat bodies, accessory glands, and the testis (Figure S1B). The specific DNA sequences corresponding to the *ks-1*, *ks-2*, and *kl-1* male fertility factors have not been identified, precluding us from indicating the gene locations in Figure 1A, or including their transcriptional expression patterns in Figure S1, A and B. The temporal and tissue expression patterns of *Mst77Y* are also not presented, due to the complications in analyzing this gene by RT-PCR, as a consequence of the many duplications of *Mst77* on the Y chromosome and on the third chromosome (Krsticevic *et al.* 2010, 2015).

To verify that all Y chromosome genes are expressed in the testis, we performed RT-PCR. In addition to examining the signals from RNA prepared from the testis, we compared expression in the male thorax, as well as from whole males and females. As a negative control, we performed RT-PCR using primers corresponding to an intergenic region on the X chromosome (*X8C*), which has been reported to be transcriptionally silent (Wen *et al.* 2016). We used *rp49* as a positive control for wide expression (Gupta *et al.* 2013), and *Mst35Ba* as a testis-specific control (Dorus *et al.* 2008). All of the Y genes were expressed in whole male flies and in testis, although *kl-2* and *ORY* were expressed at low levels (Figure 1B). We did not detect signals for any of these genes in the male thorax or whole female flies (Figure 1, B and C). In contrast, the *rp49*-positive control was expressed in all RNA samples, while we detected *Mst35Ba* signals in the testis only (Figure 1B). We did not observe signals corresponding to the transcriptional silent *X8C* region (Figure 1, B and C). These data confirm that all genes encoded on the Y chromosome are expressed in testes.

Disruption of Y chromosome genes by CRISPR/Cas9 system

Previously, *kl-3* was mutated using the CRISPR/Cas9 system (Yu *et al.* 2013). We set out to use CRISPR/Cas9 to mutate

