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dMARCH8, a *Drosophila* ubiquitin E3 ligase, regulates polymodifications of tubulin in the spermiogenic pathway

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ABSTRACT

Ciliary stability and function are regulated by the covalent addition of polyglutamate and polyglycine chains to axonemal tubulin subunits. The *Drosophila* gene CG13442 encodes a predicted ubiquitin E3 ligase involved in the regulation of tubulin glycylation and glutamylation. Homologous to mammalian MARCH8, CG13442/*dMARCH8* is required for male fertility. Sperm in *dMARCH8* mutant testes appear to undergo a normal individualization process but fail to be transferred to the seminal vesicle. This phenotype is very similar to that of mutants in the *Ntl* gene, shown here to be a glycine transporter using a [^{3}H]glycine uptake assay. Mutations in *dMARCH8* are associated with a reduction of both polyglutamylation and polyglycylation of sperm tubulin. Polyglutamylation of tubulin is significantly increased in the Ntl^- background, and recovers to wild-type levels in the Ntl^--$dMARCH8^-$ double mutant background, indicating that glycine and glutamate compete for some common site(s) on tubulin molecules in this system. By analogy to the regulation of the mammalian glycine transporter GlyT2 through ubiquitin-mediated trafficking between the plasma membrane and endosome, *dMARCH8* may target *Ntl* and glutamate transporters, or other upstream regulators of these proteins.

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Introduction

Amino acids and their chemical derivatives serve as signaling molecules in several biological contexts. Glycine and glutamate function as major inhibitory and excitatory neurotransmitters, respectively, in the central nervous system (Kandel et al., 2013). At the subcellular level, these same two amino acids regulate ciliary function and stability through a mechanism involving their competition for covalent addition of polyglycine and polyglutamate chains to tubulin subunits incorporated into stable microtubule-based structures (Raunser and Gatsogiannis, 2015). The activity of glycine transporters, controlling the levels of glycine both inside and outside of the cell, can therefore result in behavioral effects and ciliary dysfunction (Boison, 2016)

Here we use the *Drosophila* spermiogenesis system to examine the regulation of tubulin polymodifications. *Drosophila* spermiogenesis provides an opportunity for genetic analysis of many different cellular subsystems (Fabian and Brill, 2012). These include the differentiation of mitochondria (Hales and Fuller, 1997;Politi et al., 2014), polarization of the spermiogenic cyst (Wei et al., 2008), the radical restructuring of spermatid nuclei (Kost et al., 2015), and the complex process of sperm individualization, in which individual sperm are finally resolved from the syncytium in which they have developed (Arama et al., 2007;Arama et al., 2003;Fabrizio et al., 1998;Tokuyasu et al., 1977) This complexity is reflected in the large number of genes that are mutable to a male-sterile phenotype, and indicates that genes contributing to any of the many different cellular subsystems---including the polymodifications of tubulin---may be accessible through the analysis of male-sterile mutations (Wakimoto et al, 2007).

Because there is very little transcription in spermiogenic cysts(Barreau et al., 2008) posttranscriptional and posttranslational regulatory mechanisms are especially important in this process (Karr, 2007). Ubiquitination is one of these processes, affecting many different aspects of spermiogenesis (Richburg et al., 2014). Although the ubiquitin system is known primarily for marking proteins for degradation (Ciechanover, 2005;Zhi et al., 2013), recent studies indicate that ubiquitination can affect many other aspects of protein function, including intracellular trafficking (de Juan-Sanz et al., 2011),

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58 modulation of protein-protein interactions (Yang et al., 2010), and modulation of
59 transcription, DNA repair and transmembrane signaling (Metzger et al., 2012).
60
61 The substrate-specificity of a ubiquitination system is generally controlled by E3
62 ubiquitin ligases. Here we identify an E3 ubiquitin ligase, *dMARCH8,* homologous to
63 mammalian MARCH8. *dMARCH8/CG13442* function is required for the full
64 complement of polyglycylation and polyglutamylation of sperm tubulin in the *Drosophila*
65 testis. We show that the *Drosophila Neurotransmitter transporter-like (Ntl)* gene*,*
66 previously found to exhibit a similar phenotype to *dMARCH8* mutants (Chatterjee et al.,
67 2011), encodes a glycine transporter using a [^{3}H]glycine uptake assay. Loss of *Ntl*
68 function results in a large increase in polyglutamylation levels, indicating that *Ntl* and
69 *dMARCH8* activities contribute to the balance of glutamylation and glycylation of sperm
70 tubulin.
71

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Materials and Methods

Fly Husbandry

Flies were raised on standard cornmeal molasses agar at 25°C. Unless otherwise mentioned, all stocks were from the Bloomington Stock Center. Males were tested for fertility by mating in groups of 4–5 with an equal number of virgin females. Generally, w^+ or y^+ males were mated with *yw* females, with the recovery of w^+ or y^+ daughters in the F1 generation confirming fertility. Genetic constructions were carried out using standard *Drosophila* genetics as in Greenspan (Greenspan RJ, 1997). All experiments were carried out in the *yw* genetic background.

Generation *of dMARCH8* mutants

The *dMARCH8* transcript/CDR is in the 57B region on the 2R arm of the *Drosophila* chromosome. The Mi{MIC}(*Minos* Mediated Integrated Cassette) $dpr^{MI06571}$ transposon carrying the y^+ marker was generated by the *Drosophila* Gene disruption project (GDP)(Venken et al., 2011)The transposon was mobilized by crossing the flies carrying the MiMIC insertion to flies carrying stable heat shock transposase (y^1 w^*; sna^{Sco} /*SM6a,* $P\{w^{+mC}=hsILMiT\}2.4$ (Metaxakis et al., 2005;Venken et al., 2011). Three broods from each cross were generated by transferring flies to new bottles on the 3rd and 5th days after the cross was initially set up. Bottles were subjected to heat shock (39°C, 90 minutes) on the third, 5th and seventh day of each brood. Chromosomes that lost the y^+ markers carried by the transposon were recovered and established in balanced stocks using standard *Drosophila* genetics, then screened for new male-sterile mutations produced by imprecise excision of the Mi{MIC} transposon.

RNA isolation and qRT-PCR

Total RNA was isolated using TRI reagent (Sigma) according to the manufacturer's recommendations. RNA was extracted from males, females, testes, and heads and concentration was determined by measuring its absorbance.

