A Non-Coding RNA Within the Rasgrf1 Locus in Mouse Is Imprinted and Regulated by Its Homologous Chromosome in Trans

Chelsea M. Brideau *, Krista P. Kauppinen *, Rebecca Holmes, Paul D. Soloway*

Division of Nutritional Sciences, College of Agriculture and Life Sciences, Cornell University, Ithaca, New York, United States of America

Abstract

Background: Rasgrf1 is imprinted in mouse, displaying paternal allele specific expression in neonatal brain. Paternal expression is accompanied by paternal-specific DNA methylation at a differentially methylated domain (DMD) within the locus. The cis-acting elements necessary for Rasgrf1 imprinting are known. A series of tandem DNA repeats control methylation of the adjacent DMD, which is a methylation sensitive enhancer-blocking element. These two sequences constitute a binary switch that controls imprinting and represents the Imprinting Control Region (ICR). One paternally transmitted mutation, which helped define the ICR, induced paramutation, in trans, on the maternal allele. Like many imprinted genes, Rasgrf1 lies within an imprinted cluster. One of four noncoding transcripts in the cluster, AK015891, is known to be imprinted.

Methodology/Principal Findings: Here we demonstrate that an additional noncoding RNA, AK029869, is imprinted and paternally expressed in brain throughout development. Intriguingly, any of several maternally inherited ICR mutations affected expression of the paternal AK029869 transcript in trans. Furthermore, we found that the ICR mutations exert different trans effects on AK029869 at different developmental times.

Conclusions/Significance: Few trans effects have been defined in mammals and, those that exist, do not show the great variation seen at the Rasgrf1 imprinted domain, either in terms of the large number of mutations that produce the effects or the range of phenotypes that emerge when they are seen. These results suggest that trans regulation of gene expression may be more common than originally appreciated and that where trans regulation occurs it can change dynamically during development.

Introduction

Genes inherited in two copies, one from each parent, are normally functionally equivalent, however, notable exceptions to this have been known for many years [1–5]. Among the genes that are functionally distinguishable according to their parent of origin are the imprinted genes, for which one parental copy is silenced while the other is expressed. Because these genes have unique modes of regulation and are often implicated in disease syndromes, much effort has been devoted to their identification and study.

The first imprinted locus identified was an artificial transgene carrying elements of the RSV LTR and a translocated c-myc gene. This transgene was expressed in heart, but only when inherited from the father. Furthermore, the silencing that occurred upon paternal transmission was accompanied by DNA methylation of the transgene, which has been known to regulate gene expression [6]. Shortly after this discovery, two naturally occurring genes, H19 and Igf2r, were shown to exhibit parent of origin specific expression [7,8] and DNA methylation [9,10]. Since these discoveries, the role of DNA methylation in the regulation of imprinted genes was firmly established [11]. Besides DNA methylation, additional factors are known to regulate imprinted gene expression, including the covalent modifications to histone proteins associated with DNA [12–16], the CTCF protein, [17–22], and physical interactions between the two parental chromosomes [23–28].

DNA sequences that control imprinting lie within the ICR, which often regulates imprinting of several nearby genes. Rasgrf1 is paternally expressed in brain [29] and its ICR is located 30 kb upstream of the Rasgrf1 transcription start site between Rasgrf1 and four upstream non-coding (nc) RNAs (Figure 1A). One of these ncRNAs, A19 (also called AK015891), located ~10 kb upstream of the Rasgrf1 ICR, is also imprinted in brain, where expression is solely from the paternal allele [30]. As ICRs have only been identified for seven imprinted clusters [31], the Rasgrf1 system provides an uncommon opportunity to study regulation of imprinted gene expression.

The Rasgrf1 ICR contains a differentially methylated domain (DMD) as well as a series of tandem DNA repeats, consisting of
40 copies of a 41 bp repeat unit. The DMD acquires DNA methylation exclusively on the paternal allele and placement of DNA methylation is controlled by the tandem repeats (Figure 1B) [32,33]. The repeats are required for both establishment and maintenance of allele-specific DNA methylation during spermatogenesis and through pre-implantation development. Only a few other sequences have been implicated as cis-acting regulators of DNA methylation [34–36]. Imprinted expression is a result of both allele-specific binding of the methylation-sensitive enhancer blocker-binding protein, CTCF, and allele-specific DNA methylation. CTCF binds the unmethylated paternal DMD and disrupts enhancer to promoter interaction, resulting in silencing of Rasgrf1. On the paternal allele, the DMD is methylated, preventing binding of CTCF and allowing paternal allele expression [22]. The presence of the DNA repeats on the paternal allele is necessary for the establishment and for the maintenance of DNA methylation at the DMD until implantation at embryonic day 5.5 (e5.5). Deletion of the paternal repeats before this time point leads to loss of DNA methylation on the paternal allele, resulting in silencing of Rasgrf1 [33]. The DNA repeats, in combination with the DMD, constitute a binary switch that is necessary for imprinted expression of Rasgrf1.

We previously generated a Rasgrf1 mutation in which the Rasgrf1 repeats were replaced with sequences referred to as Region 2 (R2) that were implicated in the control of Ig2r imprinting [37]. When paternally inherited, this modification allowed both methylation and Rasgrf1 expression, in cis, of the paternal allele, indicating R2 could substitute for the endogenous Rasgrf1 repeats. Interestingly, the wild-type maternal allele also became methylated and expressed in trans, and once modified, the altered epigenetic state persisted in the next generation, an example of paramutation [37].

