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Imprint switch mutations at *Rasgrf1* support conflict hypothesis of imprinting and define a growth control mechanism upstream of IGF1

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Abstract *Rasgrf1* is imprinted and expressed preferentially from the paternal allele in neonatal mouse brain. At weaning, expression becomes biallelic. Using a mouse model, we assayed the effects of perturbing imprinted *Rasgrf1* expression in mice with the following imprinted expression patterns: monoallelic paternal (wild type), monoallelic maternal (maternal only), biallelic (both alleles transcribed), and null (neither allele transcribed). All genotypes exhibit biallelic expression around weaning. Consequences of this transient imprinting perturbation are manifested as overall size differences that correspond to the amount of neonatal *Rasgrf1* expressed and are persistent, extending into adulthood. Biallelic mice are the largest and overexpress *Rasgrf1* relative to wild-type mice, null mice are the smallest and underexpress *Rasgrf1* as neonates, and the two monoallelically expressing genotypes are intermediate and indistinguishable from one another, in both size and *Rasgrf1* expression level. Importantly, these data support one of the key underlying assumptions of the "conflict hypothesis" that describes the evolution of genomic imprinting in mammals and supposes that equivalent amounts of imprinted gene expression produce equivalent phenotypes, regardless of which parental allele is transcribed. Concordant with the difference in overall body size, we identify differences in IGF-1 levels, both in serum protein and as liver transcript, and identify additional differential expression of components upstream of IGF-1 release in the GH/IGF-1 axis. These data suggest that imprinted *Rasgrf1* expression affects GH/IGF-1 axis function, and that the consequences of *Rasgrf1* inputs to this axis persist beyond the time period when expression is restricted via epigenetic mechanisms, suggesting that proper neonatal *Rasgrf1* expression levels are critical for development.

Introduction

Imprinted genes are expressed preferentially from one of two parental alleles in a predetermined manner. Epigenetic mechanisms distinguish the two alleles from one another and control imprinted expression, which can be tissue-specific or restricted to certain developmental stages. Imprinted genes have been identified in a variety of eutherian and meta-eutherian mammals (Edwards et al. 2007; Suzuki et al. 2005; Weidman et al. 2004), suggesting that imprinted loci have somehow escaped the protection that diploid expression provides against recessive mutations, although the possibility exists that imprinting might evolve at loci where deleterious mutations are the least likely to be recessive (Ubeda and Wilkins 2008).

A widely accepted theory explaining the emergence of genomic imprinting in mammals is the "conflict" or "kinship" hypothesis (Moore and Haig 1991). It postulates that conflict exists between parental genomes regarding the

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transfer of maternal resources to offspring, with paternal reproduction benefiting from abundant transfer and maternal reproduction benefiting from parsimonious transfer. At loci that control this transfer, the gradual tug-of-war between the genomes over optimum expression levels ultimately results in imprinted monoallelic expression. Maternally expressed imprinted genes are predicted to act as growth inhibitors in offspring, and paternally expressed imprinted genes are predicted to act as growth promoters.

Rasgrf1 is an imprinted gene that is expressed preferentially from the paternal allele in neonatal mouse brain, with maternal allele expression identified in adults (Plass et al. 1996). Expression is high in the central nervous system, although low levels of expression have been identified in other tissues (Plass et al. 1996). RasGRF1 is a 140-kDa protein that acts as a guanine-nucleotide exchange factor and activates the small g-proteins Ras and Rac (Innocenti et al. 1999; Yang and Mattingly 2006) in a calcium-dependent manner (Farnsworth et al. 1995). As its expression pattern might suggest, *Rasgrf1* plays a role in learning and memory (Brambilla et al. 1997; Giese et al. 2001) and associates with NMDA and AMPA receptors in neurons (Krapivinsky et al. 2003; Tian and Feig 2006; Tian et al. 2004).

As predicted by the conflict hypothesis, *Rasgrf1* is also known to play a role in postnatal growth. Mice completely lacking *Rasgrf1* expression display a moderate 15–30% reduction in weight that varies depending on the mouse strain, type of mutation introduced, sex, and age assayed (Clapcott et al. 2003; Giese et al. 2001; Itier et al. 1998).

Earlier studies have all used permanent *Rasgrf1* null alleles; thus, none have considered the consequences of transiently perturbing *Rasgrf1* expression during the neonatal period, which is when *Rasgrf1* expression is imprinted. Using two previously described mouse models (Yoon et al. 2002, 2005), in which *Rasgrf1* imprinted expression is disrupted, we assayed the importance of proper neonatal imprinted expression. In addition, combining these models produced an imprinting switch in which expression is maternally derived with corresponding paternal allele silencing. This unique feature allowed us to test the equivalence of expression derived from either parental allele. More specifically, we could ask whether equivalent phenotypes arise when equivalent amounts of expression are maternally or paternally contributed, which is a critical assumption upon which the conflict hypothesis rests (Wilkins and Haig 2003). The hypothesis assumes that genomic imprinting in mammals emerged solely as a means of controlling the levels of total imprinted gene expression and that there are no qualitative differences between expression derived from the two parental alleles. Our series of mutant alleles at the *Rasgrf1* imprinted locus afforded us the opportunity to test whether this assumption is valid, in addition to asking whether neonatal misimprinting has phenotypic consequences.

