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1 # Uncoupling DNA- and RNA-directed DNA methylation at
2 # *Rasgrf1*

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18 **ABSTRACT:**

19 Long noncoding RNAs (lncRNAs) have garnered much attention as possible links
20 between DNA sequence and the protein factors that mediate DNA methylation.
21 However, the mechanisms by which DNA methylation is directed to specific genomic
22 locations remain poorly understood. We previously identified a lncRNA in mouse, the
23 pitRNA, that was implicated in the control of DNA methylation at the imprinted *Rasgrf1*
24 locus. The pitRNA is transcribed in the developing male germline antisense to the
25 differentially methylated region (DMR) that harbors paternal allele methylation, and is
26 driven by a series of tandem repeats that are necessary for imprinted methylation.
27 MitoPLD, a factor necessary for piRNA biogenesis, both processes piRNAs from the
28 pitRNA, and is necessary for complete methylation at the locus, along with piRNA
29 binding proteins. Using two independent mouse systems where pitRNA transcription is
30 driven by the doxycycline-inducible Tet Operator, we demonstrate that pitRNA
31 transcription across the DMR is insufficient for imprinted methylation, and that the
32 *Rasgrf1* repeats have additional, critical *cis*-acting roles for imparting DNA methylation to
33 *Rasgrf1*, independently of their control of pitRNA transcription. Furthermore, pitRNA
34 overexpression and oocyte loading of pitRNA is insufficient to induce transallelic and
35 transgenerational effects previously reported for *Rasgrf1*. Notably, manipulation of the
36 pitRNA with the *TetOFF* system led to transcriptional perturbations over a broad
37 chromosomal region surrounding the inserted Tet Operator, revealing that the effects of
38 this regulatory tool are not localized to a single target gene.
39

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40 **AUTHOR SUMMARY:**

41 DNA methylation is a heritable genetic modification known to impact vital
42 biological processes. While the proteins that establish, maintain, and remove DNA
43 methylation are well characterized, the mechanisms by which these proteins are directed
44 to specific genetic sequences are poorly understood. We have previously demonstrated
45 that DNA methylation at the imprinted *Rasgrf1* locus requires a DNA element with a
46 series of tandem repeats. These repeats act as a promoter for a long noncoding RNA,
47 the pitRNA, which is targeted by a small noncoding RNA pathway known to silence viral
48 elements in the male germline via DNA methylation. We queried the sufficiency of the
49 pitRNA to mediate DNA methylation at *Rasgrf1*. We show that, in the absence of the
50 repeats, the pitRNA expression is insufficient to establish imprinted methylation. This
51 work supports a pitRNA-independent mechanism for methylation at *Rasgrf1*, and a
52 critical *cis*-acting role for the tandem repeats separate from their control of pitRNA
53 transcription.

54 **INTRODUCTION:**

55 DNA methylation is essential for appropriate embryonic development. While the
56 *trans*-acting factors required to establish [1,2,3,4,5], maintain [6,7,8,9,10,11], and
57 remove [12,13,14,15] DNA methylation have been identified, little is known of *cis*-acting
58 elements that direct these *trans*-acting factors to specific genomic locations
59 [16,17,18,19,20].

60 One such *cis* element exists at the imprinted *Rasgrf1* locus. In mouse and rat,
61 *Rasgrf1* is paternally methylated and expressed in the neonatal brain. Imprinted
62 expression in mouse is controlled by a differentially methylated region (DMR) 30 kb
63 upstream of *Rasgrf1* coding sequence, and requires a 1.6 kb stretch of tandem repeats

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64 immediately adjacent to the DMR. Targeted deletion of the *Rasgrf1* repeats
65 (*Rasgrf1*$^{tm1\mathrm{PDS}}$, *tm1*) leads to loss of methylation at the *tm1* DMR in the male germline,
66 and loss of imprinted *Rasgrf1* expression [21]. The repeats also play a role in the
67 maintenance and spreading of DMR methylation in the embryonic somatic lineage after
68 fertilization, though they are dispensable past the epiblast stage [22,23].

69 Our lab previously characterized a long noncoding RNA (lncRNA), the pitRNA,
70 which is driven by the *Rasgrf1* repeats and is expressed in the embryonic male gonad
71 [24]. LncRNAs, with their ability to recruit and bind effector proteins [25,26], represent a
72 molecular class that could bridge the gap between the protein effectors of local
73 epigenetic states if they recruit the effectors while being transcribed. Indeed, lncRNAs
74 have been implicated in diverse biological processes, and have been proposed to
75 modulate gene expression via a number of mechanisms including recruitment of histone
76 modification complexes [27,28], transcriptional interference [29], and enhancer
77 regulation [30].

78 Using the *Rasgrf1* repeats as a promoter, the pitRNA is transcribed antisense to
79 the DMR, spanning an RMER4B element, an LTR-type retrotransposon. The repeats
80 and RMER4B element are conserved at the *Rasgrf1* DMR in species where *Rasgrf1* is
81 imprinted [31]. The pitRNA is processed into secondary piRNAs by the piRNA pathway,
82 which is required for DNA methylation and transcriptional silencing of retrotransposons
83 [32], and also for full methylation at the *Rasgrf1* DMR [24]. Given the apparent
84 importance of the pitRNA and piRNA pathway in controlling methylation at *Rasgrf1*, we
85 hypothesized that aberrant expression of the pitRNA could explain transallelic and
86 transgenerational effects previously reported at *Rasgrf1* [33]. Indeed, aberrations in non-
87 coding expression have been associated with such effects in other systems [34,35,36].

88 More recently, our lab targeted the *Wnt1* locus, inserting the *Rasgrf1* repeats and
89 DMR between the *Wnt1* coding sequence and its annotated enhancer (*Wnt1*^{DR}). We

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90 found that when paternally transmitted, the *Wnt1*^{DR} allele was methylated, recapitulating
91 patterns of imprinted methylation found at *Rasgrf1*. However, pitRNA expression from
92 the *Wnt1*^{DR} was extremely low (less than 2% of the pitRNA expressed from the
93 endogenous locus) [37]. These data suggested that the *Rasgrf1* repeats could impart
94 methylation to their associated DMR independent of robust pitRNA expression.

95 None of the systems described above have uncoupled the pitRNA from the
96 *Rasgrf1* repeats to ascertain necessity or sufficiency of either individual element for
97 methylation in *cis* at the endogenous *Rasgrf1* locus. Here, we directly queried the
98 sufficiency of the pitRNA to establish methylation at *Rasgrf1* using a targeted mutation in
99 mouse, *Rasgrf1*$^{tm5.1PDS}$ (*tm5.1*) where the *Rasgrf1* repeats were replaced by the Tet
100 Operator. This enabled inducible control of pitRNA expression through combination of
101 *tm5.1* with one of two transactivating proteins: *TetON*, which binds the Tet Operator and
102 drives pitRNA expression in the presence of doxycycline [38]; and *TetOFF*, which binds
103 the Tet Operator and drives pitRNA expression in the absence of doxycycline [39,40].
104 We found that induction of the pitRNA at physiologic levels in male gonocytes was
105 insufficient to impart methylation to the *tm5.1* DMR, revealing a critical role for the
106 repeats in methylation control, independent of their regulation of pitRNA transcription,
107 consistent with our findings with *Wnt1*^{DR}. Using *tm5.1* as well as a transgenic allele,
108 TetOΔ^{Tg}, we also determined that the pitRNA overexpression was insufficient to induce
109 transgenerational or transallelic effects on *Rasgrf1* expression or methylation. Finally, in
110 addition to enabling control of pitRNA expression as designed, *TetOFF* transactivation of
111 *tm5.1* activated transcription across a broad chromatin domain previously shown to
112 exhibit interactions, and that activation was not confined to the target sequences at the
113 DMR. Our data identify a role for *Rasgrf1* repeats as a *cis*-element directing DNA
114 methylation, independently of the pitRNA it drives. Furthermore, we show that

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115 expression patterns controlled by engineered Tet repressor proteins can be exerted over
116 large regions of the genome.

117 **RESULTS:**

118 **Generation of *Rasgrf1$^{tm5.0}$*^{PDS}.**

119 We successfully generated the targeted mutant *Rasgrf1*$^{tm5.1PDS}$ (*tm5.1*), where the
120 endogenous repeats were replaced with the Tet Operator (Fig 1a). Allelic structure was
121 validated by Southern blot, and Sanger sequencing of PCR products that spanned the
122 vector ends and the endogenous sequence at the target locus, as well as by copy
123 number qPCR (Fig S1a-d).

124 **The *tm5.1* allele lacks DMR methylation and *Rasgrf1* expression, like the**
125 ***Rasgrf1^{tm1}* repeat-deficient allele.**

126

127 We first characterized *tm5.1* in the absence of a transactivator. We expected
128 that, in the absence of a transactivating protein, *tm5.1* would neither accrue methylation
129 at its DMR or impart imprinted expression at *Rasgrf1*, similar to the repeat-deficient
130 allele, *Rasgrf1^{tm1}* (*tm1*). The *tm5.1* DMR was hypomethylated when paternally
131 transmitted (+/*tm5.1*, Fig S2e), leading to minimal expression of *Rasgrf1* in the brain as
132 measured by qRT-PCR (FigS2d); +/*tm5.1* animals were on average of lower body weight
133 than wild-type littermates (Fig S2f), consistent with findings that repeat-deficient animals
134 are underweight [41].

135 Also consistent with *tm1*, a portion of +/*tm5.1* animals were methylated at, and
136 expressed *Rasgrf1* at wild-type levels from the *tm5.1* allele. Also as seen with the *tm1*
137 allele, a portion of mice with the +/*tm5.1* genotype had *tm5.1* methylation and expression

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138 in the N2 and N3 generations (4 out of 14 animals in the N2 generation; 2 out of 15
139 animals in the N3 generation). Consistent with findings using repeat-deficient animals
140 [41], this was a stochastic event, and the methylation status of *+/tm5.1* offspring was not
141 dependent on the methylation state of their +/*tm5.1* fathers (Fig S2a-b).

142 **Induction of pitRNA from *Rasgrf1*$^{\mathbf{tm5.1PDS}}$ via the *TetON* and *TetOFF* systems.**

143 To ascertain transactivator-dependent induction of pitRNA, we then generated
144 *tm5.1* mice expressing one of two transactivating proteins, *TetON* and *TetOFF*. As
145 discussed in Methods, the *TetOFF* allele was generated from the commercially available
146 *pA-TetOFF* allele, where *TetOFF* is preceded by a floxed neomycin-resistance
147 polyadenylation cassette. The polyadenylation cassette was removed by embryonic day
148 6.5 by crossing pA-*TetOFF* males with females carrying a *Cre* transgene driven by the
149 *Sox2* promoter [42]. Successful Cre-mediated recombination was confirmed by Sanger
150 sequencing of PCR products spanning the pA-cassette (Fig S3). We expected pitRNA
151 transcription from the *tm5.1* allele would depend either on the *TetOFF* transgene in the
152 absence of tetracycline, or the *TetON* transgene in the presence of doxycycline. We
153 assayed pitRNA induction in several adult tissues by endpoint PCR, as well as by qPCR
154 in the neonatal male germline, adult liver, neonatal brain, and oocytes. pitRNA induction
155 from the *tm5.1* allele required a transactivating protein, and was expressed at
156 physiological levels in the neonatal male embryonic germline of males (Fig 1b), and from
157 10 to 1000-fold wild-type levels in adult tissues (Fig S4, Fig S7a) depending on the
158 tissue assayed. In all tissues, pitRNA was silent in the absence of a transactivator.

