Germ-line epigenetic modification of the murine A^{vy} allele by nutritional supplementation

Jennifer E. Cropley*†, Catherine M. Suter*‡, Kenneth B. Beckman†, and David I. K. Martin*†§¶

*Victor Chang Cardiac Research Institute, 384 Victoria Street, Darlinghurst 2010, Sydney, Australia; §School of Biotechnology and Biomolecular Sciences and ‡Faculty of Medicine, University of New South Wales, Anzac Parade, Kensington 2033, Sydney, Australia; and †Children's Hospital Oakland Research Institute, 5700 Martin Luther King Junior Way, Oakland, CA 94609

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Environmental effects on phenotype can be mediated by epigenetic modifications. The epigenetic state of the murine A^{vy} allele is highly variable, and determines phenotypic effects that vary in a mosaic spectrum that can be shifted by *in utero* exposure to methyl donor supplementation. We have asked if methyl donor supplementation affects the germ-line epigenetic state of the A^{vy} allele. We find that the somatic epigenetic state of A^{vy} is affected by *in utero* methyl donor supplementation only when the allele is paternally contributed. Exposure to methyl donor supplementation during midgestation shifts A^{vy} phenotypes not only in the mice exposed as fetuses, but in their offspring. This finding indicates that methyl donors can change the epigenetic state of the A^{vy} allele in the germ line, and that the altered state is retained through the epigenetic resetting that takes place in gametogenesis and embryogenesis. Thus a mother's diet may have an enduring influence on succeeding generations, independent of later changes in diet. Although other reports have suggested such heritable epigenetic changes, this study demonstrates that a specific mammalian gene can be subjected to germ-line epigenetic change.

agouti | inheritance

Higher eukaryotes use epigenetic modifications to reversibly suppress transcription of genes and repeat elements, often by stable silencing (1). Epigenetic marks are retained through mitosis, allowing maintenance of characteristic cell types, but they may also be transmitted from one generation to the next (2). By their nature, epigenetic modifications are susceptible to environmental influence: the interposition of epigenetic modifications between genes and the environment provides a way in which the environment can exert heritable influences on phenotype (3–5).

Mice carrying the viable yellow allele of *agouti* (A^{vy}) are a model of epigenetic variation and inheritance. The A^{vy} allele carries an insertion of an intracisternal A-particle (IAP) retrotransposon into pseudoexon 1A of the *agouti* locus, upstream of the transcribed region coding for agouti signaling protein (ASP) (Fig. 1*A*) (3, 4). When the inserted IAP is active, a cryptic promoter in its LTR usurps transcriptional control of *agouti* and drives ectopic expression of ASP (3, 6). Pancellular expression of ASP gives a neomorphic phenotype of yellow fur, obesity, type II diabetes, and predisposition to tumors; when the IAP is silent *agouti* is expressed in its normal pattern (6). A^{vy} is dominant, so that when heterozygous with *a* (*nonagouti*, a loss-of-function allele of *agouti*), the epigenetic state of the allele is readily apparent (Fig. 1*B*). The obese yellow phenotype in A^{vy} mice displays extremely variable expressivity in an isogenic background (7). The activity state of the IAP is typically mosaic and varies widely between isogenic A^{vy}/a mice, whose phenotypes (Fig. 1*B*) range from fully yellow and obese, through degrees of mottled yellow/agouti with intermediate body mass, to lean fully agouti (called pseudoagouti) (6, 7).

The observed pattern of epigenetic mosaicism of the A^{vy} allele is consistent with a somatically stable epigenetic state (either on or off) established in early embryogenesis. Phenotypic variation is a direct result of variation in the epigenotype of the IAP retrotransposon from which the aberrant A^{vy} transcript originates; thus the syndrome represents a case of a retrotransposon acting as a controlling element (2). The IAP's epigenotype correlates closely with cytosine methylation of its 5′ LTR: in pseudoagouti mice the LTR is heavily methylated, in yellow mice it is unmethylated, and in mottled mice methylation is intermediate (4, 8, 9). Whereas other retrotransposons are maintained in a state of stable epigenetic silence, the behavior of the IAP that controls the A^{vy} allele is highly unusual: it exhibits a strong tendency for reversion in the germ line from the active to the silent state (or vice versa), whereas its somatic state (active or silent) is stable. The mechanistic basis of epigenetic variation in the A^{vy} allele is not known.

