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

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

64 immediately adjacent to the DMR. Targeted deletion of the *Rasgrf1* repeats
65 (*Rasgrf1*^{tm1PDS}, *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

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

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

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*^{tm5.0PDS}.**

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

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*^{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*.**

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

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.

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

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

pitRNA loading of oocytes does not produce paramutation

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

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

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-

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

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

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

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

359

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

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

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

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

392 MATERIALS AND METHODS:

393 **Primer sequences** for all analyses are listed in Table S1.

394 **TetO vector generation:** We modified pYP1, which carries the DMR and
395 *Rasgrf1* repeats, and 4 kb of homologous flanking sequence [77] to carry seven copies
396 of the Tet Responsive Element (collectively termed TetO) in place of the *Rasgrf1* repeats
397 as follows. The *Rasgrf1* repeats were removed *via* restriction digest with *Clal* and *MluI*;
398 sticky ends were blunted with Klenow; the plasmid backbone was gel purified and then
399 ligated closed generating pDHT2. pDHT2 and pPX3, a vector containing the Tet
400 Operator, were digested with *NheI*, and linearized pDHT2 was ligated to the TetO
401 sequences, generating pDHT3, which was confirmed by Sanger sequencing. The 3'
402 homologous arm of pDHT3 was then shortened to approximately 1kb by restriction
403 digest with *BsrGI* and *SfiI* to generate pETC6, which was linearized with *PciI* prior to
404 lipofection into ES cells.

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

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

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 (95°C 2min, 40 cycles of 95°C 30s, 60°C 30s, 72°C 30s, 72°C 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*. 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 60°C 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

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

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

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

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

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

AUTHORS' CONTRIBUTIONS:

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

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COMPETING INTERESTS:

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
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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

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**

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

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

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

FIGURE 6. Working model for regulation of methylation at *Rasgrf1* in the male embryonic germline and neonatal brain. For neonatal brain, only the paternal allele is depicted. **a)** At the wildtype *Rasgrf1* DMR, the *Rasgrf1* repeats (yellow triangles) drive expression of the pitRNA antisense to the DMR (transcription depicted by green arrow) and increase the likelihood that the DMR is hypermethylated (filled lollipops), likely by Dnmt3c, in the male germline. **b)** *Rasgrf1*^{tm1PDS} (*tm1*) lacks the *Rasgrf1* repeats (open

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

SUPPORTING INFORMATION CAPTIONS:

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

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 *PstI* 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

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* sites.
683 Product digestion with *AcI* 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 *+tm5.1* animals express
690 *Rasgrf1* at WT levels, whereas biallelically expressing *+tm5.1* 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

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.

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* digestion of
 753 PDS 245-6 endpoint RT-PCR product in the offspring of a *TetON* x *tm5.1* cross. *TetON*:

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* 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* 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

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, $T_a = 60^\circ\text{C}$, $t_e = 30\text{s}$ 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 60°C 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.

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

a)

b)

Brain

c)

Testes

Color Key

♂ embryonic germline

neonatal brain