One-step qRT-PCR was performed using the iTaq Universal SYBR Green One-Step Kit (BIORAD) according to the manufacturer's recommendations. The BIORAD MyiQ Real-Time PCR Detection System was programmed as follows: 50°C for 10 min, 95°C

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for 5 min followed by the amplification steps of 95°C for 15 sec, 56°C for 30 seconds, 72°C for 30 sec. 45 cycles of PCR were run for all samples followed by 72°C for 15 min, 57°C for 10 min and held at 4°C overnight. *dMARCH8* qRT primers amplified a 112 bp fragment, while rp49 control primers amplified a 154 bp fragment. Primers for this and all subsequent PCR-based molecular biology are specified in Table 1.

Deletion PCR

Genomic DNA was isolated from males according to the Berkeley *Drosophila* Genome Project protocol (http://www.fruitfly.org/about/methods/inverse.pcr.html). 13 pairs of gene-specific primers were used to amplify the entire coding sequence of *dMARCH8*. The thermocycler (MJ Research PTC-200 Peltier thermal cycler) was programmed as follows: 94°C for 2 min followed by 30 cycles of: 94°C for 1 min, 56.3°C for 1 min, 72°C for 1 min. After a final 5 min at 72°C, samples were held at 4°C until gel analysis.

Phalloidin assay for Individualization Complex

Testes from 0–1 d old males were crudely dissected in *Drosophila* Ringers and transferred immediately to a tube of Ringers on ice. Testes were then fixed for 15 min in 4% paraformaldehyde in buffer B (16.7mM KH_2PO_4/K_2HPO_4 pH 6.8, 75mM KCl, 25mM NaCl, 3.3mM $MgCl_2$). Following fixation, testes were rinsed three times in PTx (PBS+0.1% Triton X-100), washed for 30 min in PTx and blocked for at least 1 h in blocking solution (0.01% NaAzide and 3% BSA in PTx**)**. Staining with rhodamine-conjugated phalloidin was for 30 min in blocking solution (3 µg/ml). Testes were then rinsed and washed with PTx. Testes were then finely dissected from remaining carcasses in 50% glycerol and then mounted in 90% glycerol. Slides were stored overnight at 4°C before imaging. Fluorescence images were captured by confocal microscopy (Leica TCS-2, Exton, PA)(Fabrizio et al., 2012)

Phase squashes

Testes were dissected from 0–1-day-old young male *Drosophila* mutants (unless mentioned otherwise) in 1× PBS buffer and gently squashed with a coverslip before taking phase images with Leica DM 4500B. For phase microscopy of seminal vesicles,

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wild type and mutant freshly eclosed *Drosophila* males were withheld from females for three to four days before dissecting their testes.

Protein Electrophoresis and Immunoblot

Samples were prepared from male fly testes with seminal vesicle for each genotype, from males that were withheld from females for 4–6 days in order to maximize sperm yield. Six fly testes (12 testes) worth of protein were loaded in each lane. Testes were dissected in 1× PBS, ground in 2× Laemmeli buffer (Laemmli UK., 1970) vortexed and boiled for 5 min. Samples were then spun at 15,800 g for 5 min, and supernatants were separated in a 12% SDS-Polyacrylamide gel and transferred to a PVDF membrane (Amersham, GE Healthcare,United Kingdom) using a Trans-Blot Semi-Dry transfer apparatus (BioRad, U.S.A). Membranes were incubated with primary antibodies directed against poly-glycylated tubulin (Poly-G) (1:10,00) (MABS276**,** cloneAXO49, Millipore), polyglutamylated tubulin (Poly-E) (1:1000) (mAbGT355, Adipogen Life sciences), ubiquitin {MAB1510 clone Ubi-1 (aka 042691GS), Millipore}, TSSK2 (ab172434, Abcam) and α-tubulin (1:500), (DM1A, Sigma). Protein bands were visualized with HRP-labelled anti rabbit or anti-mouse (1:5,000) secondary antibodies followed by detection with ECL immunoblot detection kit (Pierce, U.S.A). Loading control was α-tubulin.

All statistical analyses were made using two-tailed Students t-test in Microsoft Excel. The average values of relative intensity (Target antibody intensity/anti-tubulin) were plotted for each genotype. Intensities were calculated using Image J software (Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA, http://rsb.info. nih.gov/ij/, 1997–2009).

[3H]glycine uptake assays

Human GlyT1, GlyT2 and *Drosophila* Ntl cDNAs were cloned into pRK5myc or pTMR vectors as previously described (Carta et al., 2012). HEK293 cells were grown in minimal essential medium (Earle's salts) supplemented with 10% (v/v) FCS, 2 mm L-glutamine, and 20 units/ml penicillin/streptomycin in 5% CO_2, 95% air. The cells were plated on poly-D-lysine-coated 24-wells plates (Nunc), grown to 50% confluence, and transfected with 1 μg of total pRK5myc-hGlyT1, pRK5myc-hGlyT2, pRK5myc-Ntl or

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159 pTMR-Ntl plasmid DNAs using Lipofectamine LTX reagent (Invitrogen). After 24 h, the
160 cells were washed twice with prewarmed buffer (118 mM NaCl, 1 mM NH_2PO_4, 26 mM
161 $NaHCO_3$, 1.5 mM $MgSO_4$, 5 mM KCl, 1.3 mM $CaCl_2$, 20 mM glucose) pre-equilibrated
162 with 5% CO_2, 95% air. After 2 min, the cells were incubated for 5 min in 0.1 µCi/ml
163 [^{3}H]glycine (60 Ci/mmol; PerkinElmer Life Sciences) at a final concentration of 300 µM.
164 The cells were rinsed twice with ice-cold buffer pre-equilibrated with 5% CO_2, 95% air
165 and then digested in 0.1 M NaOH for 2 h. The samples were used for scintillation
166 counting and for determination of protein concentration using the Bradford reagent (Bio-
167 Rad). [^{3}H]glycine uptake was calculated as nmol/min/mg of protein and expressed as
168 percentages of that in control cells transfected with the empty expression vector. All
169 statistical comparisons used an unpaired Student's *t*-test.

170 **Generation of *Ntl-dMARCH8* double mutant**

171 *Ntl-dMARCH8* double mutants were generated by crossing *Ntl/CyO* males to
172 $dMARCH8^{7A}$*/CyO* females. Straight-winged female progeny obtained were crossed to *yw*,
173 *Gla/Sm6a* males. Isomales from this cross were crossed again to establish balanced
174 stocks. Males from these established stocks were checked for fertility by crossing to *yw*
175 female virgins. Males from sterile stocks obtained were screened for complementation
176 with Ntl^- and $dMARCH8^-$ mutants.
177

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Results

Amino acid sequence alignment of *dMARCH8 (CG13442)* with human MARCH8-E3 ubiquitin Ligases belonging to the RING protein family

A ClustalW alignment of the predicted dMARCH8 protein sequence with human MARCH8 is shown in Figure 1A. RING domain considered critical for E3 ubiquitin ligase function (Samji et al., 2014) are conserved in the predicted dMARCH8 protein. The TMpred algorithm (Hofmann and Stoffel, 1993) predicts two transmembrane domains for dMARCH8, characteristic of the MARCH family (Samji et al., 2014), whose RING domains are most closely related to the Ringv and PHD domains (Deshaies and Joazeiro, 2009).