159 **pitRNA induction in the male germline is insufficient for establishment of germline**
160 **methylation at *Rasgrf1*.**

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161 Having confirmed that pitRNA could be induced from *tm5.1* using both the *TetON*
162 and the *TetOFF* systems, that expression in the neonatal germline was at physiologic
163 levels, and that *tm5.1* was transcriptionally silent in the absence of a transactivator, we
164 tested whether artificially regulated pitRNA expression was sufficient to impart
165 methylation at the *Rasgrf1* DMR in the male germline, independently of the repeats that
166 normally effect this regulation. To perform this analysis, we used gonads from male
167 embryos heterozygous for the *tm5.1* allele that also carried the *TetOFF* or the *TetON*
168 transgenes. We assayed methylation of the *tm5.1* and WT DMRs under conditions
169 where the pitRNA is induced from the *tm5.1* allele. The use of heterozygotes enabled us
170 to monitor methylation of both the *tm5.1* allele, with artificial regulation of the pitRNA
171 from the Tet Operator, and the wild-type allele, as an internal control, which has natural
172 regulation of the pitRNA from the repeats. gDNA was prepared from the gonocyte and
173 somatic cell fractions of developing male gonads, and assayed for DMR methylation by
174 targeted bisulfite sequencing (Fig 2a-d, S5a,b lower panels) and COBRA (Fig S5a,b
175 upper panels), using assays specific for the *tm5.1* and wild-type alleles. Both assays
176 revealed that the *tm5.1* DMR was hypomethylated in gonocytes from each of six mice
177 tested, despite the proper regulation of pitRNA in the germline from the *tm5.1* allele. In
178 contrast, the wild-type DMR from the same animals was hypermethylated, as expected.
179 This pattern was observed in male gonocytes regardless of the parental modes of
180 inheritance of the two alleles, or whether the *TetON* or *TetOFF* regulator was used. As a
181 control for purity of germ cells, we performed BS-PCR and sequencing for the *Igf2r*
182 DMR, which is methylated only upon maternal transmission, and found extensive
183 hypomethylation, as expected for male germline cells [43]. We also assayed methylation
184 states of the two alleles in somatic fractions of developing gonads. As with gonocytes,
185 the *tm5.1* allele was unmethylated regardless of mode of inheritance or transactivator.
186 As expected, the wild-type allele was hypermethylated upon paternal transmission, and

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187 hypomethylated upon maternal transmission. Hypomethylation of the maternal DMR in
188 somatic fractions demonstrated that pitRNA expression from the *tm5.1* allele does not
189 act in *trans*.

190 We further assayed *tm5.1* and wild-type DMR methylation in mature sperm of
191 *tm5.1* heterozygotes, where the pitRNA was regulated by *TetOFF* in the absence of
192 doxycycline. As with gonocytes, the *tm5.1* DMR was consistently hypomethylated,
193 whereas the wild-type DMR was methylated (Fig 2e and Fig S5c). We concluded that in
194 the embryonic and mature male germlines, pitRNA expression alone was not sufficient
195 to impart methylation at the *tm5.1* DMR in *cis*, indicating that the repeats perform an
196 additional necessary function for DMR methylation, beyond controlling pitRNA
197 expression.

198 **pitRNA induction in the male germline is insufficient for somatic methylation at**
199 ***Rasgrf1*.**

200 In previous studies, exporting the *Rasgrf1* ICR to the *Wnt1* locus led to
201 hypomethylation of the mutant allele in sperm, but hypermethylation in somatic tissue
202 after fertilization [37]. Though sperm methylation at the modified *Wnt1* allele was higher
203 than sperm methylation at the *tm5.1* allele, we determined if expression of pitRNA by
204 TetO induction of the *tm5.1* allele could enable methylation in somatic tail DNA of
205 progeny after paternal transmission. In all tail samples tested, the *tm5.1* allele remained
206 unmethylated regardless of which transactivator was used to control pitRNA (Fig 3a-b).
207 These findings demonstrated that like methylation in the male germline, methylation in
208 somatic tissue of offspring after paternal transmission is not enabled by pitRNA
209 expression alone. Instead, and consistent with findings from the *Wnt1* mutant allele,
210 additional features of the repeats, beyond their control of pitRNA expression, are
211 necessary for methylation.

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212 ***tm5.1* transactivation and pitRNA induction leads to expression changes of**
213 **neighboring genes.**

214 We expanded our initial analysis of methylation by characterizing expression
215 states of *Rasgrf1* and nearby loci in mice carrying the *tm5.1* allele. *Rasgrf1* expression in
216 neonatal brain requires either methylation of the DMR, which is a methylation-sensitive
217 enhancer blocker, or ectopic insertion of an enhancer proximal to the *Rasgrf1* promoter
218 [44]. We found that *TetOFF*-mediated *tm5.1* induction led to a tenfold upregulation of
219 *Rasgrf1* in neonatal brain relative to wild-type regardless of the parental origin of the
220 *tm5.1* allele. Sequencing *Rasgrf1* RT-PCR products revealed that *TetOFF*:+/*tm5.1*
221 animals that inherited *tm5.1* paternally, expressed *Rasgrf1* solely from the paternal
222 *tm5.1* allele (Fig 4a-c). *TetOFF: tm5.1*/+ animals that inherited *tm5.1* maternally
223 expressed *Rasgrf1* from both the maternal and paternal alleles at a ratio of
224 approximately 9:1, indicating there was a dramatic upregulation of *Rasgrf1* from the
225 normally silent wildtype maternal allele, when it was replaced by *tm5.1*, and with
226 transactivation by *TetOFF*. This was accompanied by continued *Rasgrf1* expression
227 from the paternal wild-type allele (Fig 4a-c). We observed similar effects with *TetON*
228 transactivator. Though the magnitude of *Rasgrf1* induction was lower, *TetON* also
229 activated expression from the maternal *tm5.1* allele (Fig 4 d-f, Fig S6).

230 The *Rasgrf1* locus lies within two overlapping annotated regions of chromatin
231 interaction, a smaller 150 kb and encompassing 250 kb region, as shown by cohesin
232 ChIA-PET analysis of mouse embryonic stem cells [45] (Fig 5a). To define the extent of
233 *TetOFF*-mediated transactivation, and its relationship to the bounds of known regions of
234 interaction, we queried the effects of *TetOFF* on other transcripts within the interacting
235 regions. In neonatal brain, all transcripts within the minimal 150kb interaction domain
236 were upregulated in both *TetOFF*:+/*tm5.1* and *TetOFF*:*tm5.1*/+ animals, in a

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237 transactivator dependent manner, indicating the effects of transactivation extended
238 throughout the 150kb domain. Interestingly, *TetOFF*-dependent effects on brain
239 expression of transcripts within the *tm5.1* domain varied, depending on parental origin of
240 *tm5.1*, consistent with the existence of distinct chromosomal architecture for parental
241 alleles within imprinted regions (Fig 5b). The allele-specific effects were even more
242 dramatic when expression was assayed in neonatal testes: transcripts linked to *tm5.1*
243 that were upregulated by *TetOFF* when *tm5.1* was maternally transmitted were
244 downregulated when it was paternally transmitted. Additionally, the effects extended
245 over a broader chromosomal domain in testes, and on the paternal chromosome,
246 highlighting parental- and tissue-specific chromatin architecture at *Rasgrf1* (Fig 5c).

247 **pitRNA loading of oocytes does not produce paramutation**

248 Previously, our lab described a paramutation-like phenomenon at *Rasgrf1*
249 associated with the *Rasgrf1*$^{tm3.1PDS}$ allele (*tm3.1*), in which the repeats were replaced by
250 the imprinting control region (ICR) of *Igf2r* [43]. Progeny carrying a paternal *tm3.1* allele
251 exhibited a derepression of the maternal allele, as interpreted from endpoint RT-PCR;
252 and further, some wild-type offspring of +/*tm3.1* females demonstrated continued
253 depression of the maternal allele, though they lacked the original paternal allele that
254 incited the derepression. This intergenerational effect is a key feature of paramutation.
255 The *Igf2r* sequences in the *tm3.1* allele harbors the promoter for *Air*, a non-coding RNA
256 that regulates imprinted expression at the locus [46]. Its presence and orientation in the
257 *tm3.1* allele could impart novel expression patterns of the pitRNA; accordingly, we
258 hypothesized that the intergenerational, paramutation-like effects could involve oocyte
259 loading of pitRNA. To test this hypothesis, we treated females carrying the *tm5.1* allele,
260 and the TetON activator, with intraperitoneal dox for three days, which led to oocyte
261 pitRNA levels approximately 90-fold higher than in wild-type mice (Fig S7a). Females

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262 subjected to these treatments were bred to wild-type males, and maintained on dox
263 chow for the duration of pregnancy—as such, their *TetON:*+/*tm5.1* offspring were
264 informative for somatic effects of pitRNA induction in oocytes (Fig S6). Additionally, their
265 wild-type, *TetON:*+/+, and +/*tm5.1* offspring were informative for determining if pitRNA
266 loading in oocytes could induce paramutation. In none of these offspring born to mothers
267 with pitRNA preloaded in their oocytes did we observe effects on imprinting status or
268 expression levels of *Rasgrf1* (FigS7c, d). COBRA analysis of the maternal *tm5.1* allele
269 transmitted by oocytes preloaded with pitRNA showed that it remained hypomethylated
270 (Fig S7e). We concluded that preloading oocytes to nearly 100-fold levels of pitRNA
271 relative to wild-type was insufficient to induce transgenerational effects.