This was our first observation of a trans-expression effect in the Rasgrf1 genomic region.

Here, we report that a second ncRNA, AK029869, located ~5 kb upstream of the Rasgrf1 ICR is imprinted and paternally expressed in neonatal brain. We also show that deletion of the tandem repeats within the Rasgrf1 ICR perturbs imprinting of AK029869, and has the ability to do so in trans. Finally, we demonstrate that several ICR mutations on the maternal copy of Rasgrf1 lead to silencing of the normally expressed AK029869 paternal allele in trans. These constitute additional examples of trans-expression effects within the Rasgrf1 imprinted cluster and demonstrate the robust communication between Rasgrf1 alleles across chromosomal boundaries.

**Results**

**AK029869 is imprinted in brain**

To test the imprinting status of AK029869, we first identified useful SNPs between the polymorphic mouse strains PWK and C57BL/6 (B6) to distinguish the expression from the two alleles. We chose one SNP that overlapped with an AluI restriction site. AluI digestion produces distinct banding patterns for the two strains. We established reciprocal crosses between PWK and B6, extracted RNA from brains of post partum day 10 (P10) progeny and then performed RT-PCR followed by AluI digestion to identify the expressed alleles. We found that AK029869 expression was exclusively from the paternal allele at P10 (Figure 2A). Though there was a bias in the efficiency of amplification of the two alleles, paternal allele specific expression was quite clear. As imprinted expression can be developmental time point specific, we repeated the analysis using brain samples collected between embryonic day 16.5 (e16.5) through P42. We found that
AK029869 was paternally expressed throughout this interval (Figure 2B). In addition to brain, we performed the analysis on testes taken between embryogenesis and adulthood. AK029869 expression was biallelic in testes at all stages tested (not shown). Allele-specific expression of AK029869 depends on the Rasgrf1 repeats. Next, we wanted to test whether paternal allele-specific expression of AK029869 depended upon the presence of the tandem DNA repeats in the ICR, as is the case for Rasgrf1. To do this, we established reciprocal crosses between PWK animals and mice carrying a targeted deletion of the tandem DNA repeats (△rep, Figure 3A, [32]). The △rep allele, and all other Rasgrf1 ICR mutations described below were prepared on the 129S4Jae background, which shares the same AK029869 Alu I site polymorphism as B6. As with Rasgrf1, paternal inheritance of a △rep allele led to silencing, in cis, of AK029869. Expression of the normally silent maternal allele was unaffected, as was DNA methylation at the maternal DMD [32]. Unexpectedly, maternal inheritance of the △rep allele led to silencing, in trans, of the paternal AK029869 allele (Figure 3B). This trans-silencing was independent of DNA methylation, as maternal inheritance of a △rep allele did not change the DNA methylation status of either DMD, or expression of Rasgrf1 [32].

Effect of timing of repeat deletion on AK029869 expression

After fertilization, there is a developmental time before which the repeats are required to maintain methylation at the Rasgrf1 DMD, and after which they are dispensable [33]. Therefore, we wondered if the cis- or trans-silencing we observed at P10 in Rasgrf1 repeat-deficient mice was also sensitive to the developmental stage at which the repeats were deleted. To control deletion of the maternal or paternal repeats after fertilization, we used mice homozygous for an allele with loxP sites flanking the Rasgrf1 DNA repeats (flox-rep, Figure 4, [33]). This allele is functionally wild-type, preserving normal imprinted expression of both AK029869 and Rasgrf1 as well as imprinted DNA methylation [33]. We crossed mice bearing the flox-rep allele with different Cre transgenes to delete the repeats. To facilitate allele-specific expression analysis, we bred the Cre transgenes onto the PWK mouse background. The breeding strategy is shown in Figure S1.

Figure 2. AK029869 is imprinted in brain at many developmental stages. (A) Brains from post-natal day 10 (P10) or (B) embryonic day 16.5 (e16.5) mice and older (P11–P42) were assayed for AK029869 imprinted expression. Animals were progeny of C57BL/6 (B6) and PWK parents that carry the Alu polymorphism in AK029869 shown below the gel in (A), where numbers represent Alu fragment sizes in nucleotides. Maternal strain is shown first in red followed by paternal strain in blue. Where no cross is shown, the mice were inbred. (A) RT-PCR products from six independent progeny of two reciprocal crosses were digested with AluI before gel analysis (left panel). Inbred strains and mixtures of inbred cDNAs in the ratios indicated were included as markers and controls for amplification bias (right panel). Band sizes and the strain source are to the left of the gel. (B) cDNAs were analyzed as in (A) with the exception that undigested (U) RT-PCR products were also analyzed and PCR was done using mRNAs that were (+) or were not (−) reverse transcribed (RT). M identifies marker lanes.

doi:10.1371/journal.pone.0013784.g002
Using P10 brain cDNAs from progeny of reciprocal crosses between site. (B) Allele-specific expression of conditional allele leaves an frt site adjacent and in addition to the loxP mutation [32] and as a conditional allele whereby the repeats can be triangle). The repeat deficient allele was prepared as a germ line (bottom), the repeats were replaced with a single loxP site (white methylation at the DMD (white rectangle). In the repeat deficient allele kbp repeat element (black triangles) needed to program DNA of development.