Two mutated *Rasgrf1* alleles—termed *tm1* and *tm2*—transiently impart three additional patterns of imprinted expression in neonates (Yoon et al. 2002, 2005). Imprinted expression at the *Rasgrf1* locus is controlled by methylation at a differentially methylated domain (DMD) 30 kb upstream of the gene's promoter. The DMD is flanked 3′ by a series of repeats that direct the placement of methyl groups on the paternal allele DMD. When these repeats are deleted, as they are in the *tm1* allele, the DMD fails to acquire methylation and expression is silenced. An unmethylated DMD is a target for CTCF binding, which acts as an enhancer blocker and inhibits communication between the gene's endogenous enhancer and its promoter. When an extra enhancer is inserted in place of the repeats, as is the case with the *tm2* allele, expression is present even in the absence of DMD methylation because the extra enhancer bypasses the need for endogenous enhancer-promoter communication. Inheriting these two alleles in various combinations produces four different patterns of imprinted neonatal *Rasgrf1* expression: wild-type mice (+/+) exhibit monoallelic paternal (MP) expression, biallelic mice (B, *tm2*/+) express *Rasgrf1* from both alleles, null mice (N, +/*tm1*) do not express *Rasgrf1* from either allele, and monoallelic maternal mice (MM, *tm2*/*tm1*) express *Rasgrf1* from the maternal allele. The MM mice represent an inversion of imprinting because *Rasgrf1* expression is still monoallelic but maternally derived. Importantly, biallelic expression of *Rasgrf1* is maintained in adult animals from each of these genotypes, mimicking the wild-type relaxation of imprinting at the locus.

We demonstrate here that transient perturbations in *Rasgrf1* imprinted expression significantly affect overall growth, and that the phenotypic effect of imprinting mutations persists into adulthood, beyond the period during which expression is epigenetically restricted. Biallelic animals are the largest, null animals are the smallest, and monoallelic paternal and monoallelic maternal animals are intermediate between the two and indistinguishable from one another. Differences in adult size are sensitive to the overall level of neonatal *Rasgrf1* expression, not the allele from which expression is derived, which validates an important assumption of the "conflict" or "kinship" hypothesis. We also demonstrate that *Rasgrf1* produces this effect through involvement of the growth hormone/IGF-1 axis, as differences in IGF-1 serum protein and liver transcript levels exist, as well as differential expression of genes upstream of IGF-1 release that are critical for proper functioning of this neuroendocrine axis.

Materials and methods

Breeding scheme

Mice were generated as described previously (Yoon et al. 2002, 2005), with *tm1* and *tm2* alleles maintained on a C57BL/6 background. Mice used for initial weight measurements were littermate controls generated by crossing *tm2*/+ females with +/*tm1* males to facilitate maternal transmission of the *tm2* allele and paternal transmission of the *tm1* allele and produce all four genotypes in a given litter. Weaning occurred at day 21. Mice used for subsequent gene expression assays, body composition analyses, and IGF-1 measurements were generated by crossing homozygous mice: *tm2*/*tm2* females were crossed with *tm1*/*tm1* and +/+ males to generate *tm2*/*tm1* and *tm2*/+ animals, respectively; +/+ females were crossed with *tm1*/*tm1* and +/+ males generated +/*tm1* and +/+ animals, respectively.

Animal measurements

Weights were measured weekly. Length measurements were taken at the same ages using calipers and included only the body length of the mouse. Organ weights (liver, spleen, kidney, thymus) were measured immediately post dissection and normalized to predissection body weight. Body composition was measured via NMR and performed by the University of Cincinnati Mouse Metabolic Phenotypic Center. Meal sizes were assayed using singly housed postnatal day 63 (P63) adult animals by weighing the amount of food both before and after a 3-day period. Animals were housed in wire-bottom cages so that food particles could be collected from the bottoms and included in the post-trial measurements.

Rasgrf1 imprinted expression

RNA was isolated from the brains of mice between embryonic day 13.5 (e13.5) and adulthood at P42. Mice arose from crosses between 129S4Jae mothers and PWK fathers. PWK has a polymorphism in the 3′ untranslated region (UTR) of the mRNA containing a *Hha*I site which 129S4Jae lacks, so after RT-PCR, PCR products (primers: F; CTTGGTGTTCATCGAGGAGG; R; TATTCTCGGGG AAGCACAC) can be digested with *Hha*I, and digestion products will reveal which allele is expressed. PCR products generated after cDNA amplification were run on a gel without *Hha*I digestion or after cutting with *Hha*I. Just prior to digestion the PCR products were mixed with a *Hha*I containing DNA fragment to assess completeness of digestion.

RNA quantification

For *Rasgrf1* transcript quantification, brains were collected from P11 neonates. RNA was extracted, reverse-transcribed, and quantified in duplicate using ABI Taqman© probes specific for *Rasgrf1* and 18S rRNA, to which *Rasgrf1* expression levels were normalized. *Igf-1* liver transcript was quantified in triplicate at P21 using RNA extracted from whole liver. An ABI Taqman probe recognizing both transcript isoforms of *Igf-1* was used, again normalized to 18S rRNA.