272 **DISCUSSION:**

273 **A model for DNA methylation at *Rasgrf1*.**

274 Despite the fundamental roles that DNA and histone modification states have for
275 genome regulation, there have been only a handful of sequence elements identified that
276 exert *cis*-acting control over these modification states [16-21]. We previously identified
277 one such element at the *Rasgrf1* locus: It is a series of tandem repeats found 30 kb
278 upstream of the imprinted *Rasgrf1* coding sequence, and lying immediately adjacent to
279 the DMR, that are required both in the male germline for establishment of imprinted
280 methylation at the DMR in sperm [21], and also in the pre-implantation embryo for
281 maintenance of imprinted methylation in somatic lineages [23]. In addition to serving
282 these functions, the repeats serve as a promoter for expression of the pitRNA in the
283 male germline that spans the DMR, and which is processed into piRNAs. The distinct
284 effects of lncRNAs, and the *cis*-elements that regulate them, have proven difficult to
285 uncouple [47,48] with some success coming from the truncation of the lncRNA transcript

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286 while keeping the *cis* element intact [49,50,51,52]. To assess the activity of the pitRNA
287 separately from the repeats, we uncoupled the two by replacing the repeats with an
288 artificial promoter based on TetO sequences (*tm5.1*), which regulated the pitRNA to
289 physiologic levels in the developing male germline using the *TetON* and *TetOFF*
290 transactivators. Of nine male embryos with this pitRNA regulation analyzed, only one
291 displayed partial gonocyte and somatic methylation of *tm5.1*. This frequency is
292 consistent with the frequency of DMR methylation observed in mouse models lacking the
293 repeats [23, 41]. Properly regulated pitRNA expression from the *tm5.1* allele also failed
294 to enable methylation in mature sperm, or in somatic DNA of progeny inheriting the
295 *tm5.1* allele from their fathers. We therefore conclude that the pitRNA is insufficient to
296 impart DNA methylation in *cis* and that *Rasgrf1* methylation requires critical functions in
297 the repeats, separate from pitRNA expression (Fig 6).
298 Our findings that pitRNA expression is insufficient to control imprinted
299 methylation of *Rasgrf1* are consistent with other work from our lab, in which the *Rasgrf1*
300 DMR and repeats were exported to an ectopic site at the *Wnt1* locus. Paternal
301 transmission of the modified *Wnt1* allele enabled complete methylation of the ectopic
302 DMR in somatic DNA of the offspring despite germline expression of the pitRNA at only
303 1% of the levels seen from the endogenous *Rasgrf1* locus; methylation of the *Wnt1* DMR
304 in sperm DNA at only 30% of the levels found at the *Rasgrf1* DMR.
305 One interpretation of the present study, combined with the findings from the *Wnt1*
306 study is that the pitRNA is irrelevant to *Rasgrf1* methylation: that instead, the repeats are
307 both necessary and sufficient for this function, and the transcription of the pitRNA is an
308 irrelevant sequela to some inherent activity at the repeats [reviewed in 53]. However,
309 other data from our lab support a role for the pitRNA [24], likely by influencing the
310 efficiency and/or timing of *Rasgrf1* methylation in a probabilistic way rather than in a
311 deterministic way. This characterization is similar to the *Xite* element at the X-

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312 Inactivation Center, which is transcribed, and mediates the probability with which an X
313 chromosome undergoes X chromosome inactivation [54]. The probabilistic model for
314 pitRNA function is also consistent with our findings that mice deficient for piRNA binding
315 proteins MILI and MIWI2, as well as the piRNA biogenesis factor MitoPLD, have
316 diminished methylation at the *Rasgrf1* DMR, though methylation is not completely lost
317 [24]. Unless the piRNA factors are exerting their effects through the *Rasgrf1* repeats,
318 and not through the pitRNA they in fact target for piRNA processing, the pitRNA is very
319 likely to contribute to DMR methylation, despite not being absolutely required.

320 We also utilized the *tm5.1* allele to query whether previously observed
321 intergenerational effects could be due to oocyte preloading of the pitRNA. Others have
322 reported that gametic loading of RNAs could impart such effects to the next generation
323 [35,36]. We achieved oocyte pitRNA levels up to 90-fold greater than wild-type by
324 intraperitoneal doxycycline administration, however, this produced no effects on
325 imprinted *Rasgrf1* expression or methylation in the wild-type offspring of females
326 subjected to oocyte preloading of pitRNA.

327 The repeats have several features that might enable their control of local
328 methylation states, separate from their control of pitRNA expression. Besides being
329 highly repetitive, the GC-richness of the repeats might be sufficient to recruit methylation
330 [55,56] which then spreads into the DMR. This is seen at *H19* where somatic
331 methylation on the paternal arises adjacent to the germline DMR [57], and occurs when
332 CTCF is unbound to the germline DMR [58,59,60,61,20). Within the repeats are two
333 canonical binding sites for the transcription factor Sp1, which besides its known role in
334 the regulation of gene expression [62], can mediate chromatin structure through the
335 recruitment of chromatin remodeling factors [63,64] and mediating enhancer-promoter
336 interactions [65,66]. Sp1 is known to bind the secondary DNA structure G-quadruplexes
337 (G4s) [67,68], which the *Rasgrf1* repeats are predicted to form, as well as its canonical

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338 sequence. Recently, a G4 was characterized at the imprinted *H19* locus. Binding of Sp1,
339 in conjunction with the G4, suppressed *H19* transcription [69]. To our knowledge, G-
340 quadruplex formation at other imprinted loci beyond *H19* has not been investigated.
341 However, differential G4 formation in the maternal and paternal germlines could be a
342 platform upon which the distinct chromatin states observed at the maternal and paternal
343 DMRs are built.

344 Our lab previously showed that, at the *Rasgrf1* DMR, H3K27Me and DNA
345 methylation are mutually antagonistic, whereby the presence of one mark blocks the
346 deposition of the other [22]. Placement of H3K27me3 requires YY1 and PRC2, which, in
347 *Drosophila* [70] and likely in mammals [71], can be recruited to Sp1 sites. It is possible in
348 the male germline, that Sp1 binding to the repeats excludes YYI and PRC2 binding,
349 enabling paternal allele methylation.

350 Other factors required for parent-of-origin specific DNA methylation might require
351 the repeats for their recruitment. The GHKL ATPase Morc1 is a known repressor of
352 transposable elements in the embryonic male germline; *Morc1*-null mice are
353 hypomethylated at the *Rasgrf1* DMR [72]. The KRAB-domain containing zinc-finger
354 binding protein ZFP57 and its cofactor Trim28 [73] are typically thought of as an
355 imprinting maintenance mechanism. Loss of zygotic Trim28 disrupts imprinting at
356 *Rasgrf1* along with many other maternally and paternally imprinted loci [74].
357 Interestingly, KRAB-domain ZFP binding can also trigger *de novo* DNA methylation
358 during mouse embryogenesis [75].

359

360 **Additional technical considerations for the study of imprinted loci, and use of**
361 **transactivators.**

362 Our experiments highlight an important technical consideration for the imprinting
363 field, that allele-specific expression analyses must be supported with quantitative data.

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364 By end-point RT-PCR followed by allele-specific restriction digest, *TetOFF*-mediated
365 transactivation of maternally inherited *tm5.1* appears to fully reverse imprinting, with
366 maternal-only bands upon gel electrophoresis (Figure S7). Similar data have previously
367 been interpreted as a silencing of the paternal allele *in trans* [76]. However, sequencing
368 of *Rasgrf1* cDNA of *tm5.1/+:TetOFF* neonatal brain demonstrated that roughly 10% of
369 total *Rasgrf1* reads are paternal in origin, suggesting that the wild-type paternal allele
370 continued to express *Rasgrf1* at wild-type levels, and was not silenced *in trans*.

371 Similarly, end-point analysis of *+/tm5.1* animals lacking a transactivating allele
372 revealed biallelic expression. While this could be interpreted as a possible *trans* effect of
373 the paternally inherited *tm5.1* allele exerting effects on the maternally inherited and
374 normally transcriptionally silent WT allele, qPCR of *Rasgrf1* in these animals
375 demonstrated severely reduced *Rasgrf1*, suggesting instead that, in the *tm5.1* system,
376 biallelic expression reflects minimal transcription.

377 An unexpected outcome of this work was that *TetOFF*-mediated transactivation
378 of *tm5.1* influenced expression across the entire chromatin domain, defined by cohesin
379 ChiA-PET [45], where the transactivator bound, and that this influence was both allele-
380 and tissue-specific . The cohesin ChiA-PET study defined two regions of interaction,
381 which may represent the maternal and paternal alleles. These widespread transcriptional
382 changes were not observed in *TetON:+/tm5.1* tissues (data not shown), suggesting that
383 these widespread effects may be restricted to the *TetOFF* system. While this finding was
384 not an objective of our study, it emphasizes the considerations that should be taken
385 when interpreting transcriptional effects using potent transactivating systems such as the
386 *TetOFF* protein.

387 In conclusion, our data support the existence of a second, pitRNA-independent
388 mechanism for DNA methylation at *Rasgrf1*. We propose a *cis*-acting mechanism by
389 which the repeat sequences themselves are largely responsible for methylation control

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at the locus, and the pitRNA increase the probability of methylation in the paternal germline.

MATERIALS AND METHODS:

Primer sequences for all analyses are listed in Table S1.

TetO vector generation: We modified pYP1, which carries the DMR and *Rasgrf1* repeats, and 4 kb of homologous flanking sequence [77] to carry seven copies of the Tet Responsive Element (collectively termed TetO) in place of the *Rasgrf1* repeats as follows. The *Rasgrf1* repeats were removed *via* restriction digest with *Cla*I and *Mlu*I; sticky ends were blunted with Klenow; the plasmid backbone was gel purified and then ligated closed generating pDHT2. pDHT2 and pPX3, a vector containing the Tet Operator, were digested with *Nhe*I, and linearized pDHT2 was ligated to the TetO sequences, generating pDHT3, which was confirmed by Sanger sequencing. The 3' homologous arm of pDHT3 was then shortened to approximately 1kb by restriction digest with *Bsr*GI and *Sfi*I to generate pETC6, which was linearized with *Pci*I prior to lipofection into ES cells.

CRISPR/Cas9-mediated generation of TetO^{tg}, TetO□^{tg}, *Rasgrf1*$^{tm5.0PDS}$ and *Rasgrf1*$^{tm5.1PDS}$: pX330 (Addgene Plasmid # 42230) was modified to carry PDS 2195-6, a complementary primer pair coding for an sgRNA targeting the *Rasgrf1* repeats, following the Zhang lab protocol [78] to generate pX330-rep5. PDS 2195-6 was designed using the CRISPR Design Tool (http://crispr.mit.edu:8079/). To effect homology directed repair at the *Rasgrf1* locus, v6.5 embryonic stem cells [79] were lipofected with linearized pETC6 and pX330-rep5 with Lipofectamine 2000, following the manufacturer's protocol. Cells were allowed to recover overnight, then treated for 10 days with 300 ug/mL G418 (Sigma A1720). Colonies were picked and genotyped with PCR 2359-8 and PDS 2344-2263, which generate a product only from the targeted allele

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415 (*Rasgrf1*$^{tm5.0PDS}$) and PDS 2757-8, an internal PCR for TetO, to detect cells harboring a
416 randomly inserted vector that provided the transgenic (Tg) model. Targeted and
417 transgenic status for tm5.0 and TetO^{Tg} were further confirmed by Southern blot [21].

418 TetO^{Tg} and *Rasgrf1*$^{tm5.0PDS}$ ES cells were microinjected into B6(Cg)$^{Tyrc-2J}$/J
419 blastocysts by the Cornell University Transgenics Core. 22 chimeras were recovered.
420 Germline transmission was confirmed by diagnostic crosses to FVB/N females and PCR
421 with PDS 2757-8. Chimeras were crossed with mice constitutively expressing FlpE
422 recombinase (JAX Strain 003800) to generate *Rasgrf1*$^{tm5.1PDS}$ and TetO^{Tg}.
423 Recombination was confirmed by Sanger sequencing of PDS 2262-3 PCR products,
424 which spans the neo resistance cassette and frt sites.