Somatic activity of the A^{vy} allele can be affected by maternal nutrition. When pregnant dams receive a diet supplemented with methyl donors (folate, choline, betaine, and vitamin B12), the spectrum of phenotypes in A^{vy} offspring is shifted toward the epigenetically suppressed state that is termed pseudoagouti (4, 8, 10). This change correlates with an increase in cytosine methylation of the A^{vy} allele (4, 8), indicating that the epigenetic state of the allele can be influenced by environmental factors. Methyl donors may influence the pool of *S*-adenosylmethionine, which donates methyl groups to cytosine and many proteins (10); the epigenetic effects of dietary methyl donors could be direct (methylation of cytosine) or indirect.

The somatic epigenetic changes in response to methyl donor supplementation raise the question of whether methyl donor supplementation also affects the germ line, and whether any changes could be maintained into the next generation. Epigenetic marks at genes and repeat elements are usually removed and reset soon after fertilization (11), but some mammalian genes, and the A^{vy} IAP, can at least partially retain epigenetic marks so that their epigenotype is inherited (9, 12). Reports of heritable effects mediated by environmental factors (13–15), sometimes involving methylation changes (13), have not identified specific genes.

We have assessed the ability of methyl donor supplementation to alter the epigenetic state of the A^{vy} allele in the germ line. We find that the A^{vy} allele is responsive to methyl donor supplementation only when it is contributed by the sire. Supplementation of the maternal diet for a period during midgestation produces changes in the offspring exposed *in utero*, but also in pups born in the subsequent generation that was not exposed to

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Abbreviations: ASP, agouti signaling protein; E*n*, embryonic day *n*; IAP, intracisternal A-particle.

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¶To whom correspondence should be addressed. E-mail: dimartin@chori.org.

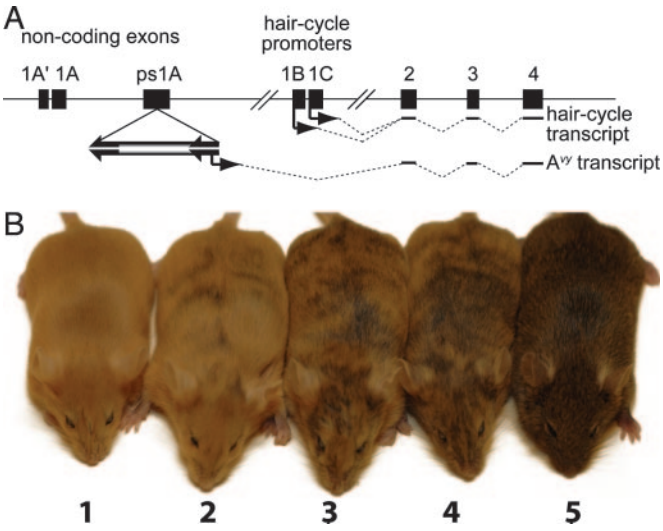

Fig. 1. The A^{vy} allele and the spectrum of A^{vy} phenotypes. (*A*) The A^{vy} allele carries an insertion of an IAP retrotransposon in an antisense direction in *agouti* pseudoexon 1A (3, 4). The A^{vy} transcript originates from a cryptic promoter in the 5′LTR of the IAP and is spliced to *agouti* coding exons 2, 3 and 4, which encode ASP (3). When the IAP is silent, *agouti* is transcribed from hair-cycle-specific promoters in exons 1B and 1C. (*B*) A^{vy} phenotypes are scored from 1 to 5 based on coat color. Fully yellow mice are scored as 1, and fully agouti mice are scored as 5. Phenotypes of mosaic mice range from mostly yellow (2) to mottled yellow/agouti (3) to mostly agouti (4).

higher levels of methyl donors. Taken together, these results demonstrate that environmental influences on phenotype may act by inducing epigenetic changes, and that such changes may be heritable and allele-specific.

Results

The A^{vy} allele is dominant: when heterozygous with *a*, the epigenetic state of the A^{vy} allele is readily apparent by inspection of the coat (Fig. 1) (6, 7). Coat color (yellow, mosaic yellow/agouti, or pseudoagouti) is closely linked to other manifestations of the viable yellow phenotype (obesity, type II diabetes, and tumor incidence) and to methylation of the IAP retrotransposon that drives ectopic expression of ASP (4, 6–9). Because *in utero* methyl donor supplementation subtly shifts the spectrum of phenotypes displayed by offspring, detection of any effect requires breeding of a sufficiently large number of mice to establish its significance. In our experiments, offspring in each treatment or control group were considered as independent variables for statistical analysis (see Methods), so that all offspring in each treatment group were compared with all offspring in the corresponding control group, without reference to their respective dams.

