Trans allele methylation and paramutation-like effects in mice

Herry Herman[1,2,3], **Michael Lu**[2,4], **Melly Anggraini**[2], **Aimee Sikora**[2], **Yanjie Chang**[2], **Bong June Yoon**[2,4], and **Paul D Soloway**[1,2]

[1]Cornell University, Division of Nutritional Sciences, Ithaca, New York, USA.

[2]Roswell Park Cancer Institute, Buffalo, New York, USA.

[3]Department of Orthopaedic Surgery, School of Medicine, Padjadjaran University-Hasan Sadikin General Hospital, Bandung, Indonesia.

Abstract

In mammals, imprinted genes have parent-of-origin–specific patterns of DNA methylation that cause allele-specific expression. At *Rasgrf1* (encoding RAS protein-specific guanine nucleotide-releasing factor 1), a repeated DNA element is needed to establish methylation and expression of the active paternal allele[1]. At *Igf2r* (encoding insulin-like growth factor 2 receptor), a sequence called region 2 is needed for methylation of the active maternal allele[2,3]. Here we show that replacing the *Rasgrf1* repeats on the paternal allele with region 2 allows both methylation and expression of the paternal copy of *Rasgrf1*, indicating that sequences that control methylation can function ectopically. Paternal transmission of the mutated allele also induced methylation and expression in *trans* of the normally unmethylated and silent wild-type maternal allele. Once activated, the wild-type maternal *Rasgrf1* allele maintained its activated state in the next generation independently of the paternal allele. These results recapitulate in mice several features in common with paramutation described in plants[4].

Rasgrf1 is methylated on the paternal allele in a differentially methylated domain (DMD) 30 kb 5′ of the promoter. Expression is from the paternal allele in neonatal brain[5]. This imprinting requires a 1.6-kb repeated element located immediately downstream of the DMD consisting of a 41-mer repeated 40 times that regulates establishment of methylation at the DMD[1,6]. The DMD is a methylation-sensitive enhancer-blocking element, which, together with the repeats, functions as a binary switch that regulates imprinting. Sequences regulating DNA methylation have been identified for one other locus, *Igf2r*. In intron 2 of *Igf2r*, region 2 controls methylation and allele-specific expression[2,3,7].

We generated mice containing *Igf2r* region 2 in place of the *Rasgrf1* repeats to determine if their activities overlap. Reciprocal crosses were done between mice heterozygous with respect to this allele (*Rasgrf1*[tm3.1Pds], Fig. 1) and PWK mates to monitor expression from the two alleles in neonatal brain[5]. Similar crosses were done with C57BL/6 mates to evaluate changes in methylation of the *Rasgrf1* DMD.

Maternal transmission of the *Rasgrf1*[tm3.1Pds] allele (*Rasgrf1*^{−/+}) had no effect on methylation or expression of the locus, which remained paternal allele–specific and was expressed at levels

comparable to those in wild-type mice (Fig. 2a and data not shown). Mice with a paternally transmitted repeat deletion lacked both methylation and expression of the paternal allele[1], but paternal transmission of the *Rasgrf1*[tm3.1Pds] mutation (*Rasgrf1*^{+/−}) permitted expression of the locus, albeit at lower levels than in wild-type mice (Fig. 2b). The paternal *Rasgrf1*[tm3.1Pds] allele also caused derepression of the normally silent wild-type maternal allele. Expression of the mutated paternal allele in *Rasgrf1*^{+/−} mice showed that region 2 was able, in part, to replace the function of the *Rasgrf1* repeats.

Trans activation of the wild-type allele was heritable and reversible. Pups with a paternally transmitted *Rasgrf1*[tm3.1Pds] allele had biallelic expression of *Rasgrf1* regardless of which grandparent transmitted the mutation. Pups that had inherited the mutated allele from the mother had normal monoallelic expression (Fig. 2c–e).