Quantification of *Sst*, *Gh*, *Ghrh*, *IgfBp3*, and *Prl-R* were performed using a SYBR® Green-based real-time assay. Transcript levels were normalized to *Rpl32*, a ribosomal subunit. Prior to quantification cDNAs and RNAs were verified to be free from genomic DNA contamination and PCR products were digested with appropriate restriction enzymes to verify identity. Primer pairs: *Gh*: (F) TCC TCA GCA GGA TTT TCA CC, (R) GCA GCC CAT AGT TTT TGA GC; *Ghrh*: (F) TGT GGA CAG AGG ACA AGC AG, (R) ACA GAG GAC GGA AAA GGT CA; *Igf-1* isoform 1: (F) CCT GCG CAA TGG AAT AAA GT, (R) ATT GAG TTG GAA GGC TGC TG; *IgfBP3*: (F) CGC AGA GAA ATG GAG GAC AC, (R) TTG TTG GCA GTC TTT TGT GC; *Prl-R*: (F) ATC ATT GTG GCC GTT CTC TC, (R) CCA GCA AGT CCT CAC AGT CA; *Rpl32*: (F) CAT GCA CAC AAG CCA TCT ACT CA, (R) TGC TCA CAA TGT GTC CTC TAA GAA C; *Sst*: (F) GAG GCA AGG AAG ATC CTG TC, (R) ACT TGG CCA GTT CCT GTT TC.

IGF-1 protein quantification

Serum IGF-1 protein was quantified by a double-labeled radioimmunoassay at Vanderbilt University's Hormone Assay Core, a mouse metabolic phenotyping center. Blood was extracted from P21 animals, clotted at room temperature for 15 min, and spun at 3000*g* for 15 min at 4°C, after which serum was collected.

Statistics

Weight data were analyzed using a repeated-measures analysis of variance (MANOVA). Expression data were analyzed using Student's *t* test or the nonparametric Mann–Whitney *U* test, and *p* values were Bonferroni-corrected to account for multiple comparisons.

Results

Rasgrf1 imprinted expression

We first characterized the temporal component of imprinted *Rasgrf1* expression because biallelic expression in adult

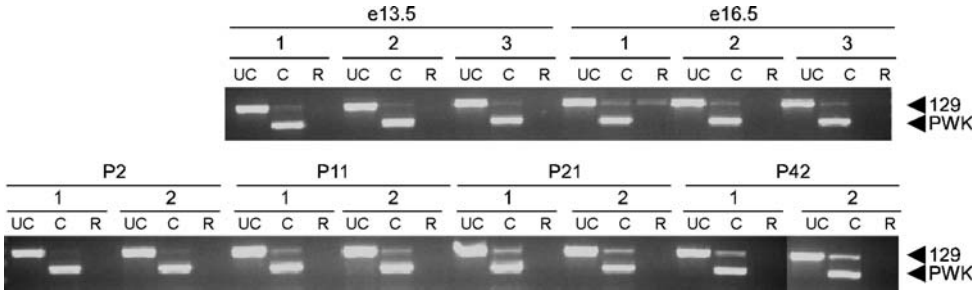

Fig. 1 Imprinting at *Rasgrf1* is relaxed during development. RNA was isolated from either two or three brains of mice between embryonic day 13.5 (e13.5) and adulthood at postnatal day 42 (P42). Mice arose from crosses between 129S4Jae mothers and PWK fathers. cDNA (UC and C) or RNA (R) was amplified by PCR. In no cases did RNA support amplification, demonstrating the RNA we isolated had no genomic DNA contamination. PCR products made after cDNA amplification were run on a gel without *Hha*I digestion (UC) or after cutting with *Hha*I (C). The PWK PCR product has a *Hha*I site lacking in the 129S4Jae product. Therefore, the quickly migrating band labeled PWK is diagnostic for paternal allele expression, while the slowly migrating species labeled 129 is diagnostic for maternal allele expression. The transition from strongly and predominant paternal allele expression to biallelic expression can be seen over time

brain tissues was previously identified (Plass et al. 1996). *Rasgrf1* imprinting in brain is relaxed during development and expression becomes biallelic around the time of weaning (Fig. 1). The transition from strongly and predominantly paternal allele expression to biallelic expression can be seen over time. In contrast to previous work in which expression was identified only in postnatal tissues (Ferrari et al. 1994), we detected transcript prenatally, as early as embryonic day 13.5. Next, we sought to quantify *Rasgrf1* levels in neonatal brains from animals of each of the four imprinted expression patterns ($n = 3$ animals/genotype) using standard qPCR with an ABI Taqman probe specific for *Rasgrf1* (Fig. 2). Expression levels are not significantly different between the two monoallelic genotypes in neonatal brain ($p = 0.30$) but are significantly increased in biallelics ($p = 0.029$) and significantly decreased in nulls ($p = 0.0024$) relative to wild-type MP mice. These data demonstrate the equivalence of *Rasgrf1* expression level in the MM and MP genotypes as well as the relative over- and underexpression of the B and N genotypes, respectively. They also identify the preweaning period as the time during which *Rasgrf1* expression is imprinted.

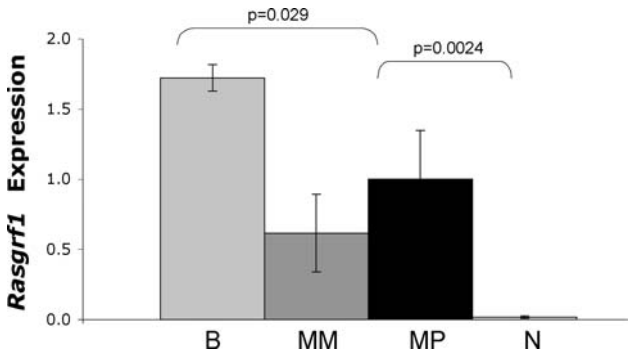

Fig. 2 Disruption of imprinted *Rasgrf1* expression causes differences in expression level. Real-time quantification of *Rasgrf1* expression in neonatal whole brain plotted relative to wild-type (MP) animals. Three animals per genotype were assayed. p values (t test) are indicated. Error bars represent standard deviations