425 **Generation of *TetOFF* mice:** To produce mice that constitutively express the tet
426 transactivator (*TetOFF*), mice carrying a tTA transgene preceded by a floxed neomycin-
427 polyadenylation cassette (pA-*TetOFF*, JAX Strain No 011008) were crossed with mice
428 constitutively expressing *Sox2*-Cre (JAX Strain No 008454). Recombination and
429 subsequent loss of the neomycin resistance cassette was confirmed by endpoint PCR
430 with PDS 2794-5, followed by Sanger sequencing. PDS 2794-5 sequences were
431 supplied by Bruce Morgan [80].

432 **Induction of pitRNA:** *TetOFF*: *tm5.1* mice were crossed to *TetOFF* mice.
433 Induction of pitRNA expression was validated in adult and neonatal tissues using PDS
434 2266-7.

435 *TetON*: Female mice were injected with 0.01 mg/g body weight of 0.01 mg/mL
436 doxycycline hyclate (Sigma D9891) intraperitoneally every 24 hours for three days as a
437 preloading phase, then bred. Breeding pairs were fed 200 mg/kg doxycycline chow
438 (BioServ S3888) for the duration of pregnancy. Induction of pitRNA expression was

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439 validated in all tissues by endpoint and qRT-PCR using PDS 2266-7 and PDS 2916-7, a
440 primer pair specific for pitRNA produced from the *tm5.1* allele.

441 **Allele-specific analysis of *Rasgrf1* expression:** Neonatal brains were
442 collected at postnatal day 2; the olfactory bulbs were visually identified under dissection
443 microscope and discarded. Other tissues were collected by gross dissection. Tissues
444 were snap frozen in liquid nitrogen, then submerged in 1 mL Trizol. Tissues were
445 homogenized using a Biospec Mini-Bead Beater-8 using 1 3mm steel bead in XXTuff
446 Microvials (BioSpec XX0TX). Total RNA for all samples was processed via the Trizol
447 protocol (Thermo Fisher Scientific 15596018). RNA was DNAse treated, random primed,
448 and reverse transcribed using Promega RQ1 DNAse and RQ RTase (Promega M6101
449 and A5003 respectively) following the manufacturer's protocol. RT-PCR was performed
450 with PDS 245-6 (95ºC 2min, 40 cycles of 95ºC 30s, 60ºC 30s, 72ºC 30s, 72ºCC 7min
451 using Promega GoTaq in a volume of 25 uL (Promega M3001). *For allele-specific*
452 *restriction digest*: 20 uL of PCR product was digested with 2.5U *Aci*I. Digestion products
453 were separated by electrophoresis on a 4% agarose gel. *For allele-specific read*
454 *quantification*: End-point PCR samples were submitted for sequencing in MiSeq libraries
455 as described below in. Targeted sequencing analysis. Read quantification for *Rasgrf1*-
456 specific expression was performed on trimmed samples with the grep and wc functions.
457 Grep sequences are listed in Table S4. MGI SNP IDs and flanking sequences are listed
458 in Table S5.

459 **qRT-PCR and heat map generation:** qRT-PCR was performed in 20 uL
460 reactions using SYBR Green Master Mix (CAT 4367659) on a Biosystems 7500 with
461 annealing temperature 60ºC for forty cycles followed by a dissociation stage. The
462 following primer pairs were used: PDS 2266-7 for general pitRNA expression; PDS
463 2916-7 for TetO-pitRNA expression; PDS 2877-8 for *Rasgrf1* expression; PDS 72-3 for

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464 *Rpl32* expression; PDS 2719-8 for *ATOHe* expression; PDS 3211-2 for *Ctsh* expression;
465 PDS 2178-9 for *Ankrd34c* expression. Heat maps were generated in R [81].

466 **Gonocyte collection**: Females were checked for plugs, weighed to confirm
467 pregnancy, and sacrificed at gestational day 16.5. Gonocytes from male embryos were
468 collected as in Watanabe *et al*, 2011 [24] with some modifications. Briefly, embryonic
469 testes were collected and incubated in 50 uL 0.25% trypsin for 10 minutes. Samples
470 were then triturated and visually inspected for tissue disaggregation. Incubation and
471 trituration were repeated up to two times until full disaggregation was achieved; any
472 remaining clumps were manually removed. Samples were then transferred to McCoy's
473 basic medium supplemented with FBS and pre-plated for 1.5 hours. Suspended germ
474 cells were harvested, pelleted at 300 x *g* for 8 minutes, then processed for RNA and
475 DNA. We validated the purity of gonocytes by bisulfite sequencing of *Igf2r*, which is
476 expected to be extensively hypomethylated in the male germline [**Error! Bookmark not**
477 **defined.**].

478 **Oocyte collection**: 28 to 42 day old females were superovulated with 5 IU
479 human chorionic gonadotropin (Millipore 367222) followed by pregnant mare serum
480 gonadotropin (Millipore 230734) 48 hours later. Oocytes were collected the next morning
481 via standard methods [82] and processed for total RNA via Trizol. Oocyte recovery was
482 evaluated using RT-PCR primers for Zp3 (PDS 2212-3).

483 **Genomic DNA extraction from tails and cells**: All DNA was collected via
484 overnight incubation at 55°C in Laird's Lysis Buffer [83] and 20 ug/mL Proteinase K
485 followed by isopropanol precipitation and resuspension in TE. Scant gDNA samples,
486 such as those from gonocytes, were co-precipitated with 20 ug glycogen (Thermo Fisher
487 R0551) and spun at 20,817 *x g* for 15 minutes.

488 **DNA extraction from sperm:** The caudal epididymis and vas deferens of adult
489 male mice were harvested and placed in PBS for 1 hr at 37°C. Large tissue chunks were

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490 manually removed and the remaining sperm were pelleted at 400 x g for 12 minutes at
491 4ºC. Supernatant was discarded; the pellet was resuspended in the remaining
492 supernatant and incubated with 1 mL somatic cell lysis buffer [84] for 1 hr on ice. Lysed
493 samples were spun at 20,817 x g for 3 minutes at 4ºC and the supernatant was
494 discarded. The sperm pellet was resuspended in remaining supernatant; 2 uL was mixed
495 with Trypan Blue and examined microscopically for remaining somatic contamination.
496 The remaining suspension was mixed with 500 uL Buffer RLT (Qiagen 79216) and 150
497 mM DTT, then homogenized with 2 2mm steel beads following the protocol of Wu *et al*
498 [85]. DNA extraction then proceeded as described above.

499 **Bisulfite conversion, BS-PCR, and COBRA:** Bisulfite conversion was
500 performed using the Zymo Research EZ DNA Methylation-Lightning Kit (Zymo Research
501 D5031). BS-PCR was performed with PDS 271-272 for the wild-type DMR, PDS 272-
502 2627 for the *tm5.1* or Tg DMR, PDS 2934-5 for the *Igf2r* DMR, and 271-287 for the *tm1*
503 DMR using NEB Epimark HotStart *Taq* DNA Polymerase (NEB M0590) following the
504 following cycling parameters: 95ºC for 30s, 40X (95ºC 15s, 55ºC 30s, 68ºC 30s) with the
505 exception of PDS 271-287, where the annealing temperature used was 58ºC. COBRA
506 was performed with primers PDS 271-272 after bisulfite treatment via direction addition
507 of 5U of *Bst*UI and digestion at 60ºC for 1 hour, followed by electrophoresis on a 4%
508 agarose gel. In this assay, digestion products arise from methylated DNA; unmethylated
509 DNA resists digestion.

510 **Targeted sequencing analysis:** PCR products were pooled, column cleaned
511 with the BioBasic EZ-10 DNA Columns (BioBasic BS427), eluted in 30 uL of Tris-HCl pH
512 8.0, and quantified via Nanodrop. NEBNext Universal Adaptors were ligated using T4
513 DNA Ligase in Quick Ligase Buffer in a total volume of 22 uL at 25□C for 15 minutes.
514 Self-ligated adaptors (adaptor dimer) was excluded with a 0.8X (2.55 uL) Agencourt
515 AMPure XP bead cleanup (Beckman Coulter A63880) followed by 1X PEG-NACl buffer

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516 (25% PEG, 2.5 M NaCl in DEPC water) cleanup to exclude self-ligated adaptors.
517 Barcodes were added via PCR amplification for 20 cycles with Phusion HF (NEB
518 M0530L) using NEBNext Multiplex Oligos for Illumina® (NEB E7335). Adaptor dimer
519 was again excluded via 0.8X AMPure bead cleanup followed by a 1X PEG Buffer-NaCl
520 cleanup using the same beads. Libraries were quantified using Qubit, and sequenced on
521 a MiSeq 2000 Paired End 2 x 250bp at the Cornell University Genomics Core. Samples
522 were evaluated for quality and trimmed to 200bp with Trim Galore!
523 (www.bioinformatics.babraham.ac.uk). Trimmed samples were probed for WT, *tm5.1*, or
524 Igf2r DMR-specific sequences using the grep function; these reads were compiled into
525 separate files and analyzed using QUMA [86]. Animals with a minimum of ten reads
526 were included for analysis. Total reads per amplicon per sample are listed in Table S2.
527 Grep sequences and reference sequences for QUMA are listed in Table S3.

528 **AUTHORS' CONTRIBUTIONS:**

529 ETC, DHT, and PDS designed experiments. ETC, MH, and DHT performed
530 experiments. ETC and PDS wrote the manuscript. All authors read and approved the
531 final manuscript.

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534 of these data. We thank Maria Garcia-Garcia for generous donation of *Sox2-Cre* mice.
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536 used to genotype the *pA-TetOFF* and *TetOFF* alleles.

537 **COMPETING INTERESTS:**

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538 The authors have declared that no competing interests exist.

539 **ETHICAL APPROVAL AND CONSENT TO PARTICIPATE:**

540 All animal studies were approved by the Institutional Animal Care and Use Committee
541 protocol 2002-0075. No studies included human subjects.

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548 to publish, or preparation of the manuscript.

549 **FIGURE LEGENDS:**

550 **FIGURE 1. Schematics and pitRNA expression of the wild-type *Rasgrf1* and**
551 ***Rasgrf1*$^{tm5.1PDS}$ alleles. a)** Schematics of the wild-type *Rasgrf1* and *Rasgrf1*$^{tm5.1PDS}$
552 alleles. Green box represents the *Rasgrf1* differentially methylated region (DMR). The
553 Imprinting Control Region (ICR) lies 30 kb upstream of *Rasgrf1* coding sequence (purple
554 box), and includes the DMR and the 1.6kbp *Rasgrf1* repeats (yellow triangles). Grey
555 blocks denote 5' and 3' homologous arms of the targeting vector pETC6, described in
556 Methods. Orange triangle downstream of *Rasgrf1*$^{tm5.1PDS}$ is a residual frt site. Figures are
557 not drawn to scale. **b)** pitRNA expression postnatal day 1 testes of wild-type,
558 *tm5.1/tm5.1*, and *TetOFF*:*tm5.1/tm5.1* animals. *TetOFF* transactivates *tm5.1* to
559 physiological levels; *tm5.1* homozygotes express undetectable levels of pitRNA in the

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560 absence of *TetOFF*. Error bars represent standard error across biological triplicates. ***,
561 p < 10-6; n.s., not significant; n.d., not detected at 40 cycles.

