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2 **The Golgi Glycoprotein MGAT4D is an Intrinsic Protector of Testicular Germ Cells From**
3 **Mild Heat Stress**

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32 **Abstract**

33 Male germ cells are sensitive to heat stress and testes must be maintained outside the
34 body for optimal fertility. However, no germ cell intrinsic mechanism that protects from heat has
35 been reported. Here, we identify the germ cell specific Golgi glycoprotein MGAT4D as a protector
36 of male germ cells from heat stress. *Mgat4d* is highly expressed in spermatocytes and spermatids.
37 Unexpectedly, when the *Mgat4d* gene was inactivated globally or conditionally in spermatogonia,
38 or mis-expressed in spermatogonia, spermatocytes or spermatids, neither spermatogenesis nor
39 fertility were affected. On the other hand, when males were subjected to mild heat stress of the
40 testis (43°C for 25 min), germ cells with inactivated *Mgat4d* were markedly more sensitive to the
41 effects of heat stress, and transgenic mice expressing *Mgat4d* were partially protected from heat
42 stress. Germ cells lacking *Mgat4d* generally mounted a similar heat shock response to control
43 germ cells, but could not maintain that response. Several pathways activated by heat stress in
44 wild type were induced to a lesser extent in *Mgat4d*^{-/-} heat-stressed germ cells (NFκB response,
45 TNF and TGFβ signaling, *Hif1α* and *Myc* genes). Thus, the Golgi glycoprotein MGAT4D is a novel,
46 intrinsic protector of male germ cells from heat stress.

47

48 **Introduction**

49 MGAT4D is designated family member D of the *MGAT4* gene family by the Human Genome
50 Nomenclature Committee based on sequence similarity to other members, including MGAT4A and
51 MGAT4B. The latter are N-acetylglucosaminyltransferases (GlcNAcTs) that add a β1,4GlcNAc to
52 complex N-glycans. However, when MGAT4D is transfected into cultured cells, it does not appear to
53 have GlcNAcT activity. Rather, it inhibits MGAT1 activity, the GlcNAcT responsible for initiating
54 complex N-glycan synthesis¹. Because of this inhibitory activity, the protein was termed GnT1IP for
55 GlcNAcT1 Inhibitory Protein. The *Mgat4d* gene is highly expressed in mouse testis with little expression
56 in other mouse tissues². Based on RNA-seq analysis, it is expressed in spermatocytes and
57 spermatids, but not in spermatogonia, sperm or Sertoli cells³. MGAT4D is the most abundant protein in
58 purified Golgi from rat testis germ cells⁴. Characterization of the interactions of MGAT4D in the Golgi
59 using a fluorescence resonance energy transfer (FRET) assay showed that it interacts with MGAT1 but
60 not MGAT2, MGAT3, MGAT4B or MGAT5³. Since knockout of *Mgat1* in spermatogonia disrupts
61 spermatogenesis and results in infertility^{5,6}, deletion or overexpression of *Mgat4d* in germ cells were
62 both expected to have effects on spermatogenesis. In this paper, we show that unexpectedly, deletion
63 of *Mgat4d* globally, or specifically in spermatogonia, or mis-expression of *Mgat4d* in spermatogonia,
64 spermatocytes or spermatids, do not appear to alter spermatogenesis in young or aged mice, and do
65 not affect fertility. However, mild heat stress of the testis in aged mice revealed that germ cells lacking

66 *Mgat4d* exhibited more damage and apoptosis following heat stress. By contrast, a *Mgat4d* transgene
67 expressed in spermatogonia, spermatocytes or spermatids, conferred partial resistance to mild heat
68 stress. This is the first report of a germ cell intrinsic molecule that protects germ cells from heat stress
69 and a novel function for a Golgi glycoprotein. Gene expression analyses showed that germ cells lacking
70 *Mgat4d* responded to heat stress by initially upregulating heat shock and related genes. However, in
71 contrast to controls, germ cells lacking *Mgat4d* did not sustain this response, nor upregulate anti-
72 inflammatory and anti-apoptotic protective genes to the same degree as wild type germ cells. The data
73 identify a new function for MGAT4D as a protector of male germ cell homeostasis, and provide new
74 insight into how male germ cells withstand heat stress.

75

76 **Results**

77

78 **Effects of global and conditional deletion of *Mgat4d* on spermatogenesis and fertility.**

79 Embryonic stem cells (ES Cells) carrying the construct *Mgat4d*^{tm1a(KOMP)Wtsi} designed to
80 conditionally delete exon 4 of the *Mgat4d* gene (Fig. 1) were obtained from the Knockout Mouse
81 Project (KOMP) repository. Following injection into C57BL/6J blastocysts, chimeras were crossed
82 to C57BL/6J to obtain mice carrying the conditional *Mgat4d*^{tm1a(KOMP)Wtsi} allele. Male progeny were
83 crossed with FVB *Stra8-iCre*⁷ or *Flp1-Cre* transgenic females (129S4/SvJaeSor-
84 *Gt(ROSA)26Sor*^{tm1(FLP1)Dym/J})⁸. *Stra8* is expressed in spermatogonia from 3 days post-partum
85 (dpp) and the *Flp1-Cre* was expressed from the ROSA26 locus. Male mice with global (*Mgat4d*[-
86 -]) or conditional (*Mgat4d*[F/F]:*Stra8-iCre*) inactivation of the *Mgat4d* gene were generated, and
87 males expressing *LacZ* from the *Mgat4d* promoter were also obtained (Fig. 1). Both strains were
88 crossed to FVB mice and maintained on a FVB background because *Mgat1* deletion was
89 performed on the FVB background⁵. Genotyping PCR identified *Mgat4d*[+], *Mgat4d*[-], *Mgat4d*[F]
90 alleles and *Stra8-iCre* (Fig. 1). Primer sequences, locations and expected product sizes are given
91 in Supplementary Table S1. Polyclonal rabbit antibodies (pAb) prepared against a C-terminal
92 peptide of MGAT4D identified the long form (MGAT4D-L) and the short form (MGAT4D-S) which
93 lacks 44 amino acids at the N-terminus of MGAT4D-L, and mice with inactivated *Mgat4d* had no
94 signal, as expected (Fig. 1). Detection of *LacZ* expression by beta-galactosidase activity showed
95 that the *Mgat4d* promoter is active mostly in spermatocytes and spermatids in testis tubules (Fig.
96 1), consistent with results of RNA-seq analysis³. Immunohistochemistry for MGAT4D on testis
97 sections from *Mgat4d*[+/-] or wild type males shows staining in the Golgi of spermatocytes and
98 round spermatids, but not in spermatogonia or spermatozoa (Fig. 1), as observed in rat testis⁴.
99 Testis sections from *Mgat4d*[-/-] males showed no staining, as expected (Fig. 1).

100 *Mgat4d*^{-/-} males and females were fertile and transmitted the inactivated gene according
101 to the expected Mendelian distribution (Table 1). Male mice with conditional deletion of *Mgat4d* in
102 spermatogonia also showed no defects in fertility on a FVB background, or after backcrossing 10
103 generations to C57BL/6J mice (Table 1). Based on histological analyses, testicular weight and
104 analysis of sperm parameters (sperm count, viability, morphology, motility and acrosome
105 reaction), no obvious defects in spermatogenesis were observed in *Mgat4d*^{-/-} males. In addition,
106 aging (up to 596 dpp for FVB and 482 dpp for C57BL/6J) did not reveal apparent histological
107 differences in spermatogenesis between mutant and control males (data not shown).

108 As discussed in the Introduction, MGAT4D was initially described as an inhibitor of MGAT1
109 activity and termed GnT1IP¹. By deleting such an inhibitor, we expected MGAT1 activity might
110 increase, and the level of complex N-glycans on glycoproteins might also increase. We
111 determined MGAT1 GlcNAc transferase activity in germ cell extracts. Germ cells were purified
112 from 28 dpp C57BL/6J wild type (n=4) and *Mgat4d*^{-/-} males (n=4) and protein extracts prepared.
113 The average activity for *Mgat4d*^{+/+}(1.86±0.38 nmol/mg/hr) and for *Mgat4d*^{-/-}(1.68±0.32
114 nmol/mg/hr) were not significantly different ($p=0.72$). This result might reflect the fact that *Mgat1*
115 is most highly expressed in spermatogonia which do not express *Mgat4d*³. However, there was
116 no evidence of a specific increase in complex N-glycan species in *Mgat4d*^{-/-} testis sections
117 subjected to MALDI mass spectrometry imaging (MALDI-IMS) for N-glycans (Supplementary Fig.
118 S1).

119

120 **Males lacking *Mgat4d* are more sensitive to mild heat stress of the testis**

121 Given the apparent lack of significant consequences for spermatogenesis of removing *Mgat4d*,
122 we investigated whether stressing testicular germ cells would reveal any effects of *Mgat4d* loss.
123 Spermatogenesis is sensitive to an increase in temperature^{9,10} and we reasoned that disturbing
124 tissue homeostasis using mild heat stress might reveal roles for MGAT4D in testis. The remaining
125 cohort of aged *Mgat4d*^{+/-} and *Mgat4d*^{-/-} FVB mice of between 592 and 596 dpp were
126 anesthetized and subjected to mild heat stress by immersing the lower half of the body in water
127 at 43°C for 25 min. Mock treatment involved the same procedure with a water temperature of
128 33°C. After recovery for 24 hr, testes were harvested. One testis was used for histological analysis
129 and the other for RNA and protein extraction. While testis sections from males treated at 33°C
130 appeared normal, 43°C treatment caused the appearance of enlarged ($\geq 10 \mu\text{m}$) multinucleated
131 cells, large vacuoles ($\geq 10 \mu\text{m}$), small vacuoles and pyknotic cells in testis tubules (Fig. 2).
132 Spermatozoa in the epididymis also included pyknotic cells following heat stress (Fig. 2).
133 Compared to controls, *Mgat4d*^{-/-} testis sections exhibited an increased number of tubules (~3.5-

134 fold) with enlarged cells, and a decrease in undamaged tubules (~2-fold). (Fig. 2). No significant
135 difference was found in testis weights of heat-treated versus control mice (Supplementary Table
136 S2).

137 Heat stress increases apoptosis in differentiating germ cells¹⁰⁻¹² and so testis sections
138 from heat- and mock-treated aged FVB males were subjected to the “Apoptag” assay and staining
139 was quantified using FIJI software (<https://fiji.sc/>). As expected, apoptosis increased in sections
140 from control heat-treated males sacrificed 24 hr after heat treatment (Fig. 3). However, testes
141 from *Mgat4d*^[-/-] mice showed ~2-fold more apoptotic germ cells than *Mgat4d*^[+/-] controls (Fig.
142 3). Thus, based on histology and levels of apoptosis, the effects of heat stress were more severe
143 for aged *Mgat4d*^[-/-] testes than for heterozygous testes.

144

145 ***Mgat4d* transgenic mice are resistant to the effects of heat stress.**

146 Mice with targeted deletion of *Mgat1* in testicular germ cells exhibit defective spermatogenesis
147 and are infertile⁵. Thus, it was expected that inhibiting MGAT1 activity by increasing the level of
148 MGAT4D in germ cells, would induce defects in mouse spermatogenesis. To investigate,
149 C57BL/6J transgenic males expressing a *Mgat4d-L-Myc* cDNA in specific germ cell types were
150 generated. This transgene has previously been shown to inhibit MGAT1 in transfected cells^{1,3}.
151 The *Stra8* (Stimulated By Retinoic Acid 8) promoter was used to express the transgene in
152 spermatogonia⁵⁻⁷, the *Ldhc* (Lactate Dehydrogenase C) promoter was used to express in
153 spermatocytes^{13,14}, and the *Prm1* (Protamine 1) promoter was used to express in spermatids¹⁵
154 (Fig. 4). The transgenic mouse strains were named *Stra8-Mgat4d-L-Myc*, *Ldhc-Mgat4d-L-Myc*
155 and *Prm1-Mgat4d-L-Myc*, respectively. They were genotyped by PCR of genomic DNA using
156 primers described in Supplementary Table S1, and transgene expression was shown to be 3-6-
157 fold greater than endogenous *Mgat4d-L* levels using quantitative RT-PCR (qRT-PCR) on cDNA
158 from testis (Fig. 4). qRT-PCR using primers specific for the *Myc* sequence gave a similar level of
159 expression based on Ct values (not shown). *Myc* transcripts could not be quantitated relative to
160 the control that has no transgene. By contrast, attempts to determine MGAT4D-L-Myc protein
161 levels in testis extracts by western blot analysis using anti-Myc monoclonal antibodies (mAb) from
162 several species were not successful, although MGAT4D-L-Myc overexpressed in CHO cells is
163 detected by anti-Myc mAb³. We generated C- and N-terminal peptide-purified rabbit pAbs that
164 detect MGAT4D-L-Myc or Myc-MGAT4D-L, respectively, in transfected CHO cells
165 (Supplementary Fig. S2). The C-terminal pAb detected Myc-MGAT4D-L much more readily than
166 MGAT4D-L-Myc (Supplementary Fig. S2). Moreover, MGAT4D-L-Myc was not detected in

167 extracts from transgenic germ cells. Nevertheless, the results that follow show that each
168 transgene was functional in the heat stress test.

169 Overexpression or mis-expression of *Mgat4d* in germ cells was expected to inhibit MGAT1
170 activity^{1,3}. However, compared to *Mgat4d*[+/+] controls (1.1 +/-0.13 nmol/mg/hr; n=10), there was
171 no significant inhibition of MGAT1 activity in germ cells from 28-42 dpp *Stra8-Mgat4d-L-Myc* (1.4
172 +/-0.16 nmol/mg/hr; n=7), *Ldhc-Mgat4d-L-Myc* (0.78 +/-0.18 nmol/mg/hr; n=4), *Prm1-L-Mgat4d*
173 (1.3 +/-0.03 nmol/mg/hr; n=4). In addition, the MALDI-IMS of testis sections showed that the
174 complement of complex N-glycan species was not reduced in testis sections from transgenic mice
175 (Supplementary Fig. S1). Rather, there was a significant increase in both oligomannosyl and
176 simple complex N-glycans.

177 Histological analysis of testis sections showed no obvious changes in spermatogenesis
178 or testicular structure in adult transgenic mice (Fig. 4). In addition, the fertility of transgenic males
179 was normal, although *Stra8-Mgat4d-L-Myc* mice showed low transgene transmission from
180 transgenic males (Table 1). Males from the three transgenic mouse strains and non-transgenic
181 littermates or wild type C57BL/6J controls were subjected to mild heat stress. No significant
182 difference was observed in testis weights of mock- versus heat-treated mice (Supplementary
183 Table S2). Importantly, however, each transgenic strain showed an ~3-fold reduction in the
184 number of tubules with enlarged germ cells, and ~2-fold fewer had tubules with large vacuoles
185 (Fig. 5). The number of undamaged tubules was also increased but small vacuoles and pyknotic
186 cells were present in heat-treated transgenic mice (Fig. 5). The “Apoptag” assay revealed an ~2-
187 fold reduction in apoptotic germ cells in all three transgenic strains (Fig. 6). We also investigated
188 previously reported gene expression changes due to heat stress. In wild type males, *Socs3*,
189 *Hspa1a* and *Degs1* were up-regulated, while *Bcl2l12*, *Crbg3* and *Dmrt1* were down-regulated
190 after heat treatment (Fig. 7), consistent with previous observations^{11,16}. Interestingly, *Mgat4d* was
191 markedly down-regulated following heat treatment (Fig. 7). Gene expression in heat treated *Stra8-*
192 *Mgat4d-Myc* males was similar to that in non-transgenic males at 33°C, up-regulated genes being
193 less up-regulated and down-regulated genes, less down-regulated compared to non-transgenic
194 at 43°C (Fig. 7). Thus, on the basis of several criteria, the presence of a *Mgat4d-L-Myc* transgene
195 in germ cells gave significant protection from heat stress.

196

197 **Molecular basis of the increased sensitivity of *Mgat4d*[-/-] germ cells to heat stress**

198 The histological and apoptotic changes induced by heat stress reported above were observed in
199 an aged cohort of 1.6 year FVB mice. We subsequently tested 7 month C57BL/6J *Mgat4d*[-/-]
200 mice and did not observe increased sensitivity to heat stress. However, protection from heat

201 stress was observed in adult C57BL/6J transgenic mice as shown here. Thus, to determine
202 whether *Mgat4d*^{-/-} mice on a C57BL/6J background exhibited a more sensitive response to heat
203 stress than controls, and to also gain insights into molecular mechanisms that underlie this
204 phenotype, microarray analyses were performed on cDNA from purified germ cells of control and
205 *Mgat4d*^{-/-} C57BL/6J mice of 2 months. *Mgat4d*^{+/+} and *Mgat4d*^{-/-} males were treated at 33°C
206 or 43°C for 25 min and sacrificed after 8 hr, a time when no visible histological changes to germ
207 cells were observed (data not shown). Testes were enzymatically dissociated and germ cells were
208 isolated and counted. RNA preparations with a RIN value >7.9 were used to make cDNA for
209 microarray analysis. Purity of germ cells was assessed by relative expression of germ cell-specific
210 and non-germ cell genes to the same genes expressed in testis RNA as previously described ⁶.
211 The Mouse Clariom™ D GeneChip™ Mouse Transcriptome Array 1.0 from Affymetrix was used.
212 Custom scripts using the R/Bioconductor tools affymetrix and limma were used to process the
213 raw (.CEL) files and to compare *Mgat4d*^{-/-} versus *Mgat4d*^{+/+} microarray data from 33°C- and
214 43°C-treated mice. The samples displayed a moderate clustering by genotype, as seen in PCA
215 plots (Fig. 8). Importantly, significant differences between genotypes were much less pronounced
216 at 33°C than at 43°C, as witnessed by the tighter correlation in the heat maps, and a lower number
217 of differentially expressed genes (DEGs) between genotypes shown in volcano plots (Fig. 8).
218 However, a clear difference was evident between wild type and *Mgat4d*^{-/-} arrays from germ cells
219 of mice treated at 43°C. Given the importance of the temperature as a confounding variable, it
220 was included in modelling differential gene expression between genotypes. DEGs in mutant
221 versus wild type germ cells at 33°C and at 43°C were determined, and the interaction between
222 temperature and genotype was evaluated to obtain gene lists for further analysis. Microarray data
223 are deposited in NCBI's Gene Expression Omnibus (GEO) and are accessible through GEO serial
224 accession number GSE137307.