Adult body size corresponds to neonatal *Rasgrf1* expression pattern

We observed a range of size differences among weaned mice of both sexes that appeared to be correlated with transient perturbations in *Rasgrf1* imprinted expression. To reliably assess these differences in size, mice were generated using a breeding scheme that produced all four genotypes in a single litter and were weighed weekly, between P8 and P63. A difference in weight was observed that is correlated with neonatal *Rasgrf1* expression level (Fig. 3), with relationships among the genotypes mirroring those identified in transcript levels. For both sexes, trends are the same: nulls are significantly lighter than the other genotypes from day 16 onward ($p < 0.0001$, MANOVA), biallelics are significantly heavier than the other genotypes from day 16 onward ($p < 0.0001$, MANOVA), and MP and MM mice are intermediate in size between the biallelics and nulls.

Furthermore, differences in weight are proportional to differences in length (Supplementary Fig. 1A, B), with biallelic mice being both heavier and longer and null mice being both lighter and shorter, suggesting that we are seeing an overall growth effect and not the effect of metabolic processes affecting weight gain alone. Further support for this conclusion is drawn from the results of a body composition analysis (Supplementary Fig. 2) indicating that differences in body composition, as reported by body fat percentage at P63, do not reflect differences in weight. The only difference identified in body fat percentage was between female biallelic and null mice ($p = 0.0018$). In addition, the ratios of various organ weights to total

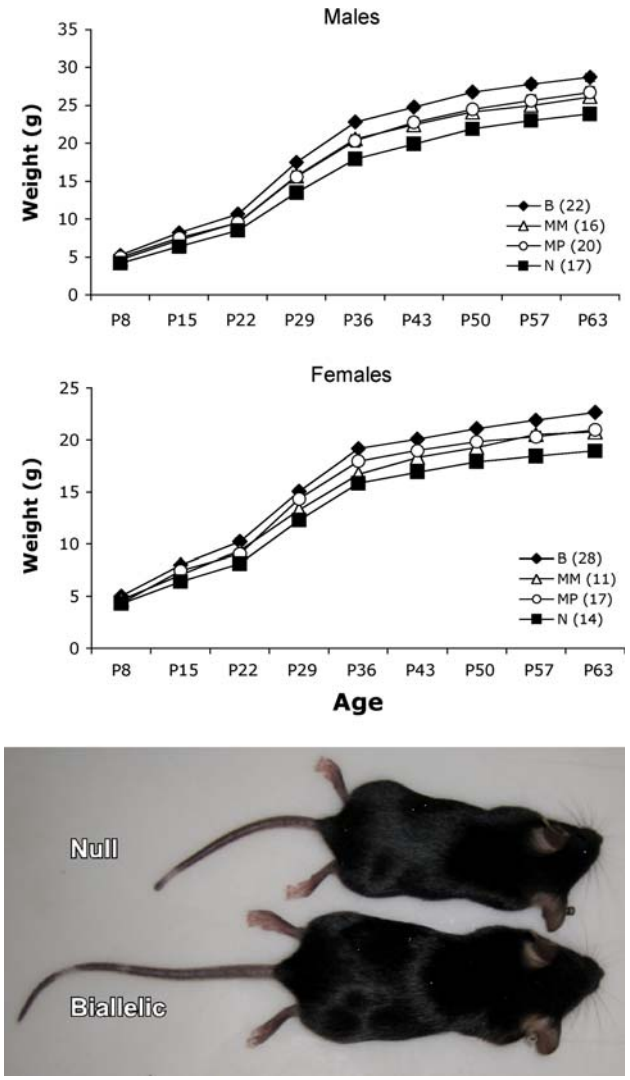

Fig. 3 *Rasgrf1* expression level, not parent-of-origin, controls body weight. Male (*top*) and female (*middle*) body weights were measured once a week, with the number of animals indicated. Error bars represent standard deviation. Compared with the MP controls across the full time interval, null mice were significantly lighter ($p < 6 \times 10^{-6}$), Bi mice were significantly heavier ($p < 2 \times 10^{-4}$), and MM were indistinguishable from controls. Differences between the null and biallelically expressing mice were highly significant ($p < 8 \times 10^{-17}$). Error bars showing standard deviations are smaller than the symbols marking the data points. Representative female mice at P50 (bottom) from the null and biallelic cohorts

body weight are similar among the four genotypes at P21 (Supplementary Fig. 3). An assessment of P63 adult meal size revealed that differences in adult body size were not proportional to the amount of food eaten by different genotypes (Supplementary Fig. 4). When food intake is normalized to body weight, null mice are eating more per gram of body weight than are biallelic mice ($p = 0.0342$), suggesting that the reduction in size is not due to a failure to eat.

Overall, these results indicate that differences in adult body size are responsive to the level of neonatal *Rasgrf1* expression, which highlights the importance of proper imprinted expression early in development. Among genotypes, adult body size corresponds to the amount of *Rasgrf1* in neonatal brain, with the larger, biallelic mice overexpressing *Rasgrf1* and the smaller, null mice underexpressing *Rasgrf1*. The monoallelically expressing genotypes are indistinguishable from one another both in transcript level and body size and are intermediate between the two extremes, indicating that equivalent levels of expression do produce equivalent phenotypes, as the conflict hypothesis assumes. Of additional importance is the observation that differences in weight persist beyond weaning, when expression from both *Rasgrf1* alleles commences, suggesting that preweaning imprinted expression functions in the setting of parameters affecting growth through adulthood by modulating overall *Rasgrf1* expression level.