Trans activation of the normally silent maternal allele in *Rasgrf1*^{+/−} mice is reminiscent of transvection in *Drosophila melanogaster*[8,9] and paramutation in maize (*Zea mays*; refs. [4,10]). In paramutation, expression of a paramutable allele may be altered by a paramutagenic allele on the homologous chromosome. Once affected, altered expression of the paramutable allele persists through meiosis independently of its homolog. In some cases, an affected paramutable allele can behave as a paramutagenic allele in the next generation, an effect termed secondary paramutation[11]

To determine if *trans* allele activation of the wild-type maternal allele in *Rasgrf1*^{+/−} mice is stable through meiosis, as is the case with paramutation, we carried out crosses using *Rasgrf1*^{+/−} females that had inherited the *Rasgrf1*[tm3.1Pds] allele from strain 129 fathers and the wild-type allele from PWK mothers. We crossed these females, which were shown to have biallelic expression of *Rasgrf1* (Fig. 2b,c,e), with wild-type C57BL/6 males. The wild-type progeny carried a C57BL/6 allele from their fathers (*Rasgrf1*^{+}) and a wild-type PWK allele from their mothers that had been biallelically expressed in the mother (*Rasgrf1*[+d]). These *Rasgrf1*[d/+] progeny fell into two clear phenotypic classes (Fig. 2f). In one, biallelic expression was preserved, indicating that aberrant expression of the maternal *Rasgrf1*[+d] allele was retained through meiosis. In the other class, the maternal allele retained its aberrant expression and the *Rasgrf1*^{+} wild-type paternal allele was inappropriately silenced.

This showed that transmission of the *Rasgrf1*[tm3.1Pds] allele from father to daughter modified the daughter's *Rasgrf1*[+d] wild-type allele in a manner that allowed it to affect expression of both parental alleles in the grandchildren. This recapitulates the two key properties of paramutation: regulation of expression of one allele by the other and stability of this phenotype through meiosis. The class of offspring with a silenced paternal allele showed secondary paramutation, a third property seen in some cases of paramutation[11]. The paramutation-like effects were observed only when the *Rasgrf1*[+d] allele was maternally transmitted, recapitulating a fourth feature of paramutation noted at the *r1* locus in maize, where paramutation was observed only on transmission of the paramutated allele from males[12]. Paternal transmission of the wild-type allele by *Rasgrf1*^{+/−} males led to normal monoallelic expression from the paternal allele (Fig. 2c).

To determine if paramutation required region 2 specifically, we tested the ability of another *Rasgrf1* allele to cause paramutation. The *Rasgrf1*[tm2Pds] allele contained the same deletion as *Rasgrf1*[tm3.1Pds] but a 2.3-kb *Pgk-neo* cassette insertion instead of the 2.8-kb insertion in *Rasgrf1*[tm3.1Pds] (B.J.Y., H.H. and P.D.S., unpublished data). None of the heterozygous progeny of *Rasgrf1*[+/tm2Pds] males expressed *Rasgrf1* from the maternal allele (Fig. 3), showing that the phenotype associated with *Rasgrf1*[tm3.1Pds] resulted from an activity specific to the *Igf2r* sequences.

We have previously shown that *Rasgrf1* expression depends on methylation of the DMD at the expressed locus, which ablates its CTCF-binding and enhancer-blocking activity (ref. [1] and B.J.Y., H.H., Y.C. and P.D.S., unpublished data). Therefore, we hypothesized that the expressed wild-type and mutated alleles of *Rasgrf1*^{+/−} mice should be methylated on the DMD. To test this, we carried out methylation-specific PCR (MS–PCR; ref. [13]) using allele-specific primer pairs and analyzed the PCR products by gel electrophoresis (Fig. 4a,b) and direct sequencing (Fig. 4c). The positive control for detecting wild-type allele methylation was DNA from wild-type mice, which is methylated on the paternal allele. The negative control was DNA from mice carrying a simple deletion of the *Rasgrf1* repeats on the paternal chromosome (*Rasgrf1*[+/tm1Pds]), which prevents establishment of methylation on either DMD[1]. MS–PCR was done using a dilution series of bisulfite-treated DNA to permit comparisons of methylation levels. Paternal transmission of the mutation resulted in methylation of the mutated paternal allele (Fig. 4b), indicating that *Igf2r* region 2 can replace the *Rasgrf1* repeats, causing methylation and expression of the paternal allele, albeit less efficiently than the native repeats. Furthermore, paternal transmission of the *Rasgrf1*[tm3.1Pds] allele imparted methylation to the wild-type maternal allele in *trans*, resulting in its aberrant expression.

Trans allele methylation has been reported in *Ascobolus immersus*[14], maize[15] and mice. Mice with a *U2af1-rs* transgene acquired aberrant methylation on the endogenous paternal allele[16]. A deletion at the maternal *H19* allele reduced methylation at the linked wild-type paternal *Igf2* allele[17]. At the *Rosa26* locus modified with a *lox*P-stop-*lox*P cassette, Cre-mediated recombination led to methylation of the modified locus that was transferred to the unrecombined *lox*P-stop-*lox*P allele on the homologous chromosome in the next generation. Transferred methylation was stable for multiple generations. This was originally characterized as transvection but may represent paramutation[18].