225 Analysis of 33°C *Mgat4d*^{-/-} versus control microarrays with FDR<0.05 and fold change
226 +/-1.5 gave 4 DEGs (3 up- and 1 down-regulated gene), including *Mgat4d* as expected. *Mgat4d*
227 transcripts were not completely lacking in *Mgat4d*^{-/-} samples due to transcription beyond the
228 deleted exon 4 (Supplementary Fig. S3). However, no MGAT4D protein was detected by western
229 analysis or immunohistochemistry (Figure 1, Supplementary Fig. S2). One of the up-regulated
230 genes, pseudogene Gm12584, maps to the locus of a testis-specific gene, adenosine deaminase
231 domain-containing 1 (*Adad1*), which encodes a nuclear RNA-binding protein ¹⁷. Upregulated
232 Gm24265 refers to an SnRNA mapped to chromosome 5. The third up-regulated gene was
233 *Tmed6* (Transmembrane P24 Trafficking Protein 6) which is enriched in the endoplasmic

234 reticulum and Golgi compartments. It is notable that all the upregulated DEGs have a log fold
235 change <1 (fold change <2), revealing a very mild effect of *Mgat4d* deletion on germ cell gene
236 expression under control conditions (Supplementary Table S3). By contrast, analysis using the
237 same stringency for data from heat-stressed *Mgat4d*^{-/-} versus control germ cells, revealed 476
238 DEGs in *Mgat4d*^{-/-} germ cells with 110 genes up-regulated and 366 genes down-regulated. The
239 top down-regulated genes were *Serpinb1a* (2.13 log fold change) followed by *Ly96* (2.12 Log fold
240 change) and S100-a11 (2.09 Log fold change) (Supplementary Table S4). Some of the down-
241 regulated genes (*Serpinb1a*, *Star*, *Osr2*, *Klk1b22*, *Itih2*) are related to the regulation of cellular
242 homeostasis, proliferation or survival¹⁸⁻²³. The top two up-regulated genes were non-coding
243 Gm26715 and Gm48565 (2.04 and 1.98 log fold change respectively), followed by *Hspa1a* and
244 *Hspa1b* heat shock proteins (1.81 and 1.75 log fold change, respectively). Most of the up-
245 regulated genes were non-coding or predicted genes (Supplementary Table S5). cDNA from the
246 43°C-treated control and *Mgat4d*^{-/-} cDNA preparations was used for qRT-PCR validation of
247 DEGs observed in microarray experiments (Fig. 9). The relevant primer sequences are given in
248 Supplementary Table S6.

249

250 **Enriched biological pathways in heat-stressed *Mgat4d*^{-/-} germ cells based on Ingenuity** 251 **Pathway Analysis (IPA).**

252 To find the most significantly represented pathways differentially altered in *Mgat4d*^{-/-} versus
253 *Mgat4d*^{+/+} germ cells following heat stress, we examined the relationship between DEGs at a
254 ± 1.5 fold change with adjusted FDR <0.05 and $p<0.05$ using IPA. Interestingly, the top canonical
255 pathways were mostly down-regulated or with “no activity pattern available” in 43°C-treated
256 *Mgat4d*^{-/-} versus control germ cells, and were related to recovery from stress conditions (Fig.
257 9). Ranked by $-\log(p\text{-value})$, the top down-regulated pathway was Acute Phase Response
258 Signaling ($p=5.2$; Z-score -2.45) followed by LXR/RXR Activation ($p=5.04$; Z-score 0.707), the
259 only pathway with a positive z-score and $-\log(p\text{-value})$ higher than 2. NRF2-mediated Oxidative
260 Stress Response ($p=4.98$; Z-score -2.111) and Glutathione-mediated Detoxification ($p=3.37$; Z-
261 score -2) were also top pathways.

262

263 **Top upstream transcriptional regulators.**

264 IPA was used to predict the top upstream transcriptional regulators in the DEGs based on their
265 gene targets. The algorithm calculates a p-value on the basis of significant overlap between genes
266 in our test dataset and target genes regulated by the same regulator in the IPA knowledge base.
267 The activation Z score algorithm was used to make predictions. This analysis identified 323

268 upstream regulators with a p-value of overlap <0.05 and a Z-score greater than or equal to ± 2 .
269 *Tgfb1*, *Tnf*, *Ifng*, *Il1b* related to immune system regulation are the top inhibited upstream
270 regulators (Supplementary Table S7). Sorting the results by Expression Log Ratio ± 1 , identified
271 13 differentially-expressed upstream regulators in our data set, 11 down-regulated and 2 up-
272 regulated (Supplementary Table S7).

273

274 **Most represented networks, toxicological functions, diseases and biological functions.**

275 DEGs in germ cells from heat-treated mice were compared by IPA with genes belonging to
276 specific biological networks or implicated in diseases. The most highly ranked network was “DNA
277 Replication, Recombination, and Repair, Nucleic Acid Metabolism, Small Molecule Biochemistry”
278 with 28 focus molecules (Supplementary Table S8). The top diseases and biological functions
279 were related to “Organismal Survival” - 19 biological functions were predicted to be increased with
280 an activation Z-score between 6.131 and 2.01, mostly related to inflammation, injury and disease
281 (Supplementary Table S9) but a higher number of diseases or functions were predicted to be
282 decreased (71). The top category was “Lipid Metabolism, Small Molecule Biochemistry, Vitamin
283 and Mineral Metabolism” and the most represented of these were related to cellular function.

284

285 **Gene set enrichment analysis (GSEA)**

286 Comparisons of DEGs at 33°C and 43°C with published, classified gene sets in the MSigDB was
287 performed using GSEA²⁴⁻²⁶. Of the eight categories of gene sets, the Hallmark collection
288 summarizes well-defined biological processes and states from v4.0 MSigDB collections C1 through
289 C6²⁷. Hallmark gene sets with a Normalized Enrichment Score (NES) of ± 2 , $FDR < 0.25$ and
290 $p < 0.05$ were examined. In *Mgat4d*^{-/-} germ cells, only 3 Hallmark gene sets were significantly
291 enriched at 43°C - E2F targets, G2M checkpoint and spermatogenesis. The Hallmark
292 Spermatogenesis gene set contains genes upregulated during the process of spermatogenesis,
293 indicating that loss of *Mgat4d* in heat-stressed germ cells leads to induction of spermatogenesis-
294 promoting genes as a response, whereas germ cells expressing *Mgat4d* were comparatively
295 protected from premature upregulation of these genes (Supplementary Fig. S4). Gene sets
296 enriched in *Mgat4d*^{+/+} germ cells at 43°C were related to immune pathways signaling,
297 inflammatory responses, apoptosis and hypoxia (Supplementary Fig. S4).

298 Gene sets of note in other collections were: negative regulation of extrinsic apoptosis
299 signaling (suppression of apoptosis) by *Mgat4d* in the C5 collection; increased inflammatory
300 response and TNF targets up in *Mgat4d*^{+/+} germ cells in the C2 collection; late ATM-dependent
301 genes induced by radiation up in *Mgat4d*^{+/+}; increased induction in *Mgat4d*^{-/-} of MYBL1 target

302 genes in spermatocytes; and genes downregulated in response to gamma-radiation were up in
303 *Mgat4d*^{-/-}. We also investigated DEGs in wild type versus mutant at 43°C versus 33°C using
304 EnrichR²⁸. Heat maps highlight some of the informative EnrichR gene sets and also show
305 illustrative gene expression differences identified (Fig. 10). The overall results suggest that *Mgat4d*^{-/-}
306 ^{-/-} germ cells have a problem responding to heat shock stress, e.g. coping with hyperthermic stress
307 through clearance of damaged proteins (*Casp8*; Fig. 10). A number of pathways and genes were
308 induced to a lesser extent in *Mgat4d*^{-/-} heat-stressed mice, including *Hif1α*, the NFκB response,
309 pro-inflammatory pathways such as TNF and TGFβ signaling, and genes that promote proliferation
310 such as *Myc* (Fig. 10).

311

312 Discussion

313 In this paper we characterize the first germ cell intrinsic molecule that protects from heat
314 stress - the Golgi glycoprotein MGAT4D. Other molecules that protect germ cells from heat stress
315 have been described, but each was overexpressed in testis under an exogenous promoter^{29,30}.
316 MGAT4D maps to mouse chromosome 8 whereas previous genetic loci linked to germ cell
317 resistance to heat stress map to mouse chromosomes 1 and 11³¹. Global deletion of the ion
318 channel *Trpv1* increases the sensitivity of germ cells to heat stress³², and this gene maps to
319 chromosome 11, albeit 5.5cM away from the heat resistant locus on chromosome 11³¹. However,
320 it is not clear which cells of the testis express *Trpv1* which is most highly expressed elsewhere in
321 dorsal root ganglia. *Mgat4d* is most highly expressed in spermatocytes and spermatids³ and thus
322 well positioned to protect germ cells from heat stress. Here we provide several pieces of evidence
323 in support of such a germ cell protective role for *Mgat4d*. First, we show that an old cohort of
324 *Mgat4d*^{-/-} males were more sensitive to mild testicular heat stress than heterozygote controls,
325 as evidenced by increased germ cell defects and apoptosis at 24 hr after heat stress. Second, we
326 found that mice expressing a *Mgat4d-L-Myc* transgene in either spermatogonia (*Stra8* promoter),
327 spermatocytes (*Ldhc* promoter) or spermatids (*Prm1* promoter) were less sensitive to testicular
328 heat stress than wild type controls, based on reduced germ cell defects and reduced apoptosis.
329 Characterization of individual gene expression changes for genes known to exhibit increased or
330 decreased expression following heat stress, showed that males expressing the *Stra8-Mgat4d-L-*
331 *Myc* transgene were comparatively resistant to heat stress and, at 43°C, behaved similarly to non-
332 transgenic germ cells treated at 33°C, whereas non-transgenic males treated at 43C showed the
333 marked gene expression changes predicted from the literature. To investigate gene expression
334 differences in more depth, we performed microarray analyses on *Mgat4d* wild type and *Mgat4d*^{-/-}

335 /-] germ cells prepared only 8 hr after males were treated at 43°C for 25 min, when no histological
336 changes were apparent. Comparisons of DEGs and bioinformatics analyses using IPA, GSEA
337 and EnrichR revealed that *Mgat4d*^{-/-} heat-treated germ cells responded initially to heat stress,
338 but did not sustain that response like wild type, heat-treated germ cells. Thus, *Mgat4d*^{-/-} germ
339 cells were less protected by autophagy or signaling pathways of inflammatory and proliferative
340 responses. In addition, heat-treated *Mgat4d*^{-/-} germ cells upregulated spermatogenic and
341 spermiogenic genes to a greater extent than controls, indicative of the loss of a regulator of
342 spermatogenesis - MGAT4D in this case. We previously showed that loss of MGAT1 in germ cells
343 gave a similar upregulation of genes that promote spermatogenesis or spermiogenesis⁶.

344 A key question for the future is to determine how MGAT4D protects against heat shock in
345 male germ cells. Interestingly, *Mgat4d* transcripts are markedly reduced by the 43°C treatment
346 and yet if MGAT4D is not present, germ cells are more sensitive to heat treatment, and if a *Mgat4d*
347 transgene is present, germ cells are comparatively protected. Thus, the presence of MGAT4D,
348 which may perdure in wild type germ cells after *Mgat4d* transcripts are reduced by heat stress,
349 appears to facilitate the sustained heat stress response observed in wild type germ cells. How
350 this is accomplished by a type II transmembrane Golgi glycoprotein may be related to the effects
351 of Golgi glycosyltransferases on Golgi fragmentation. Some Golgi glycosyltransferases of the
352 medial and trans Golgi compartments have been shown to facilitate Golgi fragmentation after heat
353 shock^{33,34}. For example, the mucin O-glycan GlcNAcT CGNT3 promotes Golgi fragmentation
354 following heat shock by interacting with myosin IIA via its cytoplasmic tail³⁴. MGAT4D is the most
355 abundant protein in rat Golgi of male germ cells⁴ and its loss after heat shock may protect the
356 Golgi from fragmentation and protect Golgi glycosyltransferases and other Golgi residents,
357 including molecules that protect from Inflammation and autophagy and that promote proliferation
358 and survival, from degradation by the proteasome³⁴.

359

360 **Materials and Methods**

361 **Mice**

362 Mice carrying a conditional *Mgat4d* allele were generated from JM8A3.N1 ES cells carrying the targeting
363 construct (Fig. 1) that were obtained from KOMP (project CSD79367). Targeted ES cells were injected
364 into C57BL/6J blastocysts by the Gene Targeting Facility of the Albert Einstein College of Medicine.
365 Chimeras were crossed to C57BL/6J mice and then to the FVB/NJ *Stra8*-iCre mice from Jackson Labs
366 (Bar Harbor, Maine) Tg (*Stra8*-iCre)¹Reb/J (Stock no. 008208 | *Stra8*-iCre) to generate *Mgat4d* deleted
367 mice carrying LacZ/Neo (*Mgat4d*-LacZ/Neo) or to Flp1-Cre mice B6.129S4-
368 *Gt*(ROSA)²⁶Sor^{tm1(FLP1)Dym}/RainJ (Stock no. 009086 ROSA26:FLPe knock-in) to obtain mice carrying a

369 conditional *Mgat4d* allele with *loxP* sites flanking exon 4 (*Mgat4d*[F/F]). The latter mice were crossed to
370 mice carrying a *Stra8*-iCre transgene to generate conditional inactivation in spermatogonia to investigate
371 spermatogenesis and fertility in males, or to generate mice with a whole body inactivation of *Mgat4d*.
372 Transgenic mice used in this study were generated in Albert Einstein College of Medicine by the
373 Transgenic Mouse Facility of the Albert Einstein College of Medicine on a C57BL/6J background. Two
374 founders were characterized for each transgenic line. The constructs used are shown in Fig. 4. C57BL/6J
375 and FVB/NJ mice were purchased from Jackson Laboratories (Stock No: 000664 and Stock No: 001800
376 respectively) and used for breeding. All mice carrying a transgene were kept as heterozygotes by
377 crossing +/Tg with homozygote wild-type (+/+) mice. Mice were sacrificed by carbon dioxide asphyxiation
378 followed by cervical dislocation. Testes were dissected free of surrounding tissue and weighed. Mouse
379 experiments were performed following Albert Einstein College of Medicine Institutional Animal Care and
380 Use Committee approved guidelines under the Institutional Animal Care and Use Committee (IACUC)
381 protocol nos. 20080813, 20110803, 20140803 and 20170709.

382

383 **Antibodies**

384 Anti-MGAT4D C-terminus pAb (Genemed, Torrance, CA) was obtained from a MGAT4D C-terminus
385 peptide conjugate CGTQSSFPGREQHLKDNYY injected into rabbits. Anti-MGAT4D N-terminus pAb
386 (Covance, Denver, PA; Genemed, Torrance, CA) was obtained with a MGAT4D-L N-terminal peptide
387 conjugate GESVGLRVTATAPWEGEQARGV injected into rabbits. Both pAbs were affinity purified on
388 respective peptide columns. Anti-Myc mouse mAb 9E10 was from Covance (Denver, PA).

389

390 **Immunohistochemistry**

391 Testes were fixed in Bouin's fixative (#100503-962, Electron Microscopic Sciences, Radnor, PA) for 48
392 hr at room temperature (RT) then processed and paraffin-embedded by the Einstein Histology and
393 Comparative Pathology Facility. Serial sections (5-6 μ m) were collected on positively-charged slides.
394 Immunohistochemistry was performed following the "IHC staining protocol for paraffin-embedded
395 sections" from Abcam (<http://www.abcam.com/protocols/>). Briefly, testis sections were deparaffinized
396 using Histo-Clear reagent Cat no. HS-200 (National Diagnostics, Atlanta, GA). We performed a heat-
397 induced epitope retrieval with citrate buffer (10 mM sodium citrate, 0.05% Tween 20, pH 6.0) at 100°C
398 for 20 min followed by 20 min period at room temperature in the same buffer. The tissue was
399 permeabilized with 0.1% Triton X-100 in Tris-buffered saline (TBS) for 10 min and blocked for 1 hr at
400 room temperature with 10% normal serum (from the same species as secondary antibody) and 1% BSA
401 in TBS. The primary antibody was diluted in TBS with 1% BSA and incubated overnight at 4°C (unless
402 otherwise indicated). Endogenous peroxidase was quenched by incubating slides in 1.5% H₂O₂ in TBS

403 for 10 min and rinsed before incubation with the Biotinylated secondary antibody diluted in TBS containing
404 1% BSA, for 1 hr at room temperature. The samples were washed and Vectastain® ABC-HRP reagent
405 (cat no. PK-6100, Vector laboratories, Inc. Burlingame, CA) was added and incubated at room
406 temperature for 30 min. After rinsing, peroxidase substrate 3,3'diaminobenzidine (DAB) (Vector
407 laboratories, Cat# SK-4100) was used to detect the antibody, following the manufacturer protocol. The
408 tissue was counter-stained with Mayer's Hematoxylin solution (cat no. MHS16-500ML, Sigma-Aldrich).
409 The specimens were dehydrated with histo-clear and mounted using Permount® reagent (cat no. SP15-
410 100, Fisher Scientific, Fair Lawn, NJ). Testis section images were produced using 3DHistec Panoramic
411 250 Flash II slide scanner obtained with the Shared instrumentation Grant SIG# 1S10OD019961-01 to
412 the Analytical Imaging Facility (AIF) of the Albert Einstein College of Medicine.

