

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

3

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11

## ABSTRACT

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

27

## 28 **Introduction**

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

38

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

51 Because there is very little transcription in spermiogenic cysts(Barreau et al.,  
52 2008) posttranscriptional and posttranslational regulatory mechanisms are especially  
53 important in this process (Karr, 2007). Ubiquitination is one of these processes, affecting  
54 many different aspects of spermiogenesis (Richburg et al., 2014). Although the ubiquitin  
55 system is known primarily for marking proteins for degradation (Ciechanover, 2005;Zhi  
56 et al., 2013), recent studies indicate that ubiquitination can affect many other aspects of  
57 protein function, including intracellular trafficking (de Juan-Sanz et al., 2011),

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 polyglutamylolation 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 [<sup>3</sup>H]glycine uptake assay. Loss of *Ntl*  
68 function results in a large increase in polyglutamylolation levels, indicating that *Ntl* and  
69 *dMARCH8* activities contribute to the balance of glutamylolation and glycylation of sperm  
70 tubulin.

71

## 72 **Materials and Methods**

### 73 **Fly Husbandry**

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

### 81 **Generation of *dMARCH8* mutants**

82 The *dMARCH8* transcript/CDR is in the 57B region on the 2R arm of the *Drosophila*  
83 chromosome. The Mi{MIC}(Minos Mediated Integrated Cassette)  $dpr^{M106571}$  transposon  
84 carrying the  $y^+$  marker was generated by the *Drosophila* Gene disruption project  
85 (GDP)(Venken et al., 2011)The transposon was mobilized by crossing the flies carrying  
86 the MiMIC insertion to flies carrying stable heat shock transposase ( $y^1 w^*$ ;  $sna^{Sco}/SM6a$ ,  
87  $P\{w^{+mC}=hsILMiT\}2.4$  (Metaxakis et al., 2005;Venken et al., 2011). Three broods from  
88 each cross were generated by transferring flies to new bottles on the 3<sup>rd</sup> and 5<sup>th</sup> days after  
89 the cross was initially set up. Bottles were subjected to heat shock (39°C, 90 minutes) on  
90 the third, 5<sup>th</sup> and seventh day of each brood. Chromosomes that lost the  $y^+$  markers  
91 carried by the transposon were recovered and established in balanced stocks using  
92 standard *Drosophila* genetics, then screened for new male-sterile mutations produced by  
93 imprecise excision of the Mi{MIC} transposon.

### 94 **RNA isolation and qRT-PCR**

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

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

101 for 5 min followed by the amplification steps of 95°C for 15 sec, 56°C for 30 seconds,  
102 72°C for 30 sec. 45 cycles of PCR were run for all samples followed by 72°C for 15 min,  
103 57°C for 10 min and held at 4°C overnight. *dMARCH8* qRT primers amplified a 112 bp  
104 fragment, while rp49 control primers amplified a 154 bp fragment. Primers for this and  
105 all subsequent PCR-based molecular biology are specified in Table 1.

### 106 **Deletion PCR**

107 Genomic DNA was isolated from males according to the Berkeley *Drosophila* Genome  
108 Project protocol (<http://www.fruitfly.org/about/methods/inverse.pcr.html>). 13 pairs of  
109 gene-specific primers were used to amplify the entire coding sequence of *dMARCH8*.  
110 The thermocycler (MJ Research PTC-200 Peltier thermal cycler) was programmed as  
111 follows: 94°C for 2 min followed by 30 cycles of: 94°C for 1 min, 56.3°C for 1 min,  
112 72°C for 1 min. After a final 5 min at 72°C, samples were held at 4°C until gel analysis.

### 113 **Phalloidin assay for Individualization Complex**

114 Testes from 0–1 d old males were crudely dissected in *Drosophila* Ringers and  
115 transferred immediately to a tube of Ringers on ice. Testes were then fixed for 15 min in  
116 4% paraformaldehyde in buffer B (16.7mM KH<sub>2</sub>PO<sub>4</sub>/K<sub>2</sub>HPO<sub>4</sub> pH 6.8, 75mM KCl,  
117 25mM NaCl, 3.3mM MgCl<sub>2</sub>). Following fixation, testes were rinsed three times in PTx  
118 (PBS+0.1% Triton X-100), washed for 30 min in PTx and blocked for at least 1 h in  
119 blocking solution (0.01% NaAzide and 3% BSA in PTx). Staining with rhodamine-  
120 conjugated phalloidin was for 30 min in blocking solution (3 µg/ml). Testes were then  
121 rinsed and washed with PTx. Testes were then finely dissected from remaining carcasses  
122 in 50% glycerol and then mounted in 90% glycerol. Slides were stored overnight at 4°C  
123 before imaging. Fluorescence images were captured by confocal microscopy (Leica TCS-  
124 2, Exton, PA)(Fabrizio et al., 2012)

