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1 Maternal Rest/Nrsf Regulates Zebrafish Behavior Through *snap25a/b*

2 Maternal Rest Regulates Behavior Through *snap25a/b*

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43 Abstract

44 During embryonic development, regulation of gene expression is key to creating the
45 many subtypes of cells that an organism needs throughout its lifetime. Recent work
46 has shown that maternal genetics and environmental factors have lifelong
47 consequences on diverse processes ranging from immune function to stress
48 responses. The RE1-silencing transcription factor (Rest) is a transcriptional
49 repressor that interacts with chromatin-modifying complexes to repress
50 transcription of neural specific genes during early development. Here we show that
51 in zebrafish, maternally supplied *rest* regulates expression of target genes during
52 larval development and has lifelong impacts on behavior. Larvae deprived of
53 maternal *rest* are hyperactive and show atypical spatial preferences. Adult male fish
54 deprived of maternal *rest* present with atypical spatial preferences in a novel
55 environment assay. Transcriptome sequencing revealed 158 genes that are
56 repressed by maternal *rest* in blastula stage embryos. Furthermore, we found that
57 maternal *rest* is required for target gene repression until at least 6 dpf. Importantly,
58 disruption of the RE1 sites in either *snap25a* or *snap25b* resulted in behaviors that
59 recapitulate the hyperactivity phenotype caused by absence of maternal *rest*. Both
60 maternal *rest* mutants and *snap25a* RE1 site mutants have altered primary motor
61 neuron architecture that may account for the enhanced locomotor activity. These
62 results demonstrate that maternal *rest* represses *snap25a/b* to modulate larval
63 behavior and that early Rest activity has lifelong behavioral impacts.

64

65 Significant Statement:

66 Maternal factors deposited in the oocyte have well-established roles during
67 embryonic development. We show that in zebrafish, maternal *rest* (RE1-silencing
68 transcription factor) regulates expression of target genes during larval development
69 and has lifelong impacts on behavior. The Rest transcriptional repressor interacts
70 with chromatin-modifying complexes to limit transcription of neural genes. We
71 identify several synaptic genes that are repressed by maternal Rest and
72 demonstrate that *snap25a/b* are key targets of maternal *rest* that modulate larval
73 locomotor activity. These results reveal that zygotic *rest* is unable to compensate for
74 deficits in maternally supplied *rest* and uncovers novel temporal requirements for
75 *rest* activity, which has implications for the broad roles of Rest-mediated repression
76 during neural development and in disease states.

77 Introduction:

78 Precise regulation of gene expression is key to proper nervous system
79 function and is influenced by both genetic and environmental factors. Central to the
80 mechanisms of gene regulation are chromatin modifications, which include
81 alterations of the acetylation and methylation status of chromatin by transcriptional
82 activators and repressors. Changes to chromatin landscapes may have both
83 immediate and lifelong consequences and are caused by environmental effects
84 including poor maternal care (Weaver et al., 2004), prenatal stress(St-Cyr and
85 McGowan, 2015; Vangeel et al., 2015), smoking(Ivorra et al., 2015), and gestational
86 diabetes(Petropoulos et al., 2015).

87 Maternal mRNAs encoding transcription factors and chromatin effectors are
88 deposited in oocytes prior to fertilization and modulate developmental gene
89 expression in many species. For example, depletion of maternal *Drosophila* Piwi
90 alters heterochromatin formation (Gu and Elgin, 2013); knockdown of VegT in
91 *Xenopus* alters embryonic cell fate and patterning (Zhang et al., 1998); loss of
92 maternal *runx2b* dorsalizes zebrafish embryos (Flores et al., 2008); and deletion of
93 maternal BRG1, arrests mouse development at early cleavage stages and reduces
94 zygotic genome activation (Bultman et al., 2006). These findings suggest a broad
95 role for maternal mRNAs in modulating chromatin landscapes in early embryos.

96 The RE1-Silencing Transcription factor (Rest)/Neuron Restrictive Silencing
97 Factor (Nrsf) recruits cofactors to modify chromatin structure to silence neural
98 specific genes in non-neural tissues (Chong et al., 1995; Schoenherr and Anderson,
99 1995) and to modulate transcription within the developing nervous system (Ballas
100 et al., 2005). Rest regulates hundreds of neural specific genes via interactions with a
101 conserved ~23bp DNA element, the RE1 site (Lunyak, 2002; Mortazavi et al., 2006).
102 The N-terminal domain of Rest interacts with Sin3 family members to recruit
103 repressor complexes that include MeCP2 and HDAC1/2 (Naruse et al., 1999;
104 Grzenda et al., 2009). The Rest C-terminal domain interacts with CoRest family
105 members, which associate with HDAC 1/2, LSD1 and H3K9 methyltransferase G9a,
106 among other factors (Ballas et al., 2001; Lunyak, 2002; Roopra et al., 2004).

107 We previously showed that zebrafish *rest* is broadly expressed in the
108 developing nervous system (Gates et al., 2010), but is not essential for neurogenesis

109 (Kok et al., 2012). Rather, Rest acts to fine tune neural gene expression(Kok et al.,
110 2012) and consequently modulate both larval and adult behaviors (Moravec et al.,
111 2015). Zebrafish *rest* mRNA is provided as a maternal transcript (Gates et al., 2010)
112 that is essential for proper regulation of gene expression in the blastula(Kok et al.,
113 2012). In addition, maternally supplied *rest* also modulates later migration of facial
114 branchiomotor neurons(Love and Prince, 2015). An early function of REST has also
115 been demonstrated in rodents, where maternal deprivation decreases REST
116 levels(Uchida et al., 2010; Rodenas-Ruano et al., 2012). Subsequent misregulation of
117 NMDA receptor gene expression leads to changes in synaptic plasticity(Rodenas-
118 Ruano et al., 2012). Conversely, increased maternal care augments REST levels,
119 which correlates with decreased expression of a stress hormone, corticotropin-
120 releasing hormone(CRH) (Korosi et al., 2010).

121 In this study, we demonstrate that in zebrafish maternal *rest* modulates
122 zygotic gene expression until at least 6 dpf and that depletion of maternal *rest*
123 results in behavioral changes in larvae including hyperactivity and atypical spatial
124 preferences. Strikingly, behavioral anomalies persist into adulthood in animals that
125 lack maternal *rest*. Affected adult males, but not females, engage in abnormal
126 swimming behaviors including atypical wall preference combined with frequent
127 vertical swimming and sharper turning angles. Importantly, disruption of the RE1
128 site of either of two target genes, *snap25a* or *snap25b*, recapitulates the larval
129 hyperactivity phenotype. This finding implicates *snap25* paralogs as key targets of
130 Rest in controlling larval behavior. Consistent with the role of Snap25 in axon
131 growth, we investigated the architecture of the primary motor neurons in the

132 mutants and observed increased branching in primary motor neurons in embryos
133 that lack maternal *rest* and in *snap25a* Re-1 mutants. Together, these results
134 demonstrate that maternally supplied Rest influences embryonic and larval gene
135 expression and lifelong behavior.

136 Materials and Methods:

137 Fish Maintenance:

138 Zebrafish embryos were obtained from natural crosses and maintained at
139 28.5° C under 13:11 hour light dark cycle. Adult fish were fed twice daily with a
140 combination of artemia and flake food. The *rest*^{sbu29} mutation was maintained as
141 previously described (Moravec et al., 2015). All *rest* mutants came from
142 intercrossing *rest* heterozygotes to control for effects caused by maintaining mutant
143 inbred stocks. Larval assays were performed at 6 days post fertilization (dpf) on
144 multiple clutches derived from different parents to minimize genetic background
145 effects.

146 Housing and genotyping:

147 Housing and genotyping were previously described in (Kok et al., 2012;
148 Moravec et al., 2015) with a slight modification. Adult fish were raised in groups of
149 8-10 in 1.8 liter tanks, moved into unisex tanks at 4 months and transferred to
150 individual 1-liter tanks two weeks before the behavioral assays.

151 Behavioral Testing Apparatus and Paradigms:

152 The Novel Environment and Visual-Motor-Behavioral Assays and the testing
153 apparatus were previously described (Moravec et al., 2015). Assays of adults, of
154 both sexes, were conducted at 6 months. All behavioral assays were performed
155 between 1 to 5 PM and approved by the Stony Brook University IACUC.

156 Deep sequencing:

157 Total RNA was extracted from pools of 10 embryos from each of the four
158 groups (*Mrest*^{SBU29/+}, *Zrest*, *MZrest* and WT) and 2 pools per a group were sent to
159 the New York Genome Center for sequencing. Samples underwent a Tru Seq V2
160 library prep and sequenced on a Hi Seq 2000 by 2X 50 bp paired end reads. The
161 reads were aligned to *Danio_rerio.Zv9.74* from Ensembl. Significance was defined as
162 $p < 0.05$ after a correction of multiple testing hypothesis using the Benjamini &
163 Hochberg procedure.

164 Expression studies:

165 Total RNA was extracted from pools of five embryos using Trizol (Invitrogen)
166 and cDNA was synthesized by using Super Script II reverse transcriptase
167 (Invitrogen). Quantitative PCR (qPCR) was carried out with a Light Cycler 480
168 (Roche) using Quanta SYBR green (Quanta bioscience). Transcript levels from each
169 sample were normalized to β -actin. Each experiment consisted of three pools of
170 embryos run in duplicate. Primer pairs are listed bellow or were described
171 previously (Kok et al., 2012).

172 npas4a F:GGGCTCAAGCACTTCTCAAC R:AGATAGCCCACTGCTTCCTG

173 *amph* F:CCAGAGGAAGAGACCAGTTCA R:CTTCTCCTGGTTGGGTCTCA

174 *sty4* F:TGGAGAAATCCCAGGACAAG R:GACAGACCATGTGCCTCCTT

175 *scn3b* F:TGATGTATGTGCTGCTGGTG R:TGTGCTTGCTCGTCAGATTT

176 *nsfa* F:TTTGACAAGTCCAGGCAGTG R:CTGAGTCGTAAGGGCTGGAG

177 *kcns3a* F:GAGGATGACCCTCAGAACCA R:GTGCCCTCAAACCTTTTCCAA

178 *cana1ba* F:ATACTGGATCGGCCCAAACCT R:ATACTGGATCGGCCCAAACCT

179 *sty10* F:TGTGGTTCGCATTCTCAAAG R:ACTTCTTTTTTGGCTCTGGA

180 *grm5(1/2)* F:TGTCCTGATGGCTTCCAGA R:TGGCTGCAGGTTTCAGGTAGT

181 *olfm1b* F:GGGACCTGCAGTACGTGGTA R:TATTGCTTGGCGATGTTTTG

182 *cadpsb* F:TTGTCTGAGGTGTTCAAGC R:CAAACCTGGCCATCCAAGAG

183 *nrxn1a* F:TAATGTGCGTGTGGAGGGTA R:GGGTGACGTTTCTGAACGAT

184 RNA whole-mount *in situ* hybridization was performed as described by

185 Thisse et al. (Thisse et al., 1993). **In cases where genotype differences could be**

186 **attributed to tube specific variations in staining, embryos were marked by tail**

187 **clips and the procedure carried out with both sets of embryos in the same**

188 **tube.** Probes were synthesized from plasmids or from 6 dpf cDNA using primer pair

189 for *amph* anti sense F:ATTTGCCAAAAACGTCCAAA

190 R:GAGTAATACGACTCACTAGGGGGCCTTTTTCAAGTCCTCT . For

191 immunohistochemistry, embryos were fixed in 4% PFA overnight at 4°C and stored

192 in methanol. ZNP-1 staining was performed as described (Wei et al., 2013).
193 Quantification of average fluorescence of the immunohistochemistry was done
194 using Image J. The same three puncta was quantified on each sample and ratio to
195 controls (WT or Zrest^{SBU29/+}).