413

414 **Western-blot analysis**

415 Testis tissue lysates were prepared using RIPA Lysis Buffer (cat no. 20-188, Millipore, Temecula, CA)
416 and following the protocol "Preparation of lysate from tissues" from Abcam with modifications. Briefly, the
417 testis tissue was homogenized in 1X RIPA, 01% SDS, 1X protease inhibitor cocktail (cat no.
418 05892791001, Roche Diagnostics GmbH, Mannheim, Germany) at a ratio of 0.5 ml buffer for 0.05 g of
419 tissue. The lysate was incubated with constant agitation (orbital shaker) at 4°C for 2 hr and then
420 centrifugated for 20 min at 12000 rpm at 4°C. The supernatant was transferred to a fresh tube and
421 supplemented with 100% glycerol to a final concentration of 20% glycerol. Protein yield was measured
422 using Bradford based colorimetric assay, (cat no. 500-0006, Bio-Rad Protein assay, Bio-Rad, Hercules,
423 CA). Isolated germ cell were lysed in buffer containing 1% IGEPAL, 1%TX-100, 0.5% Deoxycholate and
424 1X protease inhibitor cocktail in water. Briefly, 100 µl of lysis buffer was used to homogenize 10⁷ cells.
425 The lysate was incubated for 30 min on ice, then centrifugated 5 minutes at 5000 g. The supernatant was
426 transferred to a fresh tube and supplemented with 100% glycerol to a final concentration of 20% glycerol.
427 Protein levels were measured using the Bradford-based colorimetric assay. All samples were stored at -
428 80°C.

429

430 **Apoptosis assay**

431 Apoptosis induced DNA damage was measured using the ApopTag® Peroxidase *In Situ* Apoptosis
432 Detection Kit (cat no. S7100, EMD Millipore, Temecula, CA) following the manufacturer's protocol for
433 paraffin-embedded tissue. Testis sections were deparaffinized using Histo-Clear reagent (cat no. HS-
434 200, National Diagnostics, Atlanta, GA). Stained slides were scanned using a Perkin Elmer P250 high
435 capacity slide scanner and images were analyzed using FIJI software to count foci³⁵.

436

437 **Germ cells isolation**

438 Male germ cells were purified from testis following a modified protocol³⁶⁻³⁸. Mice were sacrificed by CO₂
439 asphyxiation followed by cervical dislocation and both testes were collected in 2 ml DMEM: F12 medium
440 (cat no. 11330-032, Gibco, Grand Island, NY) on ice. The tunica albuginea was removed and tubules
441 were transferred to 10 ml enzyme solution I (0.5 mg/ml collagenase Type I (cat no.C0130-1G, Sigma),
442 200 µg/ml DNase I (cat no. DN25-100 mg, Sigma) in F12 medium), briefly vortexed and incubated 30 min
443 at 33°C in a shaking water bath (100 oscillations/min). Every 10 min an additional manual shaking was
444 done to help tissue dissociation. The dispersed seminiferous tubules were allowed to sediment and the
445 supernatant was discarded. Tubules were washed with 10 ml fresh F12 medium and resuspended in
446 fresh F12 medium. The mixture was layered on 40 ml of 5% Percoll (cat no. 17-0891-02, GE Healthcare
447 Bio-sciences AB, Uppsala, Sweden) in HBSS (cat no. 55-022-PB, Mediatech, Inc. Manassas, VA) and
448 allowed to settle for 20 min at room temperature. The top 45 ml containing Leydig cells was discarded
449 and the remaining 5 ml were transferred to a new tube containing 10 ml of enzyme solution II (200 µg/ml
450 DNase I, 1 mg/ml trypsin (cat no.T4799-5G, Sigma-Aldrich, St Louis, MO) in F12 medium). The mixture
451 was incubated for 40 min at 33°C in a shaking water bath (100 oscillations/min) and every 10 min, manual
452 shaking. After tissue dissociation, 3 ml charcoal-stripped FBS were added and cells were resuspended
453 using a 10 ml pipette to dissociate clumps. The suspension was filtered sequentially through a 70 µm
454 (cat no. 352350, Falcon Corning Incorporated, Corning, NY) then 40 µm (cat no 352340) nylon cell
455 strainer and centrifugated at 500 g for 10 min at 4°C. The cell pellet was resuspended in 1 ml PBS
456 (calcium and magnesium free) and counted. Cells were stored as a dry pellet at -80°C and used for
457 protein or RNA extraction.

458

459 **RNA isolation and RT-PCR**

460 Testes or isolated germ cells were homogenized in TRIZOL reagent (cat no. 15596018, Invitrogen)
461 following the manufacturer's protocol for tissue or cell pellet, respectively. The isolated total RNA was
462 dissolved in RNase-free water, an aliquot (2 µl) was used to measure nucleic acid concentration and the
463 remainder was immediately stored at -80°C. Total RNA (3 µg) was used to synthesize cDNA (75 µl final
464 volume) with the Verso cDNA Synthesis Kit (cat no. AB-1453/A, Appliedbiosystems, Thermo scientific
465 Baltics UAB, Vilnius, Lithuania) following the manufacturer's protocol. cDNA was tested for genomic DNA
466 contamination using end-point PCR with *Actb* primers flanking an exon and intron sequence
467 (Supplementary Table S6).

468

469 **Quantitative PCR (qRT-PCR)**

470 cDNA obtained as described above was used to perform real time PCR. PowerUp™ SYBR™ Green
471 Master Mix (cat no. A25742, Applied Biosystems, Thermo Scientific Baltics UAB, Vilnius, Lithuania) was
472 mixed with each sample to a primer final concentration of 150 nm, following the manufacturer's protocol
473 and run on a master cycler (ViiA 7, Thermo Fisher). PCR conditions were 95°C for 30 sec, followed by
474 40 cycles at 95°C for 15 sec, 60°C for 15 sec and 72°C for 20 sec. Unless otherwise stated, gene
475 expression relative to *Actb* and *Rps2* was calculated by the \log_2^{ddCT} method ³⁹.

476

477 **Histological analysis**

478 Hematoxylin and eosin (H&E) counter stained testis sections were analyzed by light microscopy (Zeiss
479 Axiovert 200M, Göttingen, GERMANY) or scanned using a Perkin Elmer P250 high capacity slide
480 scanner and processed using the proprietary software CaseViewer (3D Histech P250 high capacity slide
481 scanner, Perkin Elmer, Waltham, MA).

482

483 **Mild heat stress treatment**

484 This protocol was adapted from ^{12,40,41}. Briefly, an adult male mouse was anaesthetized in an isoflurane
485 chamber with a constant oxygen flow of 2 L/min and 3 % isoflurane for 1 min followed by 2.5 % isoflurane
486 for 3 min. The mouse was quickly removed from the chamber and its nose was introduced into a nose
487 cone with the same anaesthesia parameters for another 1 min. Testes were secured in the scrotum by
488 manual massage and one third of the body (hind legs, tail and scrotum) was immersed in a 43°C or 33°C
489 (control) water bath, supported by a plastic tube for 25 min. During the experiment, the isoflurane flow
490 was reduced every 10 min by 0.5 % to reach 1.5 % at the end of the treatment (2.5 % for 5 min after
491 introduction into the water bath, then 2 % for 10 min and followed by 1.5 % for another 10 min). After the
492 heat treatment, mice were dried on paper towel, allowed to recover in a chamber with oxygen flow at
493 2 L/min and 0% isoflurane for 5 to 10 min, then returned to a cage to recover from the effects of
494 anaesthesia on a heating pad. Testes and epididymis were harvested 8 hr or 24 hr after treatment.

495

496 **Microarray**

497 Germ cell RNA (150 ng, RIN>7.9) was provided to the Genomics Core Facility of the Albert
498 Einstein College of Medicine for conversion to cDNA, labeling and hybridization to a mouse Affymetrix
499 Clariom™ D array previously known as GeneChip™ Mouse Transcriptome Array 1.0 (Affymetrix, Santa
500 Clara, CA). Raw intensity data (.CEL files) were mapped to genes using custom CDF files
501 (clariomdmousemmgencodegcdf from [http://brainarray.mbni.med.umich.edu/Brainarray/Database/Cust](http://brainarray.mbni.med.umich.edu/Brainarray/Database/CustomCDF/genomic_curated_CDF.asp)
502 [omCDF/genomic_curated_CDF.asp](http://brainarray.mbni.med.umich.edu/Brainarray/Database/CustomCDF/genomic_curated_CDF.asp)), and rma-normalized using the R/Bioconductor package affy ⁴².

503 Differential gene expression was modeled using limma⁴³. Genes with Benjamini-Hochberg-adjusted p-
504 values <0.05 and fold-change >1.5 or <-1.5 were defined as differentially expressed genes (DEGs).

505

506 **Gene set enrichment analysis and Ingenuity Pathway Analysis**

507 Gene set enrichment analysis (GSEA)^{25,44} was performed to determine enrichment of gene sets from the
508 curated (C2), GO (C5), and oncogenic signatures (C6) and Hallmark collections. Gene list enrichment
509 analysis was performed using EnrichR²⁸ and Ingenuity Pathway Analysis IPA
510 (www.qiagen.com/ingenuity, QIAGEN, Redwood City, CA) for genes with fold-change \pm 1.5, $p < 0.05$ and
511 False discovery rate $p < 0.05$.

512

513 **Statistical analysis**

514 The bar graphs in all figures represent the mean \pm SEM. Unpaired, two-tailed Student's t test or one way
515 ANOVA was used to calculate p -value using Graph Pad Prism 7.0 (Graph Pad Software Inc., La Jolla,
516 CA). Statistical significance was indicated by * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ or **** $p < 0.0001$.

517

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642

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648

649 **Author contributions**

650
651 AA performed all experiments on transgenic mice, all microarray and validation experiments, data
652 curation, bioinformatics analyses, and co-wrote the manuscript; ML characterized the original
653 cohort of *Mgat4d* control and KO mice and transgenic mice, performed heat shock experiments
654 and analyses on the old cohort and edited the paper; BB performed bioinformatics analyses and
655 interpretation and edited the paper; FB developed the *Mgat4d* conditional and global knockout
656 mice, and the LacZ mice; JA performed and interpreted MALDI-IMS data; JP made transgenic
657 constructs, and bred mutant and transgenic mice; SS characterized antibodies; PS conceived
658 and directed experiments, curated and interpreted data and co-wrote the paper.

659

660 **Additional Information**

661 **Competing Interests**

662 The authors declare no competing interests.

663

664 **Data Availability**

665 The data generated and/or analysed for the current study are available from the corresponding
666 author on reasonable request. Microarray data are deposited in NCBI's Gene Expression
667 Omnibus (GEO) and are accessible through GEO serial accession number GSE137307.

668

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697 **Figure Legends**

698 **Figure 1.** Generation of *Mgat4d* mutant mice. **(A)** Map of the targeted *Mgat4d*^{tm1a(KOMP)Wtsi} allele
699 in ES cells obtained from KOMP. Exon 4 is flanked by two *loxP* sites. LacZ and the neomycin
700 cassettes are flanked by two *Frt* sites. **(B)** PCR of genomic DNA from *Mgat4d*[+/+], *Mgat4d*[+/-],
701 *Mgat4d*[-/-] and *Mgat4d*[F/-]:Stra8-iCre pups to determine genotype. Primers are given in
702 Supplementary Table S1. **(C)** Western blot analysis of protein extracts of germ cells purified from
703 28 dpp *Mgat4d*[+/+], *Mgat4d*[+/-] and *Mgat4d*[-/-] mice. Long and short forms of MGAT4D are
704 identified. * is a non-specific band **(D)** Representative testis section from a mouse carrying the
705 *LacZ* gene under the control of the *Mgat4d* promoter after staining for β -galactosidase (blue).
706 Nuclei were stained with eosin. **(E)** Immunohistochemistry of representative testis sections from
707 *Mgat4d*[+/-] and *Mgat4d*[-/-] mice of 28 dpp. The presence of MGAT4D is shown by the brown
708 stain consistent with a Golgi localization (arrows). Nuclei were stained with hematoxylin.

709
710 **Figure 2.** Effects of heat treatment on *Mgat4d*[-/-] testes. **(A)** Representative testis sections
711 stained with H&E. Upper panels from mice whose lower body was submerged for 25 min at 33°C
712 and lower panels from mice treated similarly at 43°C. Arrows indicate enlarged cells, arrow heads
713 show vacuoles in germ cells. **(B)** Representative epididymis sections from a *Mgat4d*[-/-] male
714 treated at 33°C (upper) or 43°C (lower) and stained with H&E. Arrows in the 43°C sample indicate
715 pyknotic cells in the tubule lumen. **(C)** Quantification of different tubule categories in testis
716 sections from heat-treated (43°C) *Mgat4d*[+/-] and *Mgat4d*[-/-] males. Positive tubules were
717 counted as those with at least one cell of radius $\geq 10 \mu\text{m}$; large vacuoles were tubules with at
718 least one vacuole $\geq 10 \mu\text{m}$; small vacuoles, pyknotic cells were tubules with at least one vacuole
719 of radius $< 10 \mu\text{m}$ or tubules containing pyknotic cells ; undamaged tubules were tubules with no
720 apparent damage. Mice were from an aged cohort (592-596 days) of *Mgat4d*[+/-](n=2) and
721 *Mgat4d*[-/-](n=5) mice. Thirty (30) tubules were counted in one section per mouse. Student's t test
722 (two-tailed, unpaired) **p<0.01; *p<0.05.

723
724 **Figure 3.** Apoptosis of germ cells in heat-treated testes. Representative testis sections from 33°C-
725 or 43°C-treated aged FVB males were subjected to the TUNEL "Apoptag" assay for in situ
726 detection of DNA strand breaks. **(A)** Section from a 33°C-treated *Mgat4d*[-/-] male. **(B)** Section
727 from a 43°C-treated *Mgat4d*[+/-] male. **(C)** Section from a 43°C-treated *Mgat4d*[-/-] male. DNA
728 breaks stained brown (red arrows). **(D)** quantification of apoptotic signal in ≥ 100 tubules using
729 FIJI software. Student's t test (unpaired, two-tailed) *p<0.05.

730
731 **Figure 4.** Generation and characterization of *Mgat4d* transgenic mice. (A) Schematic
732 representation of constructs used to generate transgenic (Tg) mice. Expression of *Mgat4d-L-Myc*
733 was driven by promoters (*Stra8*, *Ldhc* and *Prm1*) specific for different germ cell types. Lower
734 diagram, position of primers used for qRT-PCR amplification. “LongFw” and “LongRev” to amplify
735 the 5’ region of *Mgat4d-L*; “TrFw” and “TrRev” to amplify the transgene junction. (B) qRT-PCR of
736 *Mgat4d-L* 5’ primer transcripts relative to *Actb* and *Rps2*. Testis RNA was isolated from males of
737 28 dpp. (C) Representative H&E stained testis sections from 120 dpp control and *Mgat4d-L-Myc*
738 transgenic males.

739
740 **Figure 5.** Effects of heat stress in *Mgat4d* transgenic testis. Quantification of heat-induced
741 damage in testes of *Mgat4d*[+/+] (n=5), *Stra8-Mgat4d-L-Myc* (n=8), *Ldhc-Mgat4d-L-Myc* (n=7),
742 and *Prm1-Mgat4d-L-Myc* (n=5) C57BL6/J males. 30 tubules were investigated per mouse in one
743 H&E stained testis section to detect (A) Enlarged germ cells; (B) Large vacuoles; (C) Small
744 vacuoles, pyknotic cells; (D) Undamaged tubules. Differences from control two- tailed, unpaired
745 Student t-test *p<0.05, **p<0.01.

746
747 **Figure 6.** Effects of heat treatment on apoptosis in *Mgat4d* transgenic mice. (A) Representative
748 images of testis sections from 43°C-treated *Mgat4d* [+/+] (n=3) and *Mgat4d* transgenic mice (n=4
749 for each) stained by the Apoptag kit to detect DNA breaks. (B) Quantification of Apoptag signal in
750 ≥100 tubules of 43°C-treated non-transgenic and transgenic mice. Statistical differences
751 determined by two-tailed unpaired Student’s t-test *p<0.05, ***p<0.001.