IGF-1 is decreased in transient *Rasgrf1* nulls at transcript and protein levels

Given the proportional effect of *Rasgrf1* expression on overall growth, we reasoned that a master growth-regulating axis might be affected by *Rasgrf1*. We investigated whether the growth hormone/IGF-1 axis (GH/IGF-1 axis), which is known to play a significant role in regulating postnatal growth (Lupu et al. 2001), might be a target. Circulating growth hormone levels are difficult to capture accurately due to the pulsatile nature of GH secretion, so circulating IGF-1 protein is generally used as an indication for how the system is functioning.

IGF-1 levels were evaluated as both protein and transcript. In serum, IGF-1 protein was measured by radioimmunoassay at P21 in males (Fig. 4a). P21 animals produce data from around the time that growth becomes GH dependent (Meyer et al. 2004) and display significant weight differences in both sexes. P21 data indicate a clear deficiency in the amount of IGF-1 circulating protein in the null mice relative to the biallelic mice ($p = 0.0012$). There is no statistically significant overabundance of IGF-1 protein in biallelic animals relative to the intermediate genotypes. However, the clear difference in IGF-1 levels between the largest and smallest animals indicates that IGF-1 is critical to the overall growth phenotype produced by *Rasgrf1* imprinting perturbation.

Decreased IGF-1 serum levels in the null mice appear to be due to a decrease in mRNA accumulation in liver. We quantified *Igf-1* expression in the livers of P21 male animals using a Taqman probe that also recognized both IGF-1 isoforms (Fig. 4b). Taqman-based data indicated the same trend present in serum, i.e., nulls underexpress *Igf-1* in relation to the other three genotypes (N/B, $p = 0.009$; N/MM, $p < 0.0001$; N/MP, $p = 0.012$), yet we cannot identify overexpression of *Igf-1* in biallelic mice.

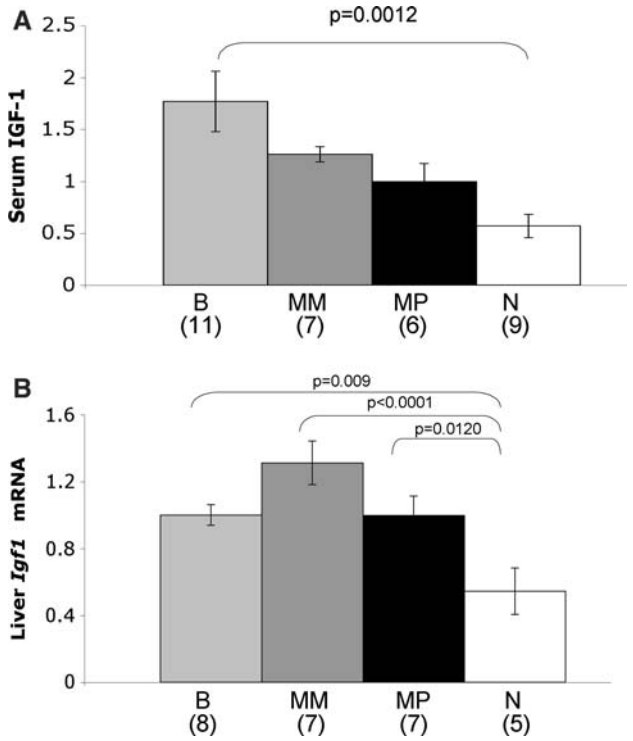

Fig. 4 *Rasgrf1* expression differences are accompanied by differences in IGF-1 levels. **a** P21 IGF-1 serum levels are reduced in transient nulls. Serum levels were quantified by radioimmunoassay in male mice from each genotype, with the number of animals indicated, and plotted relative to MP (wild-type) protein levels. Circulating IGF-1 protein is reduced in transient nulls relative to biallelics ($p = 0.0012$). Error bars represent standard error. **b** P21 IGF-1 transcript levels are reduced in transient nulls. Liver IGF-1 transcript was quantified in male mice from each genotype using a Taqman© probe specific for IGF-1 and normalized to 18S rRNA, with the number of animals indicated, and plotted relative to MP transcript levels. IGF-1 transcript is reduced in null animals relative to biallelics ($p = 0.009$), monoallelic maternals ($p < 0.0001$), and monoallelic paternals ($p = 0.0120$). Error bars represent standard errors

Rasgrf1 affects hypothalamic inputs to the GH/IGF-1 axis

Since IGF-1 levels are affected by differences in neonatal *Rasgrf1* imprinted expression (N ≪ B, at least, and the difference is in the predicted direction), we raised the question of how *Rasgrf1* expression in the brain might affect IGF-1 transcription and release from the liver. One possibility is that *Rasgrf1* levels may influence the activity of downstream transcription factors that are upstream of IGF-1 release. Given that *Rasgrf1* is expressed preferentially in the central nervous system, and not in the liver, we began an investigation into whether various neural components of the GH/IGF-1 axis are differentially expressed in our mutant mice, perhaps as a consequence of *Rasgrf1* influence on transcription factor activity. If a particular transcription factor were a downstream target of *Rasgrf1*, then differences in the transcripts dependent on that factor might indicate its identity as a target. Reasoning that the differences in *Rasgrf1* and IGF-1 levels are most robust in our two "extreme" genotypes, we limited this analysis to null and biallelic male animals and plotted transcript quantities relative to nulls.