Rasgrf1 tandem DNA repeats on both alleles at different stages of development. (A) The wild-type Rasgrf1 allele (top) includes a 1.6 kbp repeat element (black triangles) needed to program DNA methylation at the DMD (white rectangle). In the repeat deficient allele (bottom), the repeats were replaced with a single loxP site (white triangle). The repeat deficient allele was prepared as a germ line mutation [32] and as a conditional allele whereby the repeats can be deleted by Cre recombinase [33]. Deletion of the repeats from the conditional allele leaves an frt site adjacent and in addition to the loxP site. (B) Allele-specific expression of AK029869 was assayed as in Figure 2 using P10 brain cDNAs from progeny of reciprocal crosses between wild-type PWK animals and B6 mice carrying the repeat deletion in their germ line. The mutation was made on a 129S4Jae background, which has the same polymorphism as B6. The analysis was repeated using mice from which the repeats were removed by Cre transgenes at different times after fertilization. (C) Paternal repeats were deleted by a maternally transmitted Zp3-Cre transgene that can delete the repeats upon fertilization [38] or (D) by a maternally transmitted Meox2-Cre transgene that can delete the paternal repeats in the embryonic ectoderm of the e5.5 epiblast [39]. (E) The maternal repeats were deleted by a paternally transmitted Meox2-Cre transgene. Animals that lacked complete deletion of the repeats were excluded from the analysis (not shown). Rpl32 was included as a control for samples lacking detectable AK029869 expression. The strain origins of bands are shown to the left of the gels with the maternal and paternal strains shown in red and blue, respectively. doi:10.1371/journal.pone.0013784.g003

We used the Zp3 Cre transgene (Figure 3C), which is active at e0.0 and can delete the repeats at the one-cell stage [38], and the Meox2 Cre transgene (Figure 3D, E), which is active at e5.5 and deletes the repeats in the embryonic ectoderm [39]. Depending on the direction of the cross, we were able to delete the repeats at these two time points from the maternally or the paternally transmitted allele.

From these crosses, we collected P10 brain and testes from 50 litters of mice. The potentially informative animals were those bearing the Cre transgene, one 129S4Jae allele and one PWK allele at AK029869. We also genotyped each tissue sample to determine the extent of deletion of the loxP-flanked Rasgrf1 repeats because only mice that had complete Cre-mediated deletion of the repeats were informative. We prepared cDNA from all informative brain samples and performed allele-specific expression analysis of AK029869.

This analysis revealed multiple patterns of AK029869 expression control by the Rasgrf1 repeats that changed according to developmental stage. When the paternal repeats were deleted by the Zp3 Cre transgene near the time of fertilization, AK029869 expression was both silenced in cis on the paternal allele and activated in trans on the maternal allele (Figure 3C). Interestingly, this pattern changed when the paternal repeats were deleted later in the embryonic ectoderm of the e5.5 epiblast by a maternal Meox2 Cre transgene. Deletion at this later stage caused only paternal allele silencing but no maternal allele activation (Figure 3D). When the maternal repeats were deleted by a paternally transmitted Meox2 Cre transgene, paternal expression of AK029869 was silenced in trans (Figure 3E). As Zp3 Cre is not expressed in sperm, it could not be used to delete the maternal repeats upon fertilization. These results demonstrated that germ line inheritance of a Rasgrf1 repeat deletion allele silenced the normally active paternal allele in cis and in trans, whereas somatic deletion of the repeats after fertilization produced multiple cis and trans regulatory effects that included silencing and activation and that varied by developmental stage.

Additional Rasgrf1 ICR mutations result in cis and trans silencing of AK029869

In addition to the Δrep allele, we tested several other ICR alleles for their ability to influence AK029869 imprinting, when transmitted as germ line mutations. Figure 4 shows the alleles, their effects on AK029869 expression, and Figure 5 summarizes the results.

The first additional ICR mutation had an extra enhancer (enh) in place of the tandem DNA repeats [22]. When paternally transmitted, expression of AK029869 was from the paternal allele, as it is in wild-type mice (Figure 4B, lanes 4–7). However, when
Maternally transmitted, the paternal allele was silenced in trans and the maternal allele was inappropriately activated in cis (Figure 4B, lanes 8–11). The second additional ICR mutation (flox-rep-enh) carried loxP sites on either side of the repeats, with an extra enhancer 3′ of the repeats [33]. Like the enh allele, maternal transmission inappropriately activated the maternal allele in cis, and expression of the paternal allele was strongly diminished in trans (Figure 4, lanes 12–17).

The extra enhancer on the enh and flox-rep-enh alleles was from the housekeeping gene Pgk and is known to cause overexpression of Rasgrf1 [40]. We wondered whether the enhancer was so strong that it boosted AK029869 expression from the mutated allele high enough to mask otherwise normal levels of expression from the homologous wild-type PWK allele. This could lead us to conclude, erroneously, that maternal transmission of the mutated enhancer carrying alleles silenced the paternal allele in trans. To explore this possibility, we mixed brain cDNAs from a wild-type PWK mouse and a mouse with the enh allele in varying ratios and looked for evidence of amplification of both alleles. In each case, even when the mixture contained a three-fold excess of
cDNA from enh-bearing mice, we were able to easily detect PCR products diagnostic of both alleles. This provides confidence that PCR artifacts did not confound our conclusion that maternally transmitted extra enhancer alleles silenced the paternal allele in trans (Figure S2).