752
753 **Figure 7.** qRT-PCR of cDNA from testis of 33°C- or 43°C-treated control vs *Stra8-Mgat4d-L-Myc*
754 males of 7 months. Testes were isolated 24 hr after treatment. qRT-PCR was performed in
755 triplicate. Relative gene expression was normalized to *Actb* and *Rps2*. Mean ± SEM; statistical
756 analysis by two-tailed, unpaired Student’s t-test.; *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

757
758 **Figure 8.** Microarray analysis of germ cell cDNA. Two month C57BL/6J males were treated at
759 33°C or 43°C for 25 min and germ cells were harvested 8 hr after recovery. (A) Principal
760 component analysis (PCA) for 33°C-treated *Mgat4d*[+/+] (n=4) and *Mgat4d*[-/-] (n=3) arrays and
761 43°C-treated *Mgat4d*[+/+] (n=3) and *Mgat4d*[-/-] (n=5) samples. (B) Volcano plots showing the
762 distribution of DEGs based on their expressed log₂ fold-change and -log₁₀ p value. Red dots
763 represent genes with a log₂ fold-change lower than -0.6 or higher than 0.6 (equivalent to +/- 1.5

764 fold-change) and a p value below the threshold of 0.05 ($-\log_{10}(0.05) = 1.3$). **(C)** Correlation
765 analysis of microarray chip data of wild type (WT) and *Mgat4d*^[-/-] (KO) germ cells at 33°C and
766 43°C.

767

768 **Figure 9.** Validation and Ingenuity Pathway analysis (IPA). **(A)** qRT-PCR validation of up-
769 regulated genes in control (n=3) and *Mgat4d*^[-/-] (n=4) cDNA samples from the same mice used
770 for microarray analyses. **(B)** qRT-PCR of down-regulated genes in the same samples. Relative
771 expression was determined using *Actb* and *Rps2*. Assays were performed in triplicate. Error bars
772 represent mean \pm SEM; statistical analysis by two-tailed, unpaired Student's t-test * $p < 0.05$,
773 ** $p < 0.01$, *** $p < 0.001$. **(C)** Top canonical pathways in IPA significantly overrepresented in heat-
774 treated *Mgat4d*^[-/-] germ cells compared to wild type, normalized to their respective 33°C
775 counterparts, according to $-\log p$ value.

776

777 **Figure 10.** Differential gene interactions between *Mgat4d* genotype and heat shock conditions.
778 **(A)** Heat maps showing DEGs either down- or up-regulated specifically in *Mgat4d* wild type, but
779 not *Mgat4d* KO cells following heat shock, representative of significantly enriched pathways
780 identified by EnrichR (<http://amp.pharm.mssm.edu/Enrichr/>). Adjusted p values and odds ratios
781 (OR) for the respective pathways are shown. Full names of the pathways are: Single Gene
782 Perturbations from GEO: I110 KO mouse GSE25846 sample 3062; ARCHS4 TFs Coexp:
783 HIF1A_human_tf_ARCHS4_coexpression; TRRUST Transcription Factors 2019: NFKB1_
784 human; Disease Perturbations from GEO up: Infertility due to azoospermia C1321542 mouse
785 GSE3676 sample 151. Color scales represent gene-wise Z-scores. **(B)**. Box plots showing
786 expression of representative genes of the indicated pathways across genotypes and heat shock
787 conditions. These genes were much more up-regulated by heat treatment in WT compared to
788 mutant (KO) germ cells.

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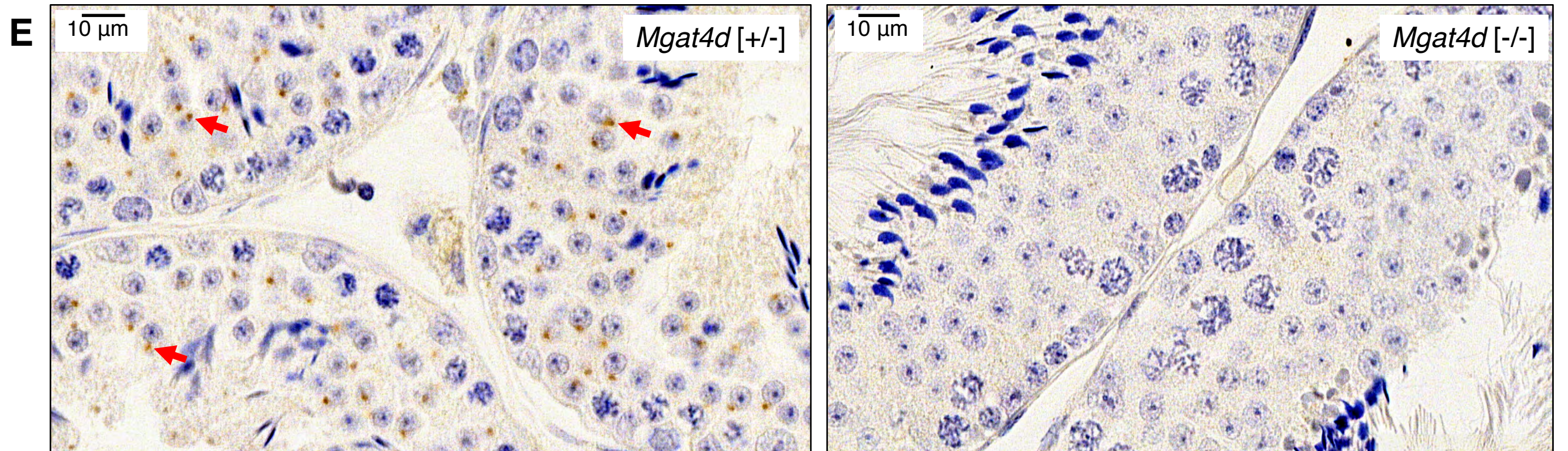
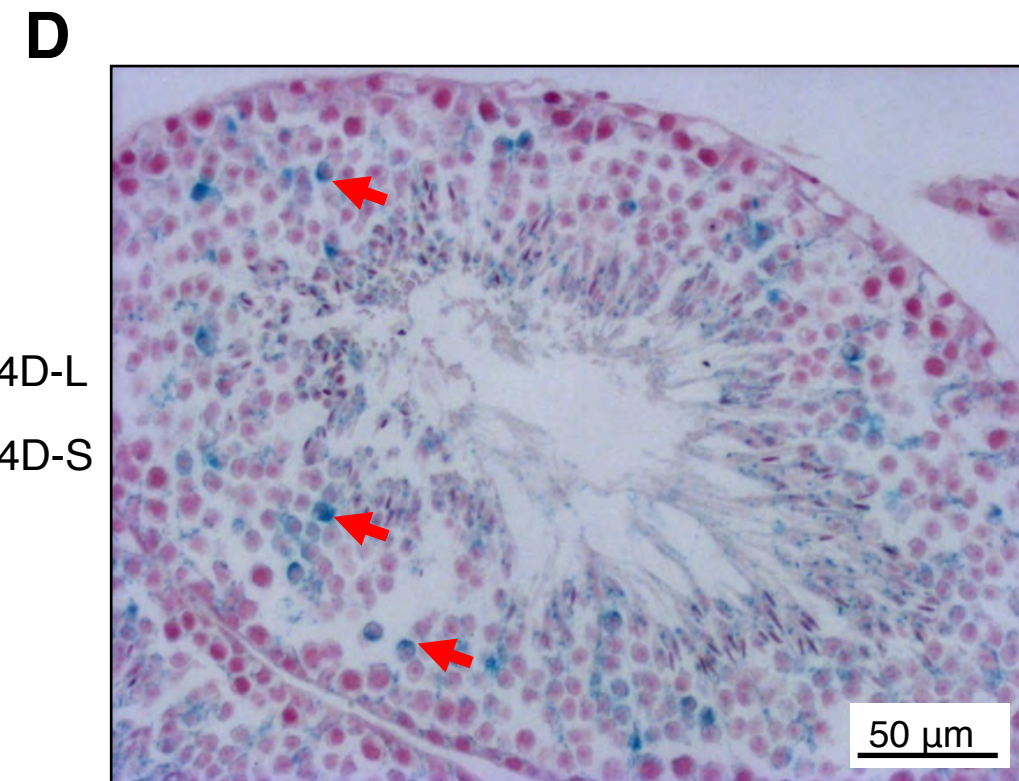
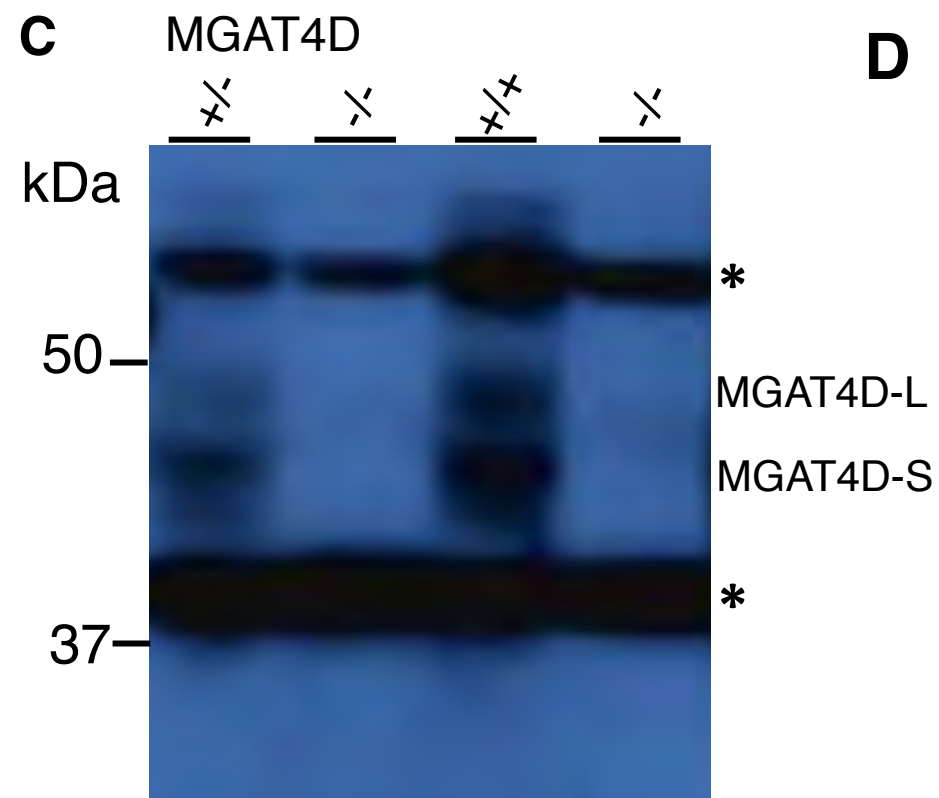
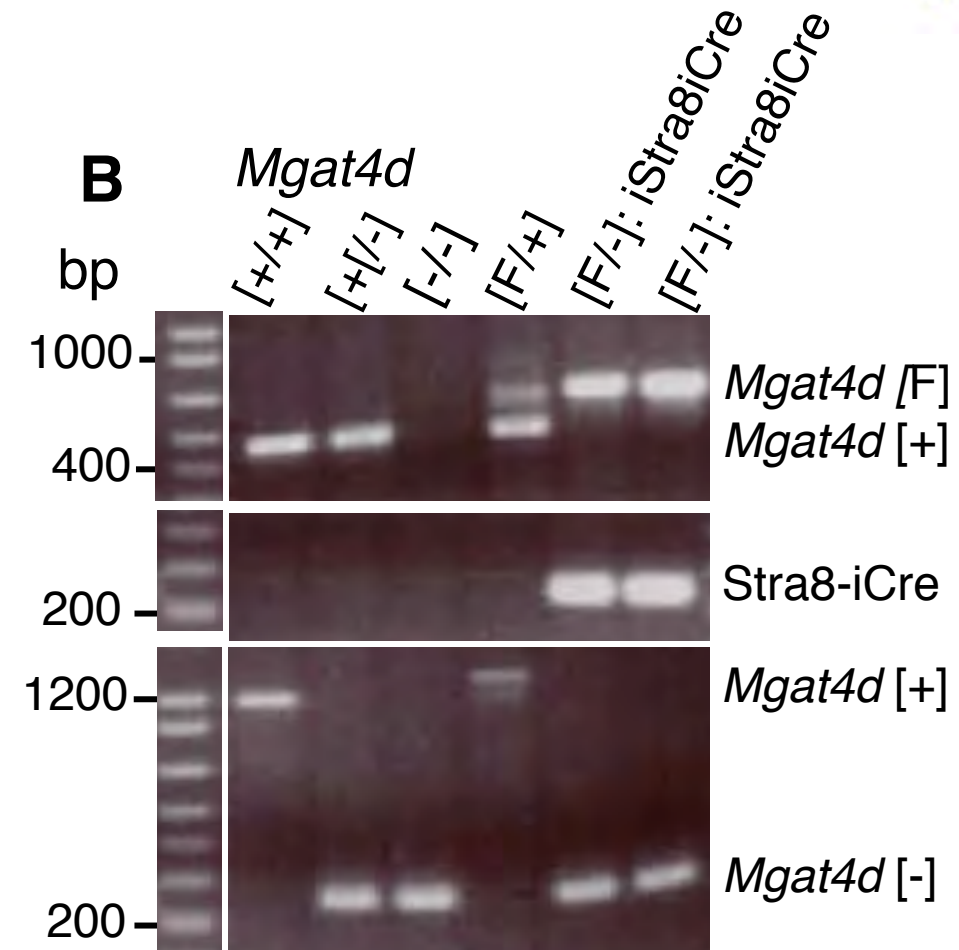
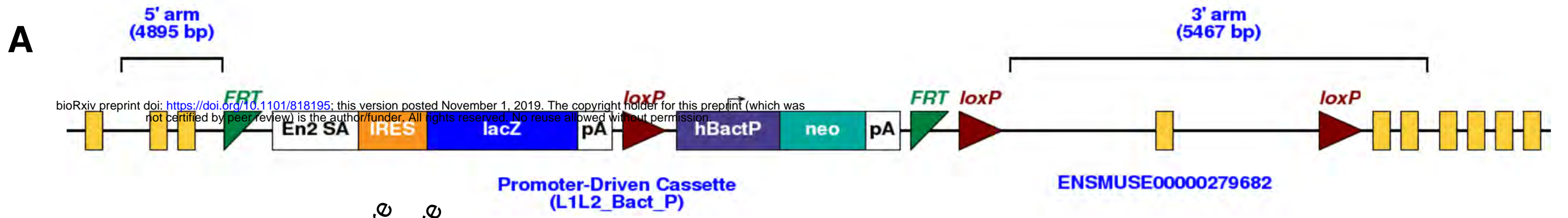
796 **Table 1.** Fertility of *Mgat4d*^{-/-} and *Mgat4d-L-Myc* transgenic male mice

797

Mouse strain	Male genotype	No. males	No. litters	No. pups	<i>Mgat4d</i> ^{-/-}	<i>Mgat4d</i> ^{+/-}	Transmission (CHI squared)
FVB	<i>Mgat4d</i> ^{-/-}	5	5	54	29	25	0.586
C57BL/6J	<i>Mgat4d</i> ^{-/-}	4	13	88	42	46	0.669
C57BL/6J	<i>Mgat4d</i> ^{[F/F]:Stra8-iCre}	2	9	59	29	30	0.896
					<i>Mgat4d-L-Myc</i>	<i>Mgat4d</i> ^[+/+]	
C57BL/6J	<i>Stra8-Mgat4d-L-Myc</i>	7	20	171	70	101	0.018
C57BL/6J	<i>Ldhc-Mgat4d-L-Myc</i>	4	9	59	26	33	0.362
C57BL/6J	<i>Prm1-Mgat4d-L-Myc</i>	6	12	77	41	36	0.568

798
 799 *Mgat4d*^{-/-} males were crossed with *Mgat4d*^{+/-} females. *Mgat4d*^{[F/F]:Stra8-iCre} males were
 800 crossed with *Mgat4d*^[F/-] females. *Mgat4d-L-Myc* transgenic heterozygote males were crossed with
 801 *Mgat4d*^[+/+] females.