In our system, *trans* methylation and activation of the wild-type maternal *Rasgrf1* allele was initiated after fertilization (Fig. 2). Initiation required a paternal *Rasgrf1*[tm3.1Pds] allele, but communication between wild-type *Rasgrf1*[+d] and *Rasgrf1*^{+} alleles persisted in the next generation in the absence of region 2 (Fig. 2f). This suggested that despite the artificial means of initiating the *trans* allelic interactions, such interactions are normal genomic events. It is not known if initiation involved physical interactions between homologous alleles[19], the sequences in region 2 implicated in *de novo* methylation and allele discrimination[2], the paternal-specific *Air* transcript from the endogenous *Igf2r* locus[3] or the extensive inverted repeat structure in region 2.

Trans allele phenomena are relevant to human disease. Susceptibility to type 1 diabetes has been shown to be sensitive to a *trans* allele effect during male meiosis[20]. Details of the mechanisms underlying methylation control and *trans* allele regulation at *Rasgrf1* may assist in understanding related processes involving alleles associated with disease.

METHODS

Preparation of mutant mice

The *Rasgrf1*[tm3Pds] vector (pML4-2) was a modified form of the *Rasgrf1*[tm1Pds] vector (pBJR3; ref. [1]). We filled in a *Sal*I site at the 3′ end of the 3′ arm of pBJR3 and replaced it with a unique *Asc*I site to produce pML3. We modified a 2.8-kb plasmid containing *Igf2r* region 2 (P4, a gift from D. Barlow[7], University of Vienna, Vienna, Austria) to include *Sal*I sites on either side of the *Igf2r* sequences and inserted the *Sal*I fragment at the single remaining *Sal*I site of pML3. In pML4-2, the *Igf2r* sequences assumed the same 5′ to 3′ orientation with respect to *Rasgrf1* that is assumed at *Igf2r*. We electroporated embryonic stem (ES) cells with pML4-2 linearized by *Asc*I, and we used homologous recombinants identified by Southern-blot hybridization[1] to prepare germline chimeras. We excised the *neo* cassette by crossing male

mutants with *Zp3-cre* transgenic mice[21] to produce the *Rasgrf1*^tm3.1Pds allele. We verified excision using three primer pairs (sequences available on request). We did all experiments with the approval of the Institutional Animal Care and Use Committees at Roswell Park Cancer Institute and Cornell University.

RNA analysis

We prepared RNA and carried out allele-specific assays for *Rasgrf1* as described previously[5,22].

Methylation analysis

We carried out nested MS–PCR on bisulfite-treated DNA, prepared from tail or limb tissue as described[1]. We amplified the wild-type allele using primers WF686 and WR1083, which amplified both methylated and unmethylated templates, followed by a second round of amplification with primers WF714M and WR1005M, which amplified only methylated DNA and resulted in a product of 292 bp. Primer sequences are available on request. We analyzed the products by electrophoresis, direct sequencing using WF714M and WR1005m as the sequencing primers and sequencing after cloning in the TopoTA vector (InVitrogen) using T3 and T7 sequencing primers. We amplified the mutated allele using either one or two rounds of PCR using primers WF803M and LuR1087M (sequences available on request).

