1   Maternal Rest/Nrsf Regulates Zebrafish 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 methyltranferase 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   <u>Fish Maintenance</u>:

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   <u>Housing and genotyping</u>:

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   <u>Behavioral Testing Apparatus and Paradigms</u>:

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/+], Z*rest* ,MZ*rest* 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:GTGCCCTCAAACTTTTCCAA

178   *cana1ba*   F:ATACTGGATCGGCCCAAACT   R:ATACTGGATCGGCCCAAACT

179   *sty10*   F:TGTGGTTCGCATTCTCAAAG   R:ACTTCTTTTTGCGCTCTGGA

180   *grm5(1/2)* F:TGTCACTGATGGCTTCCAGA   R:TGGCTGCAGGTTCAGGTAGT

181   *olfm1b*   F:GGGACCTGCAGTACGTGGTA   R:TATTGCTTGGCGATGTTTTG

182   *cadpsb*   F:TTGTCGTGAGGTGTTCAAGC   R:CAAACTTGGCCATCCAAGAG

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:GAGTAATACGACTCACTAGGGGGGCCTTTTTCAAGTCCTCT . 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* (TTCAGCACCCTGGACAGCGAC) and *snap25b*

198 (TTCAGCACCGCGGAGAGCGCT) 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 preformed deep

215   sequencing of blastula mRNA comparing *Mrest*[sbu29/+] to Z*rest*[sbu29/+] and

216   MZ*rest*[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 there for 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 (Z*rest*[sbu29/+]). MZ*rest*[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 MZ*rest*[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 MZ*rest*[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 MZ*rest*[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 Z*rest*[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   <u>Transcriptional Effects of Maternal Rest depletion persist beyond blastula stages:</u>

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 MZ*rest*[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 Z*rest*[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   <u>Depletion of maternal *rest* modulates larval locomotion:</u>

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/+, Z*rest*sbu29/+, MZ*rest*sbu29/sbu29, and

301   related wild-type control larvae were analyzed at 6 dpf in the light. Comparison of

302   *Mrest*sbu29/+ and Z*rest*sbu29/+, locomotion revealed that *Mrest*sbu29/+ larvae move

303   significantly more (n=71, average of 1511 movements) than Z*rest*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 Z*rest*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 MZ*rest*sbu29/sbu29

310   mutants (N=48) and related wild-type controls (N=72).   In this assay, the

311   MZ*rest*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. Z*rest*sbu29/+ and MZ*rest*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

15

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   <u>Depletion of maternal *rest* alters adult behavior:</u>

328   Depletion of maternal *rest* in *Mrest*$^{sbu29/+}$ or elimination of both maternal *rest*

329   and zygotic *rest* as in MZ*rest*$^{sbu29/sbu29}$ 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 Z*rest*$^{sbu29/+}$ and

336   *Mrest*$^{sbu29/+}$ movement patterns revealed a strong preference of *Mrest*$^{sbu29/+}$ males

337   for the tank walls compared to the Z*rest*$^{sbu29/+}$ males. No preference was observed

338   between *Mrest*$^{sbu29/+}$ and Z*rest*$^{sbu29/+}$ 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 Z*rest*$^{sbu29/+}$ males spend around 23% of

343   their time near the edge of the tank. The female *Mrest*$^{sbu29/+}$ and Z*rest*$^{sbu29/+}$ fish

344   spend a comparable about 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 Z*rest*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   Z*rest*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   <u>Identification of Rest target genes that modulate locomotor behavior:</u>

356   To identify the Rest target genes whose misregulation produces the

357   behavioral phenotypes we observed in the *Mrest*sbu29/+ and MZ*rest*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 with in 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   <u>RE-1 site mutant larvae are hyperactive:</u>

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 MZ*rest[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

18

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 <u>Motor neurons in *Mrest*$^{sbu29/+}$ and *snap25a* RE1$^{sbu82/sbu82}$ site mutants have</u>

413 <u>increased processes:</u>

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 Z*rest*$^{SBU29/+}$

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   <u>Transcripts regulated by maternal *rest*:</u>

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   <u>Regulation of synaptic proteins by Rest:</u>

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 MZ*rest*[sbu29/sbu29] were not apparent in the RE1 site mutants.