Generation of *dMARCH8* mutants

The *dMARCH8* gene lies within an intron of the *dpr* gene, where it is expressed from the opposite strand. *dMARCH8* mutants were generated by mobilizing the Mi{MIC}$dpr^{MI06571}$ near the 5' end of the *dMARCH8* gene, crossing it into a genetic background expressing the minos transposase (Venken et al., 2011) under heat shock control (Metaxakis et al., 2005). By standard fly genetics, chromosomes which had lost the *yellow*$^+$ (y^+) markers associated with the Mi{MIC} transposon were recovered and established in balanced stocks. Transposon excisions are often imprecise and deletions of varying size, usually extending from the insertion site in either direction, are often recovered at significant frequencies (Zhang and Spradling, 1993). A schematic of the *dMARCH8* locus showing the starting insertion, the mating scheme used to mobilize the transposon and identify new male-sterile mutations, and the location of primer pairs used to assay for deletions in the resulting male-sterile stocks is shown in Figure 1B and 1C. Of 64 chromosomes that were observed to have lost the y^+ marker, 5 were found to carry new male sterile mutations defining a single complementation group.

Deletion PCR analysis of *CG13442/dMARCH8* mutants

The mutant alleles were screened with nested gene-specific primers spanning the site of the transposon insertion (Materials and Methods and Fig. 1B). Genomic DNA from homozygous males carrying each of the 5 male-sterile alleles was used to probe for

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changes in the chromosome structure by PCR. For all of the male-sterile mutants, PCR amplicons including portions of the gene and flanking regions on either side were missing or produced products with altered size as compared to wild-type DNA (Table 2). These results indicated that the deletion carried by the *dMARCH8*^{7A} mutant removes the sequences encoding the RING domain and both transmembrane domains (Table 2). Furthermore, qRT-PCR analysis of this mutant revealed a significant reduction (80 +/- 5% in *dMARCH8* expression (Fig. 2A). The *dMARCH8*^{7A} allele was used for the remaining phenotypic analyses.

Testis Specificity of *dMARCH8* expression

qRT-PCR analysis confirmed that *dMARCH8* expression was male-specific and limited to the testes (Fig. 2B and C). Expression in females was 90 +/- 5 % lower than in males, and in the adult heads and carcass of males, it was 98 +/- 1% lower than in the testis.

Rescue by Germline Transformation

We used a P-element based construct for germline transformation with CG13442/ *dMARCH8,* to confirm that the male sterile phenotype in our mutants is caused solely due to disruption of *dMARCH8*. For this we used a pTMR-*dMARCH8* construct, which contains a full-length cDNA for CG13442 (DGRC clone AT03090) cloned downstream of the β2T-tubulin transcriptional control sequences in the *Drosophila* transformation vector pTMR (Clark et al., 2006;Huh et al., 2004). This construct provides strong germ cell-specific transcription in developing sperm (Kaltschmidt et al., 1991;Kemphues et al., 1982). A BAC genomic construct CH322-140N02 (BACPAC resources, bacpac.chori.org) extending from 9.8 kb upstream of the *dMARCH8* transcript to 8.5 kb beyond the 3' end also rescued the *dMARCH8* mutant phenotype. Two independent insertions of this construct were tested and they both rescued the mutant. All the males homozygous for the *dMARCH8*^{7A} deletion and carrying pTMR-*dMARCH8 or BAC-dMARCH8* constructs were fertile. Results from the rescued line are presented in Fig. 2D. The male-sterile mutations obtained after mobilization of the Mi{MIC} transposon therefore result from disruption of *dMARCH8*.

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Spermiogenic-defective phenotype of *dMARCH8* mutants

dMARCH8^{7A} / *dMARCH8*^{7A} males produced elongated spermiogenic cysts (Fig. 3), but we did not detect mature sperm in the seminal vesicle (SV) (Fig. 3B, arrows). In contrast, wild-type control seminal vesicles were filled with mature sperm (Fig. 3A, arrows). No motile sperm are seen in *dMARCH8*^{7A} / *dMARCH8*^{7A} squash preparations, unlike in the wild-type controls, where dense masses of mature motile sperm were evident (Fig. 3C, arrow). Instead, an extensive mass of sperm bundles accumulated at the base of *dMARCH8*^{7A} mutant testes (Fig. 3B, asterisks). Presumably because of the great physical complexity of the sperm individualization process (Bazinet and Rollins, 2003;Noguchi et al., 2008;Tokuyasu et al., 1972), a preponderance of male-sterile mutations produce elongated cysts that fail to mature into individual sperm (Wakimoto et al, 2007). In most of these cases, the individualization complex either fails to form, does not progress, or breaks down during transfer along the length of the cyst (Fabrizio et al., 1998).

When *dMARCH8*^{7A} mutant *Drosophila* testes were stained with rhodamine-conjugated phalloidin, we observed normal development and movement of the actin cones of the individualization complex (Fig. 3F). Waste bag deposition in the distal end of the testis also appeared normal, further indicating that the individualization complex was successfully navigating the entire length of the cysts (Fig 3F). These observations are similar to the phenotype seen in *Neurotransmitter transporter-like* (*Ntl*) mutants, a putative glycine transporter homologous to human glycine transporters GlyT1 and GlyT2 (Chatterjee et al., 2011).

Demonstration of Glycine transport activity from Ntl expression in mammalian cells

To test whether the *Drosophila* Ntl protein was capable of glycine uptake, we tested the capacity of recombinantly-expressed Ntl to mediate the uptake of [^{3}H]glycine, using human GlyT1 and GlyT2 cDNAs (Carta et al., 2012) as controls. As expected, both pRK5-GlyT1 and pRK5-GlyT2 constructs resulted in significant increases in [^{3}H]glycine uptake compared to vector only controls (pRK5-GlyT1: 345 ± 42; pRK5-GlyT2 299 ± 38). Both Drosophila Ntl expression constructs also resulted in statistically significant

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264 [^{3}H]glycine uptake (pRK5-NTL: 245 ± 24; pTMR-NTL: 344 ± 59) confirming that Ntl is
265 a fully-functional glycine transporter (Fig. 4).