The third additional ICR mutation we tested lacked the Rasgrf1 repeats and replaced them with the differentially methylated domain, referred to as Region 2, from the imprinted and maternally expressed Igf2r gene (R2 allele). When the R2 allele was maternally inherited, AK029869 demonstrated normal...
expression from the paternal allele only (Figure 4, lanes 20–23). However, when paternally inherited, the R2 allele silenced the paternal allele in cis. Interestingly, this differed from the effect of the paternal R2 allele on Rasgrf1 expression. When the R2 allele was paternally inherited, the paternal copy of Rasgrf1 was activated in cis and the normally silent maternal Rasgrf1 allele was activated in trans ([37] and Figure 4, lanes 25–26). This is because Region 2 positively regulates DNA methylation at the Rasgrf1 DMD, in both cis and trans, when paternally inherited. A caveat is that paternal Rasgrf1 expression is reduced, but still detectable, with an R2 paternal allele [37]. Because AK029869 expression is low in wild-type animals, it is possible that paternal transmission of the R2 allele reduced AK029869 expression below the threshold of detection. Maternal transmission of the R2 allele has no effect on Rasgrf1 expression or DNA methylation at the DMD [37].

Discussion

Although the role of DNA methylation in the control of imprinted expression is relatively well understood, advances in the understanding of other factors regulating imprinted expression have been made more slowly. The body of evidence for the involvement of trans-expression effects in imprinted gene regions is growing. At the imprinted gene, In2, a mutation on the paternal allele silenced expression of the normally expressed maternal allele in trans [41]. A mutation to the paternal allele of the normally expressed gene, Surtm, activated expression of the normally silenced maternal allele in trans [42]. At a nearby locus, a mutation on the maternal allele of the maternally expressed gene Ube3a, silenced expression of the paternally expressed antisense transcript, Ube3a-ATS, in trans [43]. At the H19/Igf2 imprinted locus, a maternal allele deletion at the H19 allele led to a reduction in the level of DNA methylation at the wild-type Igf2 paternal allele [44]. Also at the H19/Igf2 locus, replacement of a differentially methylated domain between In2 and Igf2 on the paternal allele led to activation of the normally silent Igf2 maternal allele [45]. Similarly, when this mutation was present on the maternal allele, the normally silent H19 paternal allele was activated [45]. Consistent with this trans chromosomal communication that affects expression at imprinted genes is evidence that physical interactions occur between imprinted loci and both homologous and non-homologous loci [23,27].

In addition to imprinted genes, trans-effects occur at other loci including the X-chromosome inactivation center [46], odorant receptors [47], and T-helper cytokines [48]. As with imprinted genes, the expression of these genes is also epigenetically regulated [49–51]. During mammalian X chromosome inactivation, one of the female X chromosomes is silenced to effect equal levels of X chromosome gene expression between XX and XY individuals. X chromosome inactivation is regulated by the genes Xite, Tsix, and Xist. Two of these genes, Tsix and Xite, also function in trans to regulate X chromosome counting and choice through physical pairing of the two X chromosome homologues [46,52]. Similar interactions occur between non-homologous chromosomes in T-helper cytokine gene expression and allelic exclusion of odorant receptor expression [47,48], however, the significance of these interactions for gene expression are not clear [53]. Recent data using the Hi-C method show such trans chromosomal interactions are quite common in humans [54]. Little is known about the significance of these long-range genomic interactions or if they are responsible, mechanistically, for the trans expression effects we describe. The Hi-C data were collected for human cells and Rasgrf1 is not imprinted in primates (K. Kauppinen, J.T. Brenna and PDS, unpublished) so it is not known what interactions occur with Rasgrf1 in mouse, where it is imprinted.

The Rasgrf1 imprinted cluster in mouse is uniquely positioned as a model to study trans-expression effects for several reasons. First, the Rasgrf1 ICR has been well characterized. Sequences controlling DNA methylation have been identified [32] and the mechanism by which methylation controls expression has been determined [22]. Such details are lacking for other imprinted genes. Second, a variety of ICR mutation alleles are available for this region. Third, the effect that each of these alleles has on the methylation state of the ICR (and Rasgrf1 imprinted expression) is known.

Here, we characterized a novel noncoding RNA within the Rasgrf1 imprinting cluster, AK029869, and showed it is paternally expressed in brain and that this imprinting is subject to trans regulation by various ICR mutations. Imprinted expression of AK029869 and the trans effects that regulate it are distinct in several ways from control of Rasgrf1 imprinting, which we previously described [22,32,33,37]. First, the tandem DNA repeats within the Rasgrf1 ICR were important for proper imprinted expression of AK029869, as is the case for Rasgrf1 expression. But whereas Rasgrf1 imprinted expression depends only on the paternal repeats [32], proper AK029869 imprinting depended on both parental repeats. Loss of either set silenced paternal expression of AK029869, with maternally deleted repeats causing silencing in trans (Figure 3B). Second, loss of the paternal Rasgrf1 repeats upon fertilization caused silencing of Rasgrf1 imprinted expression [33]. Strikingly, deletion of the paternal repeats during this time led to a reversal of imprinted expression of AK029869 in brain, silencing the normally active paternal allele and activating the normally silent maternal allele (Figure 3C). Third, proper imprinted expression of Rasgrf1 required the paternal repeats only prior to the epiblast stage. Deleting the repeats at the epiblast stage or later had no effect on Rasgrf1 expression [33]. In contrast, when the repeats were deleted from either the maternal or paternal allele at the epiblast stage, the paternal allele was silenced (Figures 3D and 3E).