504   <u>Regulation of Behavior by Rest:</u>

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   <u>Changes to the architecture of primary motor neurons:</u>

518   *Mrest*[sbu29/+] embryos display increased expression of Sty2b in trunk motor

519   neurons when compared to Z*rest*[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

25

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 MZ*rest*sbu29/sbu29

694   A) Venn diagram showing the overlap of upregulated and downregulated genes in

695   *Mrest*sbu29/+ and MZ*rest*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 Mrestsbu29/+ and Zrestsbu29/+ 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) M*rest*sbu29/+ (N=71) exceed Z*rest*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 MZ*rest*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   M*rest*$^{sbu29/+}$ (A) and MZ*rest*$^{sbu29/sbu29}$ (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 M*rest*$^{sbu29/+}$

728   (n=71) larva spend significantly more time in the outer well when compared to

729   Z*rest*$^{sbu29/+}$ (n=72*)* (P=0.002) D) Quantification of percentage of time spent in the

730   outer wall over 15 minutes reveals that MZ*rest*$^{sbu29/sbu29}$ (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 Z*rest*$^{sbu29/+}$ **(N=20)**controls   C-D) Analysis of

739   percentage of time spent near the walls for females **(*Mrest*$^{sbu29/+}$ (N=18) and**

740   **Z*rest*$^{sbu29/+}$ (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.

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768   znp-1 immunohistochemistry on whole mount zebrafish embryos at 56 hpf to label

769   primary motor neurons. Confocal images were acquired from the truck 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	
Mrest vs. *Zrest*	F	P	F	P	F	P
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
MZ*rest* vs. WT						
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.