Transcription factors chosen as targets were CREB, I-karos, Pit-1, and Stat5 (Fig. 5). Transcripts sensitive to CREB activity include somatostatin (*Sst*; reviewed in Montminy et al. 1996) and growth-hormone-releasing hormone (*Ghrh*; Mutsuga et al. 2001); transcripts sensitive to Ikaros activity include *Ghrh* (Ezzat et al. 2006) and growth hormone (*Gh*; Ezzat et al. 2005); Pit-1 activity is required for *Gh* transcription as well as other pituitary-derived hormones, prolactin, and TSHß (Scully et al. 2000); and Stat5-dependent transcription occurs in the liver, at *Igf1*, *Prl-R*, and *Igfbp3* (reviewed in Hosui and Hennighausen 2008). Significant differences were identified between the biallelic and null animals for somatostatin (hypothalamus; $p = 0.0161$), growth-hormone-releasing hormone (hypothalamus; $p = 0.0458$), growth hormone (pituitary; $p = 0.0037$), Igf-1 (liver; $p = 0.001$), and prolactin receptor (liver; $p = 0.0184$). IGF1BP3 (liver) did not demonstrate a significant difference.

The most upstream of these components in the GH/IGF-1 axis is growth-hormone-releasing hormone (GHRH), which is produced in the hypothalamus and signals via its receptors in the pituitary to affect GH release (Pombo et al. 2000). Efficient GHRH transcription is sensitive to Ikaros (Ezzat et al. 2006), Gsh-1, and CREB (Mutsuga et al. 2001). Testing the hypothesis that hypothalamic *Rasgrf1* expression could be influencing *Ghrh* transcription and subsequent axis functioning, we quantified the amount of *Rasgrf1* present in the hypothalamus of P21 male mice (Fig. 6). Nulls significantly underexpressed *Rasgrf1* relative to biallelic mice ($p < 0.0001$), monoallelic paternal mice ($p < 0.0001$), and monoallelic maternal mice $p = 0.0006$). This pattern of expression follows the trends in IGF-1 levels, at both protein and transcript levels, with the nulls expressing significantly less *Rasgrf1* than the other three genotypes. The correlation between the pattern of *Rasgrf1* expression in hypothalamus and circulating IGF-1 protein suggests that GH/IGF-1 axis functioning is affected by relative amounts of *Rasgrf1* at its hypothalamic input, which ultimately affects levels of several genes regulated by target transcription factors that can affect IGF-1 production in the liver. Curiously, significant correlations were identified in P21 male mice between relative *Rasgrf1* expression in hippocampus ($p = 0.0191$) and olfactory bulb ($p = 7.8e^{-7}$;data not shown) and relative body weight (Supplementary Fig. 5A, B). *Rasgrf1* expression levels in hypothalamus did not significantly correlate with body weight.

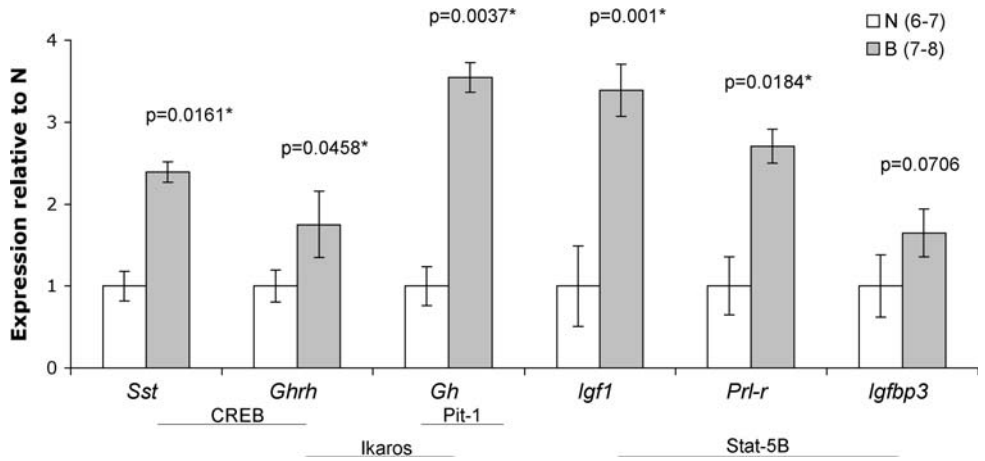

Fig. 5 Differential expression of GH/IGF-1 axis components in P21 biallelic and null males. SYBR Green-based quantification of genes involved in GH/IGF-1 axis function, normalized to Rpl32, and plotted relative to expression level in nulls. Numbers of animals are indicated; error bars represent standard error. Transcription factors responsible for each gene are indicated below. Overexpression in biallelics is seen for the following transcripts: *Sst* ($p = 0.0161$), *GHRH* ($p = 0.0458$), *GH* ($p = 0.0037$), and *Prl-r* ($p = 0.0184$), and IGF-1 ($p = 0.001$) which was included as a control. No significant difference was observed in IGFBP3 level