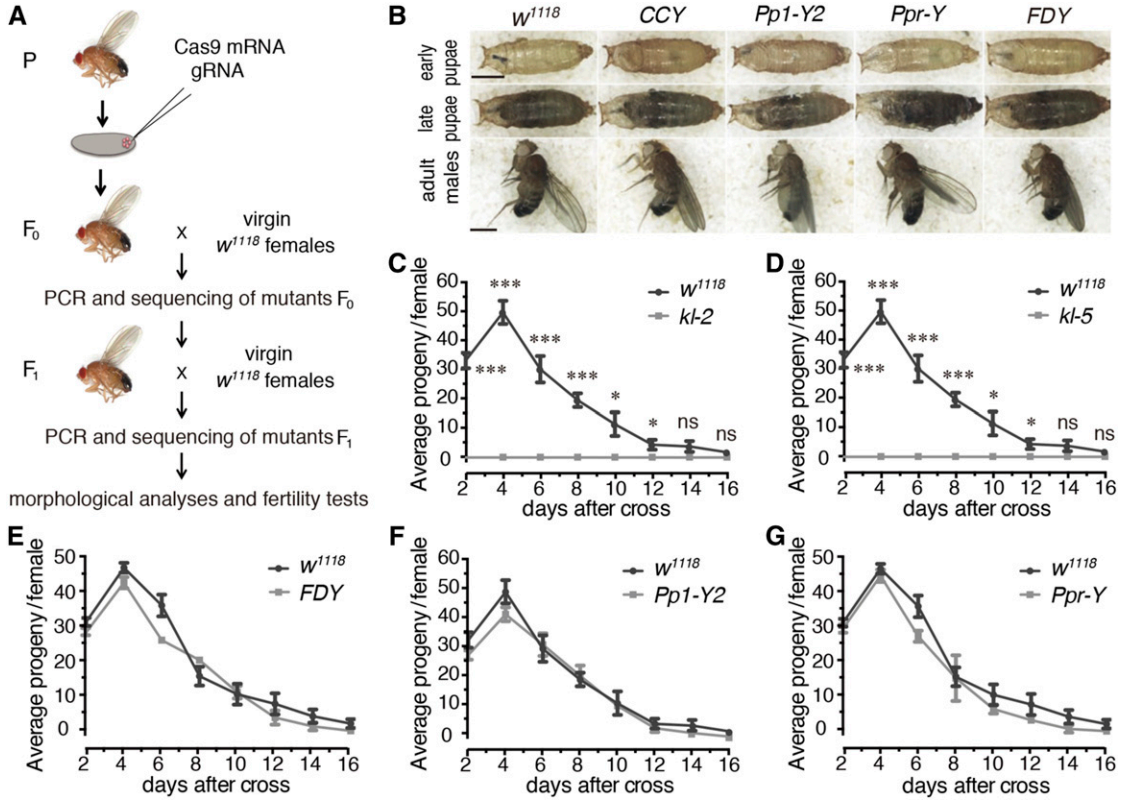

Figure 2 Disruption of several Y chromosome genes leads to defects in male fertility. (A) Schematic diagram for creating mutations in Y chromosome genes by CRISPR/Cas9. Cas9 mRNA and the gRNAs were micro-injected into embryos. The F_0 males were mated with w^{1118} virgin females. The F_1 progeny were crossed to virgin females, and the progeny of the F_1 males were used for examining morphology and male fertility. (B) Morphology of mutant early and late pupae, and adult males. The slightly dark appearances of the bodies is due to the intensity of the light level used to acquire these images. Bar, 200 μm. (C–G) Male fertility tests were performed using the indicated mutant males, and w^{1118} (control) males as the controls. *kl-2* and *kl-5* F_0 males are sterile and no F1 are produced. We established *FDY*, *Pp1-Y2* and *Ppr-Y* lines and the mutant males were examined. (C) *kl-2*. (D) *kl-5*. (E) *FDY*. (F) *Pp1-Y2*. (G) *Ppr-Y*. Means ± SEMs. Unpaired Student's *t*-tests. $n = 20$ crosses each. $^*P < 0.05$, $^{***}P < 0.001$, ns: not significant.

the other Y chromosome genes. We designed gRNAs targeting most Y chromosome genes (Table S1), and *in vitro* transcribed both the gRNA and *Cas9* RNA, which we purified and micro-injected into control embryos (w^{1118}; Figure 2A). We performed PCR and sequencing using genomic DNA from individual F_0 males, and were able to disrupt seven genes, including *kl-3*, which was mutated previously by CRISPR/Cas9 (Figure S2, Table 1: *kl-2*, *kl-3*, *kl-5*, *CCY*, *Ppl-Y2*, *Ppr-Y*, and *FDY*). All *kl-2*, *kl-3* and *kl-5* F_0 males contained the desired mutation and were sterile. Based on DNA sequencing of randomly selected F_1 males, we found that *CCY*, *Pp1-Y2*, *Ppr-Y*, and *FDY* exhibited mutation frequencies ranging from 75 to 100%. All four of these mutants exhibited normal gross morphology in pupae and adult males (Figure 2B). However, we were unable to ascertain whether any of these mutants were true nulls (no protein expression), since there are no available antibodies to examine protein levels. In the case of *CCY*, the only deletions generated were small (9 bp), and in frame, and may not disrupt function. We were unsuccessful in generating mutations in the remaining four genes that we targeted (Table 1: *ARY*, *ORY*, *PRY*, and *Pp1-Y1*), even though we tried multiple gRNAs for each gene (Table S2). We suggest that the lack of targeting by the CRISPR/Cas9 system may due to the heterochromatic characteristics of these loci, which can have a profound effect on editing efficiency (Verkuijl and Rots 2019). The gene sequences of *ks-1*, *ks-2*, and *kl-1* have not been identified, precluding attempts at disrupting these genes. Moreover, due to the many copies of *Mst77* (Krsticevic *et al.* 2010, 2015), we did not target *Mst77Y* using CRISPR/Cas9. We also did not target *WDY* due the presence of a pseudogene with very high sequence identity.