	SEX		Genotype		Sex X Genotype	
Variable	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		
Snap25a RE1site [sbu82]	F	P	WT/Het	WT/Mut	Het/Mut
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		
Snap25a RE1site [sbu82]	F	P	WT/Het	WT/Mut	Het/Mut
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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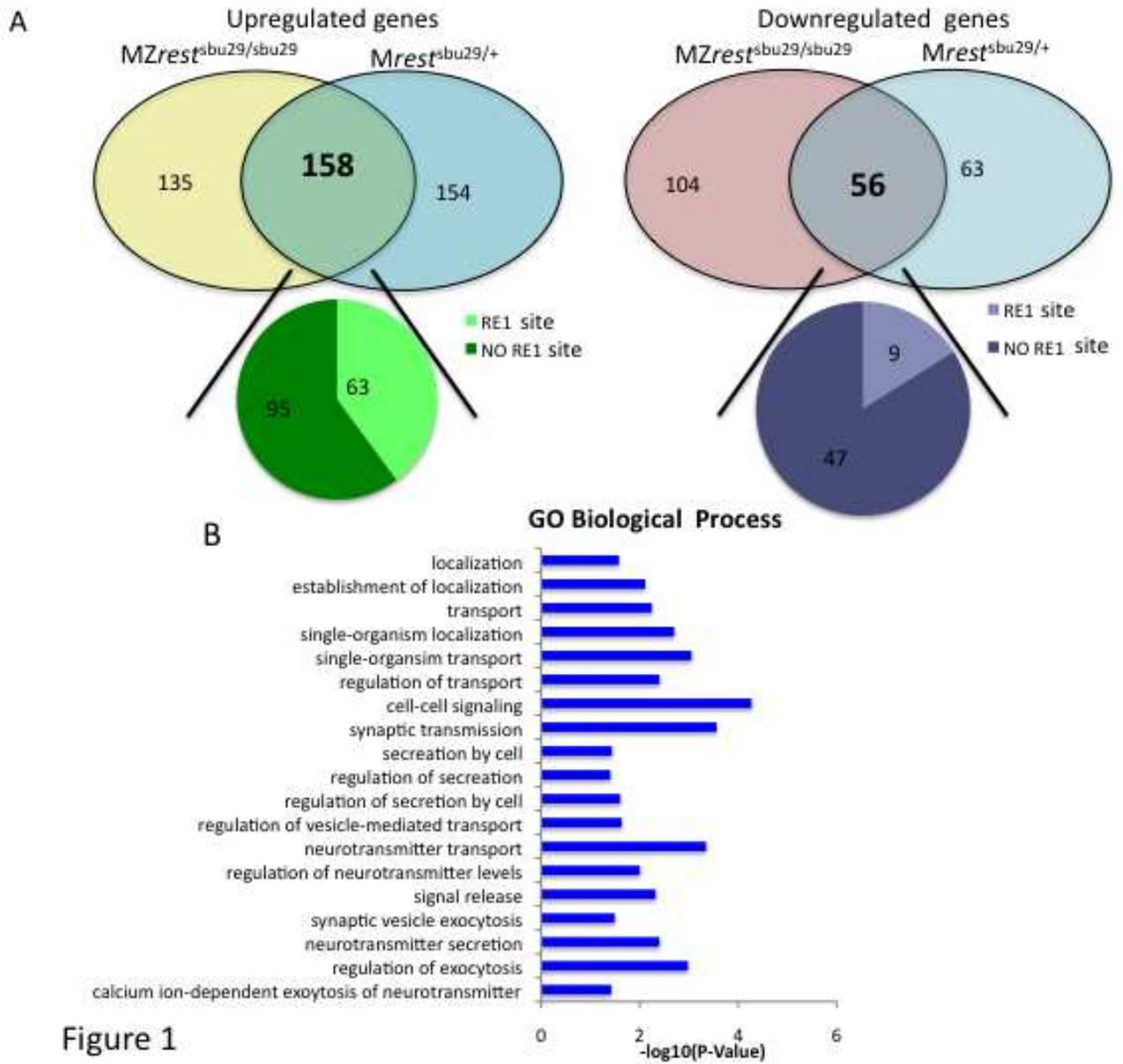
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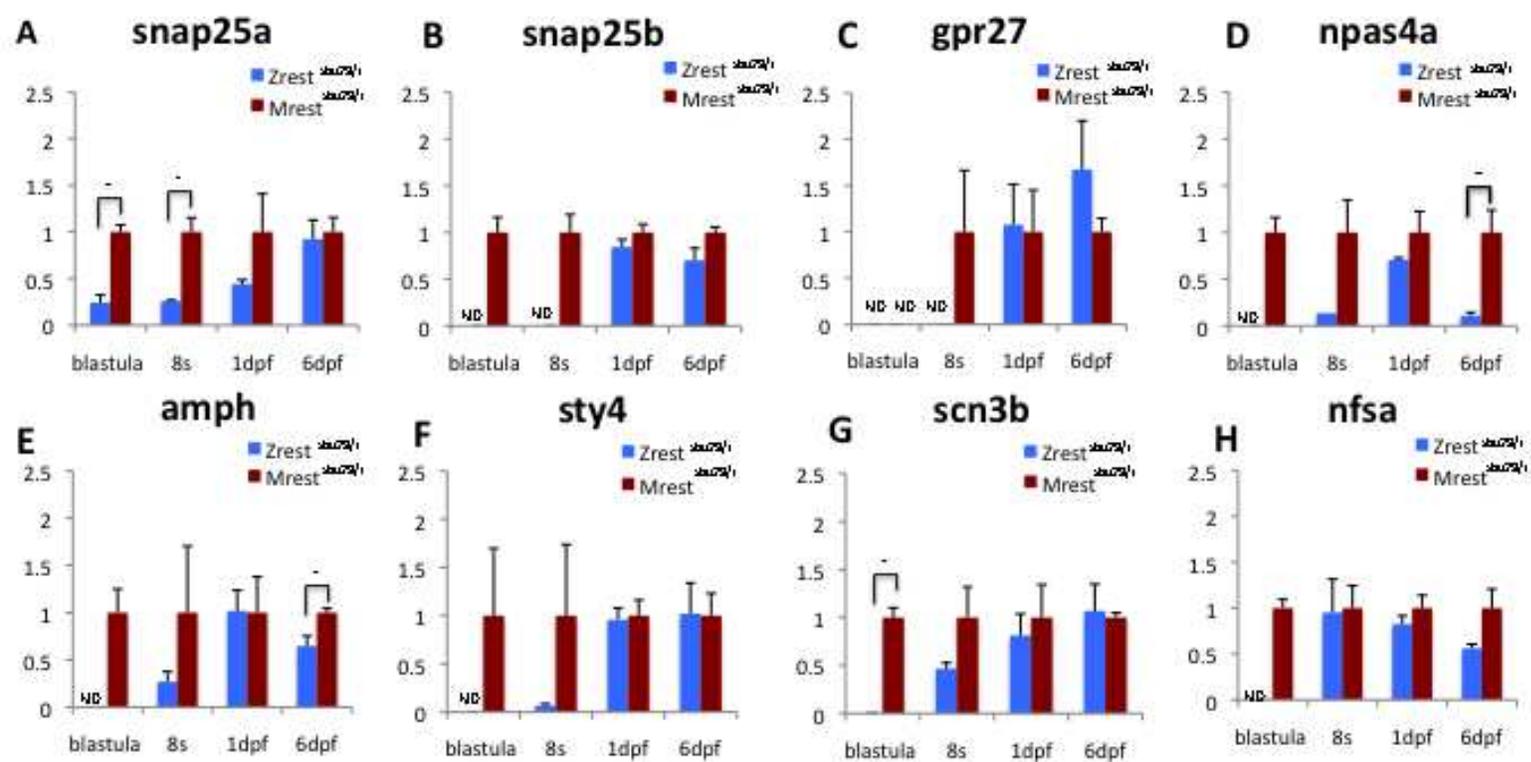