562 **FIGURE 2. Induction of pitRNA in the male germline does not impart methylation**
563 ***in cis*, at the *tm5.1* DMR, or in *trans*, at the *Rasgrf1* DMR**. DNAs were collected from
564 gonocyte and somatic fractions of male embryos (a-d) and mature sperm from adult
565 males (e). The somatic and germline fractions of one male is shown in each panel a-d;
566 panel e shows bisulfite analysis of the sperm of two males (#1 and #2). Animals were on
567 the C57BL/6 or FVB/n (FVB, c, d) backgrounds. Mothers were fed chow containing (+
568 dox), or lacking (– dox) doxycycline between mating and birth. Bisulfite PCR was done
569 using PCR primers specific to the *tm5.1* DMR or, as controls, the DMRs from wildtype
570 (WT) *Rasgrf1* and *Igf2r*. WT and *Igf2r* are respectively expected to be hyper- and
571 hypomethylated in male gonocytes; *Igf2r* is expected to be 50% methylated in soma.
572 Number of CpG dinucleotides assayed from *tm5.1*, WT, *tm5.1*and *Igf2r* totaled 15, 17,
573 and 12 respectively. MiSeq libraries prepared from PCR products were sequenced to a
574 minimum of 12 reads per sample for the two *Rasgrf1* alleles (range: 12-28,067; median:
575 135). Data are reported as the percentage of reads showing the methylation levels
576 indicated in the Bisulfite Key at lower right. *Igf2r* hypomethylation in the gonocyte
577 fractions of each sample indicate minimal somatic contamination. Consistent
578 hypomethylation of the *tm5.1* allele in each pedigree and sample indicates pitRNA
579 expression in the absence of the repeats is insufficient for DMR methylation.

580 **FIGURE 3:** *TetOFF*-mediated transactivation does not affect *tm5.1* DMR methylation in
581 neonatal tail. **a**) Targeted bisulfite analysis of WT and *tm5.1* DMRs in neonatal tail
582 gDNA; pedigree shown at top. *tm5.1* is hypomethylated regardless of parental descent
583 and presence of *TetOFF*. The WT DMR is methylated depending on parent of origin—if

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584 inherited maternally, the wildtype DMR is hypomethylated; if inherited paternally, the
585 wildtype DMR is hypermethylated. Two biological replicates for all genotypes are shown
586 (#1 and #2) except for the *TetOFF:tm5.1/+* genotype, where one animal is shown. **b)**
587 Targeted bisulfite analysis of 5.1/+, *tm5.1*/*TetON*, and *TetON*/*tm5.1* neonatal tail gDNA.
588 In all genotypes, *tm5.1* DMR is hypomethylated; as expected, the wild-type DMR is
589 methylated if inherited paternally as in *tm5.1* and *tm5.1*/*TetON* animals and
590 hypomethylated if inherited maternally as in *TetON*/*tm5.1* animals. Reads for two
591 animals (#1 and #2) per genotype are shown. **, $p < 10^{-3}$; ***, $p < 10^{-6}$; n.s., not
592 significant.

593 **FIGURE 4. Transactivation of *tm5.1* upregulates *Rasgrf1* expression from *tm5.1* in**
594 **neonatal brain**. **a** and **d)** Pedigrees of animals used respectively for **b**, **c)**, and **e**, **f)**. **b,**
595 **e)** *Rasgrf1* expression levels were assayed by qRT-PCR using neonatal brains from the
596 pedigrees, and genotypes shown directly above in **a** and **d)**. Error bars represent
597 standard error from, at minimum, biological triplicates. *Rasgrf1* levels were normalized to
598 *Rpl32*, and levels in WT mice were arbitrarily set at 1. **c** and **f)** Relative expression of
599 *Rasgrf1* from the two parental alleles (maternal pink; paternal, blue). Allelic assignments
600 were made by sequencing libraries of RT-PCR products amplified using primers that
601 span *Rasgrf1* polymorphisms, which distinguish *tm5.1*, made on the 129S4 background,
602 and the WT allele, contributed by *TetOFF* or *TetON* mice on the C57Bl/6 background.
603 Products were sequenced to a minimum of 15 reads per animal from at least two
604 biological replicates. *Rasgrf1* is dramatically downregulated in *+/tm5.1* animals, that
605 inherit *tm5.1* paternally, with most residual expression coming from the largely silent
606 maternal allele. The *tm5.1* allele increases *Rasgrf1* expression in a *Tet* transactivator-
607 dependent manner, regardless of the parental origin of *tm5.1*. No doxycycline treatments

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608 were applied for **c** and **d)**, but were applied for **e** and **f)**. *, $p < 0.05$; **, $p < 0.01$; ***, $p <$
609 10^{-6}.

610 **FIGURE 5. Regional transcription is perturbed in *TetOFF*: +/5.1 and *TetOFF*: 5.1/+**
611 **brain and testes. a)** Two regions of chromatin interactions (black bars) are annotated at
612 the *Rasgrf1* locus as predicted by cathepsin ChiA-PET in mouse embryonic stem cells
613 [45]; locations are shown relative to the genes indicated below in **b).** Lengths of each
614 predicted interaction are shown in kilobases (kb) within each bar. Relative log 2
615 expression levels in neonatal brain **b),** and neonatal testes **c)** of transcripts from
616 chr9:89,60,000-90,100,000 are shown, displayed left to right as they are located 5' to 3'.
617 In brain, *TetOFF* exerts dramatic upregulation of *Rasgrf1* and nearby transcripts
618 *AK015891*, *AK029869*, and an annotated ATOH1 binding site 3' of the *Rasgrf1* repeats
619 (*ATOHe*) in mice with both paternally and maternally inherited *tm5.1*. However, distant
620 transcripts *Tmed3* and *Ctsh* are downregulated modestly in brains of offspring inheriting
621 *tm5.1* maternally. **c)** Log10 expression of four transcripts (a subset of the nine assayed
622 in brain) in neonatal testes. While *ATOHe* and *Ctsh* are upregulated when *tm5.1* is
623 transactivated and paternally inherited, *Ankrd34c*, *ATOHe*, and *Ctsh* are downregulated
624 when *tm5.1* is transactivated and inherited maternally. All expression data are relative to
625 Rpl32. **Color Key** at lower right. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 10^{-6}$.

626 **FIGURE 6. Working model for regulation of methylation at *Rasgrf1* in the male**
627 **embryonic germline and neonatal brain.** For neonatal brain, only the paternal allele is
628 depicted. **a)** At the wildtype *Rasgrf1* DMR, the *Rasgrf1* repeats (yellow triangles) drive
629 expression of the pitRNA antisense to the DMR (transcription depicted by green arrow)
630 and increase the likelihood that the DMR is hypermethylated (filled lollipops), likely by
631 Dnmt3c, in the male germline. **b)** *Rasgrf1*$^{tm1\mathrm{PDS}}$ (tm1) lacks the *Rasgrf1* repeats (open

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632 brackets). pitRNA is not expressed and methylation is not established at the DMR in the
633 germline (open lollipops). **c)** *Rasgrf1*$^{tm5.1PDS}$ (*tm5.1*) is not methylated in the male
634 germline. **d)** *Rasgrf1*$^{tm5.1PDS}$ transactivation with *TetOFF* (orange half circle labelled TA)
635 induces pitRNA expression but does not impart methylation to the *tm5.1* DMR. **e)** In
636 neonatal brain, *Rasgrf1* is paternally methylated and expressed. **f, g)** *tm1* and *tm5.1*
637 brains have severely decreased *Rasgrf1* expression. **h)** transactivation of tm5.1 causes
638 strong upregulation of *Rasgrf1* but the *tm5.1* DMR remains hypomethylated. **Outset:**
639 *TetOFF*-mediated transactivation of *tm5.1* affects nearby transcription differentially
640 depending on parental inheritance (blue and pink lines). Upregulated and downregulated
641 genes are depicted by red and blue boxes respectively; unchanged genes are depicted
642 in gray. TetO and DMR are indicated by the pink and green boxes respectively; *TetOFF*
643 binding is indicated by the orange half circle.

644 **SUPPORTING INFORMATION CAPTIONS:**

645 **Figure S1. Validation of *Rasgrf1*$^{tm5.1PDS}$. a)** Generation of *Rasgrf1*$^{tm5.1PDS}$ from
646 *Rasgrf1*$^{tm5.0PDS}$. *Rasgrf1*$^{tm5.0PDS}$ (**a**, upper schematic) was generated via CRISPR-Cas9-
647 mediated homology-directed repair in v6.5 embryonic stem cells. Targeting was
648 confirmed by Sanger sequencing of PCR products generated with primers PDS 2359-8
649 and PDS 2344-2263, which respectively span the junctions of the 5' and 3' homologous
650 arms of the pETC6 vector (grey boxes), and the flanking sequences of the
651 *Rasgrf1*$^{tm5.0PDS}$ allele. PDS 2344 falls within the neo resistance cassette (n). PDS 2358
652 sequence falls partially within TetO. DNAs from Wild-type (WT) animals show no product
653 for either PDS 2359-8 or PDS 2344-2263. ***Rasgrf1*$^{tm5.1PDS}$** (*tm5.1*, a, lower schematic)
654 was generated by crossing *Rasgrf1*$^{tm5.0PDS}$ males to females constitutively expressing

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655 FlpE recombinase (FlpE). Recombination was confirmed with Sanger sequencing of
656 PCR products generated with primers PDS 2262-2263. PDS 2262 maps to TetO
657 sequence, and the product contains the single residual frt site (orange triangle)
658 remaining after recombination. PCR products shown below the amplicon schematics
659 arise only when using DNAs from mutant animals. **b)** Schematic for Southern blot shown
660 in **c)**; probe location, shown with pink line, is outside of the targeting vector; location of
661 *Pst*I sites are indicated. Yellow triangles indicate the *Rasgrf1* repeats. Homologous arms
662 are omitted for clarity. **c)** Southern blot of a *tm5.1* heterozygote vs. a wild-type animal
663 and a *Rasgrf1*^{tm1} homozygote (*tm1*); in these animals, the probe detects the same 3kb
664 fragment in *tm1* as is *tm5.1*. **d)** Copy number qPCR support targeting of the endogenous
665 allele: *tm5.1* heterozygotes have half the copy number of the repeat element as WT
666 animals, but the same number of DMR sequences. Error bars represent standard error
667 across biological duplicates.