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Mgat4d [+/-]

Mgat4d [-/-]

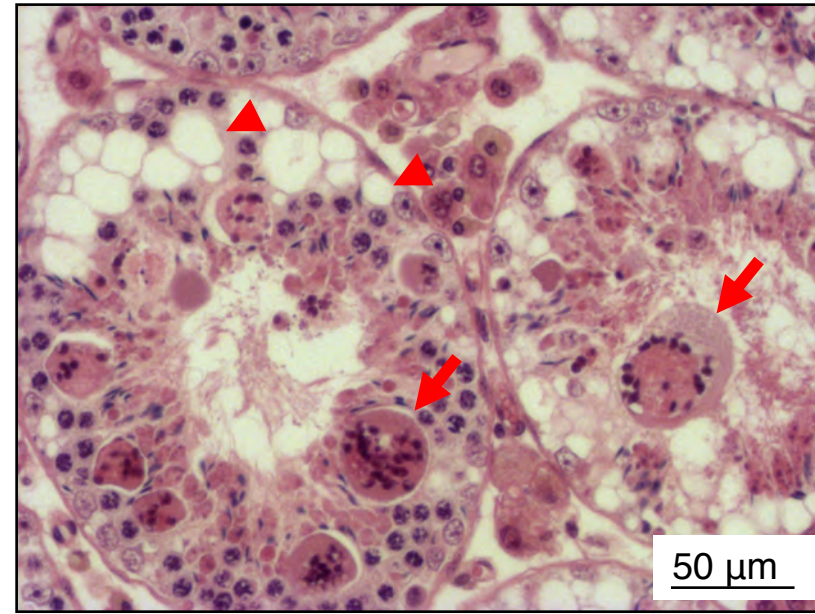
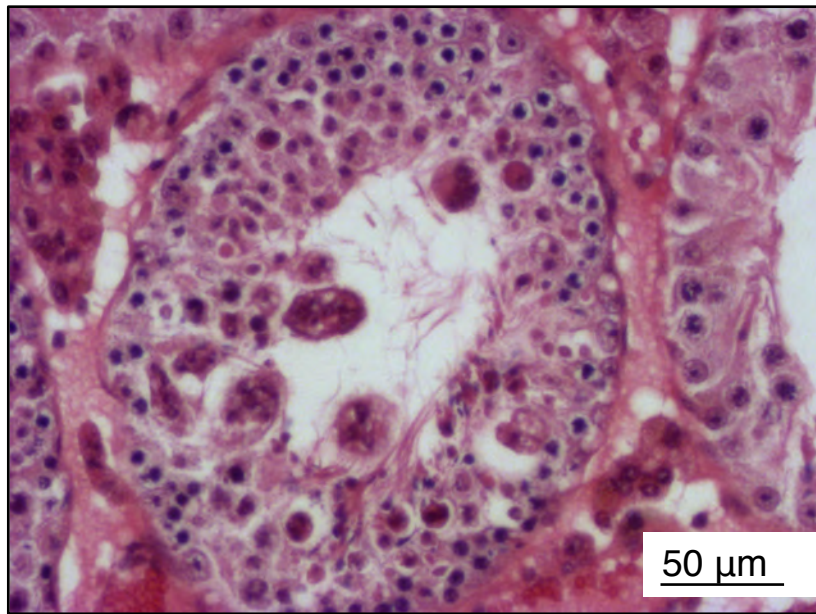
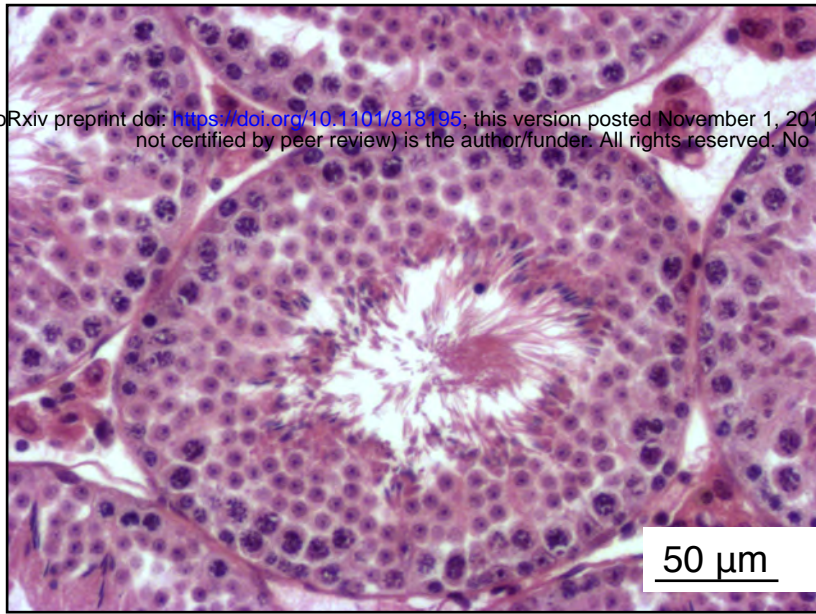
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33°C

Testis

43°C

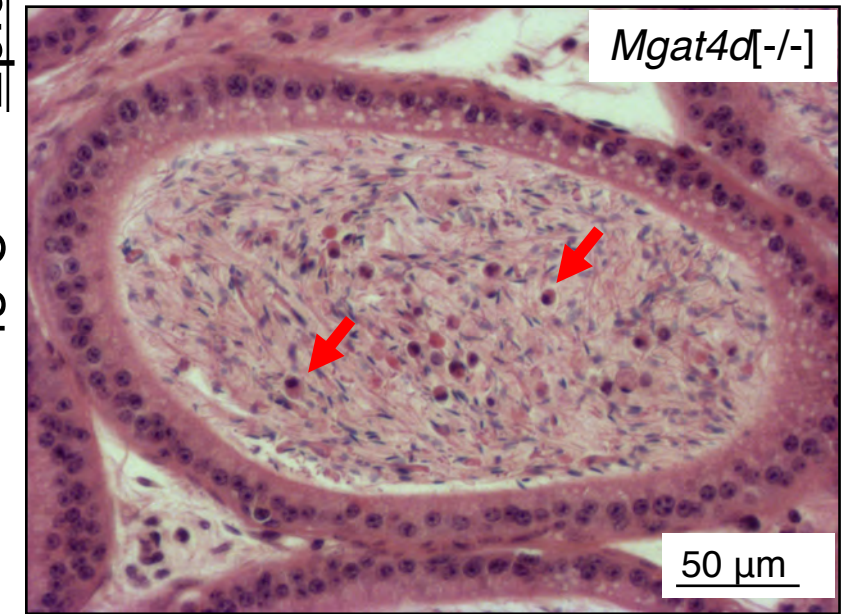
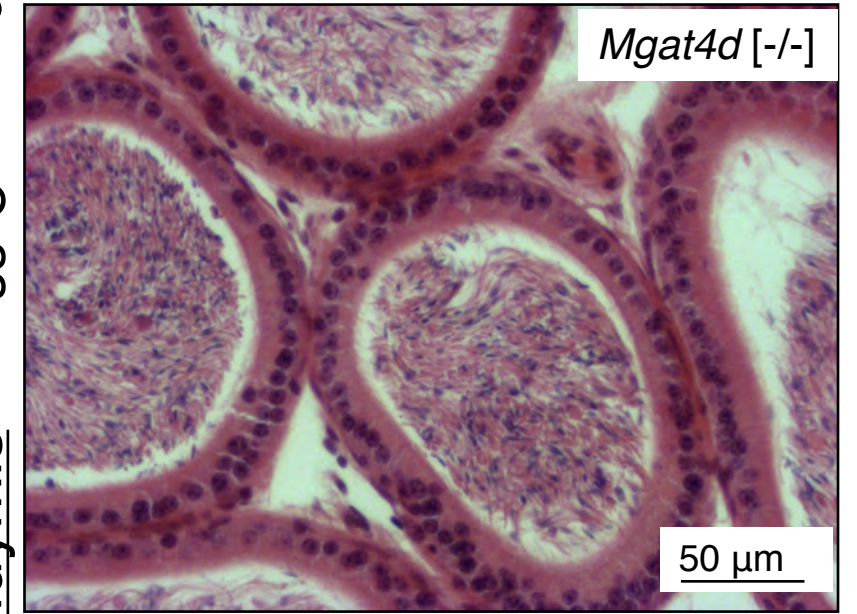


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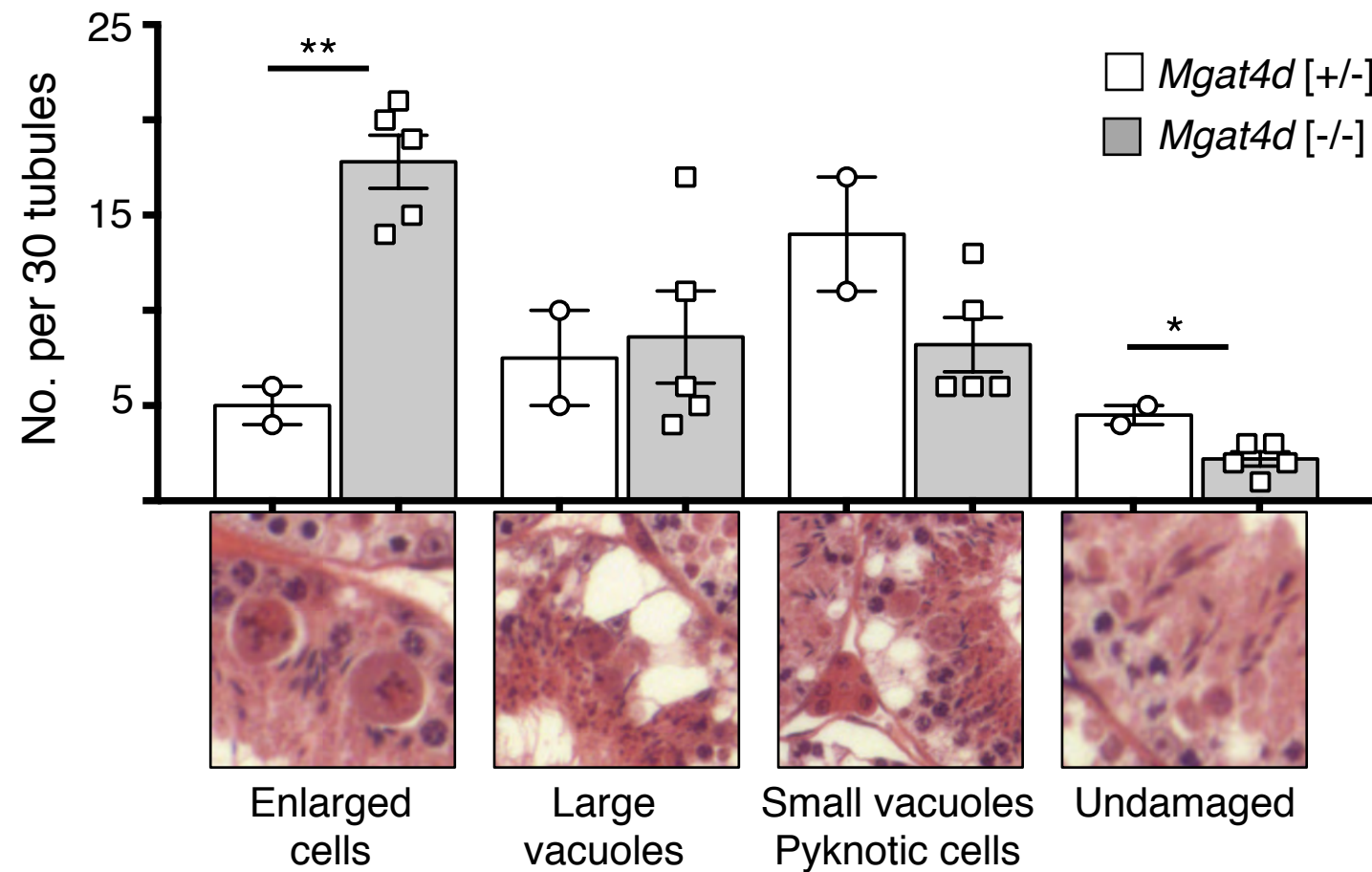
33°C

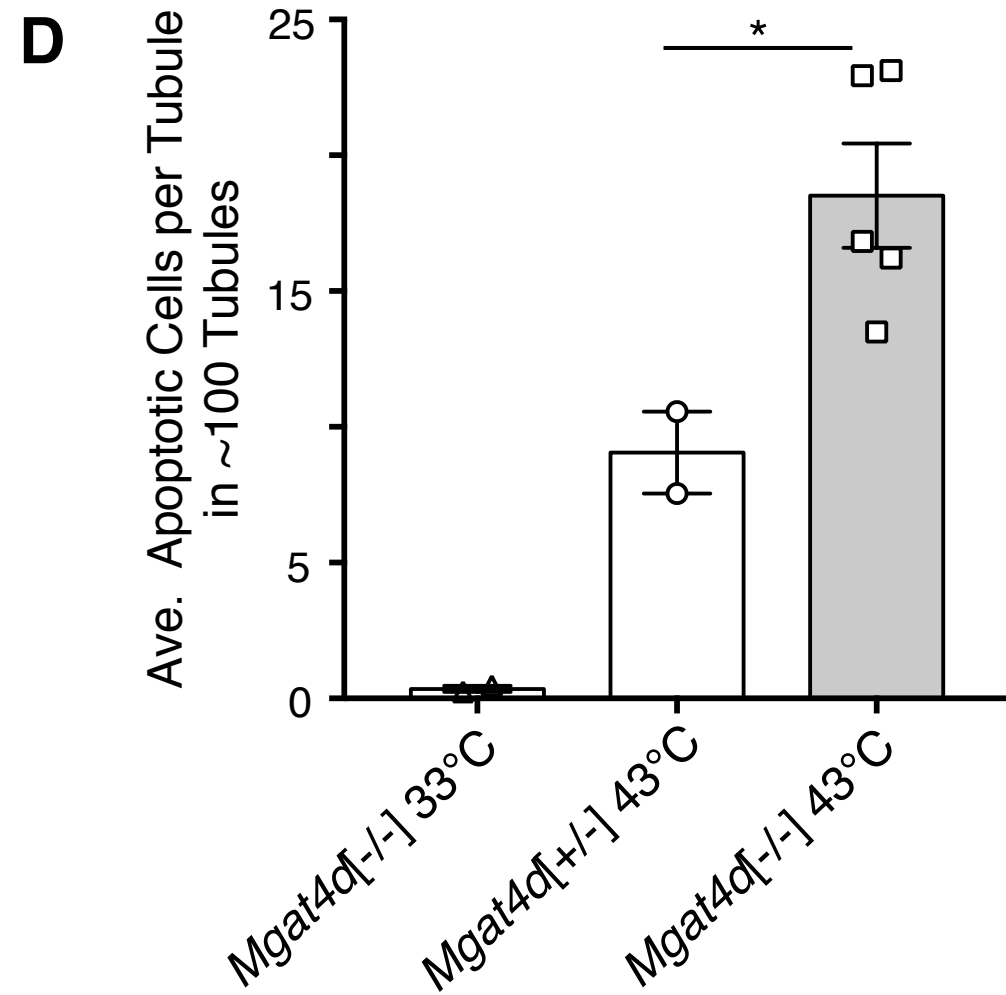
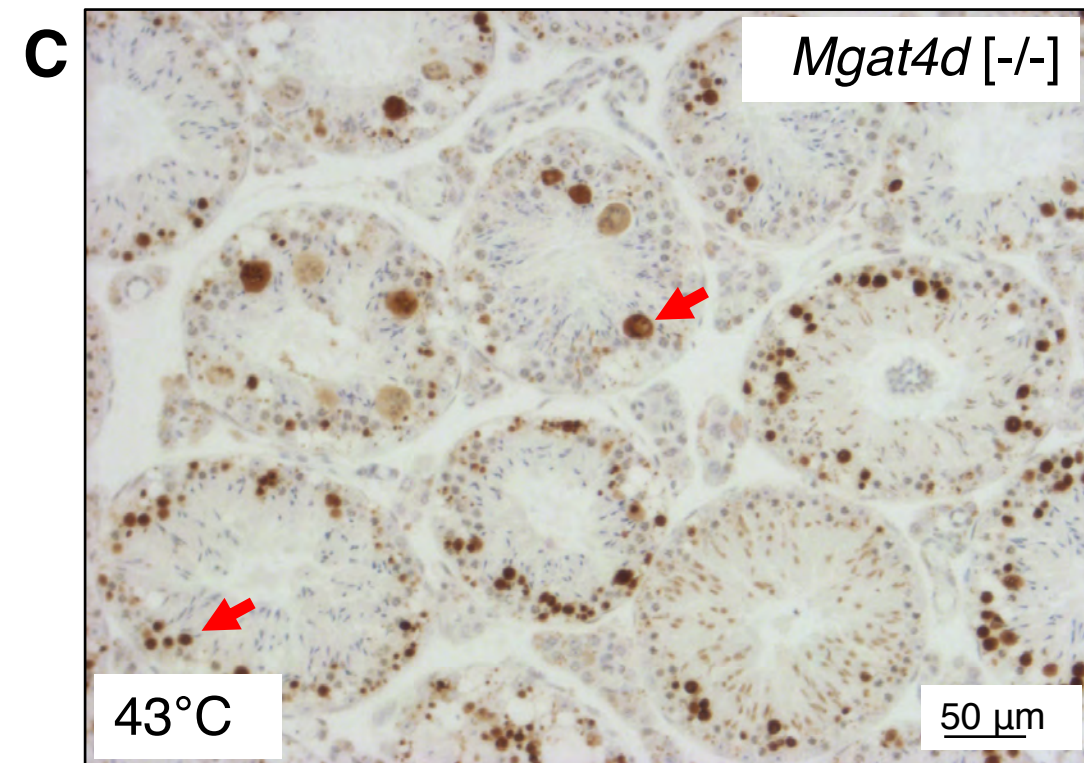
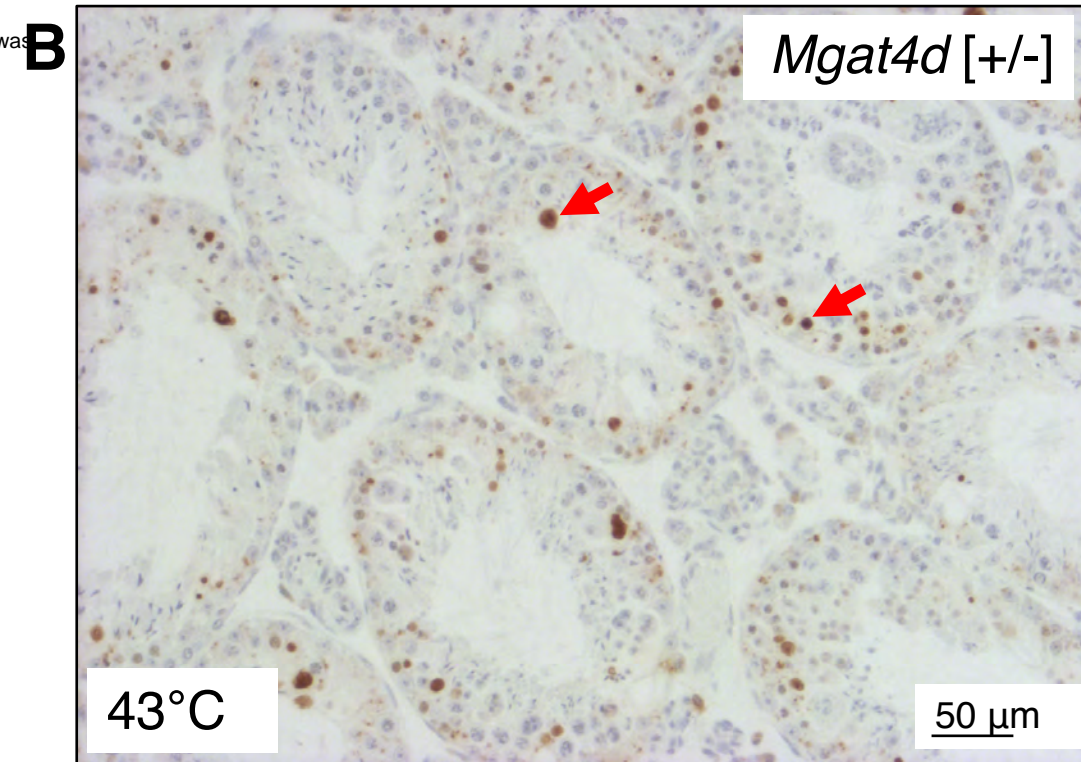
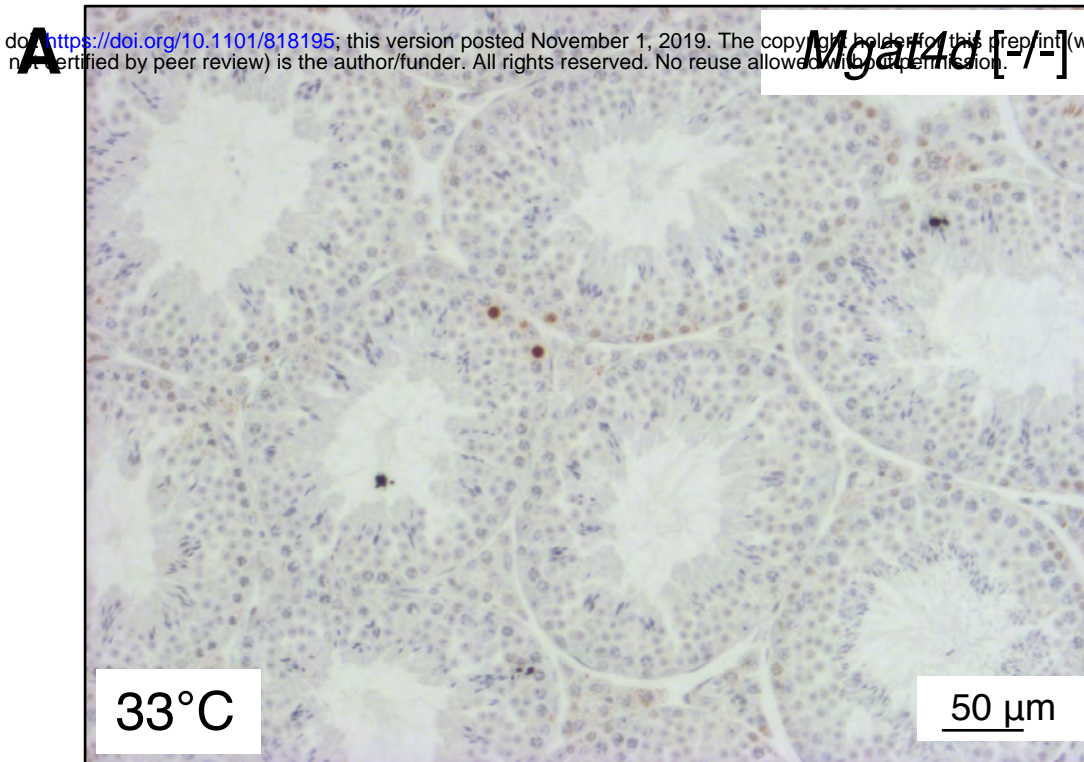
Epididymis