196 Disruption of RE1 sites:

197 RE1 sites in *snap25a* (TTCAGCACCTGGACAGCGAC) and *snap25b*
198 (TTCAGCACCGGAGAGCGCT) were disrupted using the CRISPR-CAS9 system.
199 Guide RNA targets sites: *snap25a*-GCAAACGCAGTCGCTGTCCA *snap25b* –
200 GGTGCTGAAATCCACACAAC. gRNAs were generated using Ambion *MegaScript T7*
201 kit. Guide RNA (200pg) was co-injected with 400pg of Cas9 protein (PNA Bio, Inc.)
202 into the cell of one-cell embryos. Fish were genotyped using primers: *snap25a* RE1
203 site F: ACGATGTGGGCGGTTTCT R: TGGAAATTTAGCTGCAGGAG *snap25b* RE1 site
204 F:TTGCACAGCTTTTGCATGA R:TACCATGGAGGCTCGACTTT.

205 Statistics:

206 Statistical analyses were conducted as previously described (Moravec et al.,
207 2015) using SPSS, version 21 and Graphpad software. Outliers were detected using
208 the Grubs test and removed from analysis. Significance was defined as less than 0.05
209 and trending was defined as 0.099 to 0.05. All error bars represent standard error.

210 Results:

211 **Maternal Rest regulates gene expression at blastula stage:**

212 We previously observed that depletion of maternal *rest* caused *derepression*
213 of a subset of target genes in blastula stage zebrafish *embryos* (Kok et al., 2012). To
214 better understand the role of maternal *rest* in gene repression, we performed deep
215 sequencing of blastula mRNA comparing *Mrest*^{sbu29/+} to *Zrest*^{sbu29/+} and
216 *MZrest*^{sbu29/sbu29} to related wild-type controls. *Mrest*^{sbu29/+} fish are the offspring of a
217 *rest* mutant female and a wild-type male and therefore lack maternal *rest* mRNA.
218 The corresponding controls have normal maternal contribution of *rest* and are the
219 offspring of a *rest* mutant male and a wild-type female (*Zrest*^{sbu29/+}). *MZrest*^{sbu29/sbu29}
220 lack both maternal and zygotic *rest* and are the offspring of two homozygous
221 mutants. The corresponding control wild-types were obtained from crosses of wild-
222 type siblings of the mutant parents used to generate the *MZrest*^{sbu29/sbu29} offspring.
223 Because of the temporal proximity of these embryos to the mid-blastula transition,
224 we anticipate that most of the transcriptional changes detected will be **result from**
225 direct effects of **maternal Rest depletion because the analysis occurred shortly**
226 **after the activation of the zygotic genome.**

227 Overall the deep sequencing identified a total of 26,000 transcripts, but only
228 214 were significantly misregulated in both *Mrest*^{sbu29/+} and *MZrest*^{sbu29/sbu29}
229 RNAseqs (P<0.05 after Benjamini & Hochberg correction) (Figure 1A). Of these 214
230 genes, 158 were upregulated when maternal *rest* was absent. Genuine targets of
231 maternal Rest would likely be misregulated in both *Mrest*^{sbu29/+} and *MZrest*^{sbu29/sbu29}
232 embryos. Therefore, we focused on this set of transcripts. Because Rest is thought to
233 influence gene expression over large chromosomal regions (Lunyak, 2002). We used
234 an algorithm we previously developed (Johnson et al., 2006; 2009) to determine

235 which of these genes had an RE1 site with 100kb of the transcriptional start site
236 (TSS). This analysis revealed that 63 genes (~40%) had predicted RE1 sites (score
237 >.91) located within 100kb of the TSS. This set of shared upregulated genes were
238 significantly enriched for RE1 sites (Chi square =159.989 and P<0.0001).

239 DAVID analysis of the upregulated genes revealed that 41 of the 158
240 misregulated genes are expressed in neural tissues as would be expected of
241 authentic Rest targets (Chong et al., 1995; Schoenherr and Anderson, 1995; Lunyak,
242 2002; Bruce et al., 2004). GO analysis of 158 upregulated genes indicated that their
243 functions were enriched in exocytosis, synaptic transmissions and cell-cell signaling
244 (Figure 1B). In addition, 56 significantly downregulated transcripts were identified,
245 but only 9 had associated RE-1 sites. This set of downregulated genes was not
246 enriched for RE-1 sites (Figure 1A, Chi squared= 1.990, P<.1583), although recent
247 work has suggested that *rest* might act as an activator in some contexts (Kuwabara
248 et al., 2004; Perera et al., 2015).

249 To validate the RNA-seq results, we assayed the expression of 15 upregulated
250 RE-1 associated genes by qPCR in *Mrest^{sbu29/+}* cDNA. These genes were selected
251 based on the significance of altered expression in the transcriptome analysis. Among
252 them are *amphiphysin*, the most significantly misregulated gene, known zygotic Rest
253 targets (*snap25a*, *snap25b*, *gpr27* and *syt4*) (Kok et al., 2012; Love and Prince, 2015)
254 and genes with a diversity of **functions, including an ion channel**(*scn3b*), **an**
255 **axon growth regulator** (*nsfa*) and **a transcription factor** (*npas4a*)(Bruce et al.,
256 2004).

257 At blastula stage, qPCR confirmed that 13/14 genes tested are upregulated in
258 *Mrest^{sbu29/+}* (Figure 2, Data not shown). The remaining gene, *gpr27*, was not
259 detectable by qPCR in either *Mrest^{sbu29/+}* or *Zrest^{sbu29/+}* at blastula stage. Based on
260 these results, we conclude that identification of derepressed RE1 containing genes
261 in the RNA-seq experiment had a low false positive rate.

262 Transcriptional Effects of Maternal Rest depletion persist beyond blastula stages:

263 To determine whether maternal *rest* is required to maintain gene expression
264 profiles of target genes at later stages, we assayed expression of the same target
265 genes 7.5 hours later at the 8-somite stage using qPCR. Out of the 14 genes we
266 studied, three genes, *snap25a*, *snap25b*, *gpr27*, were significantly derepressed in
267 *Mrest^{sbu29/+}* embryos at 8 somites (Figure 2, Data not shown). To determine
268 whether these effects persist, we assayed expression of a set of genes including
269 those showing earlier derepression at 6 days and observed derepression of *amph*
270 and *npas4a*, but no other differences were uncovered with qPCR (Figure 2). **The**
271 **stage specific effects on individual targets such as *amph* and *npas4a* in**
272 ***Mrest^{sbu29/+}* embryos likely stems from the presence of stage specific**
273 **transcriptional activators that play significant roles in modulating**
274 **transcription of these genes.**

275 Because domain specific differences in expression may not be detected by
276 whole embryo qPCR, we performed RNA *in situ* hybridizations on 24 hpf embryos to
277 assay gene expression in *Mrest^{sbu29/+}* embryos. It was previously shown that Rest
278 target genes are misexpressed in the hindbrain of *MZrest^{sbu29/sbu29}* mutants at 24hpf

279 (Love and Prince, 2015). We observed ectopic expression of *snap25a*, *snap25b* and
280 *syt4* in the hindbrain of *Mrest^{sbu29/+}* embryos at 24 hpf, while *nsfa* and *amph*
281 expression were not altered (Figure 3). In *Mrest^{sbu29/+}*, *snap25a* ectopic expression
282 spans the hindbrain and midbrain (as marked by the bracket) (Figure 3 A-B), while
283 *snap25b* shows ectopic expression in hindbrain cranial ganglia (arrows in Figure 3
284 E-F). *Syt4* has a restricted expression pattern in the hindbrain compared to *snap25a*
285 and *snap25b*, but the domain located rostral to the otic vesicle is broadly expressed
286 in the *Mrest^{sbu29/+}* when compared to *Zrest^{sbu29/+}* (white brackets) (Figure 3 I-J). No
287 spatial differences were observed in expression of *nsfa* or *amph* (Figure 3 M-N Q-R).
288 At 6 dpf, these genes are exclusively expressed in the brain (Figure 3). We observed
289 *Mrest^{sbu29/+}* increased expression of *nsfa* and *amph* in 6dpf *Mrest^{sbu29/+}* embryos
290 (Figure 3 O-P, S-T) but no differences in expression of *snap25a*, *snap25b* or *sty4* at
291 this stage (Figure 3 C-D, G-H, K-L).

292 Depletion of maternal *rest* modulates larval locomotion:

293 In addition to de-repression of *rest* target genes, disruption of zygotic *rest*
294 results in hypo-locomotion at 6 dpf (Moravec et al., 2015). To determine whether
295 maternal *rest* modulates larval behavior during development, we monitored
296 locomotor activity during spontaneous and evoked swimming behaviors in embryos
297 lacking maternal *rest* mRNA at 6 dpf. Larvae were placed in 24 well plates, one
298 animal per well and locomotor activity was analyzed using the Zebrabox imaging
299 system (Viewpoint).