Not all Y-chromosome genes cause defects in fertility and testis morphology

To determine whether the mutations disrupting the seven Y chromosome genes affect male fertility, we crossed individual mutant F_0 or F_1 (if the F_0 males were fertile) males with one virgin control (w^{1118}) female. We then tabulated the number of progeny every 2 days over the course of 16 days. Consistent with previous CRISPR/Cas9 gene editing experiments (Yu *et al.* 2013), mutation of *kl-3* caused male infertility (Figure S4A). CRISPR/Cas9 mediated targeting of *kl-2*, and *kl-5* also resulted in male-sterile phenotypes in the F_0 (Figure 2, C and D), confirming the predictions that these are molecularly defined genes corresponding to male fertility factors (Stern

Table 1 Mutations induced by CRISPR/Cas9

Known gene	Classical genetic region	Location	Indel-yielding F_0/total F_0 examined (n/n) progeny	Indel-yielding F_0/total F_0 examined (n/n) no progeny	Indel-yielding F_1/total F_1 examined (n/n)	Possible phenotype
kl-2	*kl-2*	h10	—	100.0% (6/6)	—	Male sterile
kl-3	*kl-3*	h7-h9	—	100.0% (8/8)	—	Male sterile[a]
kl-5	*kl-5*	h3	—	100.0% (3/3)	—	Male sterile
CCY[b]	*ks-2*	h24-h25	20.0% (1/5)	—	100.0% (7/7)	—
Pp1-Y2	—	h19	90.9% (10/11)	—	75.0% (9/12)	—
Ppr-Y	*kl-1* or *kl-2*	h10-h14	66.7% (2/3)	—	87.5% (7/8)	—
FDY	—	h16	100.0% (2/2)	—	80.0% (4/5)	—
WDY	*kl-1*	h14	—	—	—	—
PRY[c]	*kl-3* or *kl-5*	h2-h9	0.0% (0/22)	—	—	—
ORY[c]	*ks-1*	h21-h22	0.0% (0/13)	—	—	—
ARY[c]	*kl-2*	h10	0.0% (0/32)	—	—	—
Pp1-Y1	*kl-5*	h2-h3	—	—	—	—
Mst77Y	—	h17	—	—	—	—

[a] Phenotype consistent with previously published data (Yu *et al.* 2013). "—" indicates not available.
[b] Gene with in frame mutations only.
[c] Genes that could not be disrupted by CRISPR/Cas9.

1929; Kennison 1981; Gepner and Hays 1993; Carvalho *et al.* 2000). In contrast, the remaining four mutant Y chromosome genes displayed fertility indistinguishable from the w^{1118} control males (Figure 2, E–G and Figure S4B; *FDY*, *Pp1-Y2*, *Ppr-Y*, and *CCY*). These results indicate that not all Y chromosome genes are essential for male fertility.

In view of the dramatic effects of disrupting *kl-2*, *kl-3*, and *kl-5* on male fertility, we examined the morphology of the mutant testis, and of the mature sperm in the seminal vesicles. The *kl-2*, *kl-3*, and *kl-5* mutations caused defects in testicular morphology. The DAPI staining of the *kl-2* and *kl-5* mutant testes was decreased relative to the w^{1118} testes, indicating that they contain less sperm (Figure 3A). In addition, the *kl-3* testes accumulated catkin-like white flocculus in their testis (Figure 3A). We also examined the seminal vesicles, which are used to store mature sperm. The *kl-2* seminal vesicles were smaller than wild type (Figure 3B), and none of the *kl-2*, *kl-3*, or *kl-5* mutant males produced sperm (Figure 3C). The other four mutations (*CCY*, *Pp1-Y2*, *Ppr-Y*, and *FDY*) exhibited normal testes morphology, and the seminal vesicles were filled with sperm (Figure 3, A–C and Figure S5, A–C).