### 125 **Phase squashes**

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

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

### 131 **Protein Electrophoresis and Immunoblot**

132 Samples were prepared from male fly testes with seminal vesicle for each genotype, from  
133 males that were withheld from females for 4–6 days in order to maximize sperm yield.  
134 Six fly testes (12 testes) worth of protein were loaded in each lane. Testes were dissected  
135 in 1× PBS, ground in 2× Laemmli buffer (Laemmli UK., 1970) vortexed and boiled for  
136 5 min. Samples were then spun at 15,800 g for 5 min, and supernatants were separated in  
137 a 12% SDS-Polyacrylamide gel and transferred to a PVDF membrane (Amersham, GE  
138 Healthcare, United Kingdom) using a Trans-Blot Semi-Dry transfer apparatus (BioRad,  
139 U.S.A). Membranes were incubated with primary antibodies directed against poly-  
140 glycylation tubulin (Poly-G) (1:10,00) (MABS276, clone AXO49, Millipore),  
141 polyglutamylated tubulin (Poly-E) (1:1000) (mAbGT355, Adipogen Life sciences),  
142 ubiquitin {MAB1510 clone Ubi-1 (aka 042691GS), Millipore}, TSSK2 (ab172434,  
143 Abcam) and  $\alpha$ -tubulin (1:500), (DM1A, Sigma). Protein bands were visualized with  
144 HRP-labelled anti rabbit or anti-mouse (1:5,000) secondary antibodies followed by  
145 detection with ECL immunoblot detection kit (Pierce, U.S.A). Loading control was  $\alpha$ -  
146 tubulin.

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

### 152 **[<sup>3</sup>H]glycine uptake assays**

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

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<sub>2</sub>PO<sub>4</sub>, 26 mM  
161 NaHCO<sub>3</sub>, 1.5 mM MgSO<sub>4</sub>, 5 mM KCl, 1.3 mM CaCl<sub>2</sub>, 20 mM glucose) pre-equilibrated  
162 with 5% CO<sub>2</sub>, 95% air. After 2 min, the cells were incubated for 5 min in 0.1 μCi/ml  
163 [<sup>3</sup>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<sub>2</sub>, 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). [<sup>3</sup>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<sup>7A</sup>/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<sup>7A</sup>* mutants.

177

## 178 **Results**

### 179 **Amino acid sequence alignment of *dMARCH8* (*CG13442*) with human MARCH8-** 180 **E3 ubiquitin Ligases belonging to the RING protein family**

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

### 188 **Generation of *dMARCH8* mutants**

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

### 203 **Deletion PCR analysis of *CG13442/dMARCH8* mutants**

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

207 changes in the chromosome structure by PCR. For all of the male-sterile mutants, PCR  
208 amplicons including portions of the gene and flanking regions on either side were  
209 missing or produced products with altered size as compared to wild-type DNA (Table 2).  
210 These results indicated that the deletion carried by the *dMARCH8*<sup>7A</sup> mutant removes the  
211 sequences encoding the RING domain and both transmembrane domains (Table 2).  
212 Furthermore, qRT-PCR analysis of this mutant revealed a significant reduction (80 +/-  
213 5% in *dMARCH8* expression (Fig. 2A). The *dMARCH8*<sup>7A</sup> allele was used for the  
214 remaining phenotypic analyses.