300 Spontaneous movements of *Mrest*^{sbu29/+}, *Zrest*^{sbu29/+}, *MZrest*^{sbu29/sbu29}, and
301 related wild-type control larvae were analyzed at 6 dpf in the light. Comparison of
302 *Mrest*^{sbu29/+} and *Zrest*^{sbu29/+}, locomotion revealed that *Mrest*^{sbu29/+} larvae move
303 significantly more (n=71, average of 1511 movements) than *Zrest*^{sbu29/+} (n=72,
304 average of 1061.57 movements) controls (Fig 4A, P=0.0013) over 15 minutes. A
305 repeated measure ANOVA evaluated movements over one-minute time intervals
306 identified a significant main effect of genotype. On average, the *Zrest*^{sbu29/+} controls
307 traveled 70 movements/min, while the *Mrest*^{sbu29/+} larvae traveled a 100
308 movements/min (Fig 4B, Table 1). The requirement for maternal *rest* in modulating
309 larval locomotor behavior was also apparent from comparisons of *MZrest*^{sbu29/sbu29}
310 mutants (N=48) and related wild-type controls (N=72). In this assay, the
311 *MZrest*^{sbu29/sbu29} mutants significantly surpassed the related wild-type controls in the
312 number of movements, duration of movements and distance traveled (Figure 4 G-H,
313 Table 1). Both genotypes of maternal *rest* depleted larvae also show a significant
314 increased activity in additional parameters of movement including distance traveled
315 and duration of movements (Figure 4 C-F,I-L Table 1). Overall, this data revealed
316 that the loss of maternal *rest* results in larval hyperactivity.

317 Wall preference for the four groups of larvae were assessed by calculating
318 the percentage of time the larvae spent in both the center and the peripheral
319 divisions of the circular wells (enter well diameter: 150mm, center well diameter
320 62mm). Comparison of *Mrest*^{sbu29/+} vs. *Zrest*^{sbu29/+} and *MZrest*^{sbu29/sbu29} vs. related
321 wild-type controls, demonstrated that the larvae lacking maternal *rest* displayed a
322 preference to be located at the periphery of the well (Figure 5). We also examined

323 evoked responses to a light change but no significant differences were observed in
324 the absence of maternal *rest* (Data not shown). The hyperactivity and atypical
325 spatial preference behavior that is observed in the larvae lacking maternal *rest*
326 differs from that of the **zygotic** *rest* mutant (Moravec et al., 2015)

327 Depletion of maternal *rest* alters adult behavior:

328 Depletion of maternal *rest* in *Mrest*^{sbu29/+} or elimination of both maternal *rest*
329 and zygotic *rest* as in *MZrest*^{sbu29/sbu29} larvae causes hyperactivity and atypical spatial
330 preferences in spontaneous movement at six dpf. To determine whether depletion
331 of maternal *rest* changes behavior in adults, a novel environment assay was
332 employed to measure locomotion and spatial preference at six months of age.

333 To investigate whether the effects of maternal *rest* on spatial preference
334 persisted into adulthood, the amount of time that fish lacking maternal *rest* spent
335 within 2.75 cm of the walls was analyzed. A comparison of *Zrest*^{sbu29/+} and
336 *Mrest*^{sbu29/+} movement patterns revealed a strong preference of *Mrest*^{sbu29/+} males
337 for the tank walls compared to the *Zrest*^{sbu29/+} males. No preference was observed
338 between *Mrest*^{sbu29/+} and *Zrest*^{sbu29/+} females (Figure 5A). A two-way ANOVA
339 identified a significant main effect of genotype but no significant main effect of sex
340 or sex X genotype interaction, although the sex X genotype interaction was strongly
341 trending (Table 2). Our data showed *Mrest*^{sbu29/+} males spend around 40% of the
342 interval near the edge of the tank, while *Zrest*^{sbu29/+} males spend around 23% of
343 their time near the edge of the tank. The female *Mrest*^{sbu29/+} and *Zrest*^{sbu29/+} fish
344 spend a comparable amount of time near the edge of the tank, 32.8 % to 30.6%,

345 respectively (Figure 6B). A within-sex analysis of time spent near the wall in one
346 minute intervals showed that every minute *Mrest^{sbu29/+}* males spent more time near
347 the edge of the tank when compared to *Zrest^{sbu29/+}* controls (Figure 6D Table 3),
348 while no differences were observed when comparing females (Figure 6C, Table 3).

349 The *Mrest^{sbu29/+}* male fish also presented with another behavioral change,
350 erratic swimming patterns during the novel environment assay. Increased erratic
351 swimming patterns were observed in *Mrest^{sbu29/+}* males when compared to
352 *Zrest^{sbu29/+}* males as measured by distance traveled, velocity in the vertical direction,
353 turn angle and location in the tank. (Data Not Shown). This behavior is similar to the
354 movements of *rest* mutants of both sexes (Moravec et al., 2015).

355 Identification of Rest target genes that modulate locomotor behavior:

356 To identify the Rest target genes whose misregulation produces the
357 behavioral phenotypes we observed in the *Mrest^{sbu29/+}* and *MZrest^{sbu29/sbu29}* larvae,
358 we deleted the RE1 elements associated with *snap25a* and *snap25b* using the
359 CRISPR-CAS9 system. We chose these two genes because they are upregulated
360 during embryogenesis past blastula stage (Figures 2 and 3) and have key synaptic
361 functions. Both zebrafish *snap25* paralogues have RE1 sites within the first intron
362 as does mammalian *snap25* and Rest has been shown to frequently associate with
363 the *snap25* RE1 sites (Bruce et al., 2004).

364 The CRISPRs were designed to recognize a portion of the RE1 site and
365 flanking sequence to prevent cleavage events at multiple RE1 sites. RE1 sites
366 contain two highly conserved sections (Mortazavi et al., 2006) and we aimed to

367 delete at least one of these regions. The *snap25a* RE1^{sbu82} allele is an 11 base pair
368 deletion that removes one of these conserved regions, while the *snap25b* RE1^{sbu83}
369 allele is a 53 bp deletion and removes the entire RE1 site (7A-B).

370 We first determined the effects of these RE1 site mutations on gene
371 expression at multiple stages of development. qPCR analysis at blastula stage of
372 *snap25a* in the *snap25a* RE1^{sbu82/sbu82} mutant (Figure 7C) and *snap25b* in the *snap25b*
373 RE1^{sbu83/sbu83} mutant (figure7E) mirrored the upregulation of these transcripts
374 observed in *Mrest*^{sbu29/+}. RNA *in-situ* hybridization with *snap25a* and *snap25b*
375 probes at 24hpf revealed ectopic expression of *snap25a* and *snap25b* in the
376 hindbrain similar to *Mrest*^{sbu29/+} embryos. The *snap25a* RE1 heterozygotes and
377 mutants both showed increase expression in the hindbrain and midbrain (as
378 marked by the bracket) when compared to sibling wild-types (Figure 7D). The
379 *snap25b* RE1 heterozygous and mutants show medial ectopic expression in the
380 hindbrain (as marked by the arrows) when compared to sibling wild-types (Figure
381 7F).

382 RE-1 site mutant larvae are hyperactive:

383 We investigated the spontaneous and light evoked movements of both the
384 *snap25a* and *snap25b* RE-1 site mutants at 6 days. Remarkably, similar to the
385 *Mrest*^{sbu29/+} and the *MZrest*^{sbu29/sbu29} larvae the *snap25a* and *snap25b*, RE1 site
386 mutants showed hyperactivity in spontaneous movement. Specifically, the *snap25a*
387 RE1^{sbu82/sbu82} site mutants (n=24) initiated significantly more swims (an average of
388 2316 movements), when compared to sibling wild-types (n=30, an average of 1751

389 movements) and *snap25a* RE1^{sbu82/+} heterozygotes (n=74, an average of 1660
390 movements) (Figure 8A Table 4). A repeated measure ANOVA evaluated number of
391 movements across the one-minute time bins identified a significant main effect of
392 genotype. The *snap25a* RE1^{sbu82/sbu82} site mutant made an average of 154
393 movements/minute compared to the sibling wild-type and *snap25a* RE1^{sbu82/+}
394 heterozygotes who make an average of 116 movements/min and 110
395 movements/min respectively (Figure 8B, Table 5).

396 The *snap25b* RE1^{sbu83/sbu83} mutants displayed a similar behavior to
397 Mrest^{sbu29/+} larvae. These mutants engaged (n=44) in an average of 2,490
398 movements compared to the sibling wild-type(n=37) an average of 1,918
399 movements and *snap25b* RE1^{sbu83/+} heterozygotes(n=62) an average of 1924
400 movements (Figure 8G, Table 4). A repeated measure ANOVA of the number of
401 movements revealed a significant main effect of genotype. The *snap25b*
402 RE1^{sbu83/sbu83} site mutant made an average of 166 movements/min compared to the
403 sibling wild-types and *snap25b* RE1^{sbu83/+} heterozygotes that averaged
404 127movements/min and 128 movements/min respectively (Figure 8H, Table 5).
405 We also examined distance traveled and duration of movements and found that both
406 the *snap25a* and *snap2b* RE1 mutants surpassed the related wild-types and
407 heterozygotes in both parameters (Figure 8C-F,I-L, Table 4-5). Nether of these RE1
408 mutants presented with an atypical spatial preference or showed a response to a
409 light change (Data Not Shown). These results indicate that the *rest* regulation at
410 *snap25a* and *snap25b* is sufficient to controlling locomotor behavior, but not spatial
411 preference at 6 dpf.

412 Motor neurons in *Mrest*^{sbu29/+} and *snap25a* RE1^{sbu82/sbu82} site mutants have
413 increased processes:

414 Increased expression of the zebrafish *snap25* paralogs results in
415 hyperactivity, increased branching of motor neurons and changes to the synaptic
416 activity at neuromuscular junctions (Wei et al., 2013). To investigate changes in the
417 primary motor neuron architecture in the *Mrest*^{sbu29/+} and the *snap25* RE1 site
418 mutants, we performed whole mount immunostaining with Znp-1, *synaptotagmin*
419 *IIB*(*syt2b*), at 56 hpf. We observed increased expression of Znp-1 in the spinal cord
420 along with increased Znp-1 puncta associated with primary motor neurons in
421 *Mrest*^{sbu29/+} embryos (n=5) (marked by a red arrow) when compared to *Zrest*^{SBU29/+}
422 (n=4)(Figure 9A). Quantification of average fluorescence in these ZNP-1 puncta
423 showed a significant increase in fluorescence in the *Mrest*^{sbu29/+} embryos (P=0.0010)
424 (Figure 9B). We also examined the primary motor neuron architecture of the *snap*
425 RE1 site mutants and observed increased Znp-1 staining (marked by red arrows) in
426 the *snap25a* RE1^{sbu82/sbu82} site mutant (n=7), but not the *snap25b* RE1^{sbu83/sbu83} site
427 mutant (n=7) when compared to wild-type controls (*snap25a* RE1 site control =7
428 and *snap25b* RE1 site control=7) (Figure 9B,C,E). Quantification of the ZNP-1 puncta
429 in the *snap25a* RE1^{sbu82/sbu82} and *snap25b* RE1^{sbu83/sbu83} site mutants revealed a
430 significant increase of fluorescence in the *snap25a* RE1 mutant (P=0.0078), but not
431 in *snap25b* RE1 mutant (Figure 9D, F) . These results suggest that alterations of the
432 neuromuscular junction (NMJ) in *Mrest*^{SBU29/+} larvae stem from derepression of
433 *snap25a*, but that regulation of *snap25b* expression by maternally supplied Rest is
434 important elsewhere.