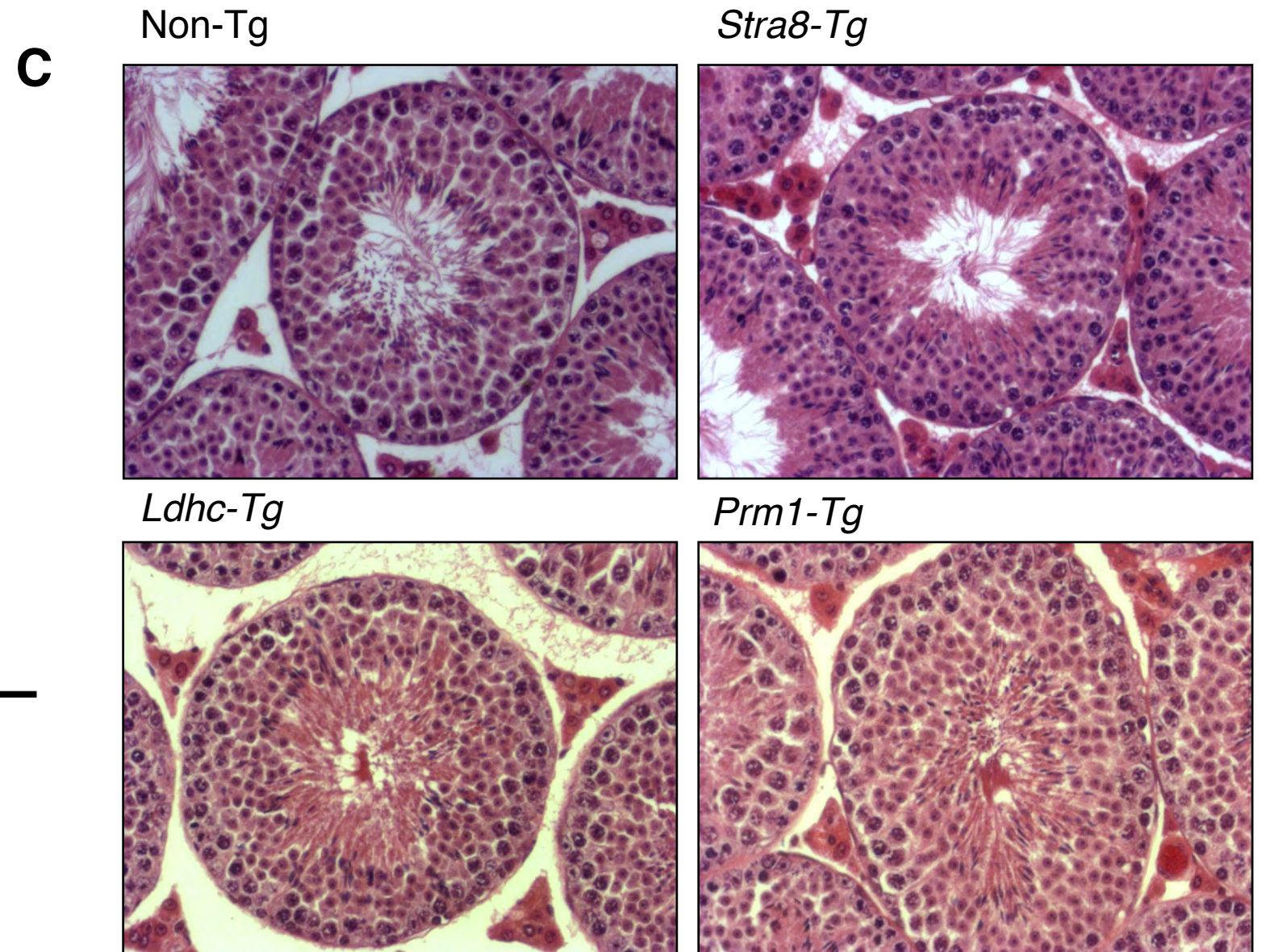
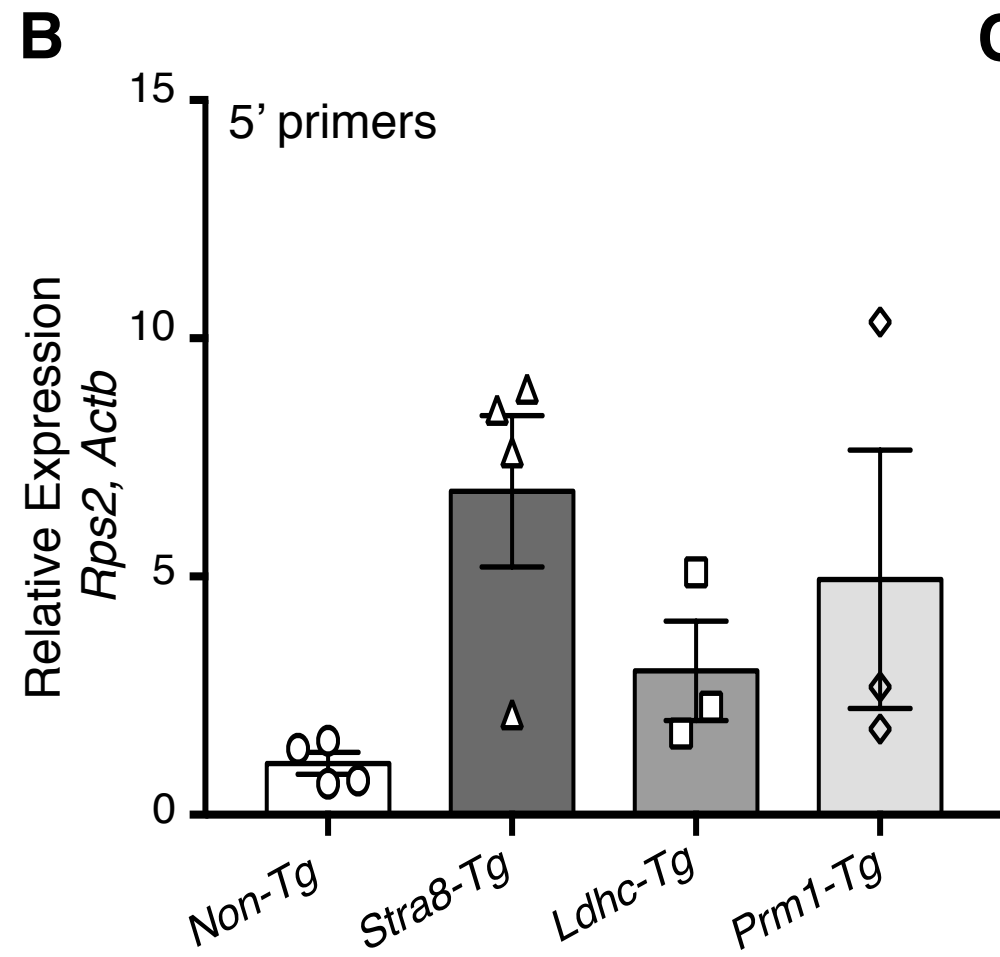
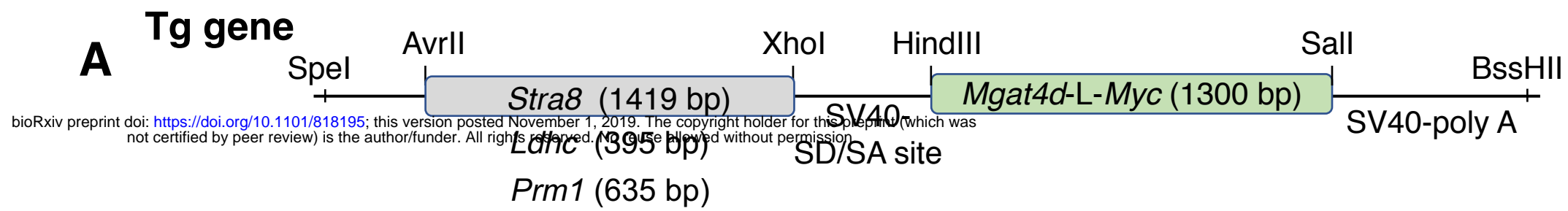
43°C