### 215 **Testis Specificity of *dMARCH8* expression**

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

### 219 **Rescue by Germline Transformation**

220 We used a P-element based construct for germline transformation with CG13442/  
221 *dMARCH8*, to confirm that the male sterile phenotype in our mutants is caused solely due  
222 to disruption of *dMARCH8*. For this we used a pTMR-*dMARCH8* construct, which  
223 contains a full-length cDNA for CG13442 (DGRC clone AT03090) cloned downstream  
224 of the  $\beta$ 2T-tubulin transcriptional control sequences in the *Drosophila* transformation  
225 vector pTMR (Clark et al., 2006;Huh et al., 2004). This construct provides strong germ  
226 cell-specific transcription in developing sperm (Kaltschmidt et al., 1991;Kemphues et al.,  
227 1982). A BAC genomic construct CH322-140N02 (BACPAC resources,  
228 bacpac.chori.org) extending from 9.8 kb upstream of the *dMARCH8* transcript to 8.5 kb  
229 beyond the 3' end also rescued the *dMARCH8* mutant phenotype. Two independent  
230 insertions of this construct were tested and they both rescued the mutant. All the males  
231 homozygous for the *dMARCH8*<sup>7A</sup> deletion and carrying pTMR-*dMARCH8* or BAC-  
232 *dMARCH8* constructs were fertile. Results from the rescued line are presented in Fig.  
233 2D. The male-sterile mutations obtained after mobilization of the Mi{MIC} transposon  
234 therefore result from disruption of *dMARCH8*.

## 235 **Spermiogenic-defective phenotype of *dMARCH8* mutants**

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

248

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

## 257 **Demonstration of Glycine transport activity from Ntl expression in mammalian cells**

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

264 [<sup>3</sup>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  $\alpha$ -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*<sup>7A/+</sup> flies are missing in the testes of homozygous  
273 *dMARCH8*<sup>7A</sup> flies. A slight reduction in several other bands (~65 and 100 kDa) was also  
274 consistently observed in the *dMARCH8*<sup>7A</sup> mutant.

### 275 **Reduction of tubulin polyglycylation and polyglutamylolation 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). Polyglutamylolation 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 polyglutamylolation 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  $\alpha$ -tubulin as  
292 a loading control showed an average of 50% reduction in poly-G signal in the  
293 *dMARCH8*<sup>7A</sup> mutant samples relative to wild-type controls (Fig 5B). Similarly, for the

294 poly-E signal, quantitation from three independent experiments exhibited an average of  
295 70% reduction in *dMARCH8*<sup>7A</sup> mutants compared to wild-type controls (Fig. 5C).

### 296 **Polyglycylation and polyglutamylolation in a *Ntl-dMARCH8* double mutant**

297 Since the *Ntl* mutant exhibits a similar phenotype to the *dMARCH8* mutant, and it also  
298 shows a predicted interaction (STITCH database) with dMARCH8, we analyzed the *Ntl*-  
299 *dMARCH8* double mutant for polyglycylation and polyglutamylolation levels of tubulin in  
300 three independent experiments. We observed that in both the *Ntl* mutant and the *Ntl*-  
301 *dMARCH8* double mutant, poly-G levels decrease while the poly-E levels increase (Fig.  
302 5D,E and F). Perhaps most strikingly, loss of *Ntl* in the *dMARCH8* genetic background  
303 restores levels of polyglutamylolation to wild-type levels (compare Fig. 5C and 5F). In this  
304 system, polyglutamylolation appears to be very sensitive to glycine levels, consistent with  
305 observations by others that glutamate and glycine compete with each other for common  
306 site(s) on tubulin (Bulinski, 2009; Rogowski et al., 2009b; Wloga et al., 2009).

### 307 **Discussion**

308 Functional spermatogenesis is particularly dependent on ubiquitin-regulated protein  
309 function and stability (Mukhopadhyay and Riezman, 2007; Richburg et al., 2014). This is  
310 evident from the spermatogenesis-specific functions of a number of E3 ligases: Bruce and  
311 Cullin3 in *Drosophila* have been studied for their role in sperm individualization (Arama  
312 et al., 2007; Arama et al., 2003; Kaplan et al., 2010; Wang et al., 2006). RNF8 and E3<sup>histone</sup>,  
313 found in mice and rats respectively, are reported to be involved in the histone degradation  
314 that occurs during histone to protamine transition in spermatid nuclei in rats (Liu et al.,  
315 2005; Lu et al., 2010). The E3 ligases Cul4A (Kopanja et al., 2011; Yin et al., 2011), Itch  
316 (Dwyer and Richburg, 2012) and Siah1a (Dickins et al., 2002) play roles during germ cell  
317 meiosis and Cullin3<sup>testis</sup> (Arama et al., 2007; Kaplan et al., 2010) and MEX (Nishito et al.,  
318 2006) are required during germ cell apoptosis. MARCH7 (Membrane-Associated Ring-  
319 CH) was found to be involved in spermiogenesis by regulating the structural and  
320 functional integrity of the head and tail of developing spermatids (Zhao et al., 2013).  
321 MARCH10 was found to be essential for spermatid maturation (Iyengar PV, Hirota T,  
322 Hirose S, Nakamura N., 2011). MARCH11 plays a role in ubiquitin-mediated protein