ACKNOWLEDGMENTS

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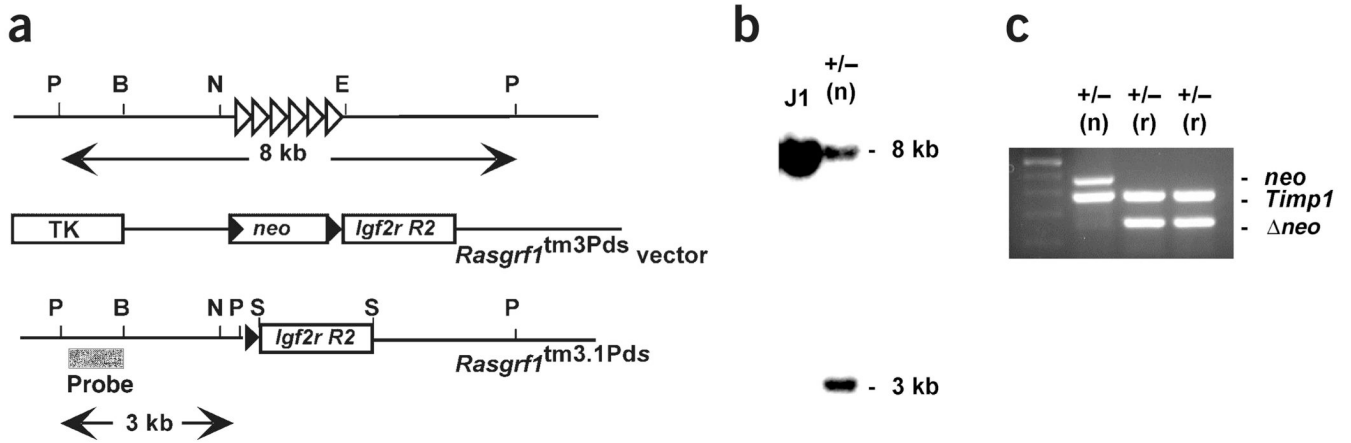
Figure 1.
Generation of mice with the *Rasgrf1*tm3.1Pds allele. (**a**) The wild-type *Rasgrf1* locus, targeting vector and structure of the *Rasgrf1*tm3.1Pds allele are shown. Open triangles represent the *Rasgrf1* repeats 30 kb 5′ of the transcription start site. Immediately upstream of the repeats is the 350-bp DMD. The *neo* cassette flanked by *lox*P sites (filled triangles) and *Igf2r* region 2 (R2) replaced the repeats in the targeting vector. Cre-mediated excision of the *neo* cassette produced the *Rasgrf1*tm3.1Pds allele used in this study. *Pst*I (P), *Bam*HI (B), *Not*I (N), *Eco*RV (E) and *Sal*I (S) sites are shown. (**b**) Hybridization of the probe in **a** to a Southern blot containing *Pst*I-digested DNA from parental J1 ES cells and a targeted clone containing the *neo* cassette (+/−(n)) produced bands of 8.0 kb and 3.0 kb from the wild-type and mutated alleles, respectively. (**c**) DNA from progeny of a cross between males with the +/− (n) allele and *Zp3-cre* transgenic females was analyzed by a PCR assay that showed loss of the *neo* cassette (Δ*neo*) and acquisition of a new band diagnostic of the *Rasgrf1*tm3.1Pds *neo*-deleted allele (+/− (r)). *Timp1* was used as a positive control for amplification.
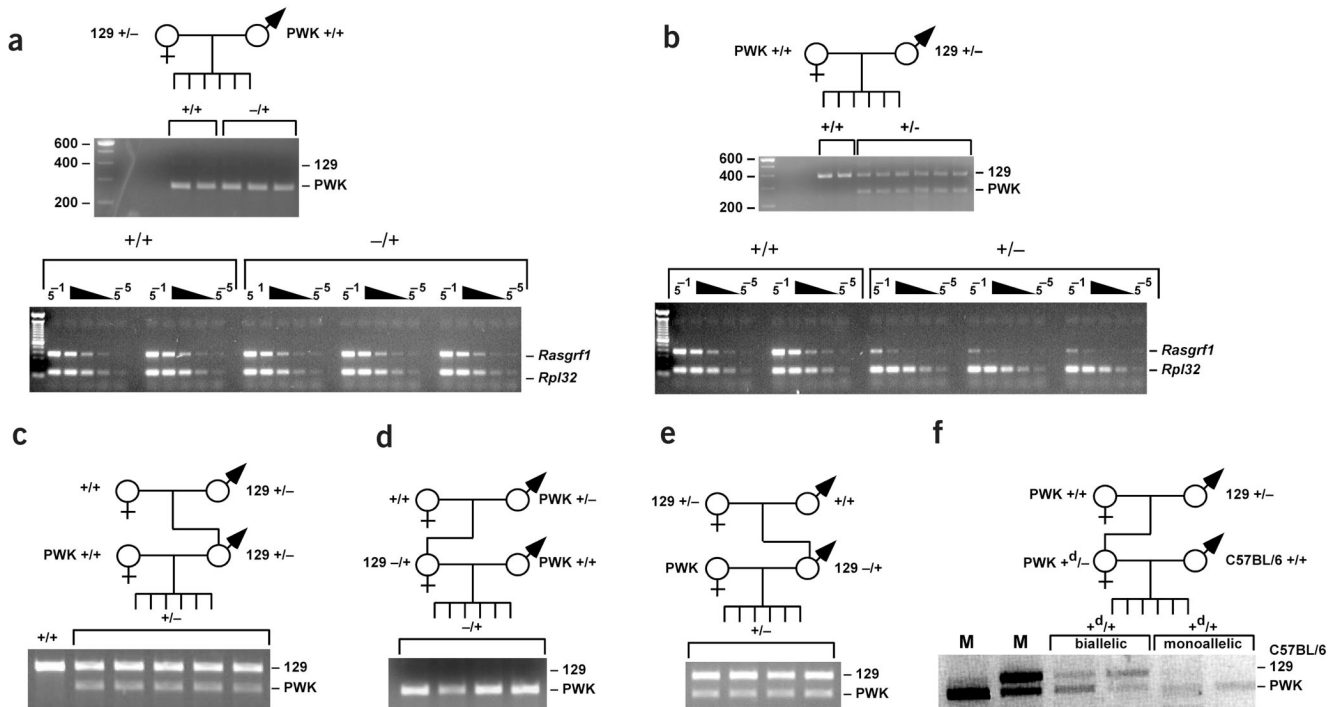
Figure 2.