435 Discussion:

436 Our previous work demonstrated that zebrafish *rest* mutants undergo largely
437 normal neurogenesis (Kok et al., 2012), but that *rest* mutant larvae show locomotor
438 defects and engage in erratic swimming as adults (Moravec et al., 2015). We now
439 present evidence that the effects of maternally supplied *rest* limits expression of a
440 subset of target genes until at least 6dpf and that larvae lacking maternal *rest* are
441 hyperactive and present with a spatial preference for outer portion of the well when
442 compared to controls. To our knowledge this is the first example of a maternally
443 supplied mRNA that modulates behavior. Remarkably, behavioral consequences of
444 the deficit in the early maternal *rest* expression persist into adulthood as observed
445 by the erratic swimming behavior and atypical place preference that was apparent
446 in adult *Mrest*^{sbu29/+} males, but not females.

447 Rest has been proposed to play important roles in stem and progenitor cells
448 to control self-renewal and differentiation in the nervous system (Ballas et al., 2005;
449 Singh et al., 2008). While we cannot conclusively rule out the possibility that
450 maternal *rest* deficit alters cell fate, we have found no evidence for major cell fate
451 changes in any of the *rest* mutants. Furthermore, because the larval hyperactivity
452 phenotype can be recapitulated by disrupting the RE1 sites in either *snap25a* or
453 *snap25b*, we favor the model that the primary effects are on gene expression of
454 these *rest* target genes. This is consistent with the observations in rodents that
455 early Rest-mediated epigenetic effects regulate the later developmental switch in
456 synaptic NMDA receptors (Rodenas-Ruano et al., 2012).

457 Rest levels in mammals are diminished by maternal deprivation and elevated
458 by augmented maternal care(Korosi et al., 2010; Uchida et al., 2010; Rodenas-Ruano
459 et al., 2012). While zebrafish do not engage in maternal care, *Mrest*^{SBU29/+} embryos
460 face a similar early deficit in Rest activity. Our transcriptome analysis did not
461 identify GRIN2b as a key Rest target, as has been demonstrated in the rat studies of
462 early Rest function (Rodenas-Ruano et al., 2012). Instead, our work implicates the
463 two *snap25* paralogues as key mediators of the observed behavioral phenotypes.
464 Nonetheless, the data in rodents and fish may point to a fundamental role for Rest in
465 establishing chromatin landscapes that have later impacts on expression of neural
466 genes and neuronal function.

467 The half-life of the protein generated from maternal *rest* RNA is unknown,
468 but the maternal mRNA is degraded by about shield stage, 6 hours after fertilization
469 (unpublished result). Because Rest protein is actively degraded(Westbrook et al.,
470 2008; Kaneko et al., 2014), it seems likely that the protein has vanished long before
471 gene expression (Fig 2,3) and behavioral defects (Fig 4,5) are observed at 6 dpf.
472 During this period, zygotic *rest* is expressed (Gates et al., 2010), yet is unable to
473 compensate for the loss of early Rest activity. The adult behavioral analysis further
474 suggests an early unique role for maternally supplied *rest* in establishing chromatin
475 states that persist lifelong. However, our data does not exclude the possibility that
476 the effects stem from consequences of cumulative transgenerational consequences
477 of Rest deficiency as has been observed in *C. Elegans* mutants for the Rest complex
478 protein, LSD1(Katz et al., 2009)

479 Transcripts regulated by maternal *rest*:

480 Bioinformatic analysis indicated that we enriched for both neural specific and RE1
481 containing genes in the upregulated set of genes in *Mrest*^{sbu29/+} identified by RNA-
482 seq. Our qPCR validation of 14 RE1 containing genes demonstrated that the
483 approach robustly identified Rest targets. The downregulated genes were not
484 enriched for RE1 sites or for neural genes, but recent work has suggested that *rest*
485 might act as an activator in some contexts (Kuwabara et al., 2004; Perera et al.,
486 2015). However, if Rest acts as an activator at blastula stages, the number of targets
487 is quite low. Alternatively, downregulation of some transcripts could be due to
488 secondary effects which are expected at a low frequency because the sequence
489 analysis was performed less than an hour (at 4 hpf) after the mid-blastula transition
490 (Kimmel et al., 1995).

491 Regulation of synaptic proteins by Rest:

492 Many of the genes regulated by maternal *rest* encode synaptic proteins. In
493 fact, the five genes that show persistent misregulation, *snap25b*, *snap25a*, *syt4*,
494 *npas4a* and *amph*, all act on presynaptic neurons. The *snap25* paralogs and *syt4*
495 enable binding of the synaptic vesicles to the presynaptic density allowing for
496 exocytosis of the neurotransmitters into the synaptic cleft, while *amph* promotes
497 recycling of empty synaptic vesicles from the presynaptic density after exocytosis.
498 *Npas4a* regulates the expression of inhibitory synapse genes to control the
499 excitatory/inhibitory balance in presynaptic cells. While disrupting the *snap25* RE1
500 sites recapitulates much of the larval locomotor observed in maternal deficient

501 larva, it is likely that misregulation of other targets produces behavioral
502 consequences. In particular, the atypical spatial preferences observed in *Mrest^{sbu29/+}*
503 and *MZrest^{sbu29/sbu29}* were not apparent in the RE1 site mutants.

504 Regulation of Behavior by Rest:

505 Zygotic *rest* mutant larvae are hypoactive (Moravec et al., 2015), while we
506 now demonstrate that fish lacking maternal *rest* are hyperactive and demonstrate
507 atypical spatial preferences, spending more time near the wall. This data suggest
508 that maternal *rest* plays a distinct role from zygotic *rest* in modulating locomotive
509 behavior at six days.

510 Adult zygotic *rest* mutants of both sexes display atypical spatial preferences
511 in a novel environment assay characterized by edge preferences and erratic
512 swimming (Moravec et al., 2015). When adult *Mrest^{sbu29/+}* fish underwent the same
513 test, only the males but not females presented with similar phenotypes to the
514 zygotic mutants. The observation that depletion of a maternal RNA effects behavior
515 in a sex specific manor is unusual and suggests that life-long effects on the
516 epigenetic genome may be strongly influenced by sex hormones.

517 Changes to the architecture of primary motor neurons:

518 *Mrest^{sbu29/+}* embryos display increased expression of *Sty2b* in trunk motor
519 neurons when compared to *Zrest^{sbu29/+}*. This suggests a possible molecular
520 mechanism for the hyperactivity observed in the *Mrest^{sbu29/+}* larvae (Figure 4) as

521 decreased locomotion has been linked to changes in axon formation and elongation
522 of the motor neurons (Granato et al., 1996).

523 We also investigated the primary motor neuron architecture in the *snap25*
524 RE1 sites mutants because they are also hyperactive (Figure 7) and increased
525 expression of Snap25 is linked to both axon growth (Wei et al., 2013; Wang et al.,
526 2014; n.d.) and hyperactivity (Wei et al., 2013). We observed increased expression
527 of Sty2b at the NMJ of the *snap25a* RE1^{sbu82/sbu82} mutant, but not in the *snap25b*
528 RE1^{sbu83/sbu83} mutant. This suggests that the increased number of processes
529 associated with primary motor neurons in *Mrest*^{sbu29/+} larvae is due to derepression
530 of *snap25a* in the absence of maternal *rest*. The behavioral phenotypes of
531 *Mrest*^{sbu29/+} are more complex because disrupting the RE1 site of *snap25b* results in
532 hyperactivity, but not overt changes of Sty2b expression in motor neurons.
533 Enhanced Snap25b levels may alter synaptic plasticity by altering
534 trafficking/exocytosis of synaptic vesicles, while not overtly altering the complexity
535 of motor neuron processes. Since neither *snap25* RE1 site mutant displays altered
536 spatial preferences, regulation of other target gene by Rest must be responsible for
537 this phenotype. It is likely that some of these genes also impact swimming
538 frequency as well.

539 We present the first evidence that maternal *rest* plays a long-term role in
540 regulation of gene expression and behavior during development. The activity of
541 maternally supplied *rest* controls expression of target genes and affects behavior not
542 only in larvae, but in adults as well. By rendering the zebrafish *snap25* paralogs

543 impervious to Rest mediated repression at these RE1 sites, we determined that
544 *snap25a/b* are key targets of maternal *rest* involved in modulating primary motor
545 neuron development and larval swimming frequency. These findings strengthen the
546 idea that a major function of Rest is to regulate synaptic activity and plasticity
547 (Rodenas-Ruano et al., 2012). The zebrafish *rest* mutant provides a unique
548 opportunity to explore the lasting requirements for maternal factors in nervous
549 system function. This study provides the first evidence that maternal *rest* is
550 necessary for long-term regulation of both gene expression and behavior.

551

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693 Figure 1: Transcriptome comparison of *Mrest*^{sbu29/+} and *MZrest*^{sbu29/sbu29}

694 A) Venn diagram showing the overlap of upregulated and downregulated genes in
695 *Mrest*^{sbu29/+} and *MZrest*^{sbu29/sbu29} blastula. The number of genes with a predicted RE1
696 site near them is indicated. B) GO analysis showing the significant biological
697 processes that are enriched in the upregulated genes.

698 Figure 2: RE1 containing genes are upregulated in *Mrest*^{sbu29/+} embryos.

699 qPCR analysis showing fold differences relative to the *Mrest*^{sbu29/+} transcript levels
700 (defined as 1). Significance was defined at P<0.05 with the use of the Student t-test.
701 All markers shown are upregulated at blastula stage in *Mrest*^{sbu29/+} embryos.
702 *snap25a*(A), *snap25b* (B) and *gpr27*(C) are upregulated at the 8 somite stage (11.5

703 hpf). *npas4a* (D) and *amph* (E) are upregulated at 6 dpf. ND = not detectable

704 Figure 3: Rest target genes are inappropriately expressed in *Mrest^{sbu29/+}* embryos.

705 RNA Whole-mount *in situ* hybridization at 24 hours and six days for Rest target

706 genes in ***Mrest^{sbu29/+}* and *Zrest^{sbu29/+}* in the same tube.** Ectopic expression

707 (marked by the white bracket or arrow) is observed with probes for *snap25a* (A-B),

708 *snap25b* (E-F) and *syt4* (I-J) in the hindbrain of *Mrest^{sbu29/+}* embryos at 24hpf.