Regarding how the expression of AK029869 is regulated, we considered three possibilities. We first considered that DNA methylation levels at the DMD might regulate AK029869. However, maternal inheritance of a Δrep allele, an enh allele, or a flox-rep-enh allele all preserved DNA methylation at the paternal DMD, but led to silencing in trans of the paternal AK029869 allele ([22,32,33], Figures 3 and 4). This indicates that paternal DMD methylation is not sufficient for imprinted AK029869 expression. In contrast, whenever the DMD was methylated, we observed expression of Rasgrf1 [22,32,33].

Second, we considered that proper imprinted expression of AK029869 may occur as long as both the maternal and the paternal alleles carry some form of the tandem DNA repeats, but this is not the case. Mice inheriting a maternal copy of a flox-rep-enh allele had both the maternal and the paternal tandem DNA repeats, but underwent an inversion of the normal imprinting pattern, with the maternal allele becoming activated and the paternal allele becoming silenced. In addition, mice with a maternally inherited R2 allele lacked one copy of the DNA repeats but exhibited apparently proper paternal allele specific expression of AK029869 (Figure 4).

Third, we considered that sequence spacing within the ICR might be critical for proper expression of AK029869. However, paternal inheritance of the R2 allele retained wild-type sequence spacing, as Region 2 and the tandem DNA repeats are both approximately 2kb, but this led to silencing in cis of the paternal allele (Figure 4). Therefore, in contrast to the binary switch model for expression of Rasgrf1, there is a distinct mechanism for AK029869 imprinting that is far more complex than the relatively simple binary switch mechanism for Rasgrf1 imprinting.
Despite the complexity of *Ak029869* regulation, there are three patterns that emerged from our data. First, any changes in spacing within the ICR on the maternal allele led to silencing in *trans* of the paternal allele. For example, the R2 allele contain an approximately 2kb deletion of the tandem DNA repeats but, since Region 2 is roughly 2kb, insertion of Region 2 retains normal sequence spacing. When maternally inherited, the R2 allele allowed expression of the paternal allele. On the other hand, the flox-rep-enh allele changed the sequence spacing of the region, and led to paternal allele silencing. The one exception is the enh allele, which kept the maternal allele spacing but silenced the paternal allele in *trans*. The enhancer insertion may produce additional changes to local chromatin structure that over ride otherwise normal sequence spacing on the ICR.

A second pattern is that deletion of the paternal repeats, regardless of the resulting sequence spacing, led to silencing in *cis* of the paternal allele. For example, paternal inheritance of the R2 allele deleted the tandem DNA repeats and preserved sequence spacing, but silenced the paternal allele. Again, the one exception was if the repeats were replaced with the extra enhancer. The enh allele also deleted the tandem DNA repeats and preserved sequence spacing, but it allowed expression of the paternal allele. This represents the third pattern we observed, any allele with an extra enhancer led to activation of that allele in *cis*, regardless of sequence spacing or the presence of the repeats on that allele.

Therefore, it appears that sequence spacing in the region of the repeats, but not presence of the repeats, on the maternal allele is important, while the presence of the repeats, but not sequence spacing, on the paternal allele is important. Also, the presence of an extra enhancer may override these two requirements to allow expression in *cis*, but not in *trans*, indicating that access to an enhancer is necessary for expression of *Ak029869*.

Other imprinted loci, including *Ig2*, undergo allele specific differences in three-dimensional conformation [24–26,28]. One consequence of the maternal allele conformation is that it might prevent distant enhancers from interacting with the *Ig2* promoters, enforcing silence of the maternal copy of *Ig2*. This mechanism may control *Rasgfl* imprinting. Additional and more complex CTCF dependent interchromosomal interactions occur between *Ig2* and other genes [27], and such regulation may be occurring to control *Ak029869* imprinting. If this is the case, it could be dependent on sequence spacing, repeat-content, or a combination of the two, which could in turn be influenced by the presence of enhancers. Experiments such as 3C or FISH are needed to address this question. Nevertheless, the results discussed above clearly demonstrate an abundance of *trans*-expression effects within the *Rasgfl* imprinted cluster.

Materials and Methods

SNP and restriction site identification

SNPs were identified in *Ak029869* using the Jackson Laboratory Mouse Genome Informatics website (http://www.informatics.jax.org). SNPs available between C57/BL6 and 129 versus PWK mouse strains were noted. In each case, potential SNPs were analyzed using NEB cutter (http://tools.neb.com/NEBCutter2/index.php) to select SNPs overlapping a restriction enzyme recognition site for identification of allele-specific expression.

Ethics statement

The Institutional Animal Care and Use Committee at Cornell University approved all research involving animals described here, as required by the United States National Institutes of Health and Department of Agriculture. Cornell University is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care.

Tissue collection

Grosses were set up between either wild-type B6 and PWK mice or B6 mice homozygous for loxP-flanked copies of the *Rasgfl* DNA repeats and PWK mice carrying either *Zfp3 Cre* or *Meox2 Cre* alleles. In each case, crosses were set up as reciprocal pairs to either rule out expression differences due to strain QTLs or to examine the effect of inheritance of both maternal and paternal repeat deletions. The progeny of each of these crosses were sacrificed at P10 (except for the wild-type imprinting time course experiment) and a small portion of the brain was collected for genotyping. The remainder of the brain was snap frozen in liquid nitrogen for later use. P11 and younger mice were killed by decapitation; P21 and older animals were killed by CO2 asphyxiation.