The A^{vy} Allele Is Affected by Dietary Methyl Donors Only When It Is Paternally Contributed. Our experiments aimed to assess the heritability of effects on the A^{vy} allele created by supplementation of the maternal diet with methyl donors; that is, the ability of methyl donors to alter the epigenotype of the A^{vy} allele in the germ line. The epigenotype of the A^{vy} allele is partially stable in the female germ line, leading to weak inheritance of the maternal phenotype, but paternal A^{vy} epigenotype does not influence the phenotype of offspring (9, 16). Thus, the spectrum of phenotypes in offspring of A^{vy}/a females is skewed toward the maternal phenotype, whereas the spectrum of phenotypes in offspring of A^{vy}/a males is the same regardless of paternal phenotype (9, 16). We thus reasoned that methyl donors would be more likely to stably alter the epigenotype of A^{vy} in the female germ line. For this reason we began by providing methyl donor supplementation to A^{vy} females mated to congenic a/a males.

A^{vy}/a dams mated to a/a sires were fed an NIH-31 diet supplemented with folate, choline, betaine, vitamin B12, zinc, and methionine (Specialty Feeds, Glen Forest, WA, Australia) (8) for 2 weeks before mating and during pregnancy and lactation. Offspring were scored for coat color phenotype (Fig. 1*B*) at weaning.

In apparent conflict with previously reported experiments (4, 8, 10) we observed no effect on the phenotypes of A^{vy}/a offspring of supplemented dams when compared with offspring of unsupplemented A^{vy}/a dams (Fig. 2 *Left*). In those experiments, however, the A^{vy} allele had been contributed by the sire (4, 8, 10). We thus repeated the supplementation experiment using A^{vy}/a sires and a/a dams, and obtained a result (Fig. 2 *Right*) consistent with the previous reports: a significant shift in the spectrum of phenotypes toward pseudoagouti (silent, methylated A^{vy}).

These results suggest that *in utero* methyl donor supplementation affects the somatic epigenetic state of the A^{vy} allele only when the allele is derived from the sire.

Methyl Donors Induce a Germ-Line Epigenetic Change in the A^{vy} Allele. We reasoned that if a germ-line change in A^{vy} epigenotype were induced by methyl donors, it could be reflected in phenotypes in a later generation that had not been exposed to methyl donor supplementation. To test this idea, we supplemented pregnant dams and bred their F_1 offspring to observe phenotypes in the F_2 generation. Because we found that methyl donor supplementation affects only the paternally contributed A^{vy} allele (see above), we mated a/a dams to A^{vy}/a sires to produce the F_1 generation. The methyl donor supplemented diet was provided only from embryonic day 8.5 (E8.5) to E15.5. This restricted period of supplementation had two related purposes. First, because it commenced well past the period

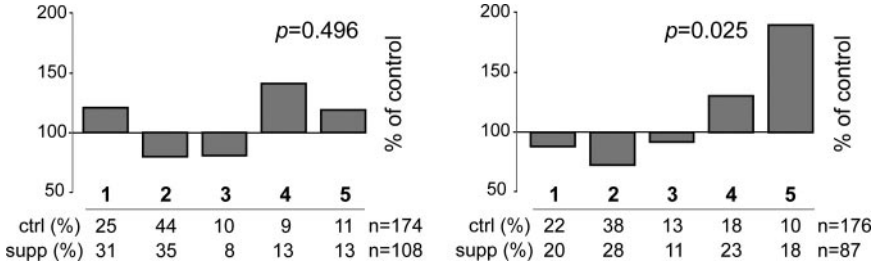

Fig. 2. Parent-of-origin effect of methyl donor supplementation. Parents were supplemented with methyl donors throughout pregnancy and offspring phenotypes scored as in Fig. 1*B*. (*Left*) Maternal transmission. Shown is the maternally derived A^{vy} allele (A^{vy}/a dam, a/a sire). (*Right*) Paternal transmission. Shown is the paternally derived A^{vy} allele (a/a dam, A^{vy}/a sire). Results are expressed as the percentage of supplemented offspring with the same phenotype as unsupplemented controls; the percentage of offspring of each coat color is shown beneath each graph.