266 **Reduction of ubiquitination of testis proteins in *dMARCH8* mutants**

267 Since dMARCH8 is a member of the E3 ubiquitin ligase family, we assayed *dMARCH8*
268 mutant males for total ubiquitination of testis proteins using an anti-ubiquitin antibody
269 (Kane et al., 2014). The results from three independent experiments, using α-tubulin as a
270 loading control, showed an apparent reduction in ubiquitination signal in mutant samples
271 relative to wild-type controls (Fig. 5A). Two distinct ubiquitinated bands of ~50 and 77
272 kDa present in the testes of *dMARCH8^{7A}*/+ flies are missing in the testes of homozygous
273 *dMARCH8^{7A}* flies. A slight reduction in several other bands (~65 and 100 kDa) was also
274 consistently observed in the *dMARCH8^{7A}* mutant.

275 **Reduction of tubulin polyglycylation and polyglutamylation in *dMARCH8* mutants**

276 The tubulin in *Drosophila* sperm axonemes undergoes a variety of posttranslational
277 modifications (Janke, 2014;Kierszenbaum, 2002;Wloga and Gaertig,
278 2010)Polyglycylation of tubulins is required for the stability of ciliary and flagellar
279 axonemes and other long-lived tubulin-based structures (Bre et al., 1996;Bressac et al.,
280 1995;Rogowski et al., 2009a). Polyglutamylation is known to regulate beating behavior
281 in motile cilia via the regulation of flagellar dynein motors (Ikegami et al., 2010;Janke et
282 al., 2005;Kubo et al., 2010;Pathak et al., 2007;Suryavanshi et al., 2010). In addition,
283 polyglycylation and polyglutamylation have been shown to affect male fertility
284 (Chatterjee et al., 2011;Lee et al., 2013).
285
286 The spermiogenic defect of *dMARCH8* mutants at the microscopic level is similar to that
287 seen in *Ntl,* which encodes a glycine transporter in whose absence polyglycylation of
288 testes tubulin is significantly reduced and sperm fail to be transferred to the seminal
289 vesicle. We therefore analyzed the levels of polyglycylated and polyglutamylated tubulin
290 in testis protein samples using antibodies directed against poly-G and poly-E.
291 Quantitation by scanning the results from six independent experiments, using α-tubulin as
292 a loading control showed an average of 50% reduction in poly-G signal in the
293 *dMARCH8^{7A}* mutant samples relative to wild-type controls (Fig 5B). Similarly, for the

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poly-E signal, quantitation from three independent experiments exhibited an average of 70% reduction in *dMARCH8*^{7A} mutants compared to wild-type controls (Fig. 5C).

Polyglycylation and polyglutamylation in a *Ntl-dMARCH8* double mutant

Since the *Ntl* mutant exhibits a similar phenotype to the *dMARCH8* mutant, and it also shows a predicted interaction (STITCH database) with dMARCH8, we analyzed the *Ntl-dMARCH8* double mutant for polyglycylation and polyglutamylation levels of tubulin in three independent experiments. We observed that in both the *Ntl* mutant and the *Ntl-dMARCH8* double mutant, poly-G levels decrease while the poly-E levels increase (Fig. 5D,E and F). Perhaps most strikingly, loss of *Ntl* in the *dMARCH8*$^{-}$ genetic background restores levels of polyglutamylation to wild-type levels (compare Fig. 5C and 5F). In this system, polyglutamylation appears to be very sensitive to glycine levels, consistent with observations by others that glutamate and glycine compete with each other for common site(s) on tubulin (Bulinski, 2009;Rogowski et al., 2009b;Wloga et al., 2009).

Discussion

Functional spermatogenesis is particularly dependent on ubiquitin-regulated protein function and stability (Mukhopadhyay and Riezman, 2007;Richburg et al., 2014). This is evident from the spermatogenesis-specific functions of a number of E3 ligases: Bruce and Cullin3 in *Drosophila* have been studied for their role in sperm individualization (Arama et al., 2007;Arama et al., 2003;Kaplan et al., 2010;Wang et al., 2006). RNF8 and E3^{histone}, found in mice and rats respectively, are reported to be involved in the histone degradation that occurs during histone to protamine transition in spermatid nuclei in rats (Liu et al., 2005;Lu et al., 2010). The E3 ligases Cul4A (Kopanja et al., 2011;Yin et al., 2011), Itch (Dwyer and Richburg, 2012) and Siah1a (Dickins et al., 2002) play roles during germ cell meiosis and Cullin3^{testis} (Arama et al., 2007;Kaplan et al., 2010) and MEX (Nishito et al., 2006) are required during germ cell apoptosis. MARCH7 (Membrane-Associated Ring-CH) was found to be involved in spermiogenesis by regulating the structural and functional integrity of the head and tail of developing spermatids (Zhao et al., 2013). MARCH10 was found to be essential for spermatid maturation (Iyengar PV, Hirota T, Hirose S, Nakamura N., 2011). MARCH11 plays a role in ubiquitin-mediated protein

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323 sorting in TGN-MVB (Trans Golgi Network-multivesicular bodies) transport in
324 developing spermatids (Morokuma et al., 2007;Yogo et al., 2012).

325 Indeed, of all organs examined in rats, ubiquitination has been reported to be highest in
326 testis (Rajapurohitam et al., 2002). Combined with the observed genetic sensitivity of the
327 spermiogenic process, these considerations indicate that details of cellular ubiquitin
328 function may be accessible through the characterization of male-sterile mutations in E3
329 ligase genes. Studies of mammalian spermatogenesis have implicated ubiquitination in
330 the regulation of multiple spermiogenic stages, including nuclear condensation, acrosome
331 formation and membrane transport (Nakamura, 2013).