668 **Figure S2. The *tm5.1* allele lacks imprinted methylation and expression in the**
669 **absence of transactivator.** Summary of *Rasgrf1* expression, *Rasgrf1* imprinting, and
670 total animals measured in *tm5.1* animals of the **a)** N2 and **b)** N3 generations. The
671 majority of animals inheriting *tm5.1* paternally (+/*tm5.1*) expressed *Rasgrf1* biallelically
672 as assayed by endpoint PCR, but levels were only 2% those seen in wild-type (WT)
673 mice, and the alleles were unmethylated. However, a subset of +/*tm5.1* animals (4 of 10
674 in the N2, and 2 of 15 in the N3 generation) expressed *Rasgrf1* at WT levels from the
675 paternal *tm5.1* allele. N3 animals expressing *Rasgrf1* paternally and at wild-type levels
676 did not arise from methylated fathers, indicating that sporadic *tm5.1* DMR methylation,
677 and *Rasgrf1* expression was not an inherited state. This is consistent with findings with
678 the *Rasgrf1*^{tm1PDS} allele which lacks the *Rasgrf1* repeats [21]. **c)** Allele-specific
679 expression analysis. A male chimera prepared using C57BL/6 blastocysts, and v6.5 ES

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680 cells with the *tm5.1* allele on the 129S4 (129) background, was crossed with C57BL/6
681 females. Neonatal brain cDNA was subjected to endpoint RT-PCR using primers
682 spanning SNPs from the 129 and C57BL/6 backgrounds that harbor distinct *Aci*I sites.
683 Product digestion with *Aci*I produces allele-specific bands, reporting the expressed
684 allele(s). The slowest and fastest migrating bands represent the 129 paternal *Tm5.1*
685 allele; the two middle bands represent the C57BL/6 allele. WT animals, inheriting the
686 C57BL/6 paternal allele, expressed *Rasgrf1* solely from the WT C57BL/6 allele(s). A
687 portion of +/*tm5.1* animals express paternally from the *tm5.1* 129 allele. The majority
688 express biallelically from the maternal C57BL/6 and paternal 129 alleles. **d)** qRT-PCR of
689 *Rasgrf1* in wild-type, and +/*tm5.1* animals. Paternally expressing +/5.1 animals express
690 *Rasgrf1* at WT levels, whereas biallelically expressing +/5.1 animals express at 2% of
691 WT, indicating biallelic expression detected in *tm5.1* by endpoint PCR was seen when
692 the normally active paternal allele was silent. Error bars represent standard error across
693 at least biological triplicate. **e)** Targeted bisulfite sequencing (bar graphs) and COBRA
694 analyses (gel images) of the WT DMR in tail gDNA of WT animals (left), and the *tm5.1*
695 DMR of +/*tm5.1* animals with paternal (middle) and biallelic (right) *Rasgrf1* expression.
696 Animals with paternal expression were methylated at the *tm5.1* DMR, whereas animals
697 with biallelic expression were hypomethylated. WT animals have two copies of the WT
698 *Rasgrf1* DMR, one hypermethylated and one hypomethylated. As such, bisulfite analysis
699 of the WT DMR in soma is 50%. Bar graphs report the percentage of total reads with the
700 levels of methylation shown in the key on the right. COBRA queries methylation at five
701 *Bst*UI sites in both the WT and the *tm5.1* DMRs. "+" and "–" denote addition or lack of
702 *Bst*UI. Methylated (+mC) and unmethylated –mC) sites are respectively sensitive or
703 resistant to *Bst*UI digestion. Digestion products are indicated by black arrowheads. The
704 PCR product of the WT DMR shows partial digestion, reporting the different methylation
705 states of the two parental alleles; paternally expressing +/*tm5.1* show full digestion of the

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706 *tm5.1* DMR; biallelically expressing +/*tm5.1* animals show no digestion. **f)** +/*tm5.1*
707 animals are lower in body weight compared to their WT littermates at several ages post-
708 weaning, consistent with previous findings that loss of paternal methylation and
709 expression at *Rasgrf1* leads to diminished body weight [41]; four to ten mice per
710 genotype at each age were measured.

711 **Figure S3. Generation of the *TetOFF* allele.** *pA-TetOFF* males were crossed with
712 females carrying a *Sox2-Cre* transgene to generate the recombined *TetOFF* allele. PDS
713 2794 maps to the splice acceptor site of the ROSA26 locus; PDS 2795 maps 3' of the
714 *TetOFF* coding sequence, within wild-type ROSA26 sequence. pA-*TetOFF* animals
715 produce a 2.2kb PCR product with PDS 2794-5, whereas *TetOFF* animals produce a
716 1.5kb PCR product due to loss of the floxed Neo-polyA cassette. Note that a portion of
717 animals are mosaic for pA-*TetOFF* and *TetOFF* (mosaic); though only fully recombined
718 mice bearing the *TetOFF* allele were analyzed in crosses with the *tm5.1* allele.

719 **Figure S4. Successful pitRNA induction in *TetON/tm5.1* and *TetOFF/tm5.1* several**
720 **tissues. a)** Endpoint RT-PCR for pitRNA in the tissues of transactivated *tm5.1* 28 to 42
721 day old animals demonstrate induction of pitRNA in several tissues of *TetON: +/tm5.1*
722 and *TetOFF: +/tm5.1* mice. Low signals from testes are likely due to the blood testes
723 barrier restriction entry of doxycycline (dox) in adult males. pitRNA is not detectable by
724 endpoint PCR in wild-type (WT) animals. **b)** qPCR for pitRNA in adult liver shows 10-fold
725 upregulation of pitRNA in *TetON/tm5.1* animals fed dox chow, and 500-fold upregulation
726 of pitRNA in *TetOFF/tm5.1* livers relative to WT. **c)** qPCR for pitRNA in neonatal brain
727 shows nearly 10,000 fold upregulation of pitRNA in *TetOFF/tm5.1* brains relative to *WT*,
728 *TetOFF/+,* and +/*tm5.1* animals. ***, $p < 10^{-6}$; n.s., not significant.

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729 **Figure S5. Additional bisulfite analysis of embryonic and adult male germline. a)**
730 COBRA of the *tm5.1* DMR for the *TetOFF/tm5.1* animal depicted in **Fig 2a** (#1) as well
731 as a littermate of the same genotype (#2). "+" and "–" designate addition of *Bst*UI. The
732 *tm5.1* DMR is hypomethylated in the gonocyte and somatic fractions of both animals.
733 Targeted bisulfite sequencing for #2 is shown below. The paternally inherited wild-type
734 (WT) DMR is hypermethylated in both gonocyte and somatic fractions; the *tm5.1* DMR is
735 hypomethylated in both gonocyte and somatic fractions. The Igf2r DMR is
736 hypomethylated in gonocyte and 50% methylated in soma. **b)** COBRA for the *tm5.1*
737 DMR for the *TetON/tm5.1* + dox animal depicted in **Fig 2c** (#1) as well as a littermate of
738 the same genotype (#2). Bisulfite sequencing results for #2 are depicted below. Note
739 that while the *tm5.1* DMR is hypomethylated in the gonocyte and somatic fractions of #1,
740 it is approximately 50% methylated in #2 by bisulfite sequencing and COBRA. This is
741 consistent with rates of stochastic *tm5.1* DMR methylation as previously described: As
742 described in **Fig S2**, 10-25% of *+/tm5.1* show evidence of *tm5.1* methylation and
743 expression in the soma. Rep #2 is one of six assayed (18%) transactivated *tm5.1*
744 embryonic gonads to display partial methylation of the *tm5.1* DMR in the gonocyte and
745 somatic fraction. **c)** COBRA for the two *+/tm5.1* and two *TetOFF/tm5.1* animals shown in
746 **Fig 2e** (#1 and #2 of each genotype). COBRA results for an additional three *+/tm5.1*
747 animals (#3, #4, #5) are shown; bisulfite results for #3 are below. COBRA from an
748 additional *TetOFF/tm5.1* animal (#3) is also shown. In all samples, the *tm5.1* DMR is
749 hypomethylated. **Bisulfite** Key at lower right.

750 **Figure S6. Transactivation of *tm5.1* with *TetON* and doxycycline induces**
751 **expression of *Rasgrf1* from the *tm5.1* allele, but does not impart methylation to the**
752 ***tm5.1* DMR or affect expression or methylation of the WT allele. a)** *Aci*I digestion of
753 PDS 245-6 endpoint RT-PCR product in the offspring of a *TetON* x *tm5.1* cross. *TetON*:

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754 +/*tm5.1* animal express from the paternal *tm5.1* (129) allele, whereas WT and *TetON*:+/+
755 animals express from the paternal WT (C57BL/6) allele, and +/*tm5.1* animals express
756 weakly and biallelically. **b)** *Aci*I digestion of PDS 245-6 endpoint RT-PCR product in the
757 offspring of a *tm5.1* x *TetON* cross. *TetON*: *tm5.1*/+ animals express *Rasgrf1* biallelically,
758 indicating activation of the maternal *tm5.1* allele in addition to the normally active
759 paternal WT allele. *tm5.1*/+, *TetON*: +/+ and WT animals express paternally from the WT
760 allele. **c)** COBRA for the *tm5.1* and WT DMRs in tail DNA of *TetOFF*: +/*tm5.1* and
761 *TetOFF*: *tm5.1*/+ animals. The *tm5.1* DMR is hypomethylated regardless of parental
762 origin. The WT DMR is unmethylated if inherited maternally (animal at left) but fully
763 methylated, as evidenced by complete digestion of PCR products, if inherited paternally
764 (animal at right).

765 **Figure S7. Oocyte preloading of pitRNA to 90X wildtype levels has no effect on**
766 ***Rasgrf1* expression in wild-type offspring. a)** pitRNA levels in oocytes after induction
767 in *TetON: +/tm5.1* females treated with doxycycline. Error bars represent standard error
768 across biological duplicates **b)** RT-PCR for *Zp3*, an oocyte-specific transcript, was used
769 to confirm isolation of oocytes. **c)** The pedigree shown was used to determine if oocyte
770 loading of pitRNA could produce intergenerational activation of the maternal *Rasgrf1*
771 allele. pitRNA was induced in oocytes of a *TetON: +/tm5.1* female by IP injection of
772 doxycycline for three days prior to mating; doxycycline-containing chow was provided
773 throughout pregnancy. Activation of the maternal allele in neonatal brains of offspring
774 was used to report intergenerational effects, with allele-specific expression assayed by
775 *Aci*I digestion of RT-PCR products. All mice tested expressed only the paternal allele. **d)**
776 *Rasgrf1* levels in brains of animals depicted directly above in c). Error bars represent
777 standard error across biological duplicates at minimum. **e)** COBRA of the wild-type allele
778 in WT, *TetON*: +/+, and *tm5.1*/+ tails from three representative animals from d) (purple

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779 trapezoids). The WT DMR is partially methylated in WT and *TetON*: +/+ animals (having
780 inherited two WT DMRs), and fully methylated in the *tm5.1*/+ animal (having inherited the
781 WT DMR paternally); the *tm5.1* DMR is hypomethylated in this animal. Pale horizontal
782 bar in the top third of each panel represents the dye front. ** $p < 0.01$; n.s., not
783 significant; IP, intraperitoneal.