Genotyping

Brain DNA samples were prepared by lysing in Laird’s lysis buffer plus proteinase K overnight followed by ethanol precipitation. Brain DNA was genotyped for the presence of one B6 allele and one PWK allele using primers either AKnewFWD (5′- CTT TCT CCA GCA ACC TAT C -3′) and AKnewREV (5′- AAG GAC CTG CCG CTT AAC T -3′) or primers PDS155 (5′- ATT CAC CGC TGC TGC TTA AA -3′) and AKR1-KPK (5′- TAG GAA AAT GGC TCG GTG TC -3′) for 40 cycles under the conditions 94°C for 30 seconds, 60°C for 1 minute, 72°C for 2 minutes. Also, for the repeat-deletion experiments, deletion of the DNA repeats was determined using the primer combination PDS16 (5′- GCA CTT CGC TAC CGT TTC GC -3′), PDS18 (5′- TTT CTG CCA TCA TCC CAG CC -3′), and PDS17 (5′- TGT CCT CCA CCC CTG CAC G -3′) and cycling conditions 94°C for 10 seconds, 61°C for 20 seconds, 72°C for 50 seconds for 40 cycles.

RNA preparation

Brain samples were isolated from F1 progeny of reciprocal crosses at P10 (except in the case of the imprinting time course experiment) and total RNA was prepared. For each neonatal brain, 2 mls of GTC RNA lysis buffer was used (4M guanidium thiocyanate, 25 mM pH 7.0 sodium citrate, 100 mM betamercaptoethanol, 0.5% sarcosyl, 0.2M pH 4 sodium acetate, and 50% acidic phenol) and each brain was homogenized for 45 seconds at 18,000 rpm. Following homogenization, RNA was extracted with 0.2 volumes chloroform followed by isopropanol precipitation. RNA was resuspended in 10 mM Tris-EDTA.

cDNA analysis

cDNA was prepared from 5 ug of RNA treated with 2.5 ul of DNaseI (Invitrogen). Amplification was done using random primers (Invitrogen) and SuperScript II reverse transcriptase (Invitrogen). Following cDNA synthesis, nested PCR was performed using 0.5 ul cDNA as template. First round PCR was done with primers PDS155 (5′ - ATT CAC CGC TGC TGC TTA AA - 3′) and AKR1-KPK (5′ - TAG GAA AAT GGC TCG GTG TC - 3′) for 19 cycles. 2 ul of first round PCR product was diluted into 18 ul of water, and 1.5 ul of this dilution was used as template for 35 cycles of second round PCR. Second round PCR primers were AKnewFWD (5′ - CTT TCT CCA GCA ACC TAT C) and AKnewREV (5′ - AAG GAC CTG CCG CTT AAC T - 3′). In each case, cycling conditions were 94°C for 30 seconds, 60°C for 1
minute, 72°C for 2 minutes. 10 ul of second round PCR product was digested 5 hours to overnight with 1U AluI (NEB). Digests were heat inactivated and run on a 3% agarose gel.

Supporting Information

**Figure S1** Crossing scheme for developmental time point specific repeat deletions. To delete the DNA repeats as specific times after fertilization, we used an allele containing a loxP-flanked copy of the Rasgrf1 DNA repeats in combination with the Cre transgenic mice. To facilitate allele-specific expression analysis, we bred specific Cre alleles onto the PWK/PhJ mouse background. These mice (PWK Cre) were mated with mice homozygous for a loxP-flanked version of the Rasgrf1 DNA repeats (Floxed), which was created on the 129S/Jae strain background and backcrossed to C67BL/6. In the presence of Cre recombinase expression, the loxP-flanked repeats can be deleted at specific time points. Zp3 Cre is active at e0.0 and deletes the repeats at the one-cell stage, while Mecox2 Cre is active at e5.5 and deletes the repeats in the embryonic ectoderm of the e5.5 epiblast around the time of implantation into the uterine wall. Depending on whether the loxP-flanked repeats were inherited maternally or paternally, we were able to delete the repeats at these time points on either the maternally or the paternally inherited allele. After genotyping to ensure that the animals were not mosaic for deletion of the DNA repeats, and to ensure that the animals carried one PWK and one 129S/Jae allele at the AK029869 locus, we carried out allele-specific expression analysis.