332 Here we have observed that loss of the E3 ubiquitin ligase *dMARCH8* results in a male-
333 sterile phenotype very similar to that observed in mutants of the transporter *Ntl.* We have
334 also shown *Ntl* mediates the uptake of [^{3}H]glycine in a cellular assay with an efficiency
335 comparable to the mammalian glycine transporters GlyT1 and GlyT2. At least one other
336 uncharacterized *Drosophila* SLC6 class transporter encoded by CG5549, gives a
337 somewhat better BLAST score against human GlyT1 (42.34% identity) and GlyT2 (35.01
338 % identity) than *Ntl* (Ntl vs GlyT1 39.15%; Ntl vs GlyT2 37.6% identity) suggesting that
339 the fly genome may encode an additional glycine transporter. Expression of CG5549 is
340 also male biased {Flybase}, although not as strongly as that of *Ntl.*

341 Modulation of the activity of the mammalian glycine transporter GlyT in mammalian
342 cells is thought to be achieved via ubiquitin-dependent endocytosis of GlyT to recycling
343 endosomes, from which it can be returned to the plasma membrane when needed (Barrera
344 et al., 2015;de Juan-Sanz et al., 2011;de Juan-Sanz et al., 2013;Fernandez-Sanchez et al.,
345 2009). Ubiquitylation of the GlyT is associated with internalization of the transporter,
346 where it can where it can no longer contribute to cellular glycine uptake. In that case,
347 one might expect constitutively higher levels of glycine in cells that had lost the ability to
348 ubiquitinate the transporter. If the Ntl transporter was similarly regulated through
349 ubiquitylation by *dMARCH8,* one might expect that loss of *dMARCH8* would not result
350 in reduced availability of glycine as a result of the intracellular sequestration of the Ntl
351 transporter. Instead, the opposite is observed, a reduction in polyglycylation and
352 glutamylation. This suggests that *Ntl* and potentially a glutamate transporter are not

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353 themselves substrates of *dMARCH8,* but that the reduced glycylation and glutamylation
354 results from the activity of *dMARCH8* on factors upstream of glycine/glutamate transport,
355 or in some parallel pathway. Future studies will seek to clarify whether Ntl is a substrate
356 of dMARCH8 and to identify and characterize any additional components of the system
357 controlling spermiogenic tubulin glycylation and glutamylation.

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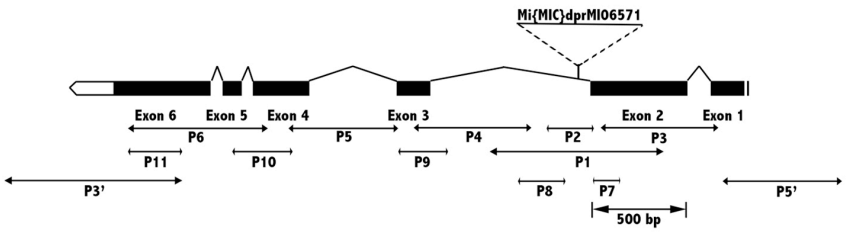
Figure 1

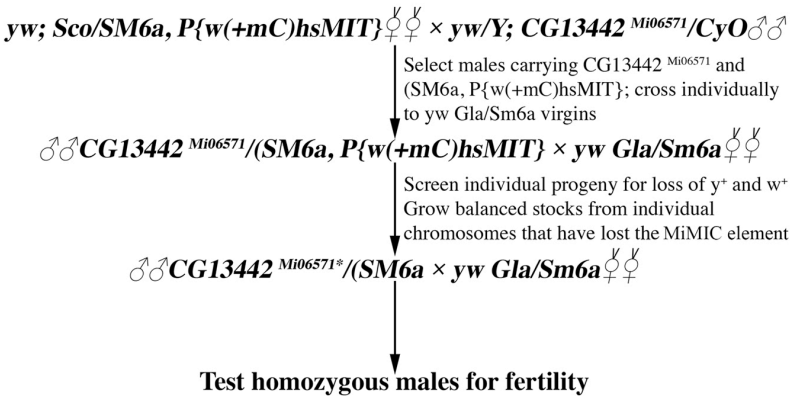
A

```
CG13442          MECEKCDITSNAGDHRLNLTRSLSDLKPETTLEMEIDEALSSSSADDTPPGMIVVETRIS 60
HU_MARCH_8       -------------------------------MSMPLHQISAIPSQDAIS----------- 18
                                                :.* :.:  : .* *  .

CG13442          SWQKVKCYDKDGTTADAEDPQIPACSLGLKPNVQFQKLPHPSHPLVSTEAVLAAATSSSY 120
HU_MARCH_8       ----ARVYRSKTKEKEREE-----------------------QNEKTLGHFMSHSSNISK 51
                     .: * .. .  : *:                        :   :   .:: ::. *
                                                                ZF-RING-CH
CG13442          SAGPPKAAATDSIATLRHCVDGATQCNNLNYESASNESMPSVGSLVCRICHN-ADNPEQL 179
HU_MARCH_8       AGSPPSASAPAPVS------------------SFSRTSITPSSQDICRICHCEGDDESPL 93
                 :..**.*:*. .::                  * *. *:.. .. :*****  .*: . *

CG13442          VSPCLCKGSLTYVHVHCLECWISTSRCTTCELCQFQYNTEQTLRYTCLQSLRLWYSRAMS 239
HU_MARCH_8       ITPCHCTGSLHFVHQACLQQWIKSSDTRCCELCKYEFIMETKLKP-----LRKWEKLQMT 148
                 ::** *.*** :**  **: **.:*    ****::::  * .*:      ** * .  *:
                                TM HELIX-1                          TM HELIX-2
CG13442          RRALQEDCQMFSLLTLVAFGIIGTLLVGIQYYALHTHSWGLSKLWTKSWMLFFLFMTITV 299
HU_MARCH_8       SSERRKIMCSVTFHVIAITCVVWSLYVLIDRTAEEIKQG--QATGILEWPFWTKLVVVAI 206
                     ::    .:: .:.   :: :* * *:  * . :.   .     .* ::  ::.:::

CG13442          YFANIYMLIKSQLTPWYRWWQSARDIKLILENRRPFPNPSRFLRSFQLETVSTTASMYTH 359
HU_MARCH_8       GFTGGLLFMYVQCKVYVQLWKRLKAYNRVIYVQN---CPETSKKNIFEKSPLTEPNFENK 263
                  *:.  :::  * . : : *:  :  : ::  :.    *.   :.:  ::  * ..: .:

CG13442          HDQQEAPVPSSSPVIVITSSEEETEDKLAHKWGTRLDSEVLAAVVAATVESIADASARES 419
HU_MARCH_8       HGYGICHSDTNS---SCCTEPEDTGAEIIHV----------------------------- 291
                 *.   .   :.*      :. *:*  :: *

CG13442          DKQDED 425
HU_MARCH_8       ------
```

B



C



358

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Figure 1: Amino acid sequence alignment of *Drosophila melanogaster CG13442 (dMARCH8)* with human MARCH 8 using ClustalW alignment and generation of mutants in the *dMARCH8* (CG13442) gene.