784 **Table S1. Primer sequences for all analyses described.** All qPCR primers are
785 between 87 and 113% efficient. Unless otherwise noted, all endpoint PCRs were
786 performed using Promega GoTaq in 25 uL reactions, Ta = 60C, te = 30s for 35 cycles.
787 All qPCRs are performed in 20 uL reactions using SYBR Green Master Mix (ABI
788 4367659) on a Biosystems 7500 with annealing temperature 60ºC for forty cycles
789 followed by a dissociation stage.

790 **Table S2. Total reads per DMR by Sample ID.** Amplicons for which less than ten reads
791 were recovered were excluded from analysis. Total number of reads correlates in part to
792 total amplicons for each barcode (variable). n.a., not applicable; n.m., not measured.

793 **Table S3. Sequences used for QUMA.** In preparation for analysis, raw files were
794 probed for reads containing amplicon-specific sequences using the grep function in
795 Linux. QUMA was performed on these reads.

796 **Table S4. Total reads broken down by C57 and FVB fractions for MiSeq**
797 **sequencing of PDS 245-6 RT-PCR product in neonatal brain**. The *tm5.1* allele was
798 generated on a 129 background; *Rasgrf1* expressed from the *tm5.1* allele carries 129
799 SNPs. *TetON* and *TetOFF* are carried on a C57Bl/6 (B6) background; "+" always
800 designates a B6 allele. A B6 x FVB gDNA sample is sequenced as a control. n.i., not
801 included in paper.

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802 **Table S5. SNP IDs and grep sequences for allele-specific PDS 245-6 digestion and**
803 **sequencing**. SNPs were probed for using the grep function and quantified using the wc
804 function in Linux.

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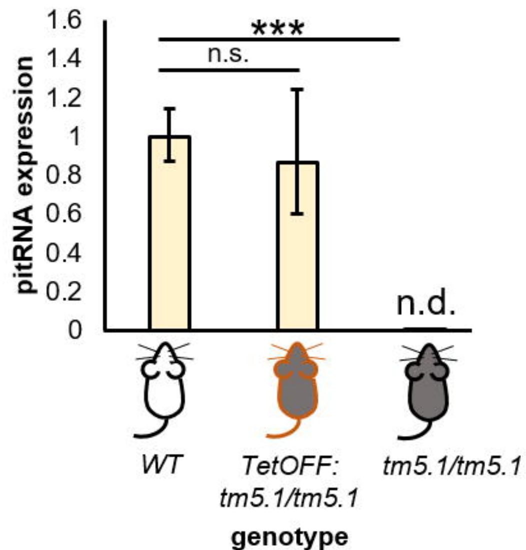
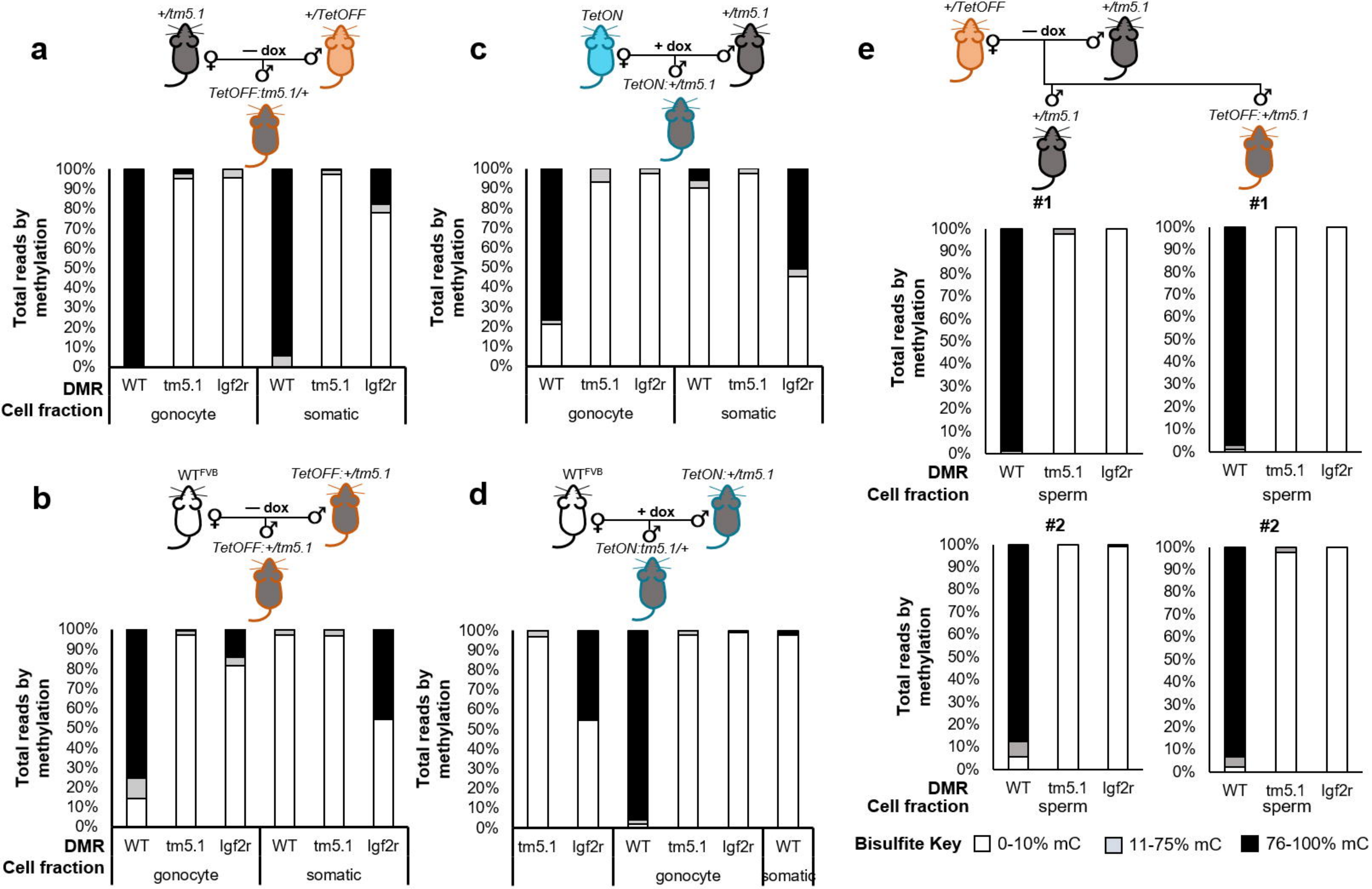
a *Rasgrf1*^{WT}

Rasgrf1$^{tm5.1PDS}$

b





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+/TetOFF
— dox
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WT
tm5.1
Igf2r
WT
tm5.1
Igf2r
Cell fraction
gonocyte
somatic
b
WTFVB
TetOFF:+/tm5.1
— dox
TetOFF:+/tm5.1
c
TetON
+/tm5.1
+ dox
TetON:+/tm5.1
d
WTFVB
TetON:+/tm5.1
+ dox
TetON:tm5.1/+
e
+/TetOFF
+/tm5.1
— dox
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#2
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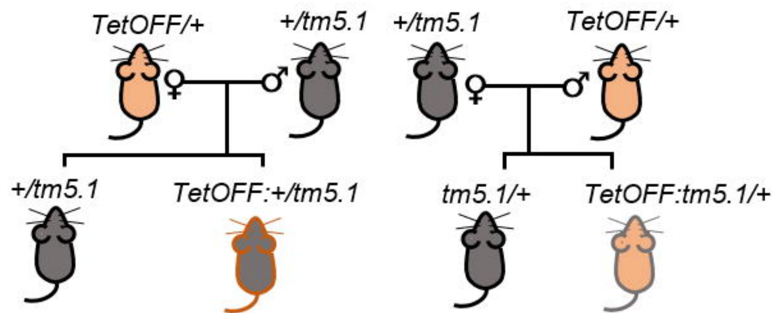
11-75% mC
76-100% mC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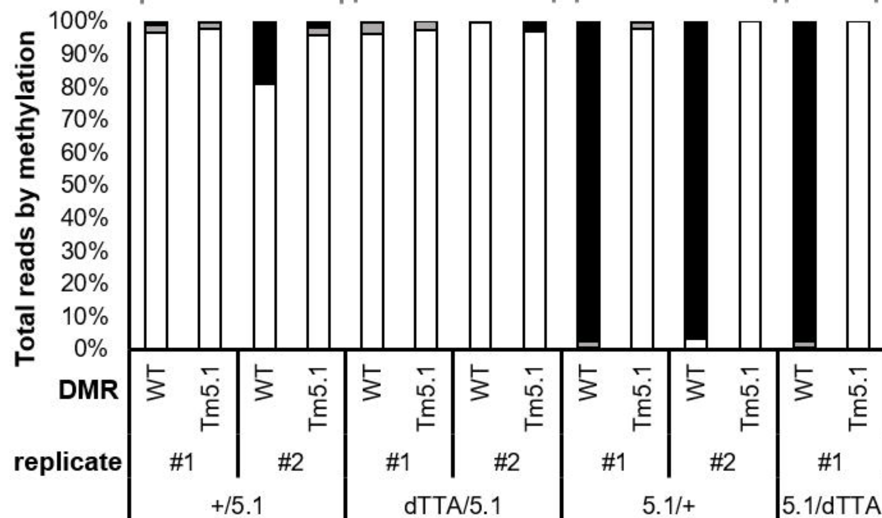
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90%
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70%
60%
50%
40%
30%
20%
10%
0%
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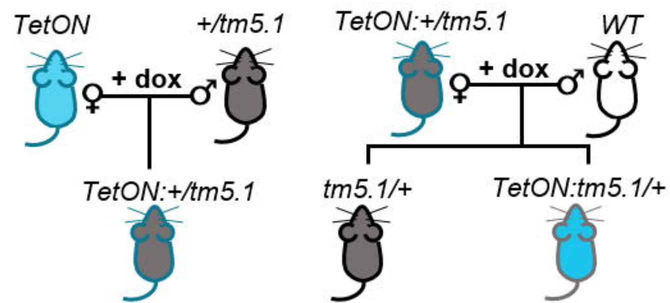
WT
Tm5.1
replicate
#1
#2
+/5.1
dTTA/5.1
5.1/+
5.1/dTTA