709 Increase expression in *Mrest^{sbu29/+}* observed at six days in with *nsfa* (O-P) and *amph*

710 (S-T) probes. OV= otic vesicle MB: Midbrain

711 Figure 4 Larvae lacking maternal *rest* are hyperactive at 6dpf

712 A-F) *Mrest^{sbu29/+}* (N=71) exceed *Zrest^{sbu29/+}* (n=72) in total movements (A-B) and

713 total distance (C-D) and total duration (E-F) over 15 minutes. G-L) Similarly,

714 *MZrest^{sbu29/sbu29}* (n=48) exceed related wild-type controls (n=72) in total movements

715 (G-H) and total distance (I-J) and total duration (K-L) over 15 minutes. All graphs

716 represent average mean with error bars representing standard error measurement.

717 Significance was defined with the use of a student t-test for the entire testing

718 periods and a 2-way ANOVAs with repeated measures designs, with genotype serving

719 as the independent factor and time serving as the repeated measure for the one minute

720 analysis. # = genotype P < 0.05

721

722 Figure 5: Larvae lacking maternal *rest* show an atypical spatial preference at 6dpf

723 A,C) Representative locomotion diagrams of movement in one minute ,

724 *Mrest*^{sbu29/+} (A) and *MZrest*^{sbu29/sbu29} (C) larva display a preference for the outer
725 well. Green represents small velocity movements and red represents large velocity
726 movements during a spontaneous locomotion assay in the light. B) Quantification
727 of percentage of time spent in the outer well over 15 minutes shows *Mrest*^{sbu29/+}
728 (n=71) larva spend significantly more time in the outer well when compared to
729 *Zrest*^{sbu29/+} (n=72) (P=0.002) D) Quantification of percentage of time spent in the
730 outer wall over 15 minutes reveals that *MZrest*^{sbu29/sbu29} (n=48) larva spend more
731 time in the outer well compared to related wild-type controls (n=72) (P=0.0307)
732 Significance was defined with the use of a student t-test.

733

734 Figure 6: *Mrest*^{sbu29/+} males, but not females showed increased wall preference in
735 the novel environment assay.

736 A) Locomotion diagrams for individual fish over 5 minutes showing the *Mrest*^{sbu29/+}
737 male wall preference. B) During the assay, *Mrest*^{sbu29/+} (**N=21**) males spent more
738 time near the wall compared to *Zrest*^{sbu29/+} (**N=20**) controls C-D) Analysis of
739 percentage of time spent near the walls for females (***Mrest*^{sbu29/+} (N=18) and**
740 ***Zrest*^{sbu29/+} (N=20)**) (C) and Males (D) in one-minute intervals reveals that
741 *Mrest*^{sbu29/+} males but not females tend to swim near the side of the tank over the
742 entire assay. Significance was defined with the use of a multivariate analysis of
743 variance (MANOVA) to identify main effects of sex and/or genotype and significant
744 interactions between the two over the testing period. A two 2-way ANOVA with
745 repeated measures design was also used to compare within-sex data collected in 1-minute
746 bins across the 15 minute testing period.

747 #= genotype P-value <0.05

748 Figure 7: CRISPR-CAS9 targeting of RE1 sites

749 A-B) A sequence alignment of wild-type and *snap25* RE1 site mutations (A)

750 *snap25a*^{sbu82} or (B) *snap25b*^{sbu83}. The genomic sequence surrounding the RE1 site is

751 marked black and RE1 site in red. C,E) qPCR analysis showing fold differences

752 relative to the RE1 mutant transcript levels (defined as 1). Significance was defined

753 at P<0.05 with the use of the Student t-test. D-F) RNA whole-mount *in situ*

754 hybridization with D) *snap25a* probe on a *Snap25a* RE1 site ^{sbu82/+} inx or F) *snap25b*

755 probe on a *snap25b* RE1 site ^{sbu83/+} inx . Ectopic expression is marked by the white

756 bracket or white arrow. OV= otic vesicle MB: Midbrain

757 Figure 8: *snap25a* and *snap25b* RE1 site mutants are hyperactive at 6 dpf.

758 A-F) The *snap25a* RE1^{sbu82/sbu82} mutants (N=24) exceeded sibling wild-type (N=30)

759 controls and *snap25a* RE1^{sbu82/+} heterozygotes(N=74) in A-B) number of

760 movements, C-D) distance and E-F) duration at 6dpf. G-L) The *snap25b*

761 RE1^{sbu83/sbu83} mutant (N=44) exceeded sibling wild-type (N= 37) and the *snap25b*

762 RE1^{sbu83/+} heterozygotes (N=62) in G-H)number of movements, I-J) distance and K-

763 L) duration at 6 dpf. Significance was defined with the use of a one-way ANOVA

764 over the entire test period; when the data were compared on per min bases, the data were

765 compared using 2-way ANOVAs with repeated measures designs, with genotype serving

766 as the independent factor and time serving as the repeated measure.

767 Figure 9. Rest regulates primary motor neuron development.

768 znp-1 immunohistochemistry on whole mount zebrafish embryos at 56 hpf to label
769 primary motor neurons. Confocal images were acquired from the trunk using the
770 yolk extension as a landmark (10um stacks). (A,C) Changes in the primary motor
771 neuron architecture are apparent in *Mrest*^{sbu29/+} and *snap25a* RE1 site mutant
772 embryos when compared to controls. (B,D) Significant increase in fluorescence was
773 observed in the *Mrest*^{sbu29/+} and *snap25a* RE1 site mutant. Significance as defined
774 with the use of the student t test and control was set to one. No changes are
775 apparent in primary motor neuron architecture or fluorescence was observed in
776 *snap25b* RE1 site mutant embryos (E-F).

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786 Table 1: 2-way ANOVA with repeated measures to compare genotypes in 1-minute
 787 intervals spontaneous movements

Variable	Genotype		Time		Time X Genotype	
	F	P	F	P	F	P
<i>Mrest vs. Zrest</i>						
Total Distance	19.936154	0.000017	1.669738	0.103	0.915994	0.50099
Total Duration	12.167352	0.000652	2.002607	0.036533	0.711894	0.69668
Total Movements	13.437356	0.00035	2.072364	0.029648	0.836665	0.58133
<i>MZrest vs. WT</i>						
Total Distance	11.085346	0.001179	0.449008	0.829216	0.88972	0.494282
Total Duration	11.380426	0.001015	0.514421	0.782803	0.902606	0.48616
Total Movements	16.616386	0.000085	0.639221	0.670649	1.007349	0.412699

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789 Table 2: MANOVA value from the novel environment assay to identify main effects
 790 of sex and/or genotype and significant interactions.

Variable	SEX		Genotype		Sex X Genotype	
	F	P	F	P	F	P
Edge of tank	0.011028	0.916645	6.22918	0.014763	3.472049	0.066329

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793 Table 3: 2-way ANOVA with repeated measures design for the novel environment
 794 assay to compare within-sex data collected in 1-minute intervals across the testing
 795 period

Edge of tank	Time		Genotype		Time X Genotype	
Sex	F	P	F	P	F	P
Female	3.993977	0.000423	0.04363	0.835755	1.32888	0.238444
Male	5.731319	0.000001	16.835916	0.000223	1.002538	0.432771

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812 Table 4: 1- way ANOVA values for spontaneous movements fore RE-1 mutants
 813 comparing genotypes

Variable	One-way ANOVA		LSD Post Hoc		
	F	P	WT/Het	WT/Mut	Het/Mut
Snap25a RE1site ^{sbu82}					
Total Counts	6.520921	0.002024	0.591579	0.009079	0.000481
Total Distance	7.413463	0.000907	0.477887	0.013031	0.000469
Total Duration	6.485344	0.00209	0.60111	0.004972	0.000212
Snap25b RE1site ^{sbu83}					
Total Counts	5.724394	0.004077	0.976402	0.006448	0.002353
Total Distance	6.68793	0.001683	0.992516	0.003439	0.001012
Total Duration	6.259185	0.002491	0.964836	0.004341	0.00152

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826 Table 5: 2-way ANOVA with repeated measures to compare genotypes in 1-minute
827 intervals during spontaneous movements

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Variable	One-way ANOVA		LSD Post Hoc		
	F	P	WT/Het	WT/Mut	Het/Mut
Snap25a RE1site ^{sbu82}					
Total Counts	6.520921	0.002024	0.591579	0.009079	0.000481
Total Distance	7.413463	0.000907	0.477887	0.013031	0.000469
Total Duration	6.485344	0.00209	0.60111	0.004972	0.000212
Snap25b RE1site ^{sbu83}					
Total Counts	5.724394	0.004077	0.976402	0.006448	0.002353
Total Distance	6.68793	0.001683	0.992516	0.003439	0.001012
Total Duration	6.259185	0.002491	0.964836	0.004341	0.00152

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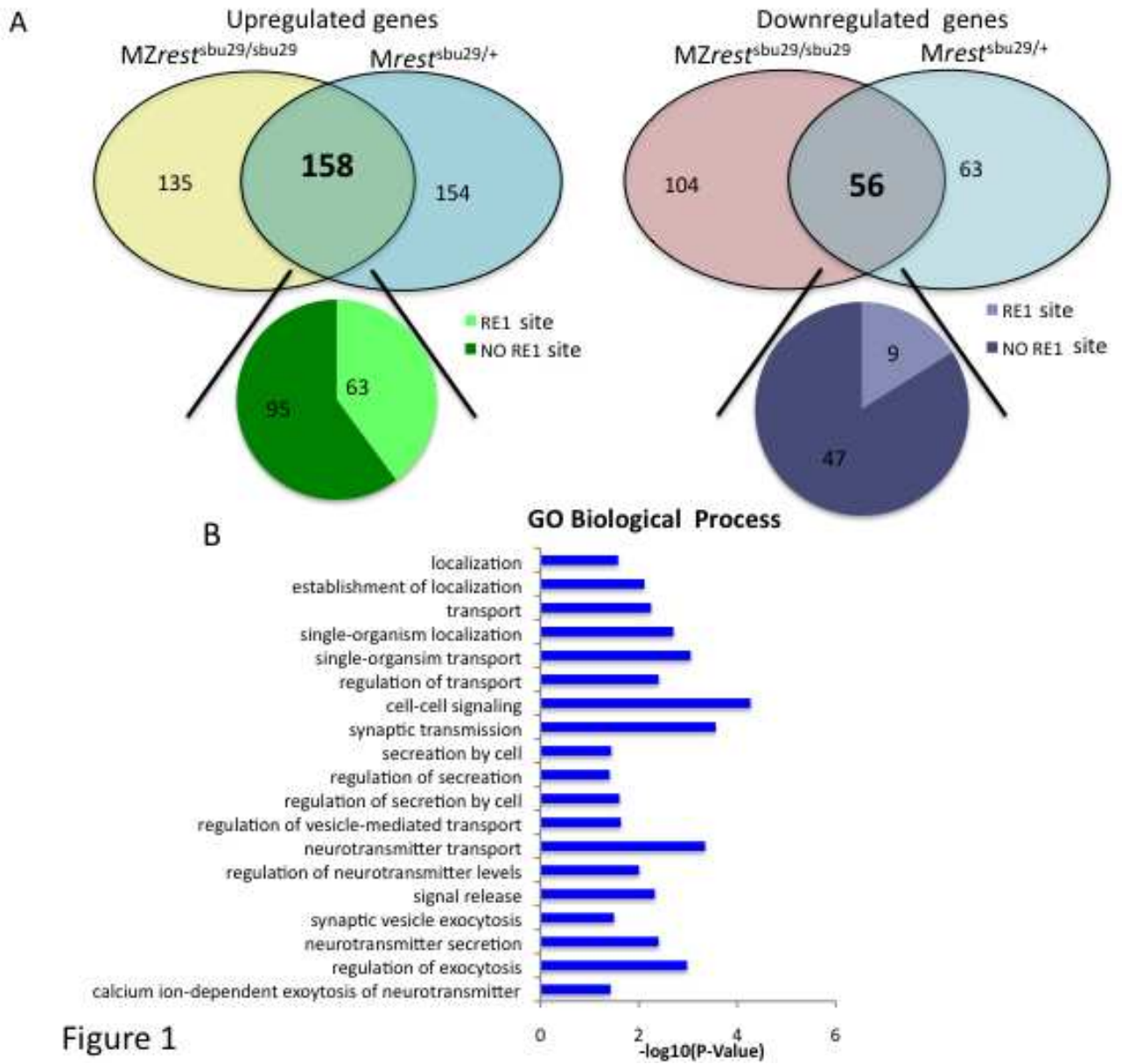