Disruption of Y chromosome fertility genes causes defects in spermatogenesis

The preceding data raise the possibility that the *kl-2*, *kl-3*, and *kl-5* genes function in spermatogenesis. Therefore, we stained squashed testes with DAPI, and examined the morphology of the spermatids. Spermatogenesis takes place in cysts comprised of 64 spermatids, which differentiate synchronously. During spermatogenesis, the round nuclei from w^{1118} males (Figure 3D) become thinner, the chromatin condenses, and the nuclei elongate until they are $\sim$10 μm long (Figure 3, D–I). Due to these morphological changes, the developmental stages are referred to as early canoe (Figure 3F), late canoe (Figure 3G), and needle-shaped (Figure 3H) stages (Fabian and Brill 2012). After elongation and nuclear shaping, the 64 needle-shaped sperm during the individualization stage shed organelles and cytoplasm that are not needed, and they are invested with their own membrane. After individualization, mature sperm becomes coiled and are released into the base of testis (Figure 3I). We found that the *kl-2*, *kl-3*, and *kl-5* mutants exhibited defects in spermatid morphology after the individualization process (Figure 3I). They all had short and curled sperm during spermatogenesis (Figure 3I). In contrast to the abnormal spermatid nuclear morphology exhibited by the *kl-2*, *kl-3*, and *kl-5* mutant males, the *CCY*, *Pp1-Y2*, *Ppr-Y*, and *FDY* mutants produced spermatids similar to control males (Figure 3, D–I and Figure S5, D–I), and the seminal vesicles in these males contained mature sperm (Figure S5C).

Disruption of Y chromosome fertility genes affects sperm individualization

Sperm development culminates with the 64 spermatids in each cyst giving rise to mature, individual sperm. Individualization proceeds after formation of the individualization complex (IC), which consists of 64 actin-based investment cones. These initially form around the nuclei, and then synchronously migrate along the spermatids. During individualization, spermatids strip excess cytoplasm in the mature sperm and invest their own plasma membrane (Tokuyasu *et al.* 1972; Fabrizio *et al.* 1998; Ma *et al.* 2010). Individual, mature sperm are ultimately released into seminal vesicles, which is a prerequisite for transferring sperm during copulation.

In order to clarify the potential reason for the defects in spermatogenesis in the *kl-2*, *kl-3*, and *kl-5* mutant males, we used phalloidin to label the actin in the testis, since actin is a major component of the IC. In control testis (w^{1118}), the IC tightly assembles in a highly organized fashion right below the spermatid nuclei, and forms a cone-like structure (Figure 3, J–L). However, the ICs of the *kl-2*, *kl-3*, and *kl-5* mutants were highly disorganized, and the cone structures were not