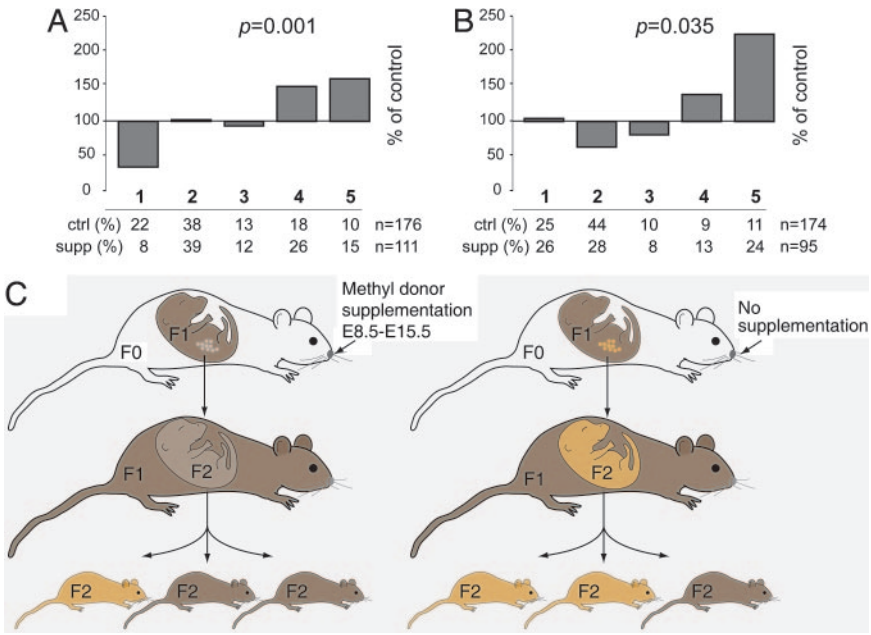

Fig. 3. Midgestation and germ-line effects of methyl donor supplementation. (*A*) F_1 phenotypes. Shown is the effect of methyl donor supplementation on mid-gestation supplemented offspring. Dams were supplemented with methyl donors from E8.5 to E15.5, and offspring phenotypes were scored as in Fig. 1*B*. Results are expressed as the percentage of supplemented offspring with the same phenotype as unsupplemented controls, and the percentage of offspring of each coat color is shown beneath each graph. (*B*) F_2 phenotypes. Shown is the heritable/grandparental effect of methyl donor supplementation. Phenotypes of F_2 offspring from pseudoagouti female mice shown in *A* are expressed as a percentage of control offspring (offspring from pseudoagouti dams that had never been supplemented) with the same phenotype. (*C*) A schematic diagram illustrating the effect of methyl donor supplementation in the germ line. Epigenetic changes to A^{vy} in primordial germ cells exposed to methyl donors during differentiation (*Left*) are maintained throughout gametogenesis and embryogenesis. Thus, pseudoagouti F_1 mice that are genetically and phenotypically identical but were subject to different diets *in utero* (*Left* vs. *Right*), can produce phenotypically different F_2 offspring.

when somatic A^{vy} epigenotype appears to be set, we supposed that it might have no effect on the phenotypes of F_1 mice. Second, because the supplementation encompasses the period when primordial germ cells differentiate and reset epigenetic marks (11), it may be the optimum point to induce an epigenetic change in the germ line.

The pattern of mosaicism in A^{vy} mice is consistent with an epigenetic state that is set during early embryogenesis and is stable (in somatic cells) thereafter. This stability resembles that seen with other epigenetic phenomena such as parental imprinting and X-chromosome inactivation. By recording phenotypes of F_1 mice in our experiment, we were able to observe any effects of *in utero* methyl donor supplementation on the epigenotype of the A^{vy} allele. Despite the restricted period of supplementation, the spectrum of phenotypes in F_1 mice was shifted significantly (Fig. 3*A*), indicating that the A^{vy} allele is susceptible to induced epigenetic change even after the early embryonic period when epigenetic resetting takes place.

We selected (F_1) pseudoagouti A^{vy}/a females that had been exposed to methyl donors *in utero* from E8.5 to E15.5, and mated them to a/a males without any further methyl donor supplementation; this strategy takes advantage of the tendency for the A^{vy} epigenotype to be partially stable in the female germ line (9, 16). The phenotypes of the second generation (F_2) offspring were compared with phenotypes of pups born to pseudoagouti females with no history of exposure to methyl donor supplementation. Phenotypes of these F_2 mice were significantly shifted toward the pseudoagouti (Fig. 3*B*). Thus a pseudoagouti dam who was exposed to methyl donor supplementation only when she was *in utero* gives rise to phenotypically different offspring than does an otherwise (genetically and phenotypically) identical female who had no exposure to methyl donor supplementation (Fig. 3*C*); this grandparental effect is directly attributable to the epigenetic state of the A^{vy} allele.