b
TetON
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TetON:+/tm5.1
WT
+ dox
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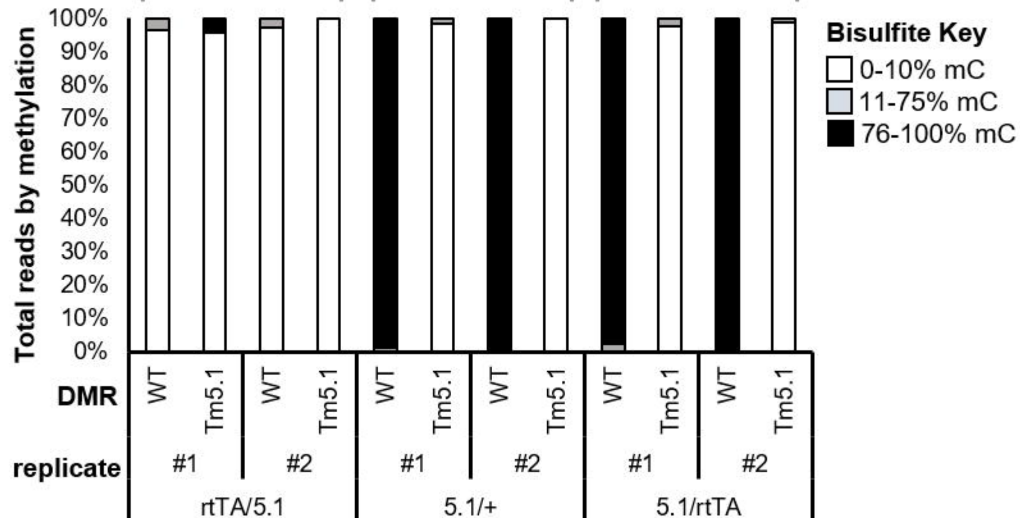
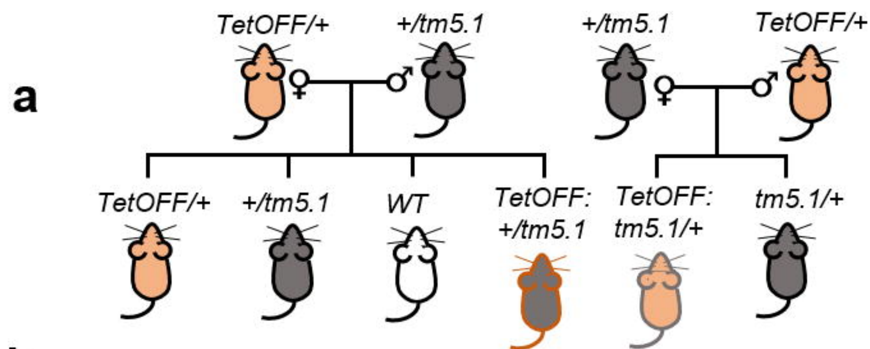
TetON:tm5.1/+


Total reads by methylation
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
DMR
WT
Tm5.1
replicate
#1
#2
rtTA/5.1
5.1/+
5.1/rtTA
Bisulfite Key
0-10% mC
11-75% mC
76-100% mC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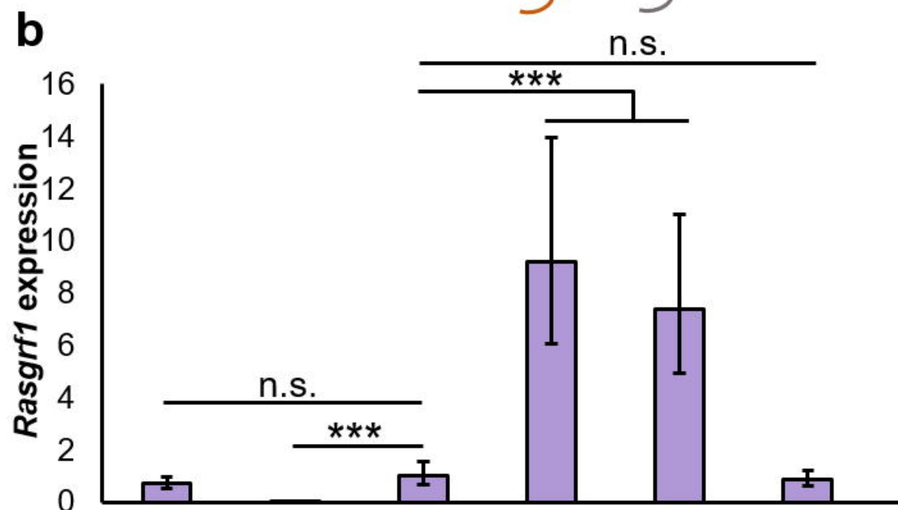
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***
n.s.
***
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10
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6
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2
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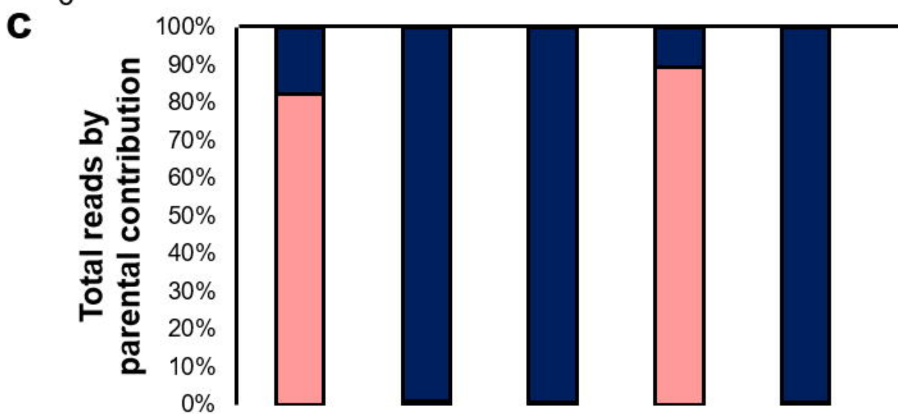
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Total reads by parental contribution
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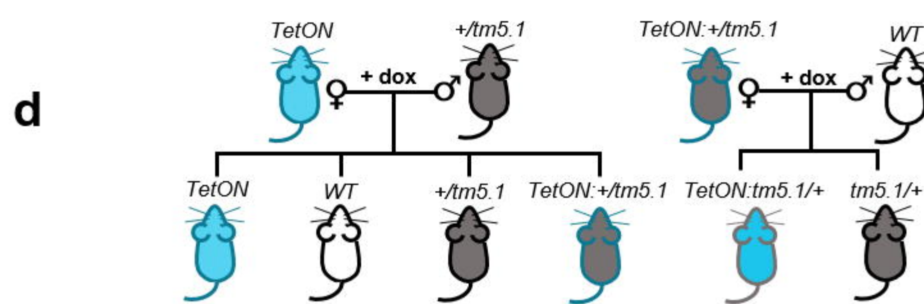
20%
10%
0%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+ dox
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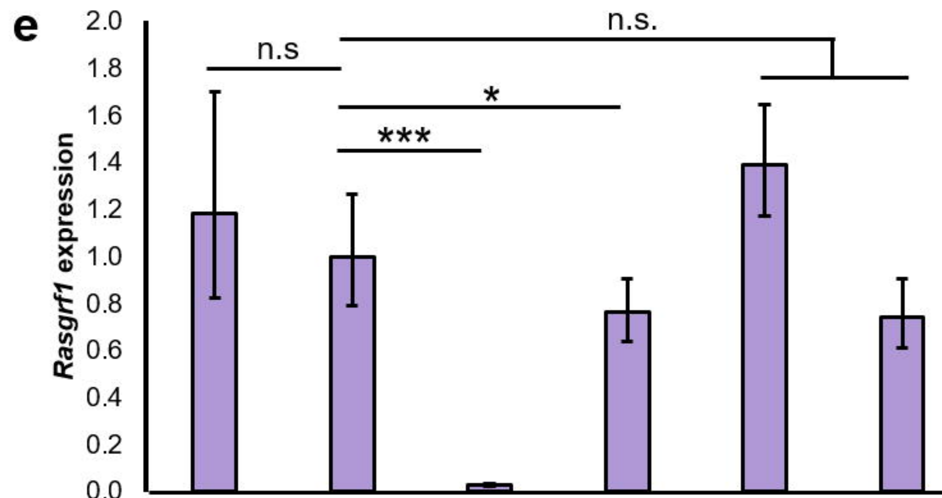
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Rasgrf1 expression
n.s
n.s.
*
***
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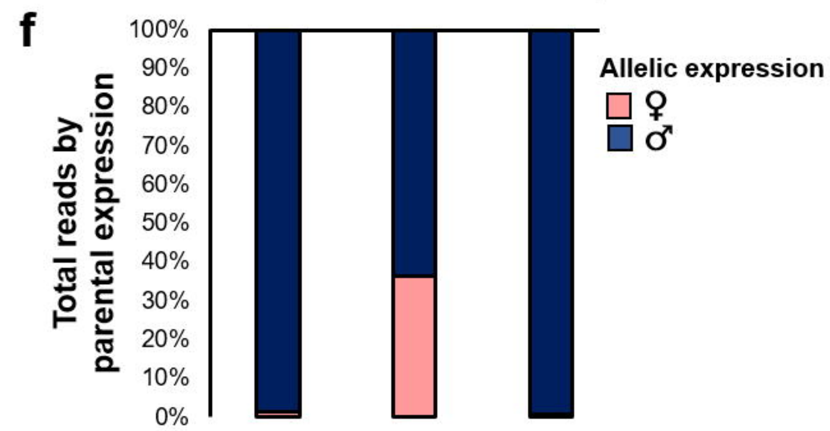
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0

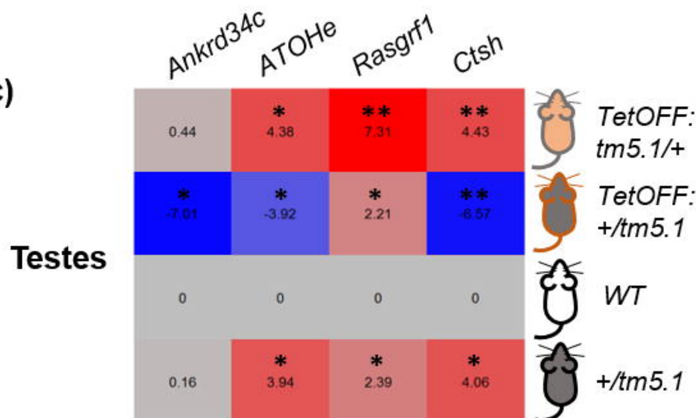

f
Total reads by parental expression
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
Allelic expression
♀
♂

a)

b)

Brain

	Tmed3	Ankrd34c	AK015891	AK029869	ATOHe	Rasgrf1	Ctsh	Morf4l1	Adamts7
TetOFF: tm5.1/+	-0.69	0.7	*** 7.42	*** 11.46	*** 8.93	*** 3.69	-0.64	0.34	0.15
TetOFF: +/tm5.1	*** -1.62	** 1.15	** 5.08	*** 9.23	** 4.53	*** 3.36	** -1.44	-0.14	-0.34
WT	0	0	0	0	0	0	0	0	0
+/tm5.1	-0.13	-0.16	-1.77	0.31	2.88	*** -5.43	-0.17	0.3	1.45



Color Key

-5 0 5

Value

♂embryonic germline

neonatal brain