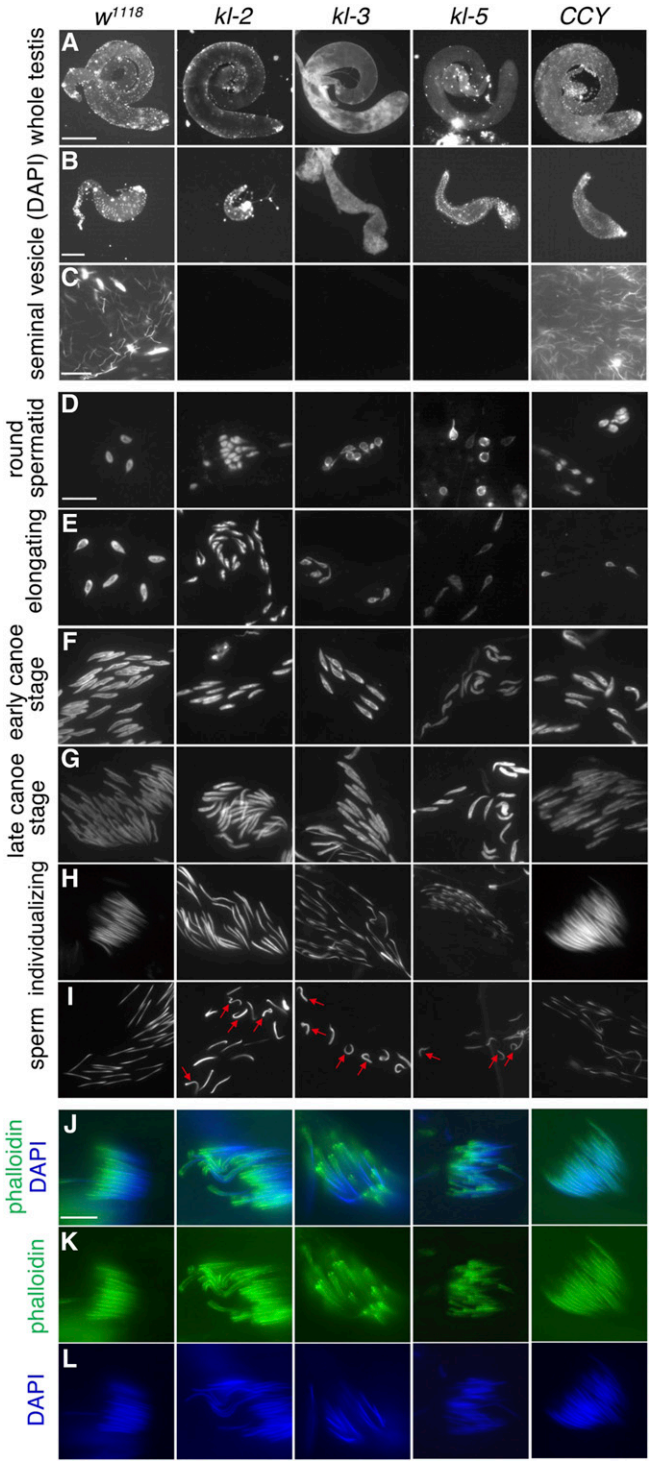

Figure 3 Disruption of Y chromosome genes causes defects in spermatogenesis. Testes were isolated from mutant males and w^{1118} (control) males and stained with: (A–L) DAPI, and (J–K) phalloidin. (A–C) Morphology of testes. (A) Whole testes. (B) Seminal vesicles. (C) Mature sperm in seminal vesicles. (D–I) Different stages during spermatogenesis. The arrows in (I) indicate abnormal, curved sperm. (J–L) Actin and nuclei in individualization complexes (ICs) were stained with phalloidin (green) and DAPI (blue), respectively. Bars, (A) 200 μm, (B) 100 μm, (C) 20 μm, (D–L) 10 μm.

well defined (Figure 3, J and K). In addition, their nuclei failed to remain tightly clustered (Figure 3L) and the ICs lost synchronization (Figure 3, J and K). However, the *Pp1-Y2*, *Ppr-Y*, and *FDY* mutants exhibited tightly assembled and synchronized ICs (Figure S5, J and K). In addition, their nuclei were organized (Figure S5L).

RNAi-mediated knockdown of CCY disrupts male fertility and spermatogenesis

In contrast to the seven Y chromosome genes that we targeted successfully using CRISPR/Cas9, four genes (*ARY*, *ORY*, *PRY*, and *Pp1-Y1*) were resistant to mutagenesis. In addition, the only mutations that we obtained in *CCY* were small, in-frame deletions, raising the possibility that these mutations did not knock out *CCY* function. To interrogate the functions of these five genes, we set out to knockdown their expression by RNAi. We drove expression of *UAS-RNAi* transgenes in testes under the control of the *tubulin-Gal4*, and performed semi-quantitative RT-PCR using gene-specific primers. We found that the expression of all five of these genes was reduced. The suppression of the *ARY* and *CCY* RNAs was significant, while the reductions in the other three genes were just above the threshold for statistical significance (Figure 4A; *ORY*, $P = 0.07$; *PRY*, $P = 0.09$; *Pp1-Y1*, $P = 0.06$).

We examined whether knockdown of *ARY*, and *CCY* impaired male fertility or spermatogenesis. We found that knockdown of *CCY* eliminated male fertility (Figure 4B), while suppression of *ARY* had no effect (Figure 4C). The reductions in *ORY*, *PRY*, and *Pp1-Y1* RNAs also had no impact on fertility (Figure 4, D–F). To determine whether RNAi knockdown caused morphological defects in the spermatid nuclei, we stained the tissue with DAPI. We found that the *CCY* RNAi caused a delay in nuclear elongation, which was most evident during the early canoe stage (Figure 5C). *CCY* RNAi resulted in nuclei that were short and curled at the early canoe stage (Figure 5C). In addition, the seminal vesicles were smaller than the control (Figure 5, G and I), and no mature sperm was produced (Figure 5J). In contrast, RNAi knockdown of *ARY*, *ORY*, *PRY*, and *Pp1-Y1* did not impact on the morphology of the nuclei during spermatogenesis (Figure 5, A–F).