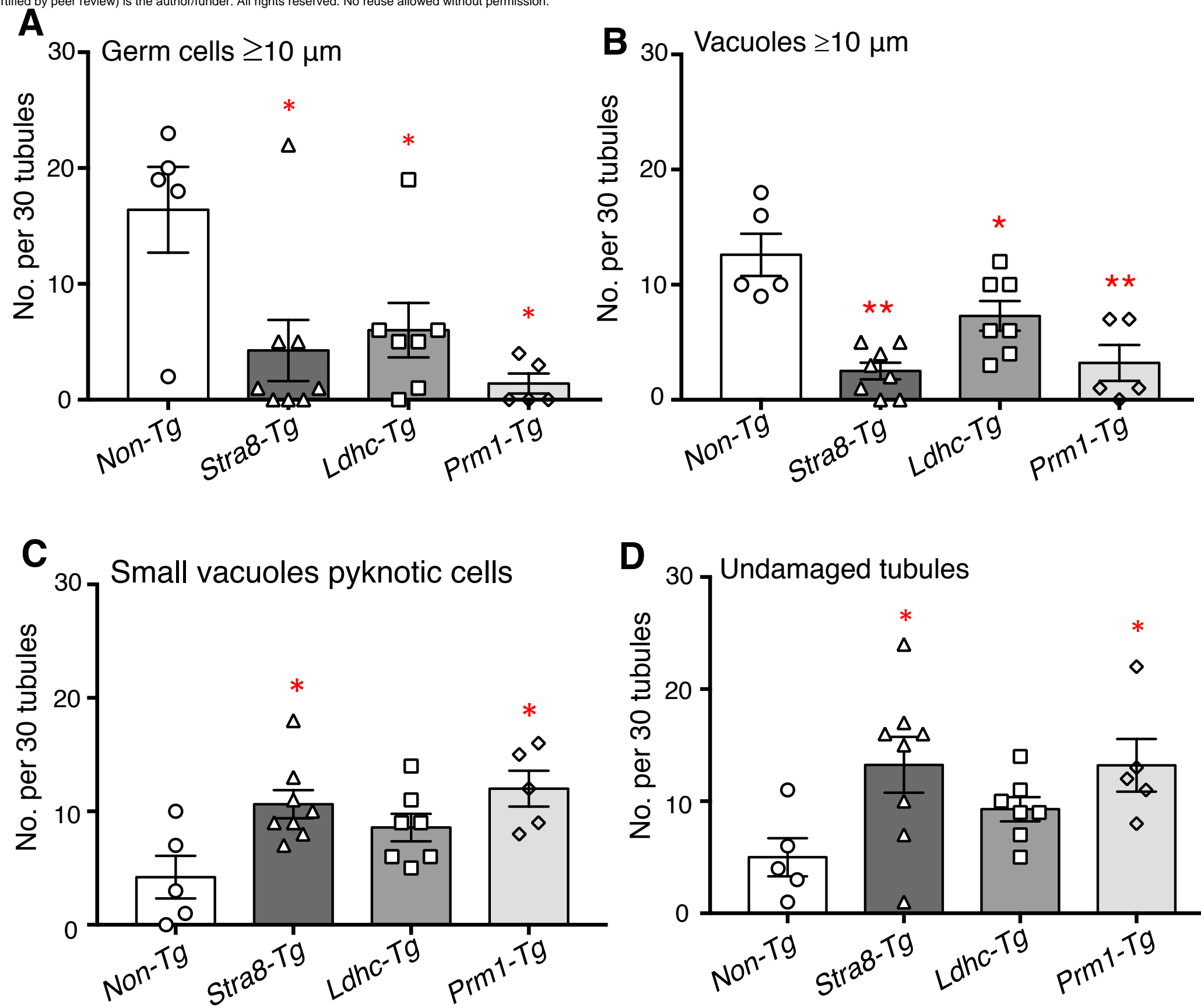


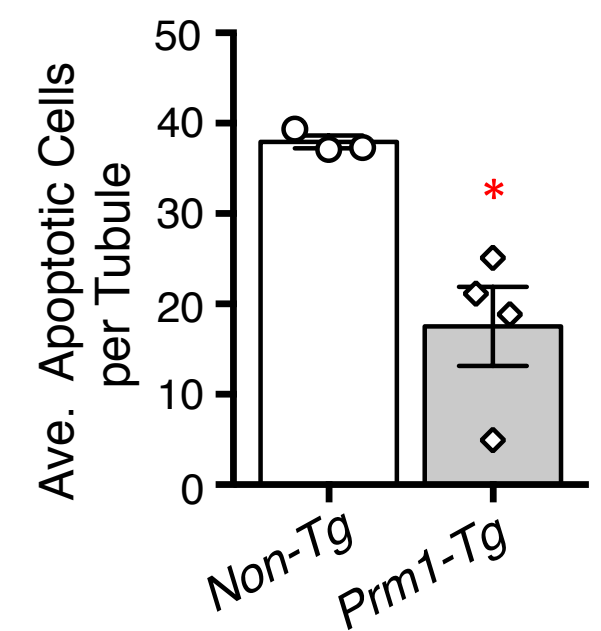
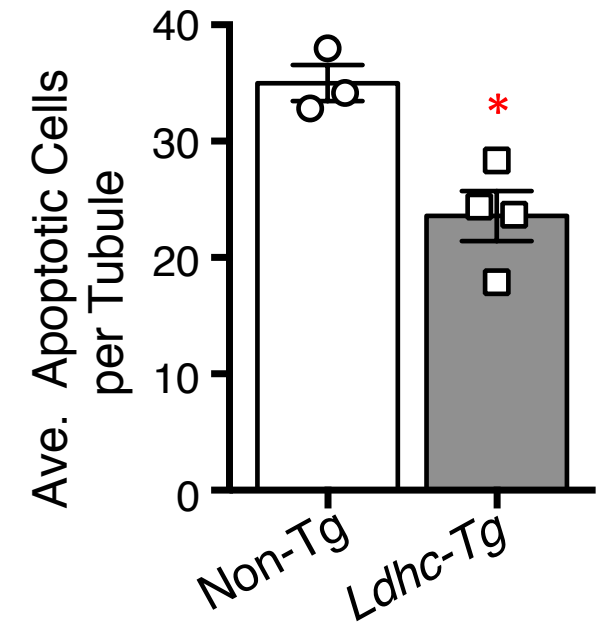
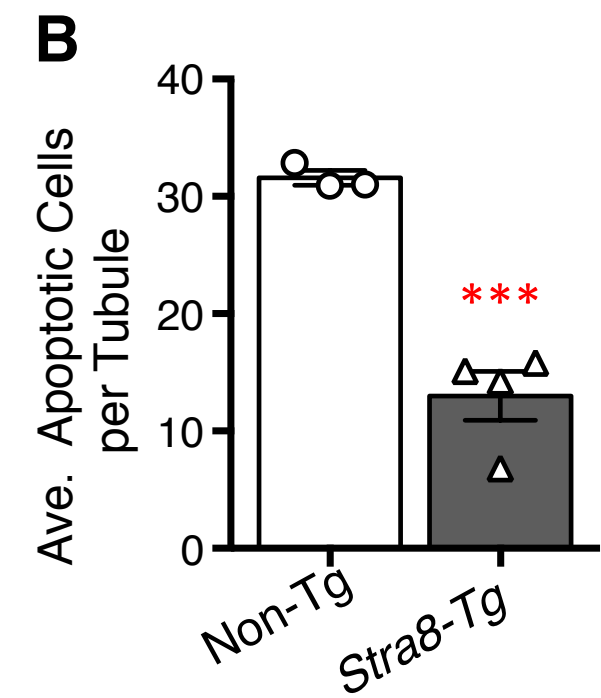
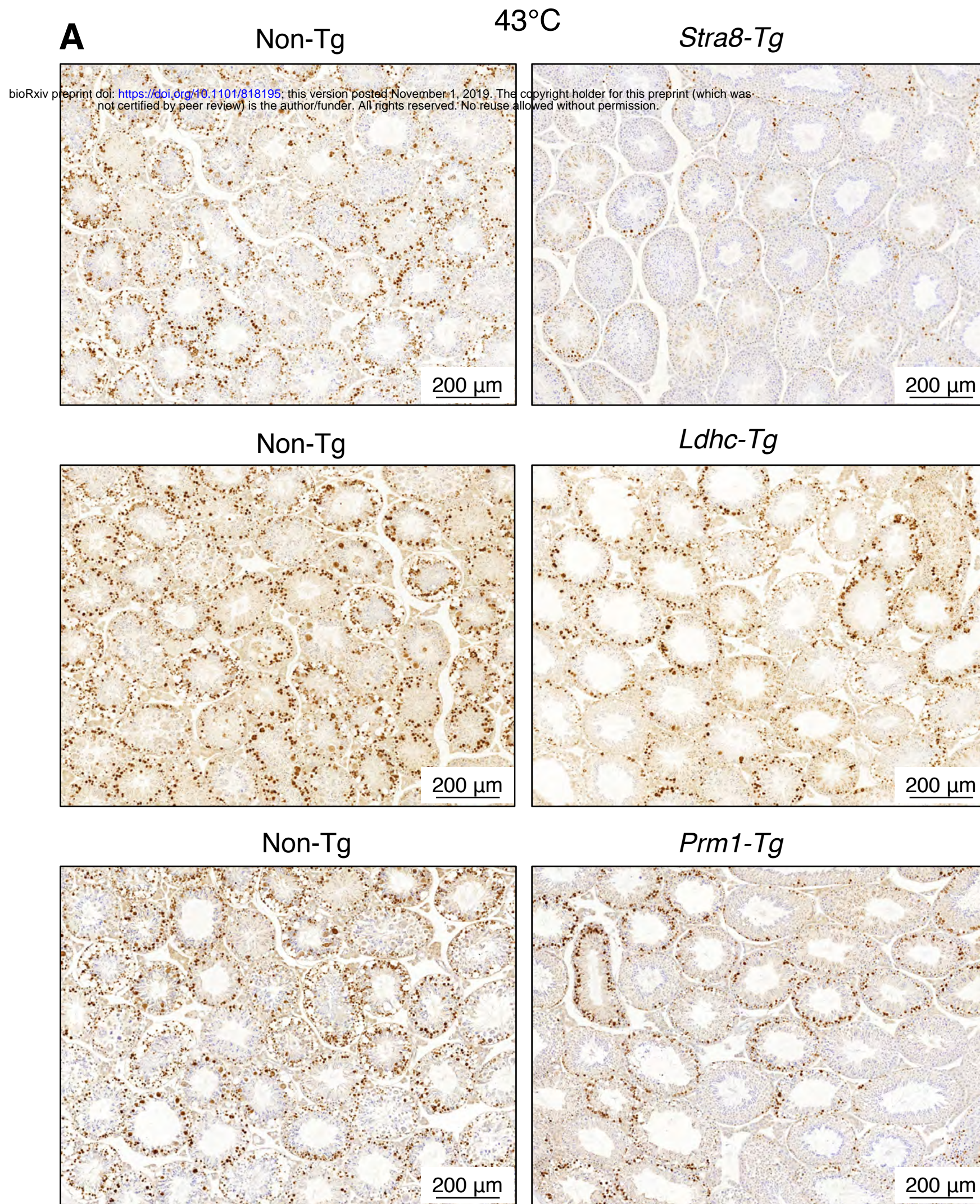
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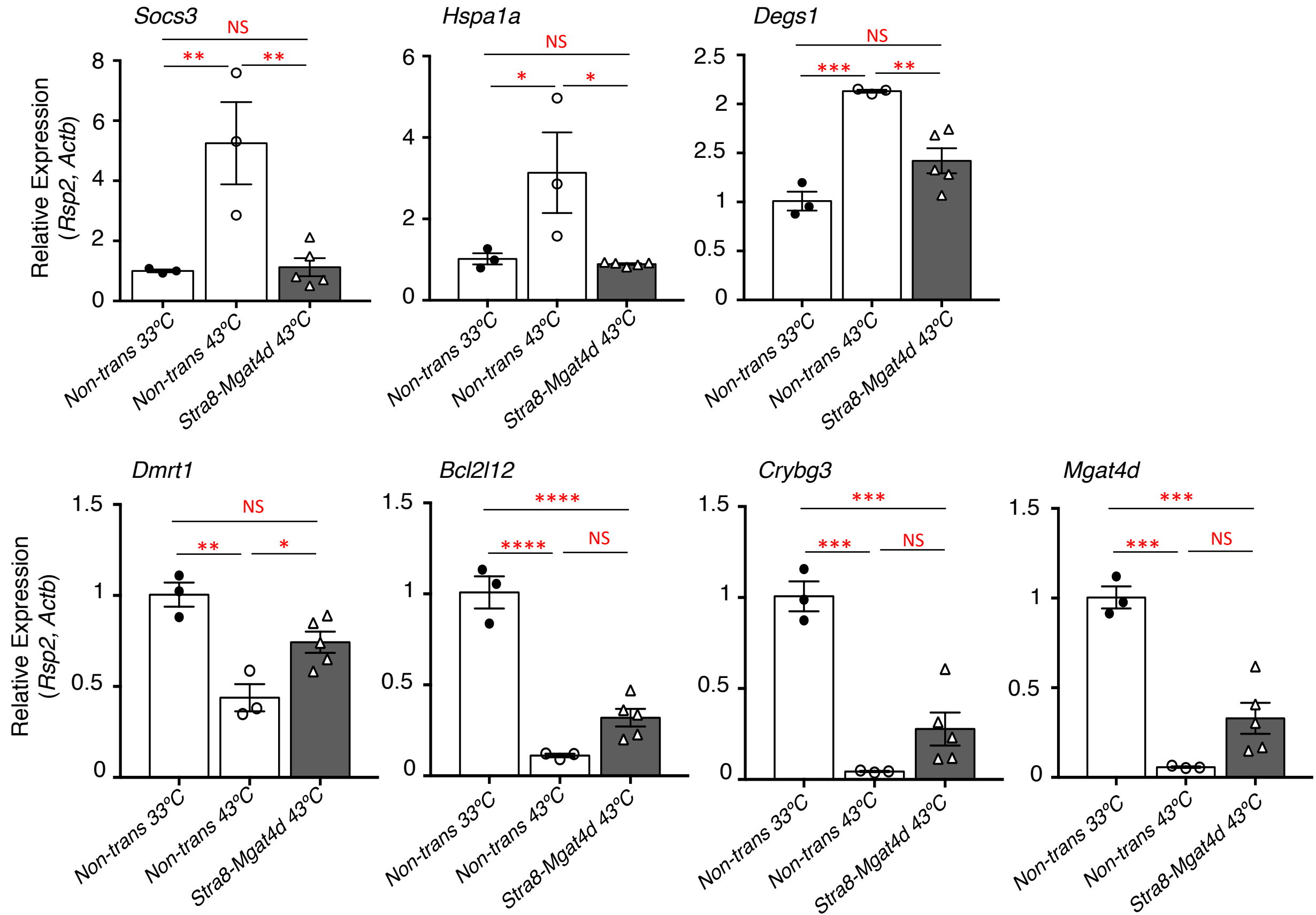


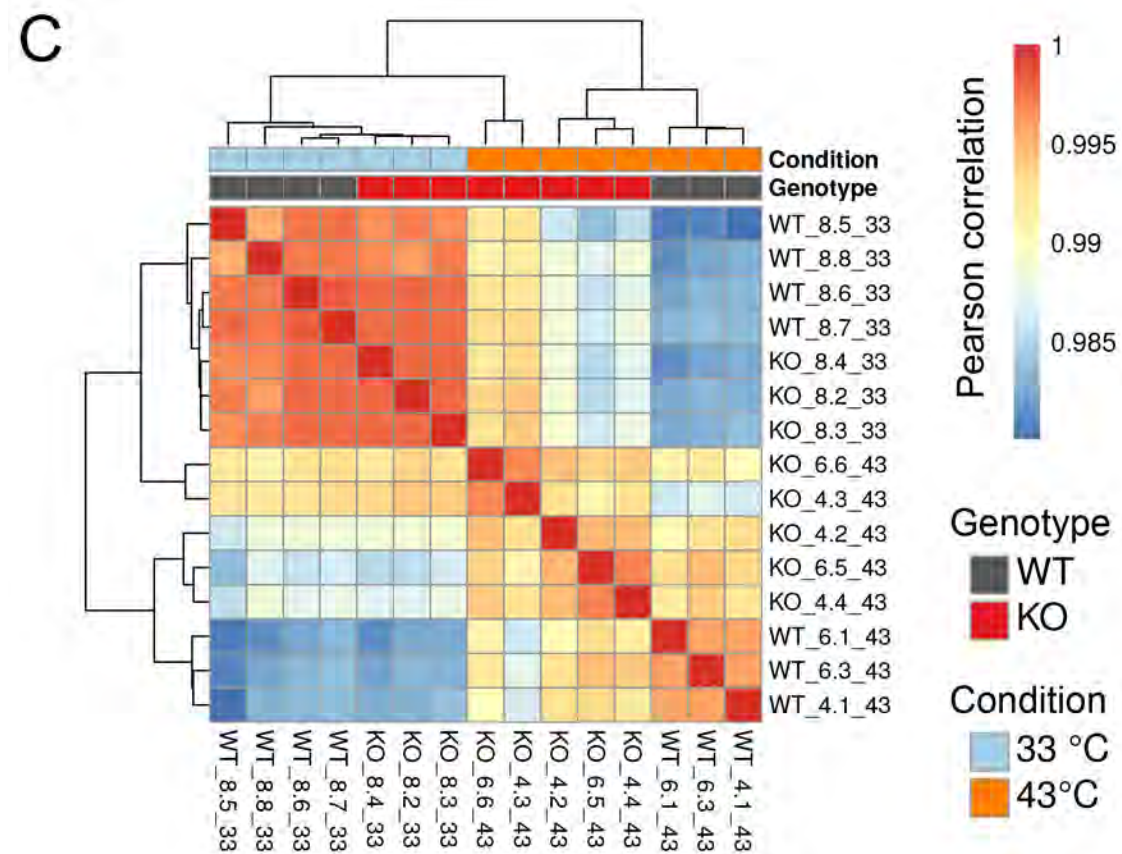
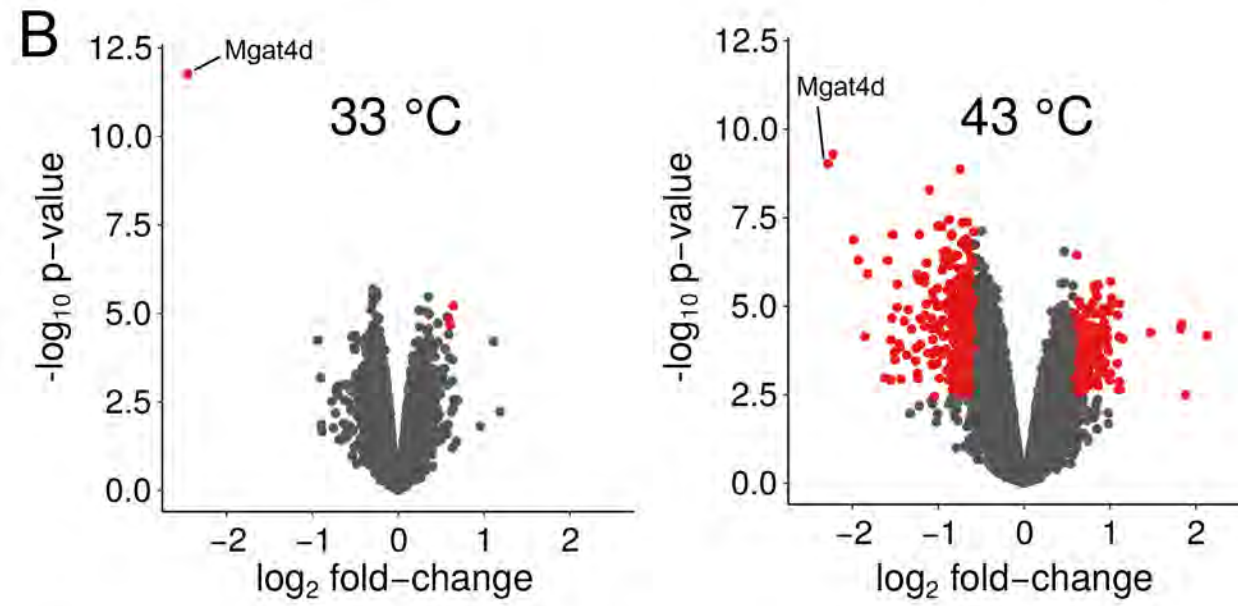
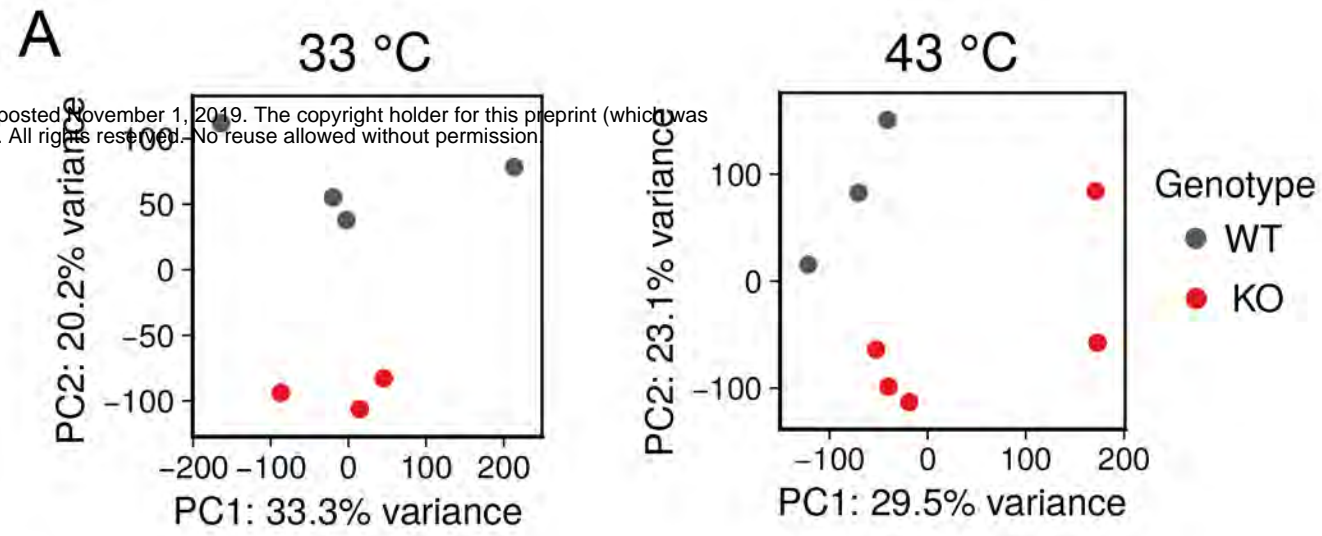