Figure 1

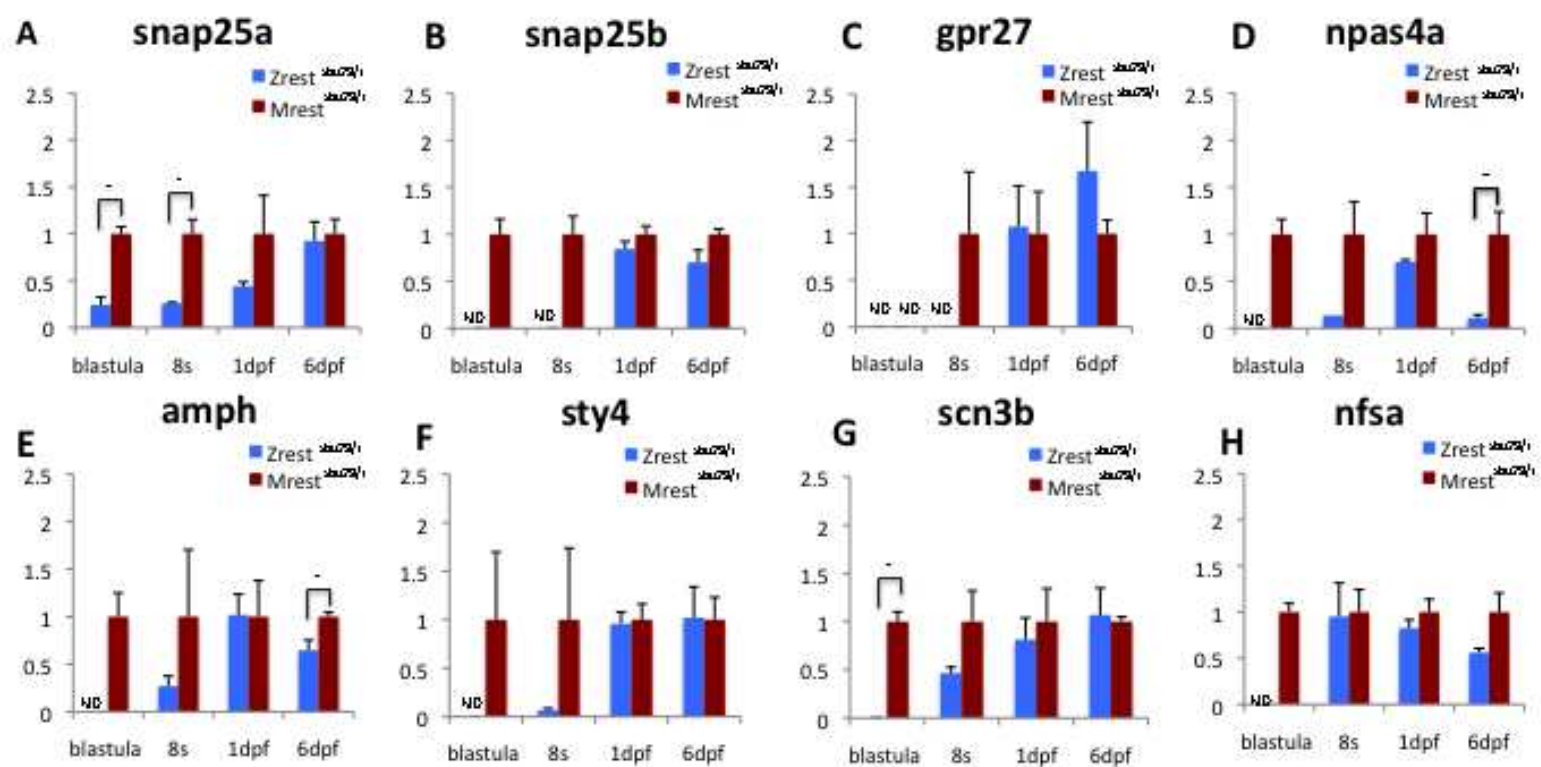


Figure 2

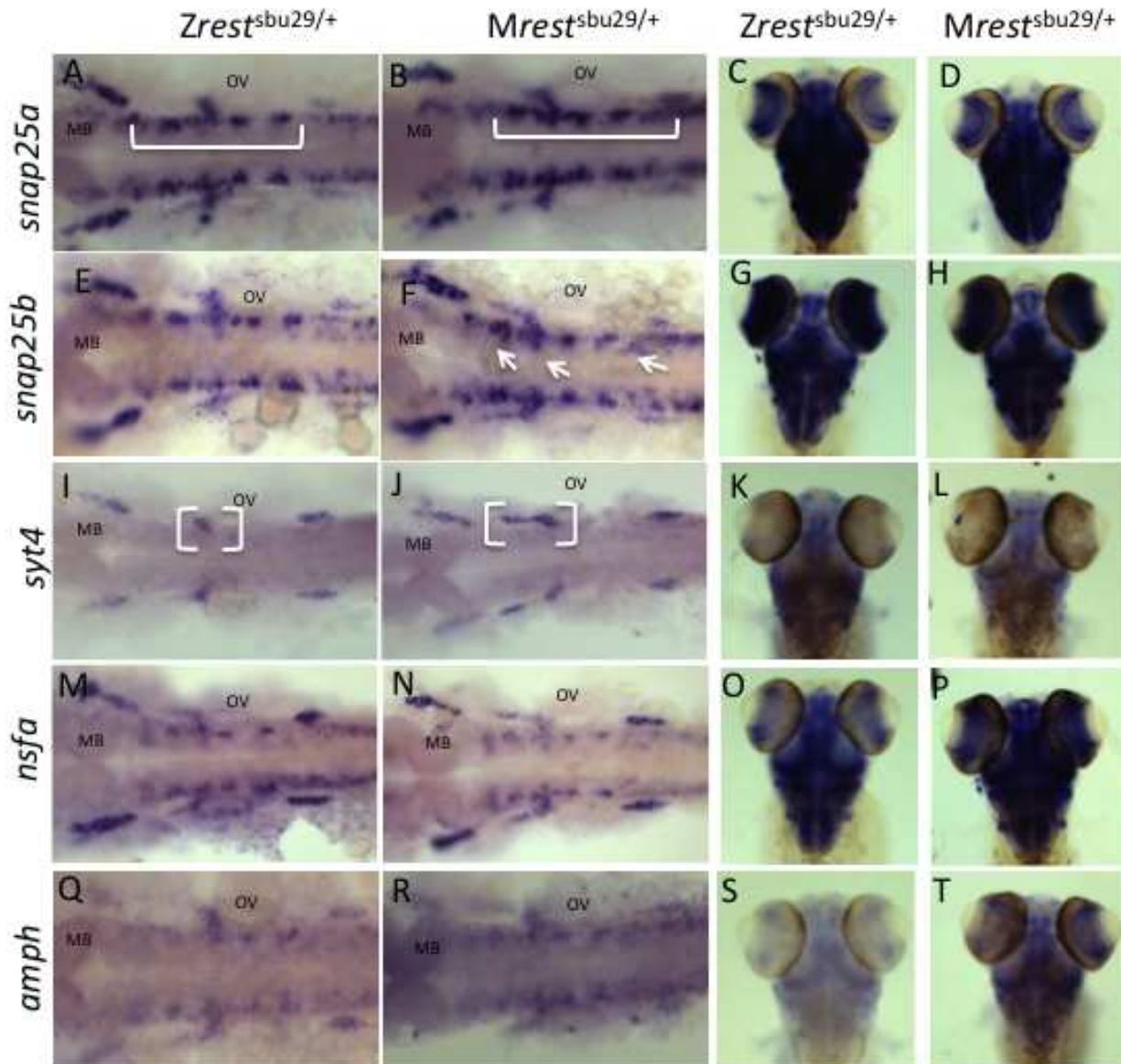


Figure 3

24hpf

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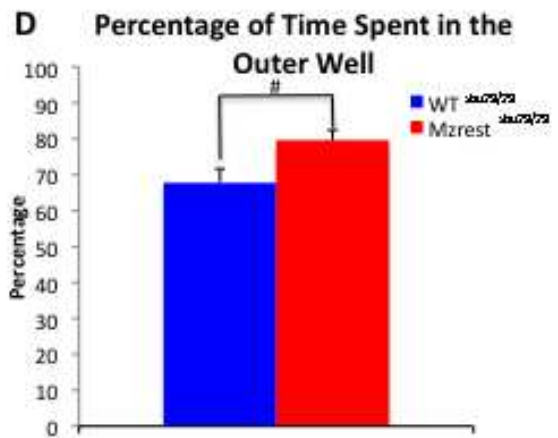
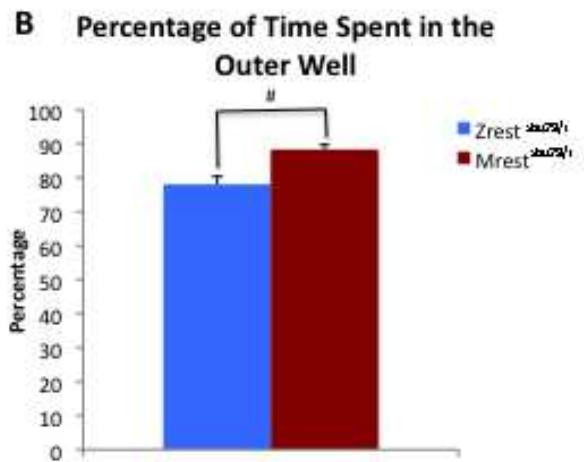