Discussion

Systematic targeting of Y chromosome genes via CRISPR/Cas9

Due to the heterochromatic nature of the Y chromosome, the abundance of highly repetitive sequences, and the incomplete assembly of the Y chromosome sequence, it has been technically challenging to conduct functional analyses of genes encoded on the Y chromosome. Nevertheless, six male fertility factors have been identified using classical genetic approaches, such as X-irradiation, ethyl methanesulfonate (EMS) mutagenesis, X-Y translocations, and *P*-element insertion

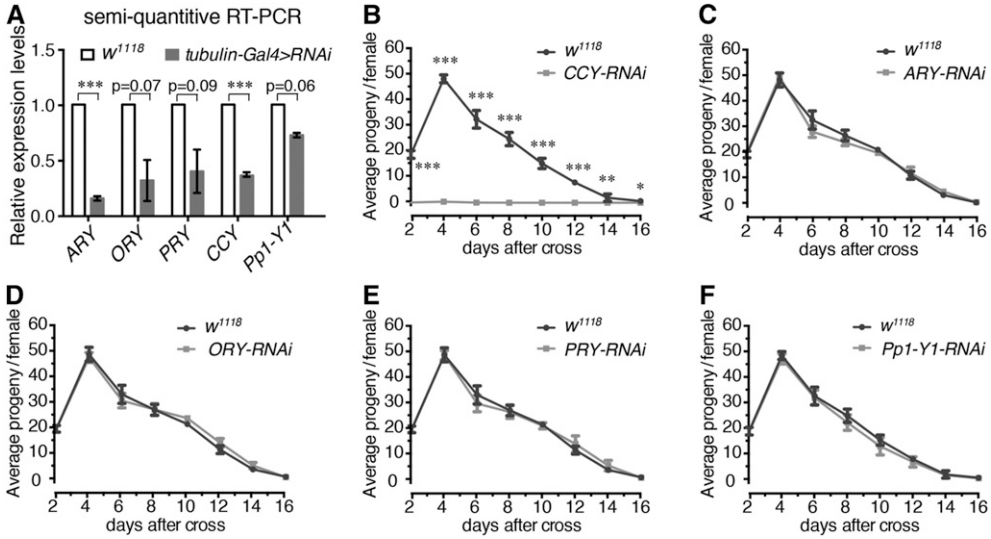

Figure 4 RNAi knockdown of *CCY* impairs male fertility. (A) Semiquantitive RT-PCR analyses of RNA derived from male testes from controls (w^{1118}), and from w^{1118} harboring RNAi transgenes (*ARY*, *ORY*, *PRY*, *CCY*, and *Pp1-Y1*) expressed in testes under control of the *tubulin-Gal4*. *rp49* was used as the internal control for normalization; $n = 3$. (B–F) Male fertility tests using male flies expressing the indicated RNAi transgenes under control of the *tubulin-Gal4*. w^{1118} served as the control. Means ± SEMs. Unpaired Student's *t*-tests. $n = 20$ crosses each. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

mutagenesis (Brosseau 1960; Williamson 1970; Kennison 1981; Zhang and Stankiewicz 1998). In addition to the six male fertility factors, at least 10 other protein coding genes are encoded on the Y chromosome. Whether any of these latter genes are required for male fertility was not known.

We employed CRISPR/Cas9 to explore the requirements for Y chromosome genes for male fertility. Among the 11 genes that we targeted, we were able to create mutations in seven, demonstrating the feasibility of this system for gene modification of the majority of Y chromosome genes, despite the heterochromatic state of this chromosome. The observation that we were unable to generate null mutations in *ARY*, *ORY*, and *PRY*, and *Pp1-Y1*, is unlikely to be due to requirements for these genes for survival, since animals without a Y chromosome (XO individuals) are viable males (Bridges 1916a; Bridges 1916b; Voelker and Kojima 1971). Rather, the lack of any mutations in these genes might be a consequence of the specific features of these heterochromatic regions, rendering them less amenable to CRISPR/Cas9-mediated gene disruption (Verkuijl and Rots 2019). Consistent with this latter possibility, we found that the flies were viable and appeared healthy following RNAi knockdown of *ARY*.