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

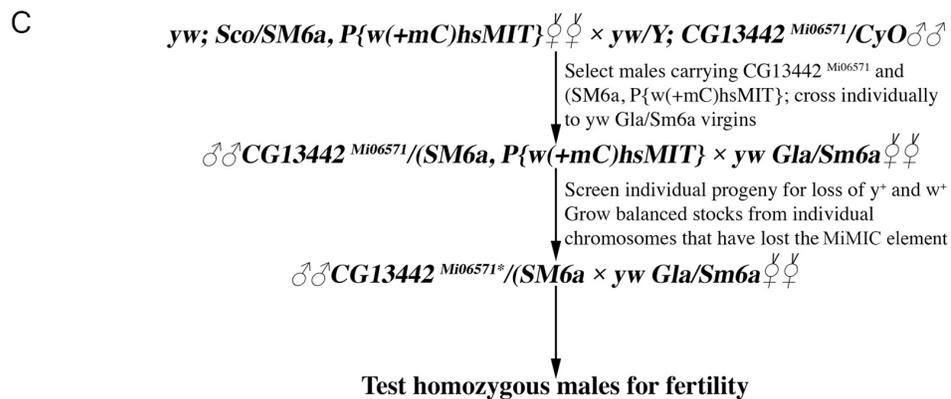
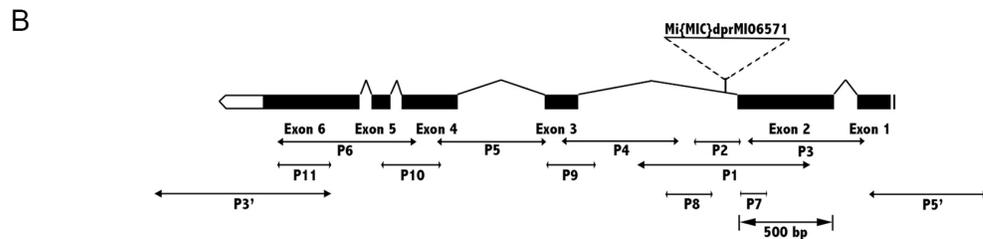
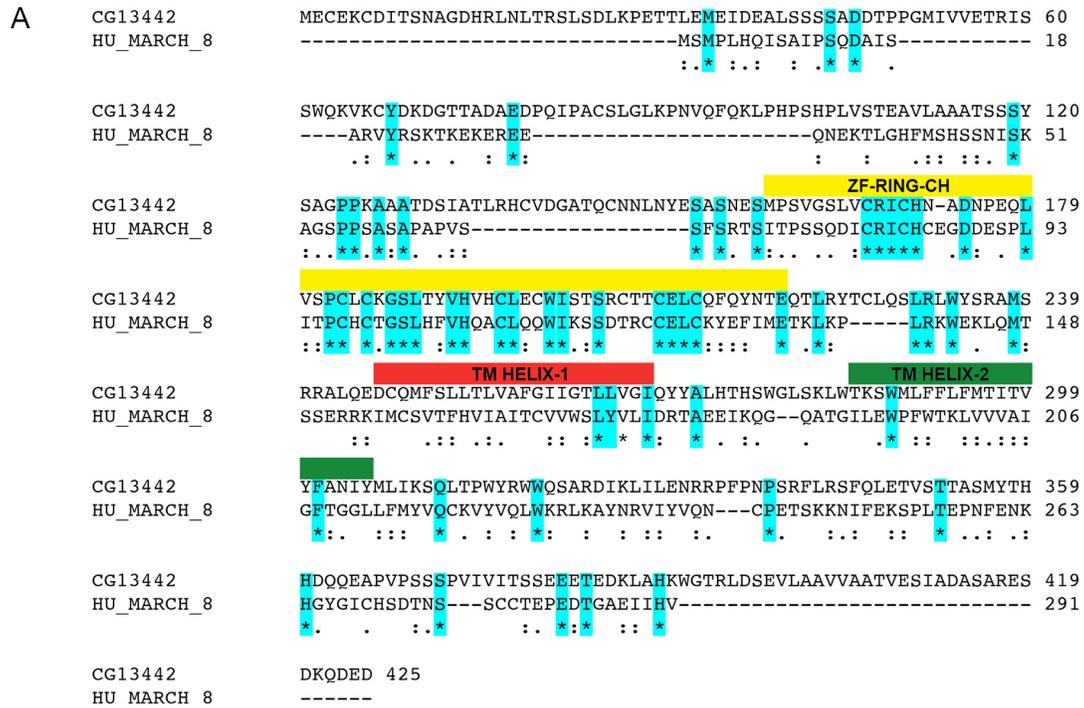
325 Indeed, of all organs examined in rats, ubiquitination has been reported to be highest in  
326 testis (Rajapurohitam et al., 2002). Combined with the observed genetic sensitivity of the  
327 spermiogenic process, these considerations indicate that details of cellular ubiquitin  
328 function may 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 [<sup>3</sup>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