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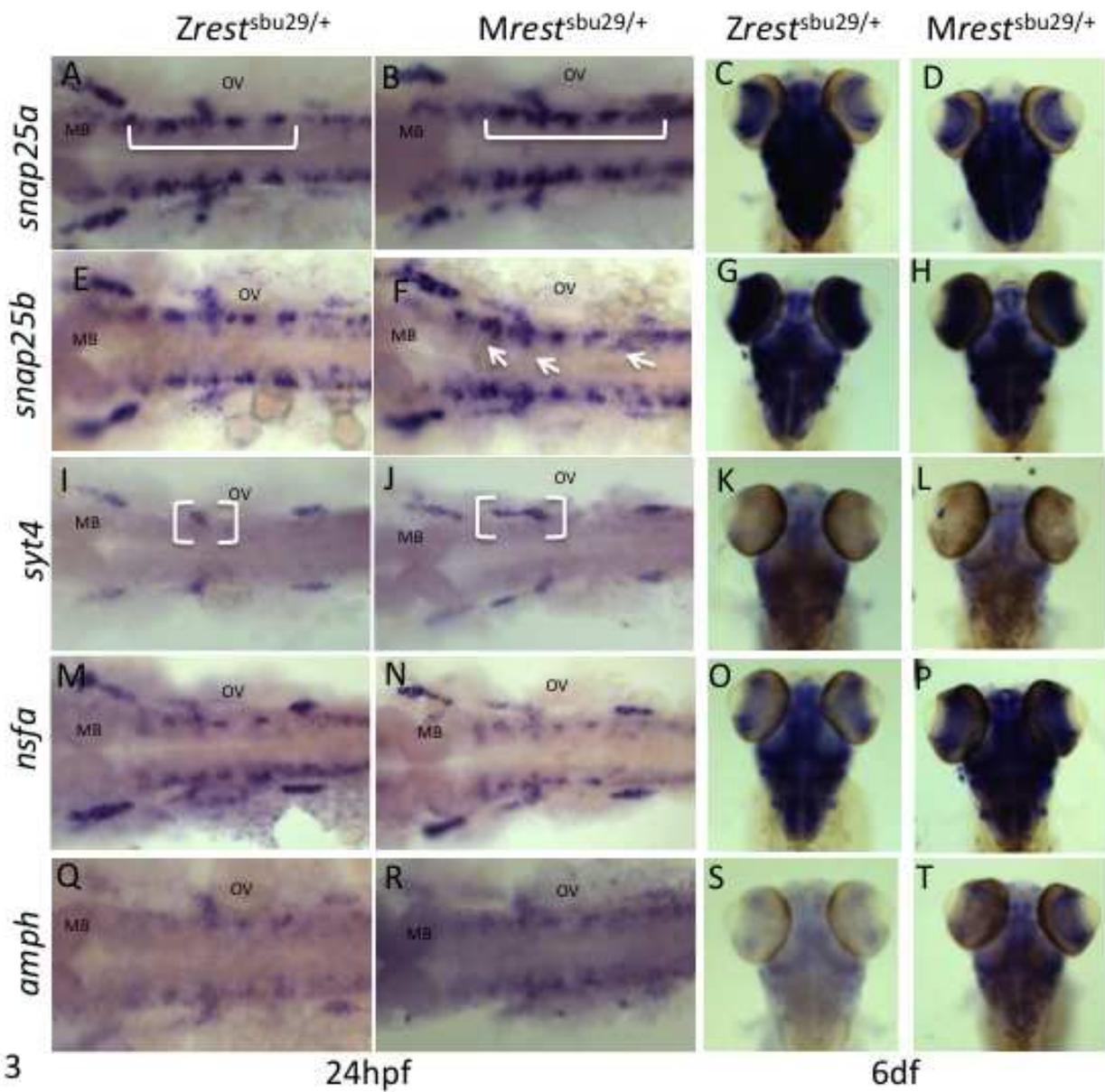
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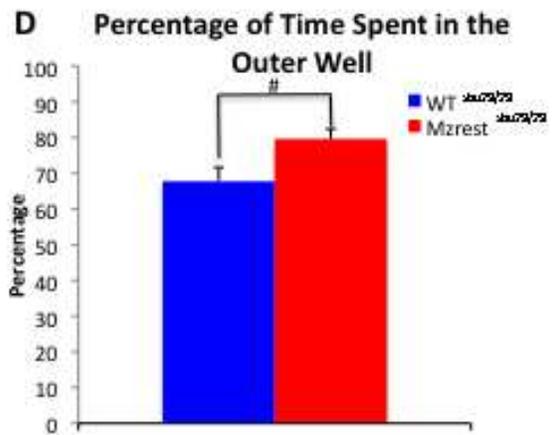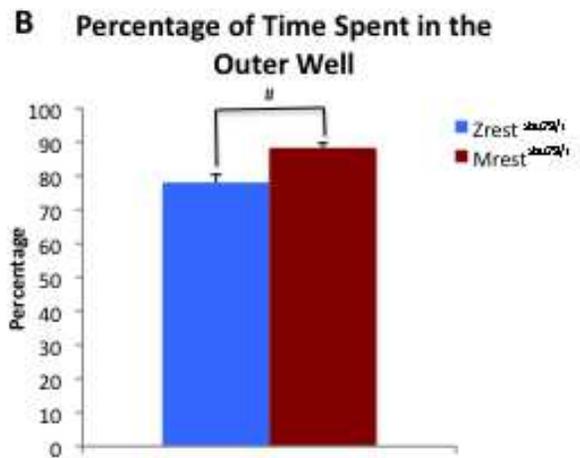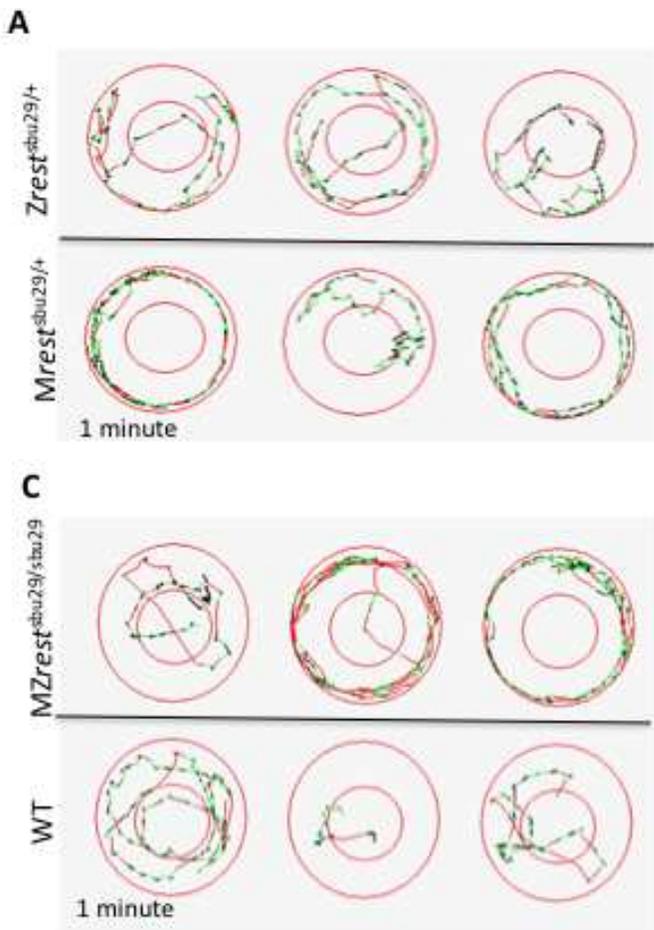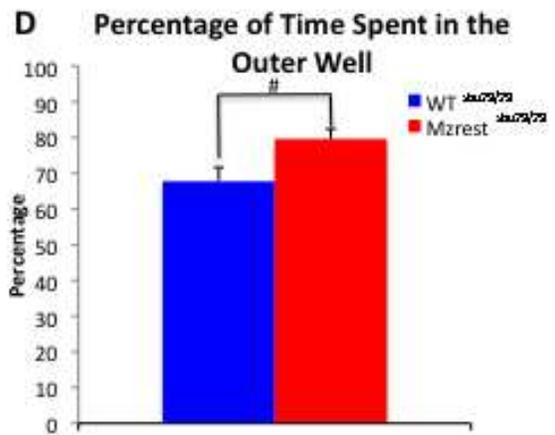