Not all Y chromosomal genes required for male fertility

A key question is whether all Y chromosome genes are required for male fertility. In this work, we establish that CRISPR/Cas9-mediated mutations in *kl-2* and *kl-5* cause male sterility, strongly supporting the prior proposals that these are the molecular sequences corresponding to the cognate male fertility factors (Stern 1929; Hardy *et al.* 1981; Kennison 1981; Gepner and Hays 1993; Carvalho *et al.* 2000). In addition, the *kl-3* coding sequence is essential for male fertility, as previously reported (Yu *et al.* 2013). We also provide evidence that *CCY* is required for male fertility. The *CCY* gene is located near the telomere of the YS arm, in the region that includes the *ks-2* locus. Thus, *CCY* may be the gene corresponding to the *ks-2* male fertility factor.

Knockout or knockdown of six other Y chromosome genes (*ARY*, *FDY*, *ORY*, *Pp1-Y2*, *Ppr-Y*, and *PRY*) did not impact on male fertility. While it appears unlikely that these six genes are absolutely essential for male fertility, we do not exclude that they contribute to male fertility, but their roles are too subtle to identify in our current assays. It also plausible that the four genes that we knocked down by RNAi, but which did not cause male sterility (*ARY*, *ORY*, *PRY*, and *Pp1-Y1*), were not sufficiently suppressed to elicit this phenotype. While suppression of *ARY* was significant, the RNAi knockdowns of *ORY*, *PRY*, and *Pp1-Y1* were just above the cutoff for statistical significance ($P = 0.06$–0.09). Nevertheless, our data raise the possibility that *ORY*, which is contained in the *ks-1* interval, may not be the molecular entity corresponding to this male fertility factor. *Ppr-Y* maps to the *kl-1* region, and CRISRP/Cas9 targeting of this sequence does not cause male sterility, indicating that it is not the gene in the *kl-1* interval that is required for male fertility. The lack of an obvious impact on male fertility due to editing of *FDY*, *Pp1-Y2*, and *Ppr-Y*, or knockdown of *ARY* indicates that some Y chromosome genes may contribute to a male-specific behavior other than fertility.

During the initial review of this work, another group reported the results of their analysis targeting Y chromosome genes using CRISPR/Cas9 and RNAi (Hafezi *et al.* 2020). Similar to our RNAi results, they show that editing of *CCY* caused male sterility, while males with targeted mutations in *FDY* and *PRY* did not, although they found that *PRY* mutant males were subfertile (Hafezi *et al.* 2020). Also consistent with our CRISPR/Cas9 results, and expectations based on classical genetic studies (Stern 1929; Brosseau 1960; Kennison 1981), their RNAi knockdowns of *kl-2* and *kl-5* caused male sterility. Concordant with our finding that *Ppr-Y* is not the *kl-1* factor, RNAi knockdown of the other gene in the *kl-1* interval (*WDY*), leads to male sterility. The combination of these findings support the conclusion that *WDY* (Hafezi *et al.* 2020), but not *Ppr-Y*, is the *kl-1* fertility factor. In the future, it would be