It is likely that the inherited modification induced by methyl donor supplementation is placed on the A^{vy} allele at the point, during mid- to late gestation, when epigenetic marks are reset in the differentiating primordial germ cells that later give rise to the F_2 generation (11). Because nutritional supplementation ceased when the F_1 mice were still *in utero*, our evidence indicates that the effect on A^{vy} epigenotype in these primordial germ cells is retained throughout gametogenesis as well as during the fertilization and development of the F_2 embryo (Fig. 3*C*).

Discussion

Our results indicate that methyl donors can affect the germline epigenetic state of the A^{vy} allele, and that this effect is stable for at least one generation without further exposure to the supplementary methyl donors. Previous work had shown that mice exposed *in utero* to higher levels of methyl donors are more likely to display the pseudoagouti phenotype that is associated with methylation and transcriptional silence of the IAP retrotransposon that drives the viable yellow phenotype (4, 8, 10). We find that this effect on the somatic epigenotype of A^{vy} occurs only when the allele is derived from the sire, and that it does not require exposure during early embryogenesis. The spectrum of A^{vy} phenotypes is altered in mice in the generation (F_2) that succeeds the one exposed to methyl donors during midgestation. The implications of this finding are worth considering.

The germ-line alteration of the A^{vy} epigenotype is definite evidence that an environmental factor can produce a phenotypic effect by inducing an epigenetic modification in the mammalian germ line, and that such a modification can persist through the epigenetic resetting that takes place during gametogenesis and

embryogenesis. A number of reports have described heritable phenotypic effects in mammals induced by environmental agents, some well defined and others less so (13–15, 17, 18). There has been wide speculation that such effects are epigenetic in origin, and in one system, heritable changes in methylation were detected in response to endocrine disruptors (13). But because the specific loci that mediate the described phenotypic effects are not known in any of these cases, the evidence that the phenotypic effects result from epigenetic modifications is inconclusive. On the other hand, the effect of dietary methyl donors on A^{vy} is indisputably one in which the inheritance of the environmental effect is based on epigenetic modification of a specific locus.

The parent-of-origin-specific effects of methyl donor supplementation on A^{vy} (Fig. 2) may provide an insight into the inheritance of A^{vy} epigenetic state. In isogenic A^{vy} mice, the epigenotype is weakly heritable through the female: the spectrum of coat colors in offspring of A^{vy}/a females differs with maternal phenotype, whereas offspring of A^{vy}/a males exhibit the same spectrum of coat colors regardless of the sire's phenotype (9, 16). That is, the epigenotype of the paternal A^{vy} allele undergoes complete epigenetic resetting during embryogenesis, whereas the maternal A^{vy} allele partly retains its epigenotype. This relative stability of the A^{vy} epigenetic state in the female germ line may relate to its resistance to the influence of increased methyl donors. The paternally inherited A^{vy} allele, being more epigenetically labile in the germ line, may be more vulnerable to environmental influence. Because we examined the effects of methyl donors on the A^{vy} allele only in offspring of pseudoagouti mothers (carrying a silent A^{vy}), further investigation will be required to determine whether all epigenetic states of the maternal A^{vy} allele are resistant to methyl donor supplementation.

We find that the somatic state of the A^{vy} allele is susceptible to methyl donor supplementation even when exposure takes place after the period of epigenetic resetting in early embryogenesis. This result was surprising, because the pattern of mosaicism in A^{vy} mice indicates stability of the epigenetic state in later embryogenesis and thereafter. Taken together with a recent finding that an imprinted locus is sensitive to nutritional intervention even post partum (19), this finding implies that epigenetic states may be sensitive to environmental effects throughout the life cycle. It is notable that the evidence for epigenetic resetting in preimplantation embryogenesis, and the stability of epigenetic states thereafter, is principally based on analysis of CpG methylation (20–23). If, as discussed below, the effect of methyl donor supplementation is mediated by another pathway, this finding might indicate an unsuspected plasticity of somatic epigenetic states.