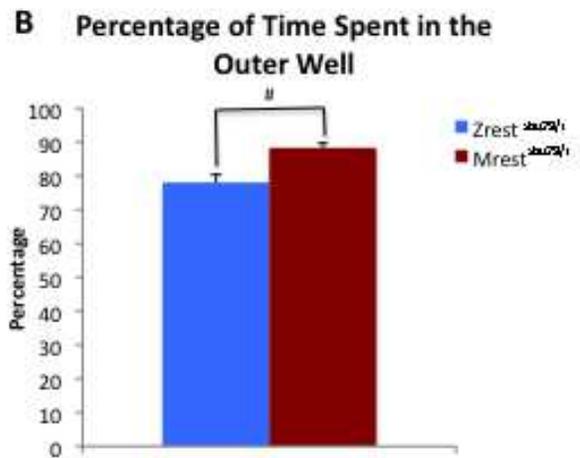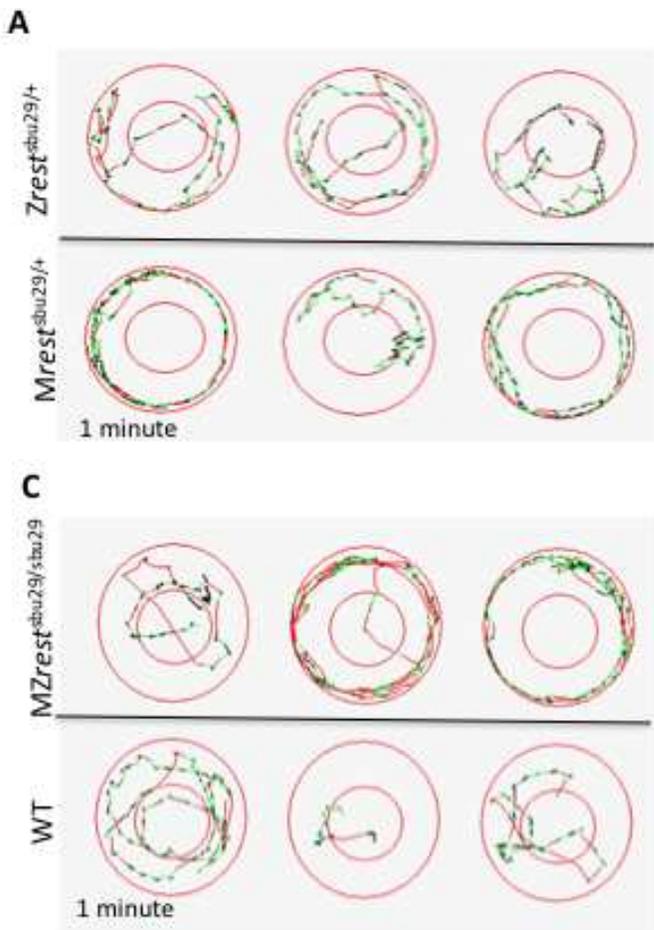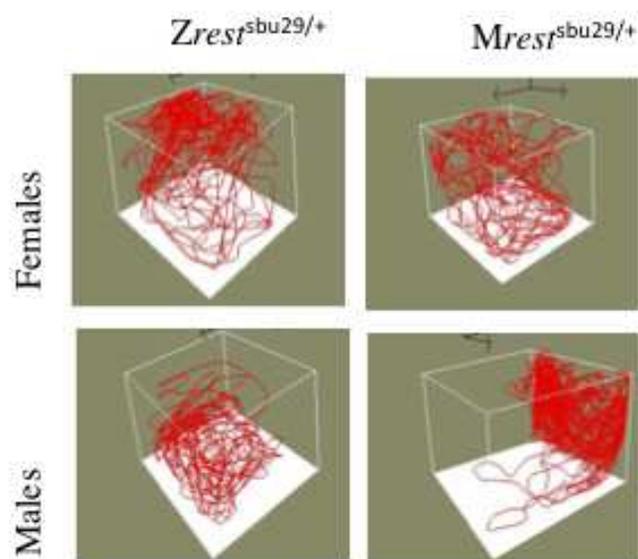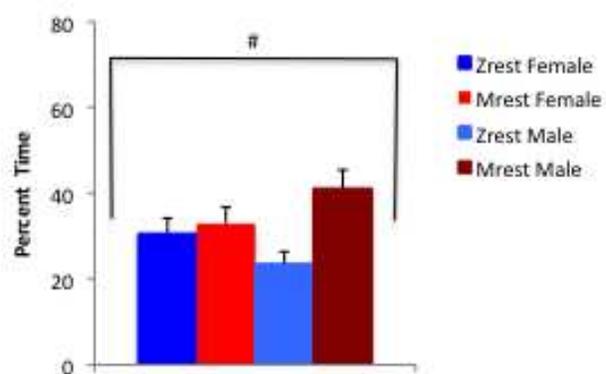
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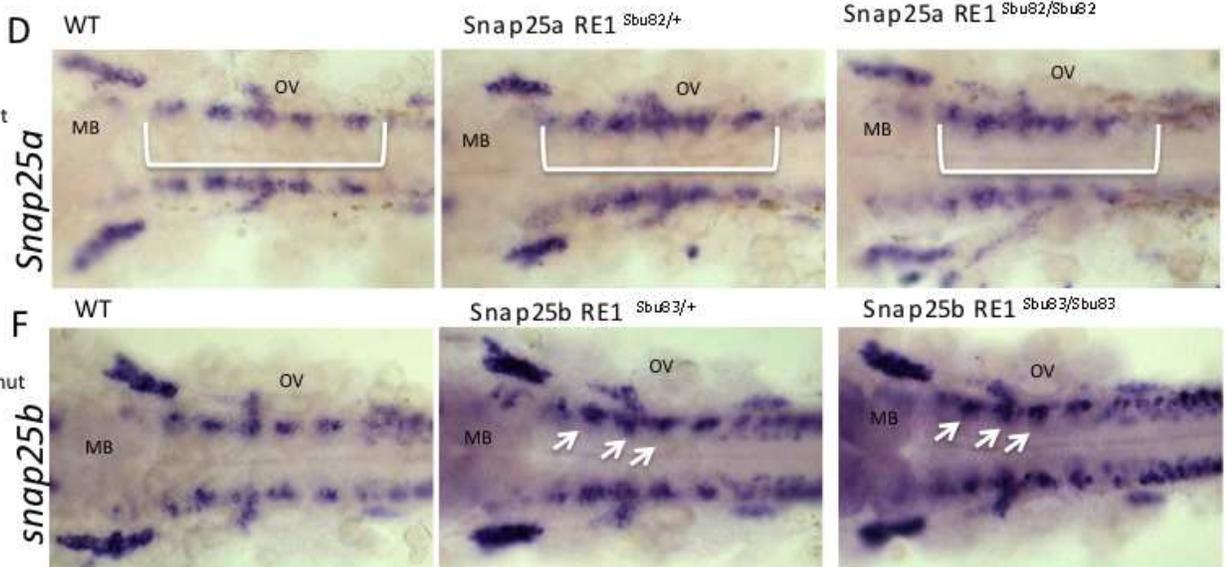
Figure 5