A) Strictly conserved residues are highlighted in blue. Conserved RING-CH domain is highlighted in yellow and transmembrane domains 1 and 2 are highlighted in red and green respectively.

B) *dMARCH8* (CG13442) transcript/CDR at 57B1 on the right arm of chromosome 2. MiMIC transposon element is inserted between Exon2 and Exon3, closer to Exon2.

C) Scheme for generation of *dMARCH8* (CG13442) deletion mutations by imprecise excision of the MiMIC transposon

Figure 2

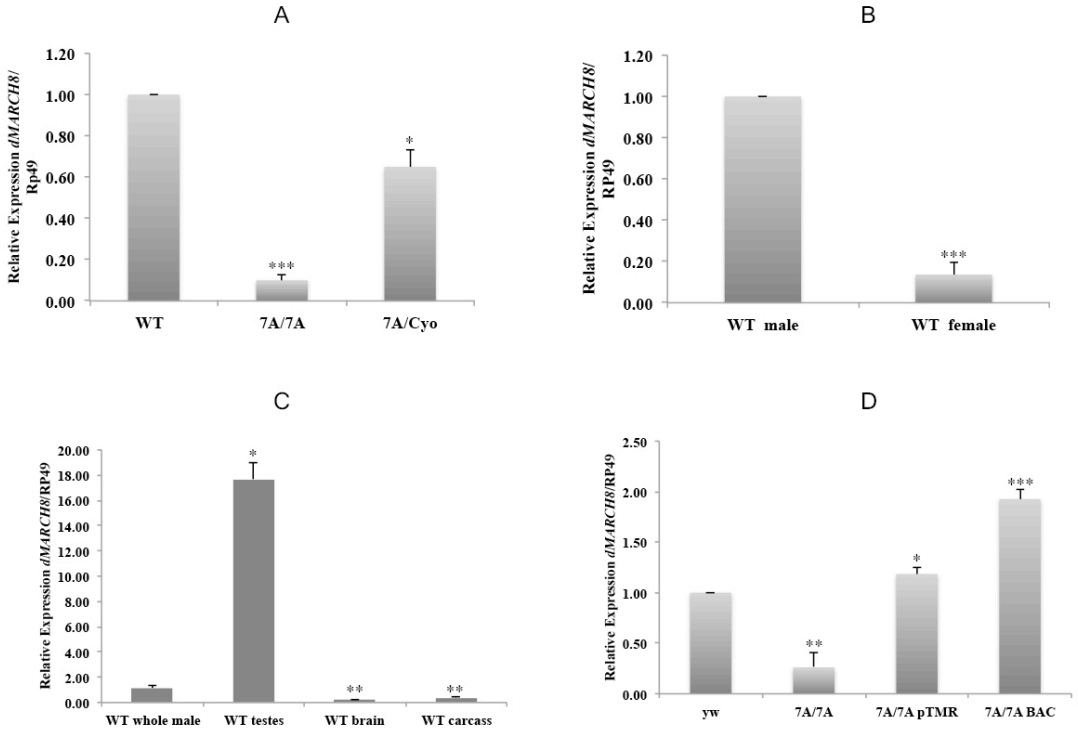

Figure 2: Analysis of *dMARCH8* mRNA Expression

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A) qRT-PCR analysis shows decrease in *dMARCH8* expression levels in *dMARCH8*^{7A}/*dMARCH8*^{7A} (7A/7A) mutants as compared to wild-type(*yw*) and heterozygous *dMARCH8*^{7A}/*CyO* flies. Bar, standard error of the mean. N=3 (* p<0.05, ** p<0.01, **p<0.001 by two-tailed t-test).

B) qRT-PCR analysis compares the relative expression of *dMARCH8* in wild-type (*yw*) male and female (whole adult flies). Bar, standard error of the mean. N=3 (* p<0.05, ** p<0.01, **p<0.001 by two-tailed t-test).

C) qRT-PCR products from wild-type (*yw*) whole male, (*yw*) male testes, (*yw*) male brain, and (*yw*) male carcass. Bar, standard error of the mean. N=3 (* p<0.05, ** p<0.01, **p<0.001 by two-tailed t-test).

D) *dMARCH8* qRT-PCR products from *dMARCH8*^{7A}/*dMARCH8*^{7A} (7A/7A) mutant males carrying a pTMR-*dMARCH8* cDNA construct (*yw*; *dMARCH8*^{7A}/*dMARCH8*^{7A}; pTMR- *dMARCH8*) (7A/7A;pTMR) and a genomic BAC construct (*yw*; *dMARCH8*^{7A}/*dMARCH8*^{7A}; BAC-*dMARCH8*) (7A/7A;BAC) compared to mutant *dMARCH8*^{7A}/*dMARCH8*^{7A} and wild-type controls (*yw*). Bar, standard error of the mean. N=3 (* p<0.05, ** p<0.01, **p<0.001 by two-tailed t-test).