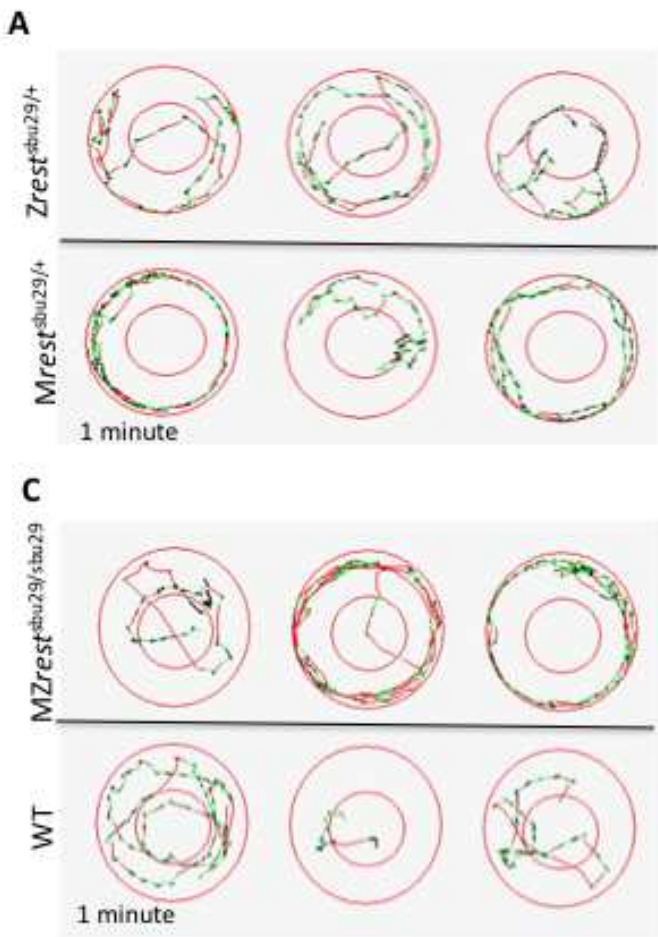


Figure 5

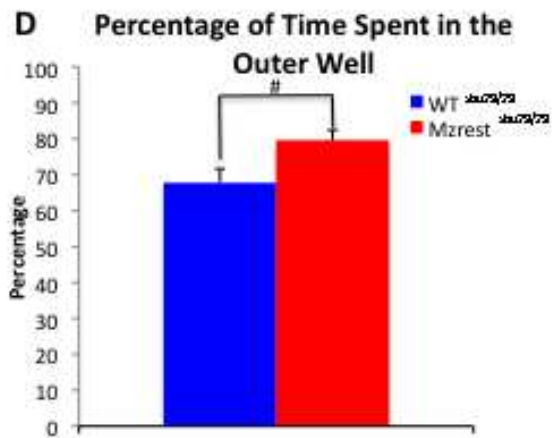
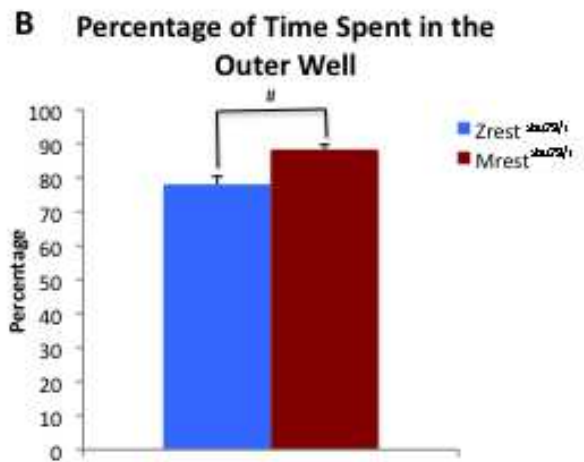
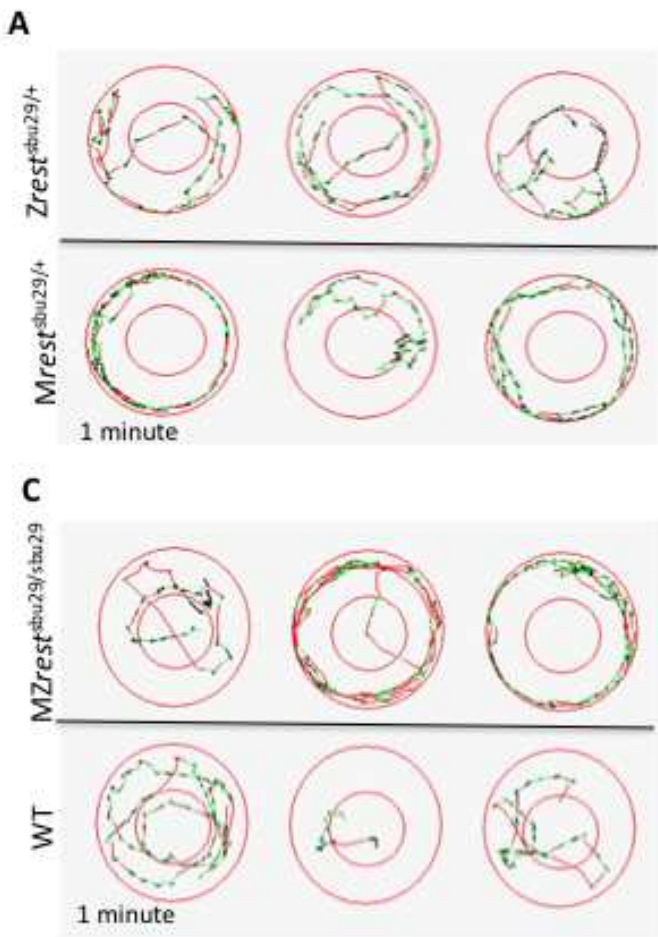
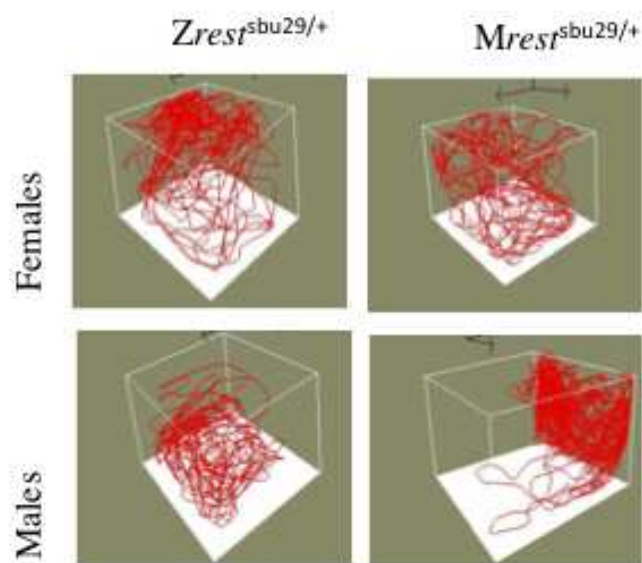
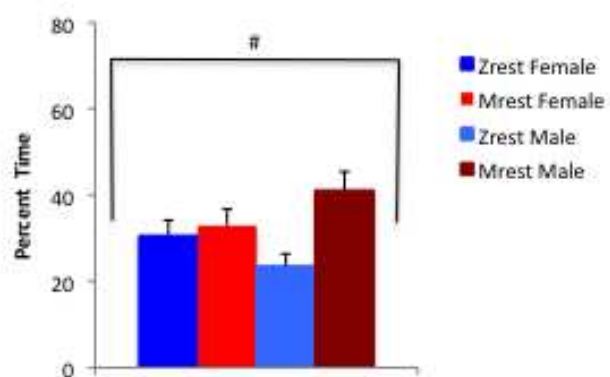