A

Zrest^sbu29/+

Mrest^sbu29/+

1 minute

B Percentage of Time Spent in the Outer Well

Percentage

100
90
80
70
60
50
40
30
20
10
0

#

■ Zrest ^sbu29/+
■ Mrest ^sbu29/+

C

MZrest^sbu29/sbu29

WT

1 minute

D Percentage of Time Spent in the Outer Well

Percentage

100
90
80
70
60
50
40
30
20
10
0

#

■ WT ^sbu29/29
■ Mzrest ^sbu29/29
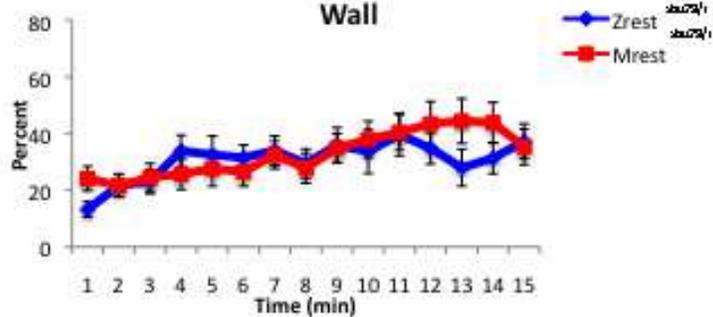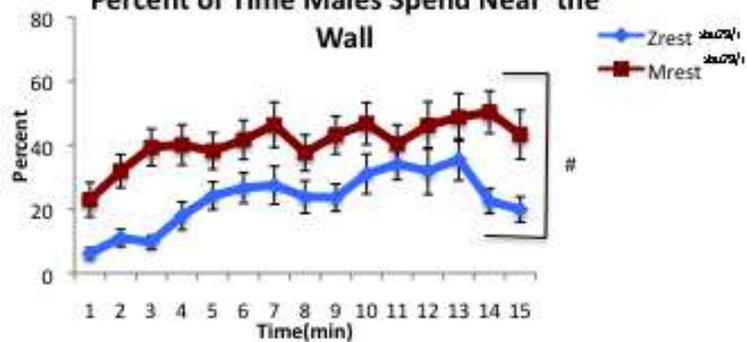
Figure 5