Epigenetic silencing at A^{vy} is linked to increased CpG methylation of the IAP (4, 8, 9, 24). It is tempting to suppose that methyl donors act directly to increase cytosine methylation on the IAP. CpG methylation is, however, merely one part of a complex of epigenetic modifications that are typical of silent chromatin; available evidence indicates that it is placed on DNA subsequent to epigenetic silencing, and it serves to consolidate and maintain the silent state through interaction with chromatin proteins (24). Studies of the somatic effects of dietary methyl donor supplementation have shown that although supplementation increases the propensity of the allele to silence, the level of CpG methylation on the IAP in supplemented mice is equivalent to that in unsupplemented mice of the same phenotype; i.e., when adjusted for phenotype, the methylation states are equivalent (4). Methyl donors are expected to increase the pool of *S*-adenosylmethionine, which can donate a methyl group to a variety of proteins as well as cytosine. Thus, the epigenetic effects of dietary methyl donors may be mediated by effects on, for example, histones, which would have the effect of increasing CpG methylation due to silencing of the A^{vy} allele in a higher proportion of cells (24).

The IAP in the A^{vy} allele is a "controlling element": a transposable element that exerts transcriptional control over a gene near its insertion site, as first described by McClintock (2, 25). Although it is not known how many retroelements act as controlling elements in the mammalian genome, several published examples are available (2, 12, 26), and our work indicates that there may be many more (D.I.K.M. and G. Thomson, unpublished work). Thousands of retroelements have the potential, if active, to behave as controlling elements, with unpredictable effects on phenotype (2). The susceptibility of these elements to perturbation by environmental agents provides another way in which epigenetics can mediate environmental influence on phenotype.

The increasing evidence for the heritability of environmental exposure introduces an added degree of complexity into attempts to disentangle so-called "gene–environment interactions," often interpreted to signify the interaction of fixed (Mendelian) inheritance with a dynamic environment. It is apparent that the heritability of "environment" through epigenetic settings will make such an effort more difficult. Moreover, in light of the roughly 20-year generation time of humans, our results suggest that current dietary habits may have an influence on grandchildren who will be born decades from now, independent of the diets that their parents consume.

Materials and Methods

Mice and Diets. The A^{vy} allele arose in the C3H/HeJ strain (27) and was backcrossed into C57BL/6 at The Jackson Laboratory (Bar Harbor, ME). The mice used in this study are descended from the isogenic C57BL/6 A^{vy} colony maintained at Oak Ridge National Laboratories and were rederived at the Victor Chang Cardiac Research Institute in 2001.

Mice were fed ad libitum on NIH-31 diet (control) or methyl-donor supplemented NIH-31 [plus (per kg) 15 g of choline, 15 g of betaine, 7.5 g of L-methionine, 150 mg of $ZnSO_4$, 15 mg of folic acid, and 1.5 mg of vitamin B_{12}] (Specialty Feeds) (8, 10). For mice supplemented throughout pregnancy, methyl donor supplementation of the dam was started 2 weeks before mating and continued throughout pregnancy and lactation. For mice supplemented during midgestation, the date of conception was determined by observing vaginal plugs. Methyl donor supplementation was started on E8.5 and discontinued at E15.5.

Female A^{vy}/a mice selected for breeding were always pseudoagouti, because the epigenotype of the dam influences that of the offspring. Male A^{vy}/a mice selected for breeding were either mottled or pseudoagouti: although the epigenotype of the sire does not influence that of the offspring, yellow mice are inefficient breeders. Control groups consisted of mice bred in exactly the same way but without any methyl donor supplementation.

Phenotype Scoring. Coat color in A^{vy} mice is tightly linked to the other manifestations of the obese yellow phenotype and to methylation of the A^{vy} allele (4, 6, 9). Coat color in A^{vy}/a offspring was assessed at mouse weaning age (3 weeks) by two trained, independent observers. We used a numerical scale from 1 to 5 such that 1 was completely yellow, 2 was mostly yellow with slight agouti mottling, 3 was ≈50% yellow/agouti mottling, 4 was mostly agouti, and 5 was complete agouti (called pseudoagouti); representative phenotypes are illustrated in Fig. 1*B*. The scores from the two observers correlated >85% of the time; when they did not agree, the coat color was randomly assigned to one or the other of the scores.

Statistical Analyses. For statistical analysis, offspring in each treatment group were considered as independent variables. We used the Kruskall–Wallis test to establish that offspring coat color phenotype was independent of the dam by comparing the means of the coat colors of offspring from all dams in a treatment group. This method allowed us to compare all offspring in each treatment group with all offspring in the corresponding control group without reference to the dam. For comparison of treatment groups with controls, we used a Mann–Whitney test ($\alpha = 0.05$). Parametric analysis (one-way ANOVA and Student's t test) gave very similar results, probably due to our large sample size, allowing parametric testing of ordinal, non-normally distributed data.

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