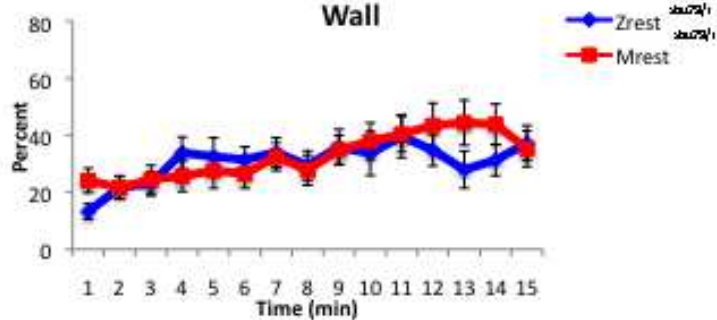
Figure 5



B Percent of Time Spent Near the Wall



C Percent of Time Females Spend Near the Wall



D Percent of Time Males Spend Near the Wall

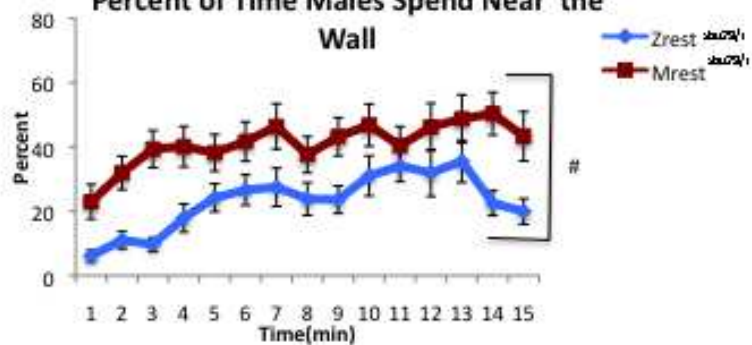


Figure 6

A Snap25a RE1 Site mutation

WT GCTTCAGCACCTGGACAGCGACTGC

SBU82 GCTTCAGCACCC-----TGC

B Snap25b RE1 Site mutation

WT GCCTGTTGTGTGGATTTTCAGCACCGCGGAGAGCGCTCATTAAGAGGGCGCGCGCCAAA

SBU 83 GCC-----AAA

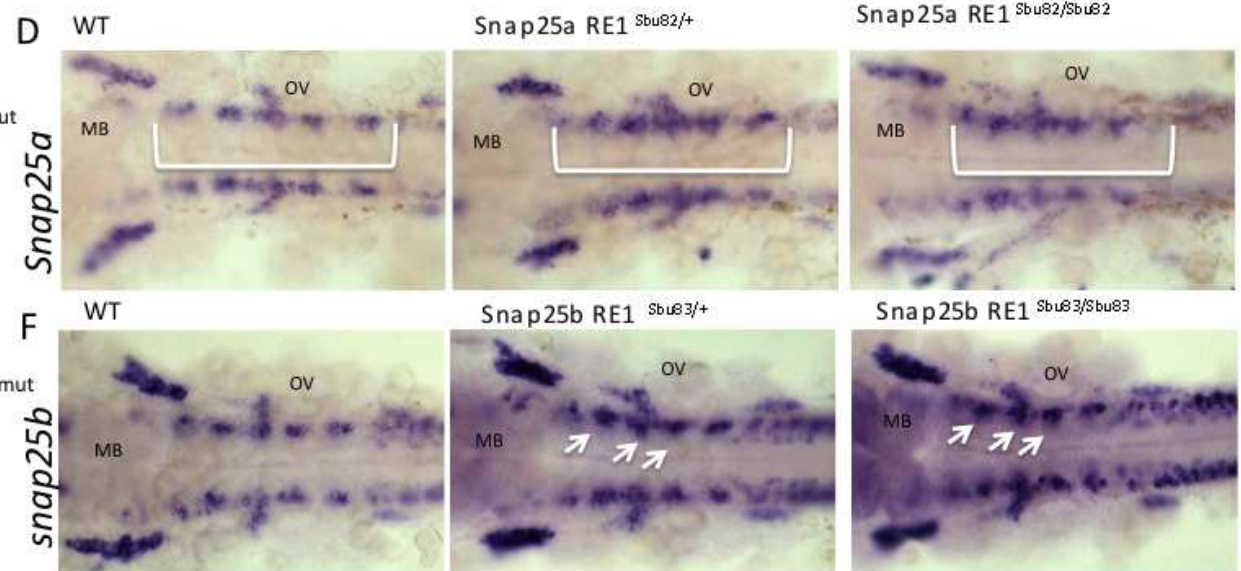
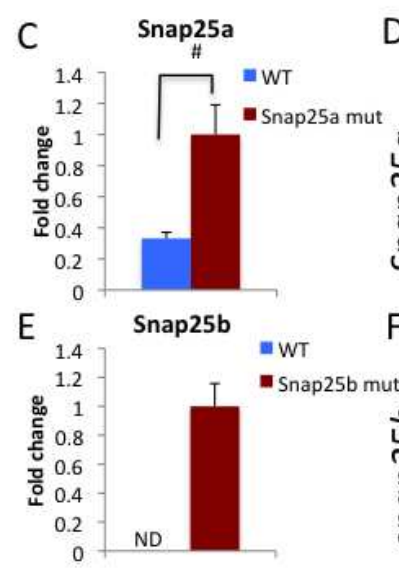


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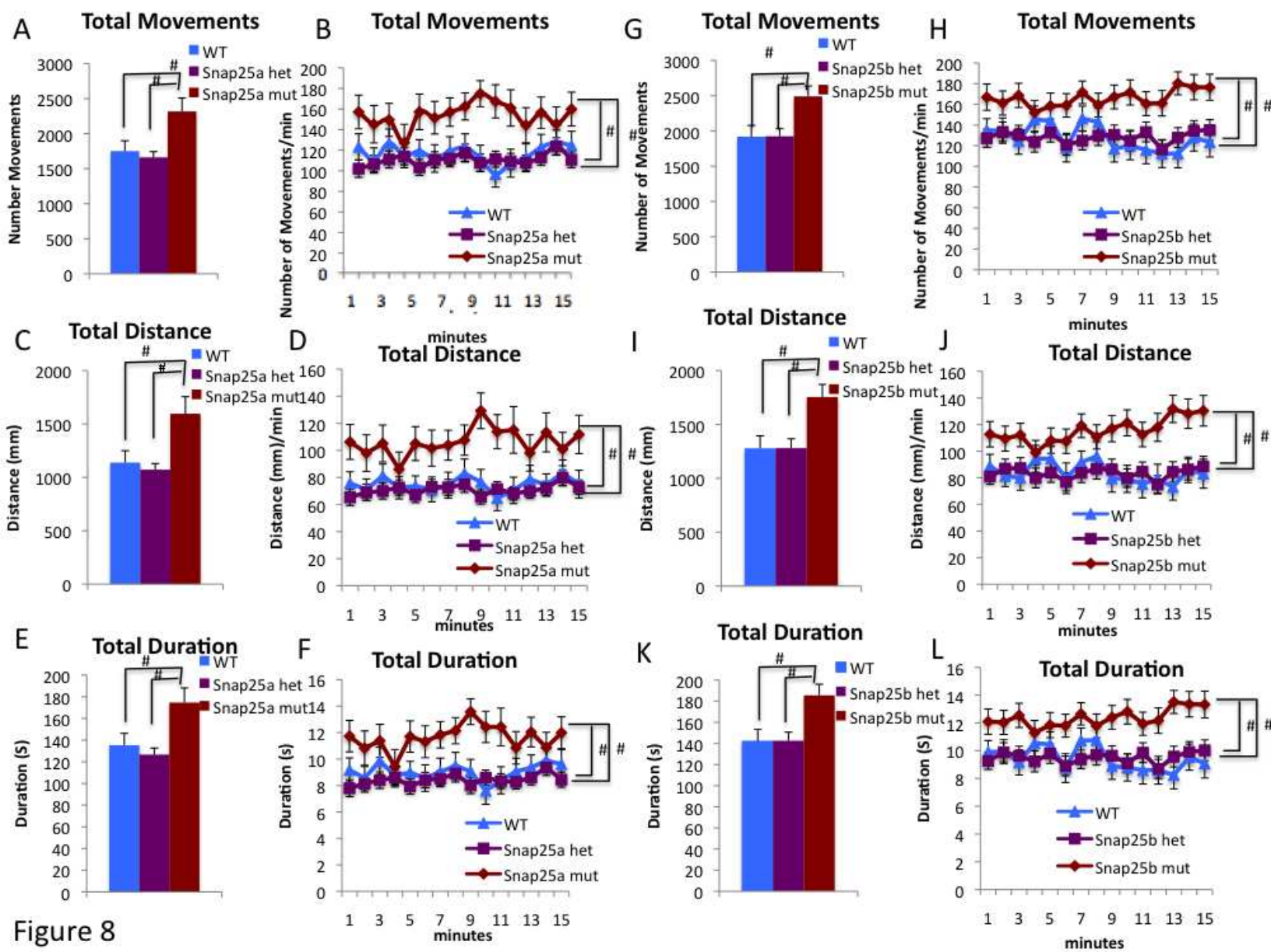


Figure 8

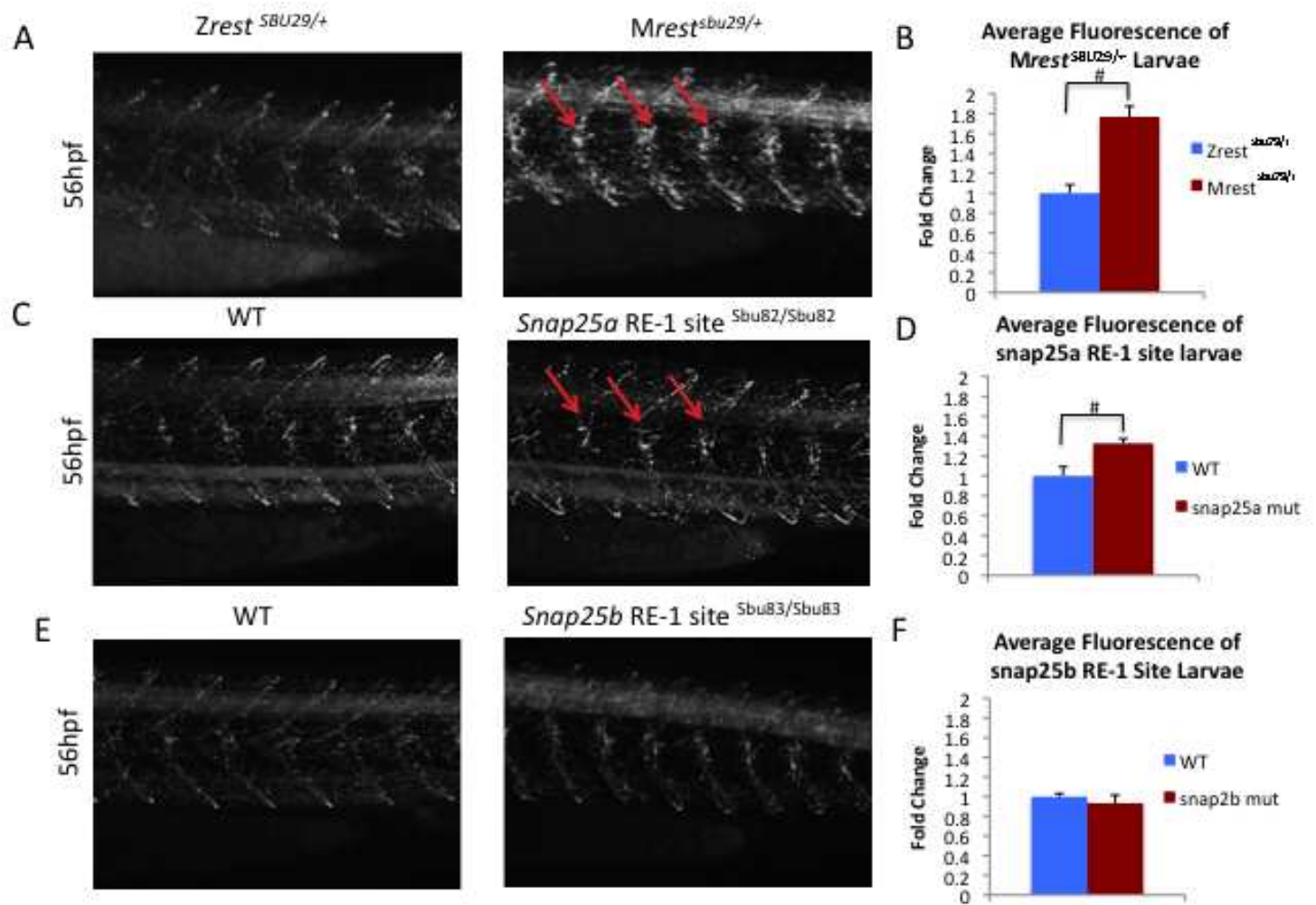


Figure 9