Discussion

Weight data

The trends in size differences demonstrate two important points: that *Rasgrf1* expression levels between birth and weaning, when *Rasgrf1* shows imprinted expression, are critical determinants of body size through adulthood, and that equivalent phenotypes are produced when equivalent amounts of expression are generated from either parental allele at this imprinted locus. That the contribution of neonatal paternal allele-derived *Rasgrf1* is necessary for normal development has been demonstrated in work using *Rasgrf1* complete-null alleles, where the mutant allele is inherited either maternally or paternally (Clapcott et al. 2003). Maternal inheritance of a permanently null allele produces no phenotype, suggesting that the absence of maternal allele expression in both neonates and adults is not critical for normal development. Conversely, paternal inheritance of a permanently inactivating null mutation produces a reduction in weight similar to that observed in our transient nulls and suggests that normal activity of the maternal allele in adulthood is insufficient to rescue the loss of neonatal paternal allele expression. Thus, we can conclude that the weight differences observed in our animals are sensitive to the relative levels of preweaning *Rasgrf1*, which is when imprinting occurs and expression levels appear to be critical. It does appear that the engineered *tm2* allele produces a noticeable but statistically insignificant difference in *Rasgrf1* expression level, both in whole brain at P11 and hypothalamus at P21. Lack of statistical significance may be due to small sample size; if this is indeed the case, it indicates that the GH/IGF-1 axis is tolerant of small differences in *Rasgrf1* expression level such that equivalent size and level of IGF-1 are still characteristic of the two monoallelically expressing genotypes. Larger perturbations in the expression level of *Rasgrf1* produce significant differences in size and IGF-1 levels, as evidenced by the null and biallelic animals.

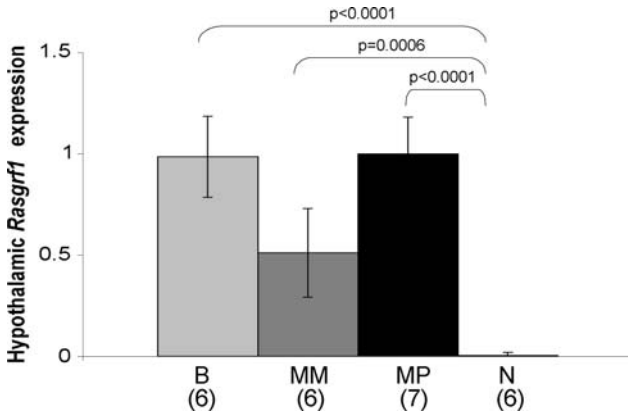

Fig. 6 Differences in hypothalamic *Rasgrf1* expression level mirror trends in weight, IGF-1 level, and axis components. *Rasgrf1* was quantified in P21 male hypothalamus using a Taqman© probe specific for *Rasgrf1* and normalized to 18S rRNA. Expression is plotted relative to wild-type animals, with the number of animals indicated; error bars represent standard error. Null mice significantly underexpress *Rasgrf1* relative to biallelic mice ($p < 0.0001$), monoallelic paternal mice ($p < 0.0001$), and monoallelic maternal mice ($p = 0.0006$)

Second, the equivalence of the monoallelic maternal and paternal phenotypes with respect to size and growth provides novel and valuable support for the conflict hypothesis describing the evolution of genomic imprinting in mammals. Because the two monoallelically expressing genotypes exhibited identical size phenotypes, our data validate

the central assumption underlying the conflict model, i.e., growth-controlling functions of an imprinted gene are dependent on only levels of expression regardless of the parental allele from which expression is derived. The experimental support for the conflict model provided by our engineered imprinting inversion at *Rasgrf1* could not emerge from studies of currently existing loss- or gain-of-function mutations at other imprinted loci. However, support for the predictions that the hypothesis affords has been derived from other experimental systems, most notably for the reciprocally imprinted *Igf2* and *Igf2r* genes, which act together in a single system to modulate expression levels and produce offspring of optimal size for both parental genomes (Filson et al. 1993; reviewed by Smith et al. 2006).

Proportional differences in size

Differences in size are proportional, meaning that overall size corresponds to overall weight. In the absence of GH/IGF-1 axis involvement, these data suggest that we might be seeing the effects of variations in metabolism or feeding behavior. To rule out these possibilities, we assayed the body composition of male and female mice from each of the four genotypes and found that no significant differences existed that were consistent between the sexes. Feeding behavior is difficult to assay accurately in neonates, when animals are still nursing. Because size differences still existed in adult animals, we reasoned that feeding differences might still be detectable in adults; however, our data indicate that a difference in food intake is not responsible for overall difference in body size.

GH/IGF-1 axis involvement

The trend toward reduced IGF-1 levels in the smaller, transient null animals and elevated IGF-1 levels in the larger biallelic animals is clear. Serum IGF-1 and liver *Igf-1* transcript quantification both reflect this relationship. Transcript-level analysis also identifies a significant reduction in mRNA accumulation in transient null livers relative to the two monoallelic genotypes. These data identify the GH/IGF-1 axis as an effector of misimprinted *Rasgrf1* expression on growth.

GH/IGF-1 axis components are essential for normal postnatal growth and development. We observed size differences as early as the second week of life, earlier than that observed in GH or GH receptor knockout models (Meyer et al. 2004; Zhou et al. 1997), which suggests that *Rasgrf1* affects the release and activity of IGF-1, at least preliminarily, in a GH-independent manner. IGF-1 and IGF-1 receptor knockout mice exhibit differences in size at birth, yet their phenotypes are much more severe, with a body weight 45–60% that of wild type and a host of other characteristics, including infertility and increased rates of perinatal lethality (Liu et al. 1993, 1998). That we have generated a transient imprinting mutant that does not fully eliminate IGF-1 expression explains the less severe phenotype we observed. GHRH knockout mice display reductions in size detectable as early as 2 weeks after birth, reduced IGF-1 in circulation, and reduced pituitary growth hormone levels (Alba and Salvatori 2004), similar to our transient null mice. We also note that differences in preweaning *Rasgrf1* imprinted expression levels affect growth into adulthood, which is expected if GH/IGF-1 activity levels are established early in development and maintained through maturation.