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Figure 3

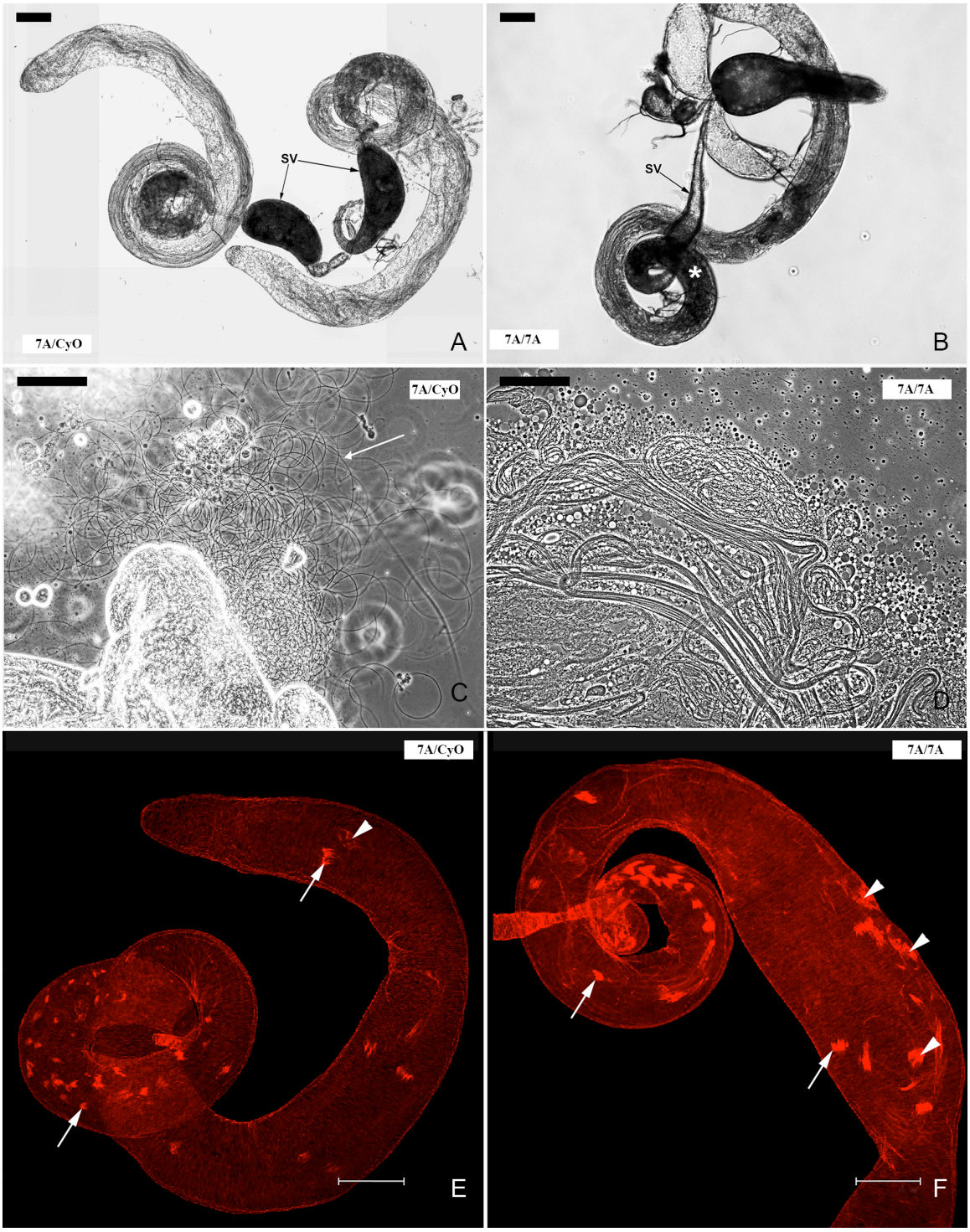

Figure 3: Immotility of *dMARCH8* mutant sperm, failing to transfer into seminal vesicles and individualization in *dMARCH8* mutants.

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Panels A, B, C and D: Phase contrast images of testes from *dMARCH8*$^{+}$ (A, C) and *dMARCH8*$^{-}$ (B, D) males. The major phenotypic feature of the mutants is the accumulation of coiled cysts at the base of the testis (asterisks), and the empty/shrunken state of the seminal vesicle (SV) (arrows). In panel C, the letter M denotes dense masses of mature motile sperm, which is not seen in the mutants. Left hand panels: heterozygous *(dMARCH8*^{7A}*/Cyo)*; right hand panels: *(dMARCH8*^{7A}*/dMARCH8*^{7A}*)* mutants. Bars, 100 µm.

E: Heterozygous *dMARCH8*^{7A}*/Cyo* testis stained with rhodamine-conjugated phalloidin to visualize the actin cone-based individualization complexes. Arrows mark the actin cones of the complex. Arrowheads mark the waste-bags.

F: *dMARCH8*^{7A}*/ dMARCH8*^{7A} mutant testis preparations stained with rhodamine-conjugated phalloidin to visualize the actin cone-based individualization complexes. Formation and movement of actin cones/individualization complex along the mutant cysts appears normal. Bars, 100 um.

Figure 4

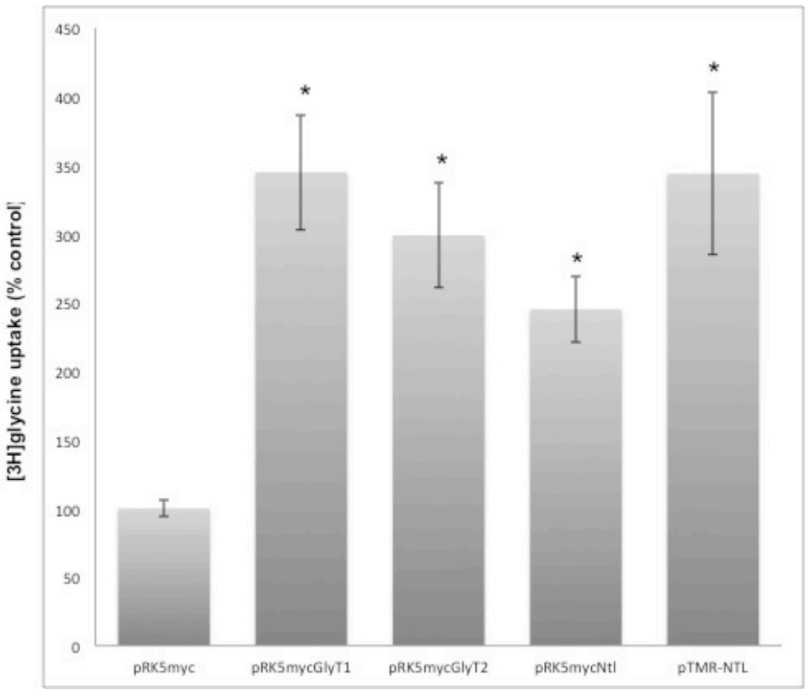

Figure 4: Drosophila Ntl is a functional glycine transporter.