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.

Figure 1



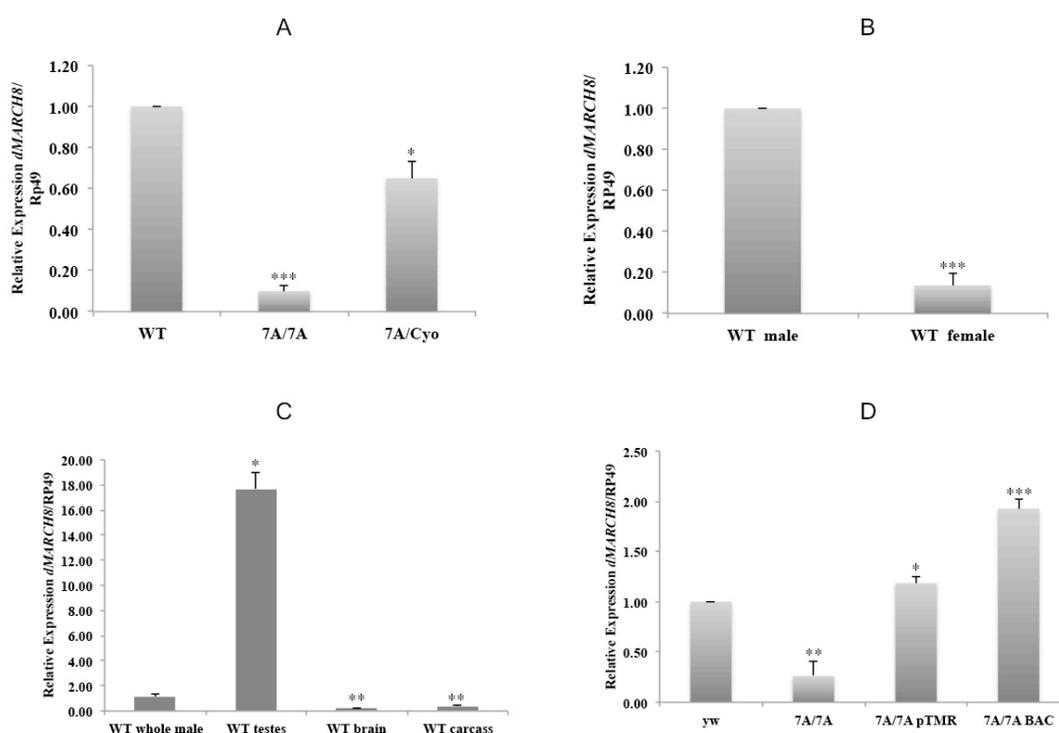
359 **Figure 1: Amino acid sequence alignment of *Drosophila melanogaster* CG13442**  
360 **(*dMARCH8*) with human MARCH 8 using ClustalW alignment and generation of**  
361 **mutants in the *dMARCH8* (CG13442) gene.**

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

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

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

Figure 2



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370 **Figure 2: Analysis of *dMARCH8* mRNA Expression**