IGF-1 and *Rasgrf1*

No clear link has been identified between IGF-1 and *Rasgrf1*, to our knowledge, though prior to this study there were suggestions that one influences the other. Similar to our transient null phenotype, Itier et al. (1998) identified reduced body weights in complete *Rasgrf1* null mice that were accompanied by a reduction (32–40%) in circulating IGF-1 levels at 4 weeks of age and a decrease in pituitary growth hormone (42–53%) assayed at several ages. We have identified both of these characteristics in P21 transient nulls. A second suggestion of a link between Igf-1 and *Rasgrf1* is derived from data gathered using pancreatic β cells from *Rasgrf1* null animals (Font de Mora et al. 2003). In wild-type islets, IGF-1 stimulation activates Akt and Erk, but it is not effective in stimulating signaling in islets derived from *Rasgrf1* null animals, suggesting that IGF-1 signaling is mediated by *Rasgrf1*. However, there have been no reports of association of *Rasgrf1* with IGF-1 receptors, and our data suggest that the directionality of the relationship between *Rasgrf1* and IGF-1 is opposite that above, with a lack of *Rasgrf1* expression leading to a decrease in IGF-1, although IGF-1 signaling relies on feedback pathways.

We have demonstrated by the detection of somatostatin (*Sst*) overexpression in biallelic hypothalamus that feedback loops appear to be intact in our animals. Somatostatin is an inhibitor of GH release and its expression is stimulated by proper GH/IGF-1 axis function (Muller et al. 1999). Thus, we would expect to see increased levels of *Sst* in response to increased levels of IGF-1.

One potential pathway connecting *Rasgrf1* to IGF-1 involves the Ikaros-dependent transcription of hypothalamic GHRH. Ikaros is an epigenetically regulated transcription factor originally identified as playing an important role in hematopoietic differentiation, and it exists in eight different isoforms (Molnar and Georgopoulos 1994; Molnar et al. 1996). Ikaros-deficient mice share phenotypic similarities with our transient null mice and with GHRH null

mice, including a preweaning reduction in size, reduction in circulating IGF-1, smaller pituitary glands, and a decrease in the amount of hypothalamic GHRH (Ezzat et al. 2006). Importantly, heterozygote-deficient animals display a less severe phenotype than homozygous null animals. Thus, one possibility is that Ikaros itself is a target for *Rasgrf1* signaling and is sensitive to reductions in *Rasgrf1* expression, which produces the reduction in hypothalamic GHRH in our transient nulls.

Another potential connection between *Rasgrf1* and IGF-1 is illuminated by mice that are null for neurofibromatosis-1 (NF1), a Ras-GAP that catalyzes the opposite reaction as *Rasgrf1*. These mice also display a phenotype similar to both the Ikaros-deficient mice and our transient null mice, with reductions in body weight, reduced hypothalamic GHRH, pituitary GH, and liver IGF-1 (Hegedus et al. 2008). This phenotype is due to the Ras-independent loss of intracellular cAMP levels, a known factor in pathways leading to CREB activation, which is necessary for efficient GHRH transcription (Mutsuga et al. 2001). CREB is an effector of RasGRF1-mediated Ras pathways, which supports the hypothesis that differences in *Rasgrf1* expression may affect the transcription of axis components and produce the observed reductions in somatostatin and GHRH in our transient nulls.

That the severity of the phenotype observed here is less than that found in previous work is explained by the transient effect on *Rasgrf1* expression. We are working with a gentler perturbation of expression during a specific developmental period, which suggests again that preweaning *Rasgrf1* expression is critical for normal growth and development, with clear consequences that persist through maturation.

The present study has expanded on these relationships by identifying more specifically where in the GH/IGF-1 pathway *Rasgrf1* has an input. The precise mechanism by which *Rasgrf1* affects GHRH levels is still unclear; it could be via an effect on Ikaros, CREB, or Gsh-1 transcription factor activity, or an effect on neuronal connectivity between the structures responsible for the brain-derived portion of the neuroendocrine GH/IGF-1 axis, perhaps through *Rasgrf1*'s mediation of Rac signaling, which is known to play an important role in neurite outgrowth and extension (Baldassa et al. 2007).

Concluding remarks

We have demonstrated that normal growth is dependent on the presence of *Rasgrf1* and that body size is sensitive to neonatal *Rasgrf1* in a dose-dependent manner. Furthermore, this sensitivity does not depend on the transcribed parental allele. This provides valuable and novel support for the conflict hypothesis describing genomic imprinting in mice. We also demonstrated that mice null for *Rasgrf1* expression as neonates display a decrease in IGF-1, both in circulating protein and in liver transcript. In addition, we demonstrated that the loss of *Rasgrf1* affects GH/IGF-1 components in structures as upstream as the hypothalamus, where we identify a decrease in *Ghrh* transcript levels. Overall, our data indicate that IGF-1 is a target for signaling pathways downstream of *Rasgrf1* and that preweaning imprinted expression of *Rasgrf1* is critical for proper sustained growth and development.

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