**References**

1. Allen WR (1969) Factors influencing pregnant mare serum gonadotrophin production. Nature 223: 64–65.
2. Engel E (1980) A new genetic concept: uniparental disomy and its potential effect, isodisomy. Am J Med Genet 6: 137–143.
3. Barton SC, Surani MA, Norris ML (1984) Role of paternal and maternal genomes in mouse development. Nature 311: 574–576.
4. McGrath J, Solter D (1984) Inability of mouse blastomere nuclei transferred to enucleated zygotes to support development in vitro. Science 226: 1317–1319.
5. Surani MA, Barton SC, Norris ML (1984) Development of reconstituted mouse eggs suggests imprinting of the genome during gametogenesis. Nature 308: 548–550.
6. Scriver CJ, Stearman TA, Leder P (1987) Parental legacy determines methylation and expression of an autosomal transgene: a molecular mechanism for parental imprinting. Cell 50: 719–727.
7. Barlow DP, Stoger R, Herrmann BG, Saito K, Schweifer N (1991) The mouse insulator-like growth factor type-2 receptor is implanted and closely linked to the Time locus. Nature 349: 84–87.
8. Bartolomei MS, Zemel S, Tilghman SM (1991) Parental imprinting of the mouse H19 gene. Nature 351: 153–155.
9. Ferguson-Smith AC, Saaki H, Cattanach BM, Surani MA (1993) Parental-origin-specific epigenetic modification of the mouse H19 gene. Nature 362: 751–755.
10. Stoger R, Kubicka P, Liu CG, Kafrit T, Razin A, et al. (1993) Maternal-specific methylation of the implanted mouse Igf2 locus identifies the expressed locus as carrying the imprinting signal. Cell 73: 61–71.
11. Li E, Beard C, Jaenisch R (1993) Role for DNA methylation in genomic imprinting. Nature 366: 362–365.
12. Wu MY, Tsai TF, Beaudet AL (2006) Deficiency of Rhbp1/Arhda and Rhbp3/Arh3 alters epigenetic modifications and suppresses an imprinting defect in the PWS/AS domain. Genes Dev 20: 2859–2870.
13. Delaval K, Givon J, Cerqueira F, Rousseaux S, Khochbin S, et al. (2007) Differential histone modifications mark mouse imprinting control regions during gametogenesis. Embry J 26: 720–729.
14. Lindroth AM, Park YJ, McLean CM, Dokshin GA, Persson JM, et al. (2008) Antagonism between DNA and H3K27 Methylation at the Imprinted Rasgrf1 Locus. PLoS Genetics 4: e1000145.
15. MikkleSEN TS, Hansen J, Zhang X, Ko M, Wernig M, et al. (2008) Dissecting direct reprogramming through integrative genomic analysis. Nature 454: 49–55.
16. Naganos T, Mitchell JA, Santa LA, Pauler FM, Ferguson-Smith AC, et al. (2008) The Air Noncoding RNA Epigenetically Silences Transcription by Targeting G9a to Chromatin. Science 322: 1717–1720.
17. Bell AC, Felsenfeld G (2000) Methylation of a CTFC-dependent boundary controls imprint expression of the Igf2 gene [see comments]. Nature 405: 482–485.
18. Hark AT, Schoenherr CJ, Katz DJ, Ingram RS, Leovane JM, et al. (2000) CTCF mediates methylation-sensitive enhancer-blocking activity at the H19/Igf2 locus [see comments]. Nature 405: 486–489.
19. Hikichi T, Kohda T, Kaeo-kuboishi T, Ishino F (2003) Imprinting regulation of the murine Meg1/Grb10 and human GRB10 genes; roles of brain-specific promoters and mouse-specific CTFC-binding sites. Nuclear Acids Res 31: 1398–1406.
20. Kanduri C, Pany V, Louskine D, Pogacheva E, Qif CF, et al. (2000) Functional association of CTFC with the insulator upstream of the H19 gene is parent of origin-specific and methylation-sensitive.Curr Biol 10: 853–856.
21. Takada S, Pausen M, Tavendale M, Tsai CE, Kelsey G, et al. (2002) Epigenetic analysis of the Dlk1-Gtl2 imprinted domain on mouse chromosome 12: implications for imprinting control from comparison with Igf2/H19. Hum Mol Genet 11: 77–86.
22. Yoon BJ, Herrman H, Hu B, Park YJ, Lindroth AM, et al. (2005) Rasgrf1 Imprinting is Regulated by a CTCF-dependent Methylation-Sensitive Enhancer Blocker. Mol Cell Biol 25: 11184–11190.
23. LaSalle JM, Lalande M (1996) Homologous association of oppositely imprinted chromosomes. Science 272: 725–728.
24. Murrell A, Heesen S, Reik W (2004) Interaction between differentially methylated regions partitions the imprinted Igf2 and H19 into parent-specific chromatin loops. Nat Genet 36: 849–893.
25. Kato Y, Saaki H (2005) Imprinting and looping: epigenetic marks control interactions between regulatory elements. Bioessays 27: 1–4.
26. Liang JQ, Li T, Hu JF, Vu TH, Chen HL, et al. (2006) CTCF mediates interchromosomal colocazation between Igf2/H19 and Wsb1/Nf1. Science 312: 269–272.
27. Zhao Z, Tavoosidana G, Sjolinder M, Gondor A, Mariano P, et al. (2006) Circular chromosome conformation capture (4C) uncovers extensive networks of epigenetically regulated intra- and interchromosomal interactions. Nat Genet 38: 1341–1347.
28. Qin X, Vu TH, Lu Q, Ling JQ, Li T, et al. (2008) A complex deoxyribonucleic acid looping configuration associated with the silencing of the maternal Igf2 allele. Mol Endocrinol 22: 1476–1485.
29. Plass C, Shibata H, Kalcheva I, Mullins L, Kotelevtseva N, et al. (1996) Identification of Grf1 on mouse chromosome 9 as an imprinted gene by RLOGS-M. Nat Genet 14: 106–109.
30. de la Pautere A, Hall J, Wu YZ, Leone G, Peters J, et al. (2002) Structural characterization of Rasgrf1 and a novel linked imprinted locus. Gene 291: 287–297.
31. Peters J, Robson JE (2008) Imprinted noncoding RNAs. Mamm Genome 19: 493–502.
32. Yoon BJ, Herrman H, Sikora A, Smith LT, Plass C, et al. (2002) Regulation of G9a to Chromatin. Science 322: 1717–1720.