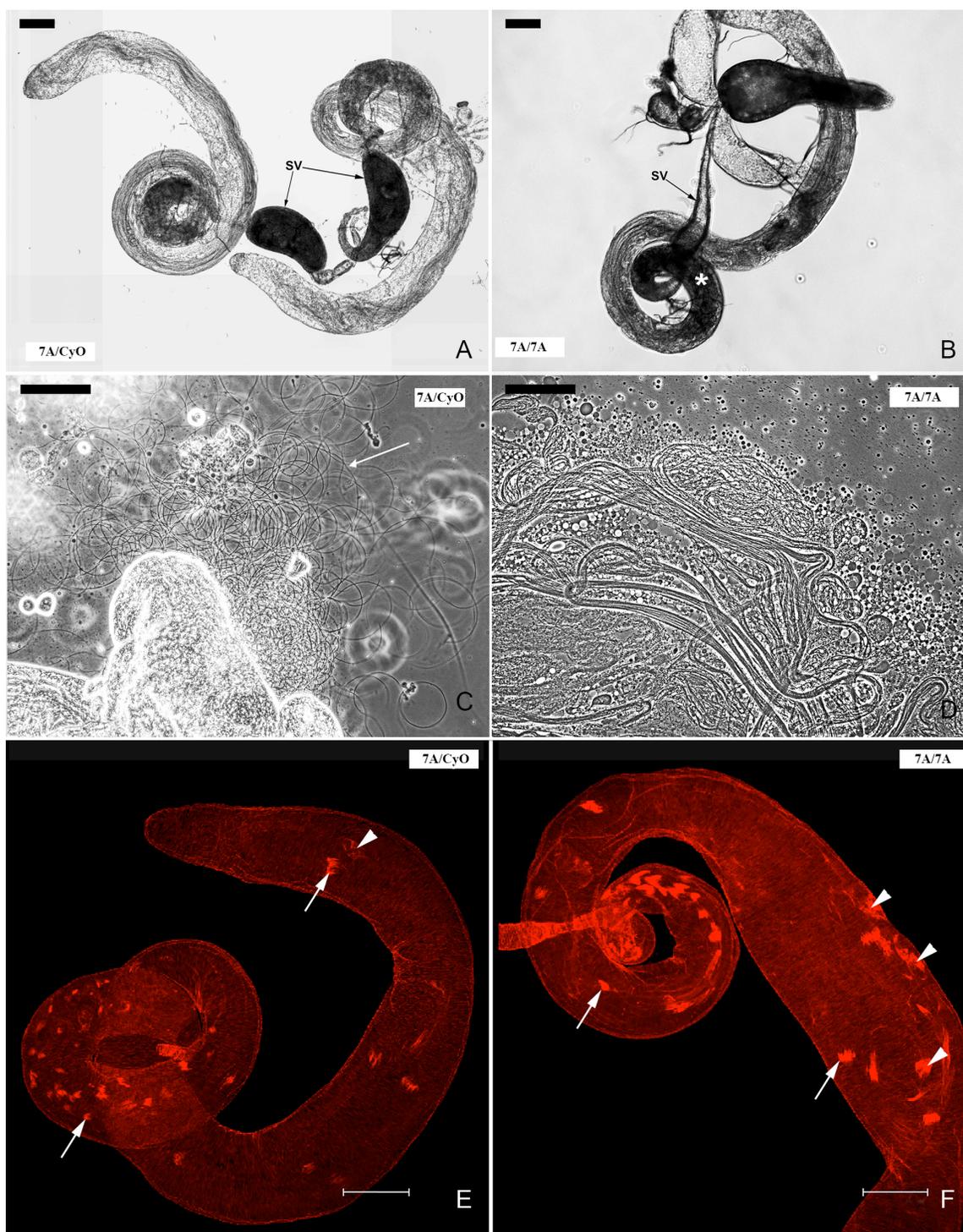
371 A) qRT-PCR analysis shows decrease in *dMARCH8* expression levels in  
372 *dMARCH8<sup>7A</sup>/dMARCH8<sup>7A</sup>* (7A/7A) mutants as compared to wild-type(*yw*) and  
373 heterozygous *dMARCH8<sup>7A</sup>/CyO* flies. Bar, standard error of the mean. N=3 (\* p<0.05, \*\*  
374 p<0.01, \*\*p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