Paternal transmission of the *Rasgrf1*tm3.1Pds allele caused derepression of the normally silent wild-type maternal allele and paramutation. Allele-specific and quantitative expression analysis of *Rasgrf1* was done using neonatal brain from (**a**) wild-type (+/+) and mutant (−/+) progeny of heterozygous mothers and (**b**) wild-type (+/+) and mutant (+/−) progeny of heterozygous fathers. Assays identifying the expressed allele (middle) were done as described[1,6]. RNA levels (bottom) were measured by RT–PCR using a dilution series (5^{-1} to 5^{-5}) of cDNA and primers for *Rasgrf1* and *Rpl32* (an internal control) as described[1]. Allelespecific expression was determined after (**c**) transmission of the mutated allele for two generations through the male germ line; (**d**) when transmission for the second generation was through the female germ line; and (**e**) after a two-generation cross where the mode of inheritance was through the female germ line for the first generation and through the male germ line for the second generation. A two generation cross served as a test for paramutation (**f**). Wild-type PWK females were crossed with +/− 129 males. Female progeny with a wild-type maternal PWK allele that had been derepressed (*Rasgrf1*+d) were crossed to wild-type C57BL/6 males. The maternal and paternal alleles in resulting wild-type (*Rasgrf1*+d/+) mice were from PWK and C57BL/6, respectively. Allele-specific expression analysis was done on *Rasgrf1*+d/+ mice. Mice expressing both alleles (biallelic) or predominantly the maternal allele (monoallelic) are indicated. Markers (M) were from mice with monoallelic PWK (left lane) or biallelic PWK and 129 expression (adjacent lane). In **a** through **f**, the top panels depict the cross.