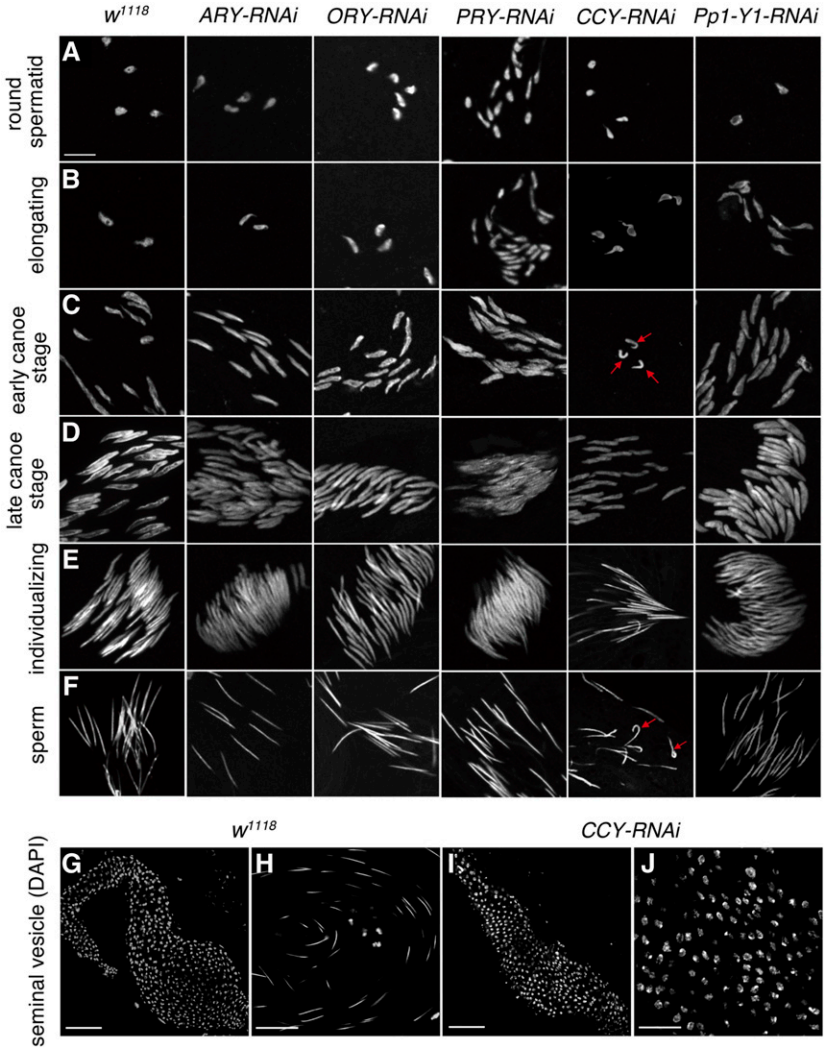

Figure 5 RNAi knockdown of *CCY* causes defects in spermatogenesis. Testes were prepared from w^{1118} (control) and from *tubulin-Gal4 > RNAi* lines, and were stained with DAPI. (A–F) Different stages of spermatogenesis. The arrows indicate abnormal, curved sperm. (G–J) Morphology of seminal vesicles from w^{1118} (control) and *tubulin-Gal4 > CCY-RNAi* males (*CCY-RNAi*). (G and I) Seminal vesicles. (H and J) Mature sperm from seminal vesicles. Bars, (A–F) 10 μm. (G and I) 50 μm, (H and J) 20 μm.

worthwhile to test for functional redundancy of genes that do not cause male sterility (*e.g.*, *ARY*, *FDY*, *Pp1-Y2*, *Ppr-Y*, and *PRY*) by injecting one or more gRNAs into another mutant background to generate double or triple mutant alleles and assaying for male fertility.

Y chromosome male fertility genes cause defects in spermatogenesis

Mutation or knockdown of the genes required for male fertility (*kl-2*, *kl-3*, *kl-5*, and *CCY*) impair spermatogenesis. The phenotypes include short and curled sperm during spermatogenesis, and a lack of mature sperm in the mutant seminal vesicles. In addition, mutation (*kl-2*, *kl-3*, and *kl-5*) or RNAi knockdown (*CCY*) of these male fertility genes led to defects in individualization. The ICs were disorganized and lagged, and the actin cone structures were scattered. The nuclei were not tightly clustered, and the ICs did not migrate in synchrony in the spermatid bundles.

The flagella axoneme is composed of microtubules (MTs), and MTs are essential for driving the shape of the nuclei (Fabian and Brill 2012). The observations that *kl-2*, *kl-3*, and *kl-5* encode dynein, which is the main component of the axoneme in flagella (Gibbons *et al.* 1991; Gepner and Hays 1993; Carvalho *et al.* 2000; Piergentili 2010), is consistent with the requirements for these genes for spermatogenesis. We suggest that the morphological defects during spermatogenesis, due to disruption of *kl-2*, *kl-3*, *kl-5*, or *CCY*, prevents the sperm from entering the seminal vesicles, which, in turn, leads to male sterility. An important future question concerns the functions of the other Y chromosome genes that do not appear to be strictly required for male fertility.

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