Figure 6

A Snap25a RE1 Site mutation

WT   GC**TTCAGCACCCTGGACAGCGAC**TGC

SBU82 GC**TTCAGCACCC**-----------TGC

B Snap25b RE1 Site mutation

WT   GCCTGTTGTGTGGAT**TTCAGCACCGCGGAGAGCGCT**CATTAAGAGGCGCGCGCGCCAAA
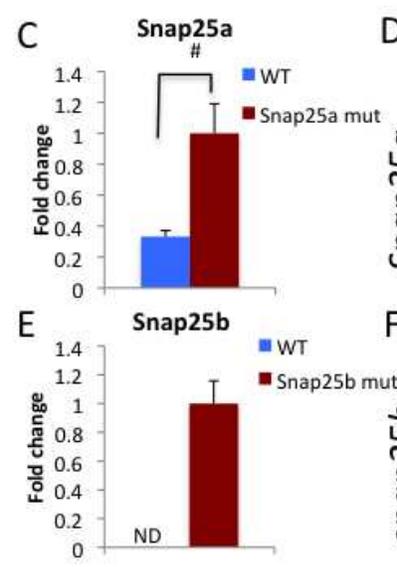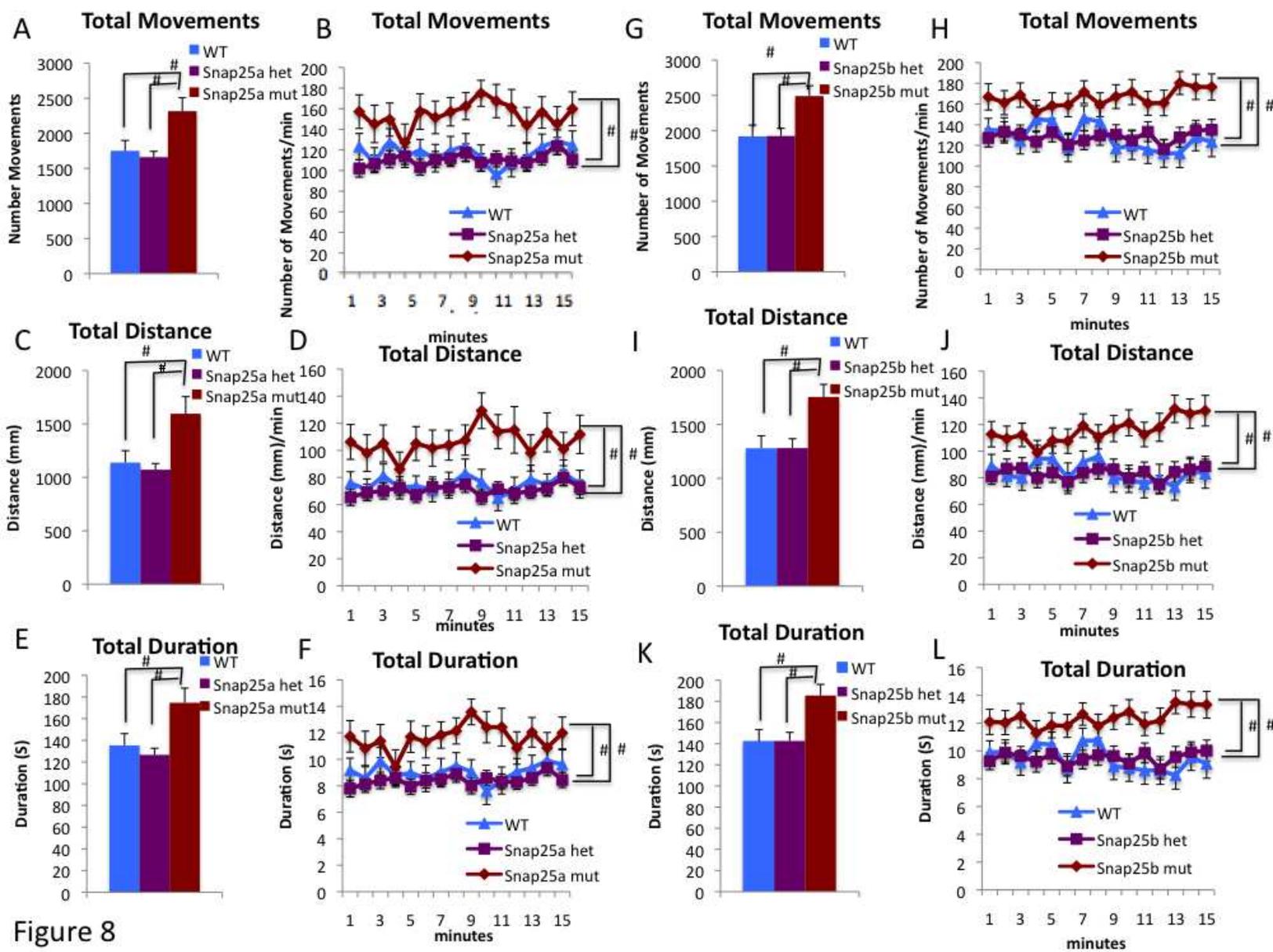
SBU 83 GCC--AAA

Figure 7

Figure 8

A   Zrest $^{SBU29/+}$ Mrest$^{sbu29/+}$

56hpf

B   Average Fluorescence of Mrest$^{SBU29/-}$ Larvae

C   WT   Snap25a RE-1 site $^{Sbu82/Sbu82}$

56hpf

D   Average Fluorescence of snap25a RE-1 site larvae

E   WT   Snap25b RE-1 site $^{Sbu83/Sbu83}$

56hpf

F   Average Fluorescence of snap25b RE-1 Site Larvae

Figure 9