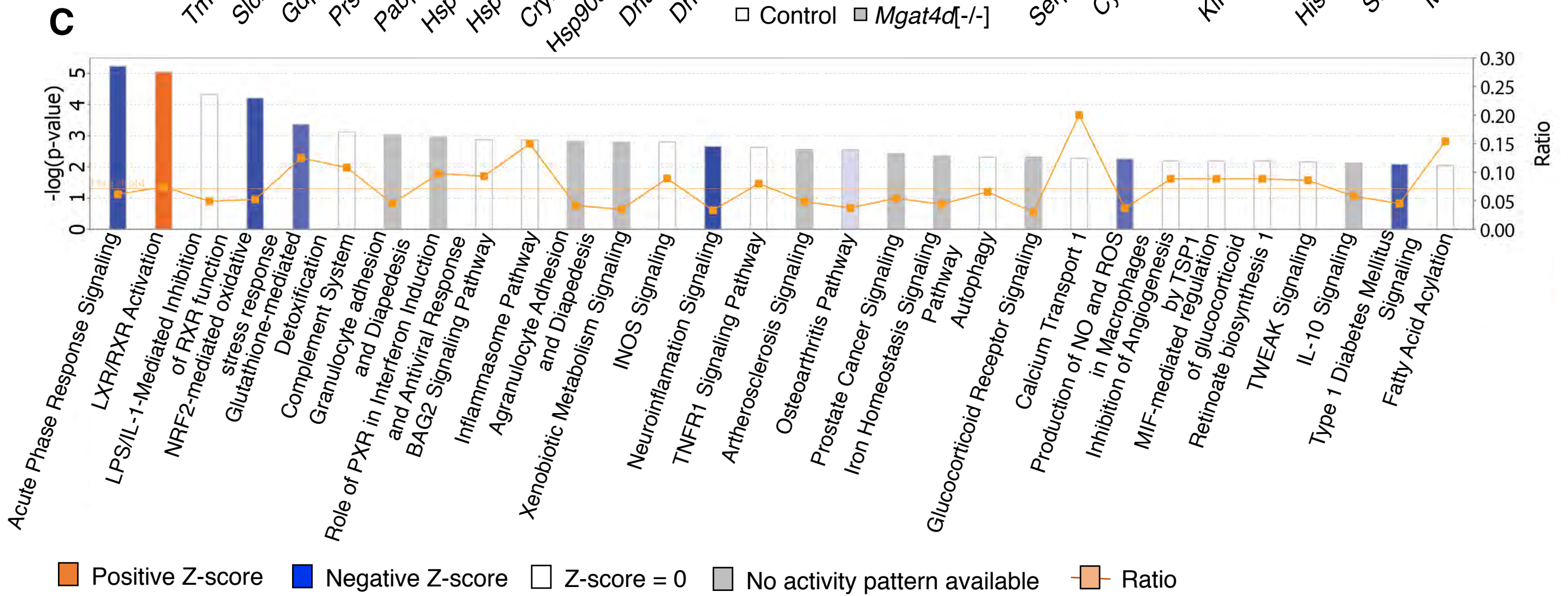
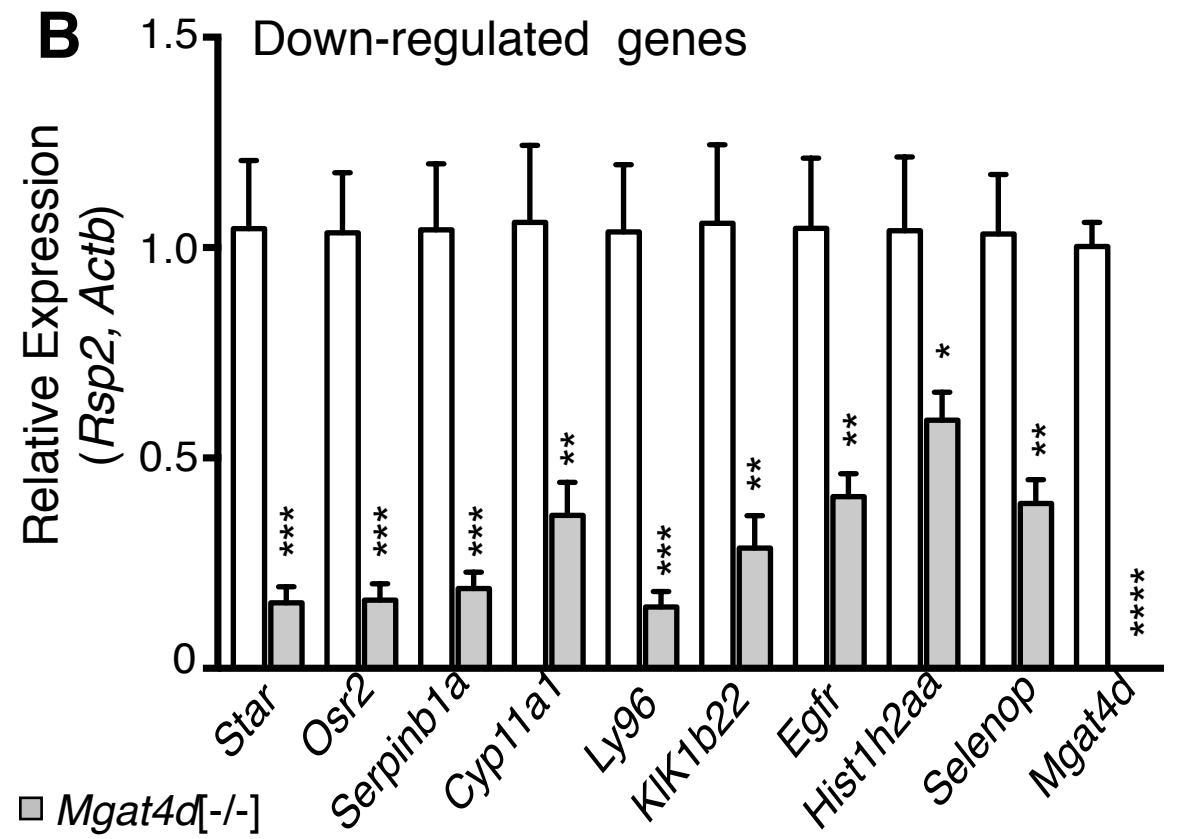
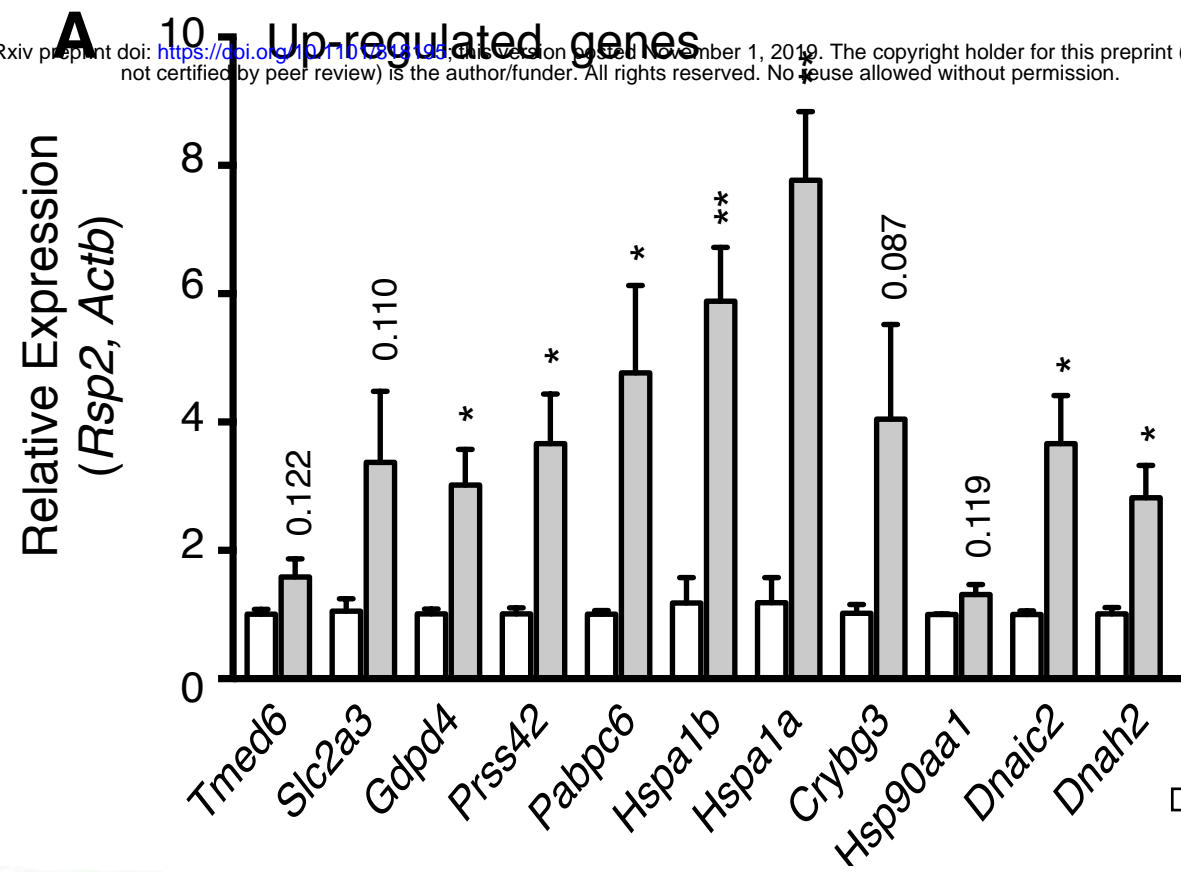












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Down-regulated

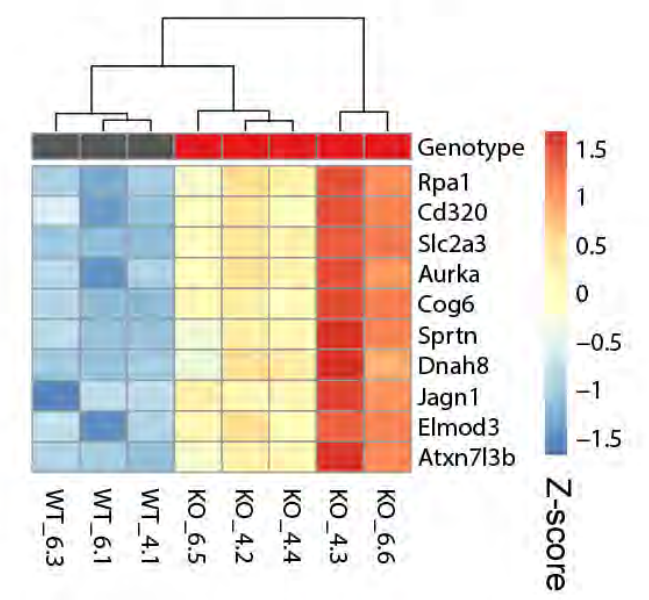
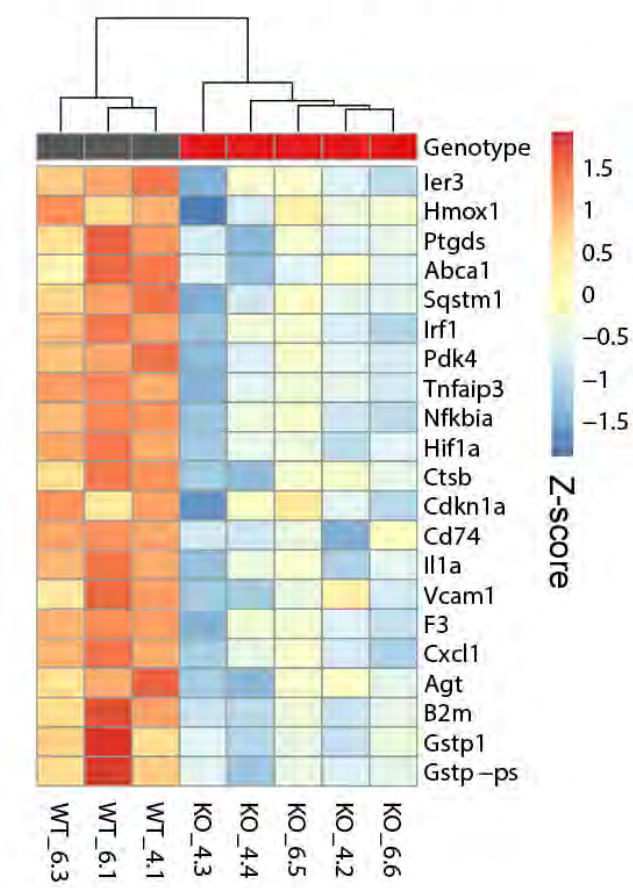
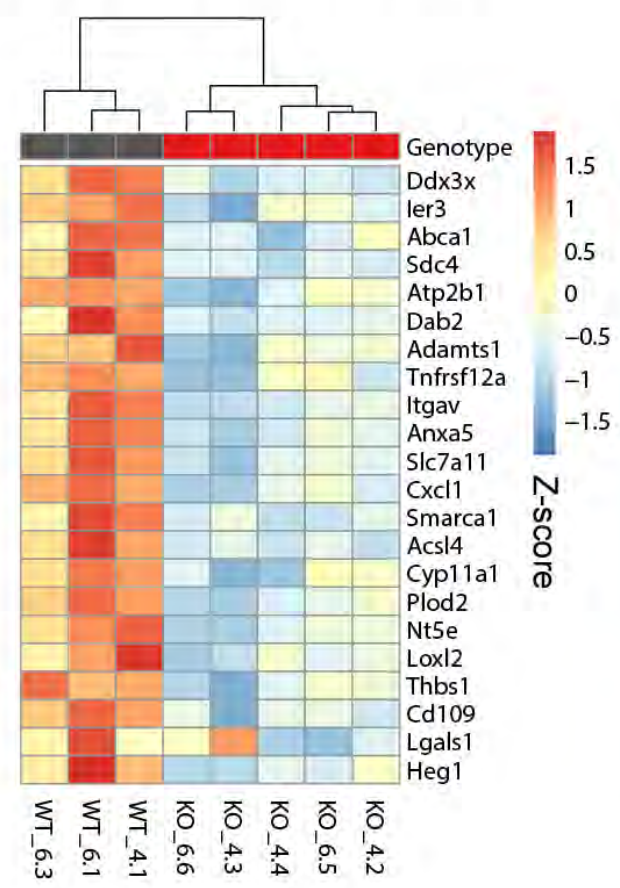
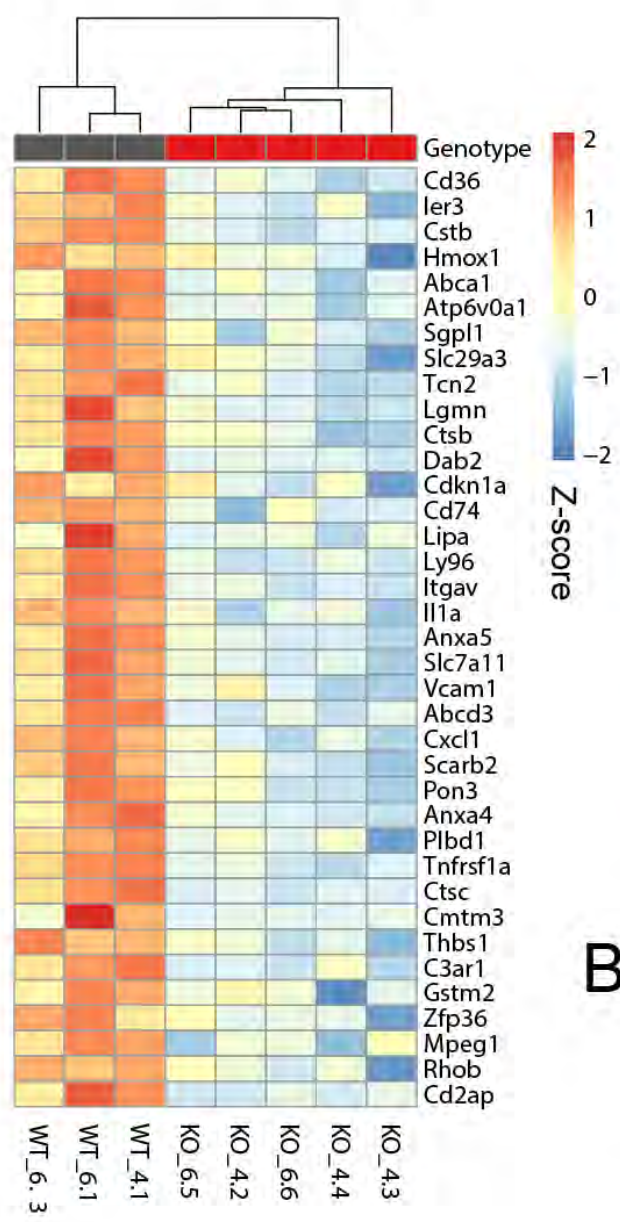
Up-regulated

Il10 KO mouse
(adj.p = 1.085e-11, OR=4.28)

HIF1A human co-expression
(adj.p = 0.00001349, OR=4.20)

NFKB1 (human)
(adj.p = 0.00002677, OR=3.97)

Infertility due to azoospermia
(adj.p = 0.01460, OR=4.84)



B