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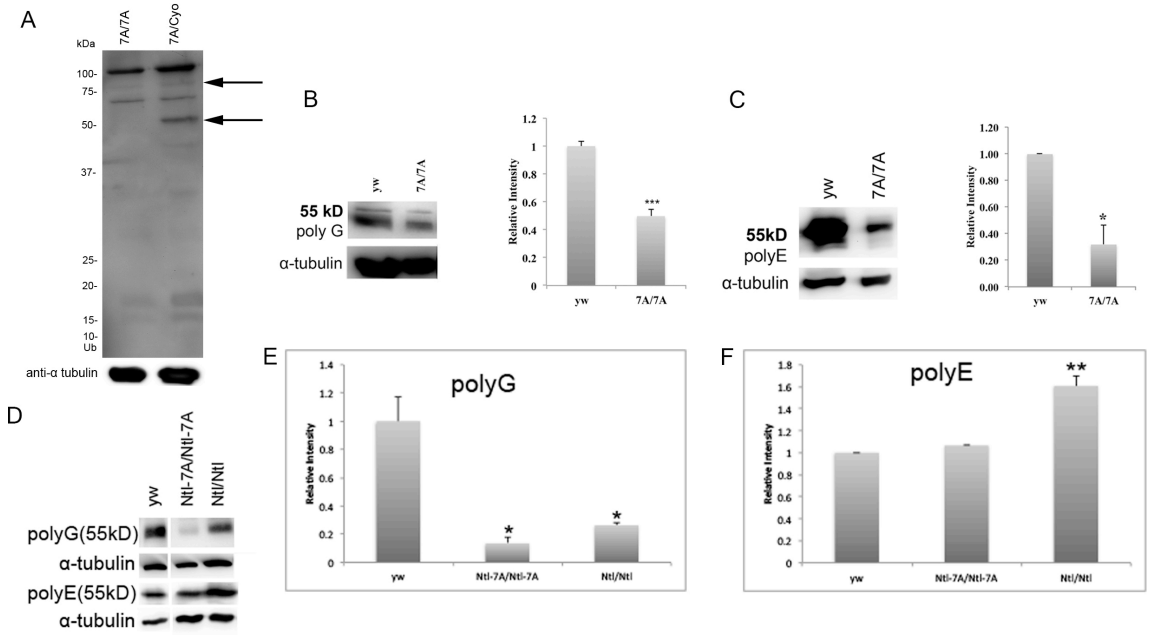
406 Glycine uptake in HEK293 cells transiently expressing hGlyT1, hGlyT2 and *Drosophila*
407 Ntl expressed from two different expression vectors after 5 min of incubation with
408 [^{3}H]glycine at a final concentration of 300 µM. Because low levels of glycine uptake are
409 found in HEK293 cells (Carta et al., 2012), [^{3}H]glycine uptake was calculated as
410 nmol/min/mg of protein and then expressed as a percentage of the empty expression
411 vector (pRK5-myc) transfected control. The data are the means ± S.E. (n = 7-8).
412 Statistical comparisons were made using an unpaired Students t-test. The *asterisk*
413 indicates significantly different from empty vector control ($p < 0.01$).

Figure 5



414

415 **Figure 5: Western Blot Analysis of *dMARCH8^{7A}/dMARCH8^{7A}* , *Ntl/Ntl* and *Ntl-***
416 ***dMARCH8^{7A}* mutant testes.**

417 A) Total ubiquitination levels of testis proteins are reduced in dMARCH8 mutant testes.
418 Western blot of dMARCH87A/dMARCH87A (dMARCH8-) males compared to
419 dMARCH87A/CyO (dMARCH8+) heterozygous males probed with anti-ubiquitin
420 antibody.

421 B) Polyglycylation of tubulin is partially decreased in mutant testes. Western blot of
422 dMARCH87A/dMARCH87A (dMARCH8-) males compared to yw (dMARCH8+/+)

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probed with anti Poly-G antiserum. Quantitation of three independent replicates of the Western analysis. Bar, standard error of the mean. N=3 (* $p<0.05$, ** $p<0.01$, **$p<0.001$ by two-tailed t-test).

C) Polyglutamylation of tubulin is partially decreased in mutant testes. Western blot of dMARCH87A/dMARCH87A (dMARCH8-) males compared to yw (dMARCH8+/+) controls probed with anti Poly-E antiserum. Quantitation of three independent replicates of the Western analysis. Bar, standard error of the mean. N=3 (* $p<0.05$, ** $p<0.01$, **$p<0.001$ by two-tailed t-test).

(D, E, F) Polyglycylation (polyG) and polyglutamylation (polyE) levels of tubulin in Ntl/Ntl and Ntl-dMARCH87A mutant testes. Western blot showing tubulin polyglycylation and polyglutamylation levels in Ntl/Ntl (lane 3) and Ntl-dMARCH87A/ Ntl-dMARCH87A (lane 2) mutant testes compared to yw (WT) (lane 1) probed with anti Poly-G and Poly-E antiserum. Quantitation of three independent replicates of the polyG and polyE Western analysis respectively. Bar, standard error of the mean. N=3 (* $p<0.05$, ** $p<0.01$, **$p<0.001$ by two-tailed t-test).

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452

453

454

Primer pair	**Sense**	**Antisense**
Interval P1	GGGCATGATCGTTGTGGAGA	AAATAAATGCATTCGCTCGC
Interval P2	CCGATAATCCCGAACAGTGA	GAAAAACCCCACTGGTGATG
Interval P3	CGTCGAACCGATCAAAGAAT	ATGCGGACTCGTAATTCAGG
Interval P4	TCCCCGCATTTGAATATTGT	GCGAGGTGCTAATCCAACAC
Interval P5	AAGGTGAGCAGCGAGAACAT	CGGAACAGACTCTGCGGTA
Interval P6	CCTGCTGGTAGGCATCCAGTACTA	GAAAGCCCAGCAAAATCCTT
Interval P7	CCACCAGCTCCTCCTATTCC	GGGATTATCGGCATTGTGAC
Interval P8	TTTTTGGTTTGCCCTTTTTG	GCTGCACGTGCGTAATTAAA
Interval P9	CCCGTCCACTGAAACTTCC	CCGCAGAGTCTGTTCCGTAT
Interval P10	AGGACTGCCAGATGTTCTCG	AGCTGGGACTTGATCAGCAT
Interval P11	GAGGATAAGTTGGCCCACAA	GAAAGCCCAGCAAAATCCTT
Interval P5'	TGCTGAACAGAACAGATCCA	TGGGGTACCGAAATTAACCA
Interval P3'	GAGGATAAGTTGGCCCACAA	GCGTCCGTGTTATCAGCTTT
***CG13442* RT PCR**	TGCAATAACCTGAATTACGAGT	GTGTTTGTGCAAGGGCTCGC
RP49 RT PCR	AGCGCACCAAGCACTTCATC	GTGCGCTTGTTCGATCCGT
CG13442	GAGTCCGCATCCAACGAATCGAT	GCGAGCCCTTGCACAAACAC

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qRTPCR

Table 1: Primers

Alleles	P1	P2	P3	P4	P5	P6	P7	P8	P9	P10	P11	P5'	P3'
7A	-	-	+	-	-	-	+	-	-	-	-	+	-
18A	-	-	+	+	+	+	+	+	+	+	+	+	+
16A	-	-	-	-	+	+	-	-	-	+	+	-	+
19A	-	-	+	-	-	+	-	-	-	+	+	+	+
18B	-	-	+	-	-	+	+	-	-	+	+	+	+
WT(yw)	+	+	+	+	+	+	+	+	+	+	+	+	+

Table 2: Deletion Analysis of CG13442 mutants

+/- Refer to presence or absence of the band respectively

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