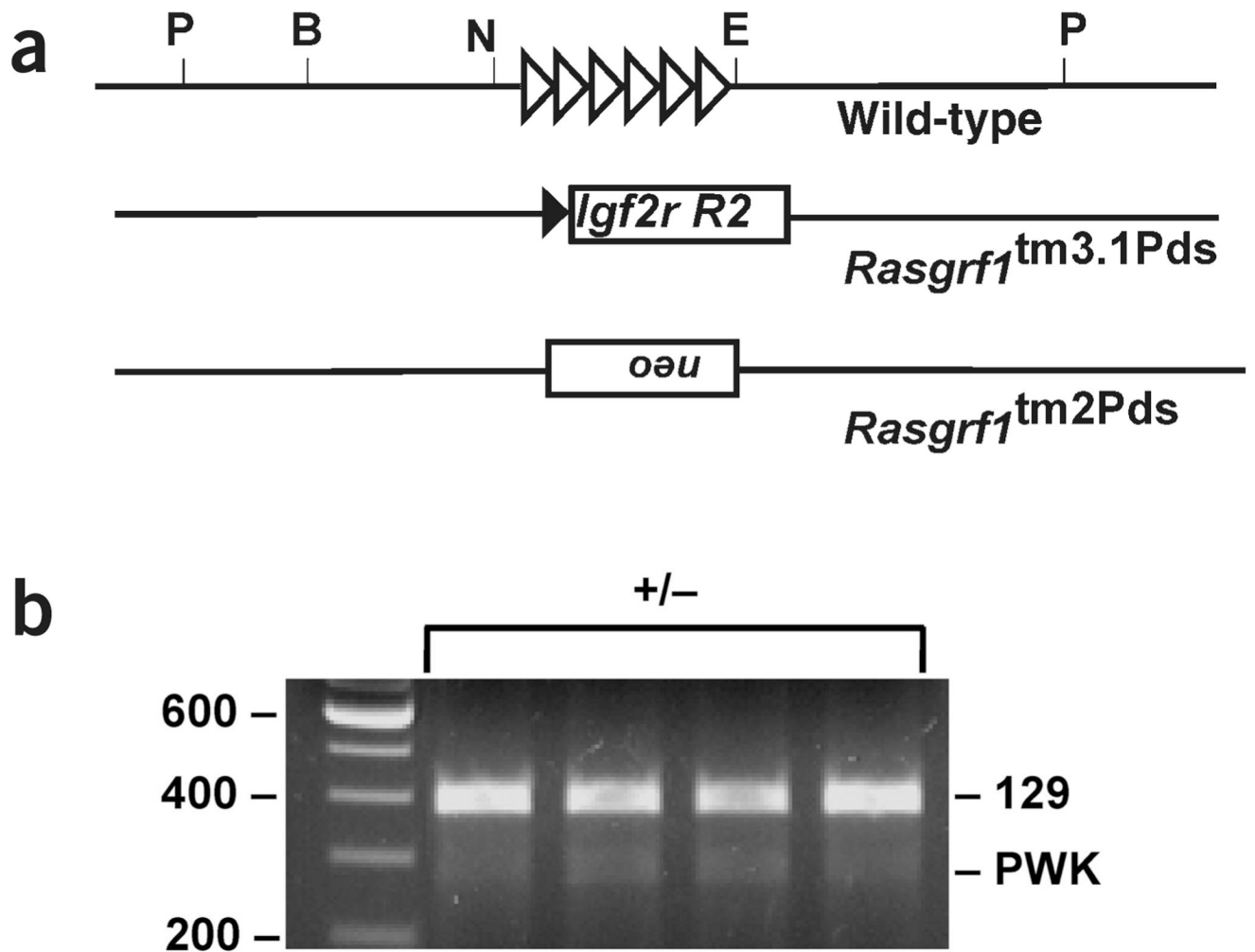
Figure 3.
Trans allele effects caused by insertion of *Igf2r* region 2 are due to the specific sequence changes. (**a**) Males with the *Rasgrf1*tm2Pds allele (bottom line) were crossed with PWK females. (**b**) Allele-specific expression analysis was done on the *Rasgrf1*+/tm2Pds progeny.
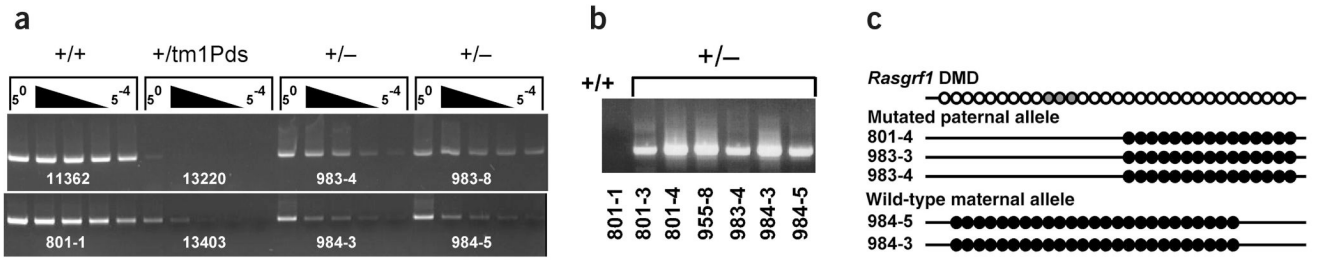
Figure 4.
Paternal transmission of the *Rasgrf1*[tm3.1Pds] allele leads to methylation of both alleles. (**a**) Wild-type allele–specific MS–PCR was done using a dilution series (5^0 to 5^{-4}) of bisulfite-treated DNA isolated from wild-type mice (+/+), mice with a paternally transmitted *Rasgrf1*[tm3.1Pds] allele (+/−) or mice with a paternally transmitted allele previously shown to cause a complete loss of *Rasgrf1* methylation[1] (+/tm1Pds). (**b**) MS–PCR was done using primers specific for the mutated paternal allele. (**c**) Selected products from **a** and **b** were sequenced directly or cloned and sequenced to verify that bands in **a** and **b** arose from methylated DNA. Numbers identify specific mice. The 31 CpG dinucleotides in the DMD are shown as open circles, and the three lightly shaded circles represent CpGs in the *Not*I site (top line). The CpGs assayed on the mutated and wild-type alleles are shown as circles on the lower five lines. Methylated CpGs are depicted as filled circles.