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

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

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

Figure 3



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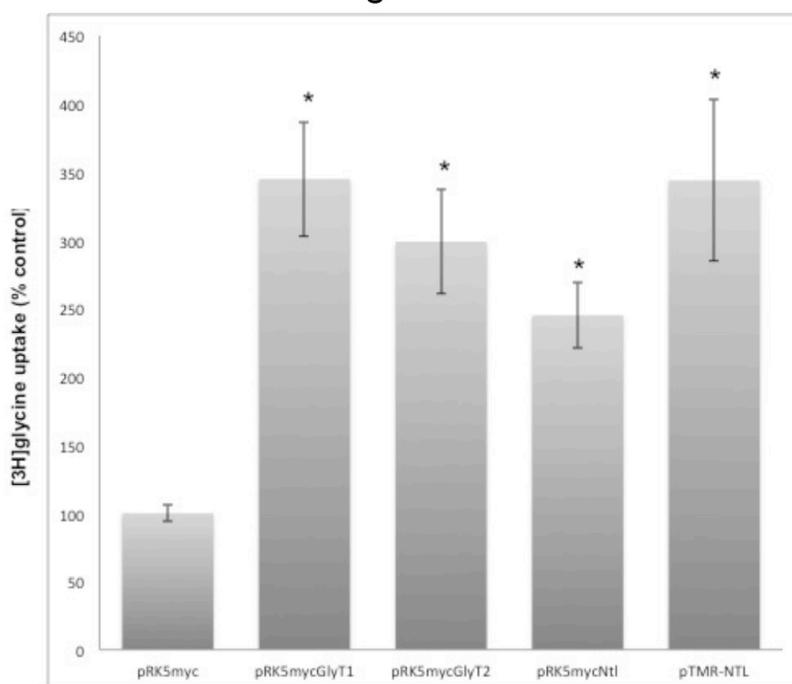
388 **Figure 3: Immotility of *dMARCH8* mutant sperm, failing to transfer into seminal**  
389 **vesicles and individualization in *dMARCH8* mutants.**

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

397 E: Heterozygous *dMARCH8*<sup>7A</sup>/*Cyo* testis stained with rhodamine-conjugated phalloidin  
398 to visualize the actin cone-based individualization complexes. Arrows mark the actin  
399 cones of the complex. Arrowheads mark the waste-bags.

400 F: *dMARCH8*<sup>7A</sup>/*dMARCH8*<sup>7A</sup> mutant testis preparations stained with rhodamine-  
401 conjugated phalloidin to visualize the actin cone-based individualization complexes.  
402 Formation and movement of actin cones/individualization complex along the mutant  
403 cysts appears normal. Bars, 100  $\mu$ m.