Found at: doi:10.1371/journal.pone.0013784.s001 (0.42 MB TIF)

**Figure S2** The presence of an extra enhancer allele does not mask detection of expression from wild-type alleles in trans. cDNAs from a wildtype and an extra enhancer allele were mixed in varying ratios. The mixed cDNAs were subjected to allele specific expression analysis of AK029869 as described in the main text. Strains and band sizes are shown to the right (see main text, Figure 2A). In each case, even when the extra enhancer allele (enh) was present in a 3:1 ratio to the wild-type allele (WT), banding patterns from both alleles were present indicating that expression from the extra enhancer allele did not obscure expression from the wild-type allele during amplification. “+” = with reverse transcriptase, “-” = without reverse transcriptase, (-) = water, B6 = C57Bl6/J, PWK = PWK/PhJ.

Found at: doi:10.1371/journal.pone.0013784.s002 (0.17 MB TIF)

Acknowledgments

James R. Putnam for mouse husbandry support; Patrick Murphy and Jonathan Flux for critical reading of the manuscript.

Author Contributions

Conceived and designed the experiments: CMB KPK RH PDS. Performed the experiments: CMB KPK. Analyzed the data: CMB KPK PDS. Wrote the paper: CMB PDS.
33. Holmes R, Chang Y, Soloway PD (2006) Timing and Sequence Requirements Defined for Embryonic Maintenance of Imprinted DNA Methylation at Rasgrf1. Mol Cell Biol 26: 9564–9570.
34. Birger Y, Sherer R, Perk J, Razin A (1999) The imprinting box of the mouse Ig2r gene. Nature 397: 84–88.
35. Sherer R, Hershko AY, Perk J, Mostoslavsky R, Tsuheri B, et al. (2000) The imprinting box of the Prader-Willi/Angelman syndrome domain. Nat Genet 26: 440–445.
36. Srivastava M, Frolova E, Rottinghaus B, Boe SP, Grinberg A, et al. (2003) Imprint control element-mediated secondary methylation imprints at the Igf2/H19 locus. J Biol Chem 278: 5977–5983.
37. Herman H, Lu M, Anggraini M, Sikora A, Chang Y, et al. (2003) Trans allele methylation and paramutation-like effects in mice. Nat Genet 34: 199–202.
38. Lewandoski M, Wassarman KM, Martin GR (1997) Zp3-cre, a transgenic mouse line for the activation or inactivation of loxP-flanked target genes specifically in the female germ line. Curr Biol 7: 148–151.
39. Tallquist MD, Soriano P (2000) Epiblast-restricted Cre expression in MORE mice: a tool to distinguish embryonic vs. extra-embryonic gene function. Genesis 26: 113–115.
40. Drake NM, Park YJ, Shirali AS, Cheadle TA, Soloway PD (2009) Imprint switch mutations at Rasgrf1 support conflict hypothesis of imprinting and define a growth control mechanism upstream of IGF1. Mammm Genome 20: 654–663.
41. Duville B, Bucchini D, Tang T, Jamj J, Palei A (1998) Imprinting at the mouse Ins2 locus: evidence for cis- and trans-allelic interactions. Genomics 47: 52–57.
42. Tsai TF, Bresuler J, Jiang YH, Beaudet AL (2003) Disruption of the genomic imprint in trans with homologous recombination at Surp in ES cells. Genesis 37: 151–161.
43. Landers M, Calciano MA, Colosi D, Glatt-Davey H, Wurtzel J, et al. (2005) Maternal disruption of Ube3a leads to increased expression of Ube3a-ATS in trans. Nucleic Acids Res 33: 3976–3984.
44. Forne T, Oscalde J, Dean W, Saam JR, Bailleul B, et al. (1997) Loss of the maternal H19 gene induces changes in Igf2 methylation in both cis and trans. Proc Natl Acad Sci U S A 94: 10243–10248.
45. Hu JF, Vu TH, Hoffman AR (1997) Genomic deletion of an imprint maintenance element abolishes imprinting of both insulin-like growth factor II and H19. J Biol Chem 272: 20713–20720.
46. Xu N, Tsai CL, Lee JT (2006) Transient homologous chromosome pairing marks the onset of X inactivation. Science 311: 1149–1152.
47. Lonvaria S, Barchu G, Pisonia JI, Mendelsohn M, Kirkland J, et al. (2006) Interchromosomal interactions and olfactory receptor choice. Cell 126: 403–413.
48. Spilianakis CG, Lalioti MD, Town T, Lee GR, Flavell RA (2005) Interchromosomal associations between alternatively expressed loci. Nature 435: 637–645.
49. Shykin NM (2005) Regulation of odorant receptors: one allele at a time. Hum Mol Genet 14 Spec No 1: R35–39.
50. Lee GR, Kim ST, Spilianakis CG, Fields PE, Flavell RA (2006) T helper cell differentiation: regulation by cis elements and epigenetics. Immunity 24: 369–379.
51. Wutz A, Griebnau J (2007) X inactivation Xplained. Curr Opin Genet Dev 17: 387–393.
52. Augui S, Filon GJ, Huart S, Nora E, Guggiari M, et al. (2007) Sensing X chromosome pairs before X inactivation via a novel X-pairing region of the Xic. Science 318: 1632–1636.
53. Fuss SH, Oumra M, Mombaerts P (2007) Local and cis effects of the H element on expression of odorant receptor genes in mouse. Cell 130: 373–384.
54. Lieberman-Aiden E, van Berkum NL, Williams L, Imakaev M, Ragoczy T, et al. (2009) Comprehensive mapping of long-range interactions reveals folding principles of the human genome. Science 326: 269–293.