Figure 4

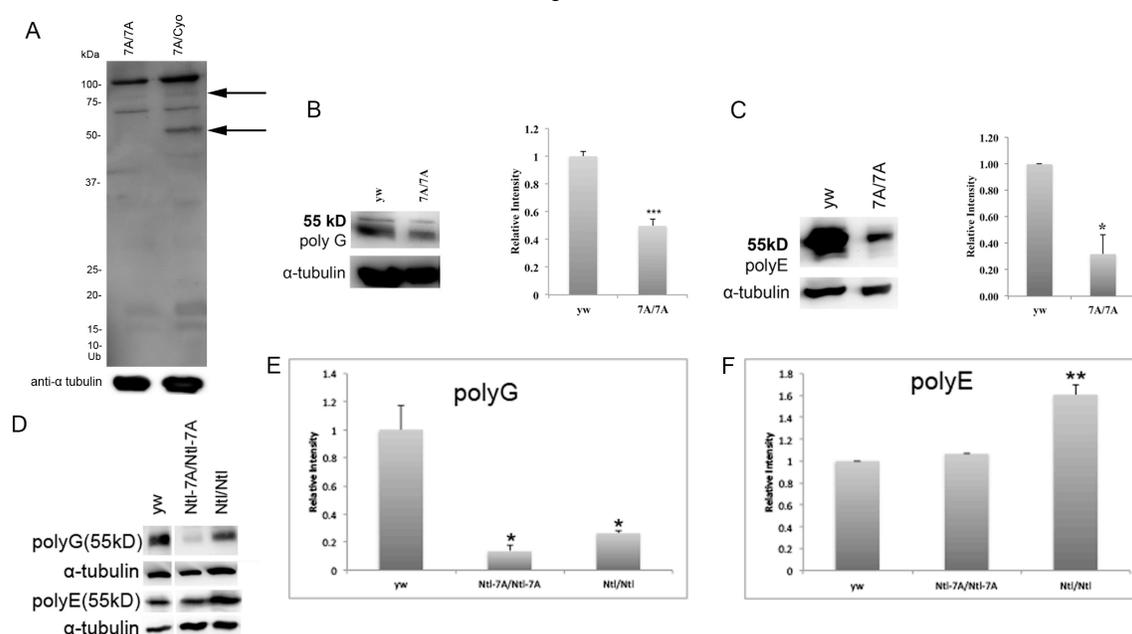


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405 **Figure 4: Drosophila Ntl is a functional glycine transporter.**

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 [<sup>3</sup>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), [<sup>3</sup>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



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415 **Figure 5: Western Blot Analysis of *dMARCH8*<sup>7A</sup>/*dMARCH8*<sup>7A</sup>, *Ntl/Ntl* and *Ntl-***  
 416 ***dMARCH8*<sup>7A</sup> mutant testes.**

417 A) Total ubiquitination levels of testis proteins are reduced in *dMARCH8* mutant testes.  
 418 Western blot of *dMARCH8*<sup>7A</sup>/*dMARCH8*<sup>7A</sup> (*dMARCH8*<sup>-</sup>) males compared to  
 419 *dMARCH8*<sup>7A</sup>/*CyO* (*dMARCH8*<sup>+</sup>) heterozygous males probed with anti-ubiquitin  
 420 antibody.

421 B) Polyglycylation of tubulin is partially decreased in mutant testes. Western blot of  
 422 *dMARCH8*<sup>7A</sup>/*dMARCH8*<sup>7A</sup> (*dMARCH8*<sup>-</sup>) males compared to yw (*dMARCH8*<sup>+/+</sup>)

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

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

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

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<b>Primer pair</b>	<b>Sense</b>	<b>Antisense</b>
<b>Interval P1</b>	GGGCATGATCGTTGTGGAGA	AAATAAATGCATTCGCTCGC
<b>Interval P2</b>	CCGATAATCCCGAACAGTGA	GAAAAACCCCACTGGTGATG
<b>Interval P3</b>	CGTCGAACCGATCAAAGAAT	ATGCGGACTCGTAATTCAGG
<b>Interval P4</b>	TCCCCGCATTTGAATATTGT	GCGAGGTGCTAATCCAACAC
<b>Interval P5</b>	AAGGTGAGCAGCGAGAACAT	CGGAACAGACTCTGCGGTA
<b>Interval P6</b>	CCTGCTGGTAGGCATCCAGTACTA	GAAAGCCCAGCAAAATCCTT
<b>Interval P7</b>	CCACCAGCTCCTCCTATTCC	GGGATTATCGGCATTGTGAC
<b>Interval P8</b>	TTTTTGGTTTGCCCTTTTTG	GCTGCACGTGCGTAATTA
<b>Interval P9</b>	CCCGTCCACTGAACTTCC	CCGCAGAGTCTGTTCCGTAT
<b>Interval P10</b>	AGGACTGCCAGATGTTCTCG	AGCTGGGACTTGATCAGCAT
<b>Interval P11</b>	GAGGATAAGTTGGCCACAA	GAAAGCCCAGCAAAATCCTT
<b>Interval P5'</b>	TGCTGAACAGAACAGATCCA	TGGGGTACCGAAATTAACCA
<b>Interval P3'</b>	GAGGATAAGTTGGCCACAA	GCGTCCGTGTTATCAGCTTT
<b>CG13442 RT PCR</b>	TGCAATAACCTGAATTACGAGT	GTGTTTGTGCAAGGGCTCGC
<b>RP49 RT PCR</b>	AGCGCACCAAGCACTTCATC	GTGCGCTTGTTTCGATCCGT
<b>CG13442</b>	GAGTCCGCATCCAACGAATCGAT	GCGAGCCCTTGACAAAACAC

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qRTPCR

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455 **Table 1: Primers**

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

457 **Table 2: Deletion Analysis of CG13442 mutants**

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

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