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Effects of Post Weaning Diet on Metabolic Parameters and DNA Methylation Status of the Cryptic Promoter in the A^{vy} Allele of Viable Yellow Mice

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Abstract

Mice carrying the A^{vy} allele are epigenetic mosaics. If the majority of cells have an active (demethylated) intracisternal A particle (IAP), mice have a yellow coat color and develop adult-onset obesity and diabetes, while mice whose mosaicism predominantly reflects an inactive (methylated) IAP are pseudoagouti (brown) and less prone to metabolic disease. Brown and yellow coat color A^{vy}/a post-weaning mice were placed on one of three diets (AIN, and two lower calorie diets NIH and methyl-supplemented, NIHMe) to determine whether coat color, weight gain, blood glucose, and methylation of hepatic IAP became altered. None of the diets altered A^{vy}/a mice coat color. NIHMe did not protect against increasing obesity or the usual onset of hyperglycemia in males. Nor did it promote increased methylation of A^{vy} IAP in liver tissue. By contrast, AIN, despite its higher content of fat and carbohydrate and ability to promote greater weight gains than the NIH and NIHMe diets, protected males better against hyperglycemia than either the NIH or NIHMe diets. This diet led to a significantly reduced ($\sim 50\%$; $P = 0.003$) average methylation state of all CpG sites within the hepatic IAP for the pseudoagouti mice. On AIN, but not on the other diets, extent of hepatic IAP methylation was negatively correlated ($R = 0.97$, $P = 0.001$) with body weight of pseudoagouti mice. The findings indicate that post-weaning

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diet might influence interpretation of studies with A^{vy}/a mice because IAP methylation patterns may be malleable in certain organs and influenced by post-weaning diet.

Keywords

Agouti; DNA methylation; Epigenetics; Methyl diet; Obesity; Hyperglycemia

1. Introduction

The various coat color patterns of A^{vy}/a mice originate from differences in the control of expression of the *agouti* (*A*) gene, and specifically the A^{vy} allele [1–4]. Mice [5] and various animals, including humans [6, 7], possess at least one *agouti* gene locus, which encodes for the agouti signaling protein (ASIP). Mouse ASIP is a 131 amino acid that acts in a paracrine manner to bind to the melanocortin-1 receptor (MC1R), which prevents alpha-MSH signaling. The suppression of this pathway down-regulates synthesis of brown/black pigments and increases synthesis of yellow/red (pheomelanin) pigments during the mid-stage of the hair follicle cycle [8]. The coat color patterns in the siblings range from pseudoagouti (brown), mottled, to full yellow. These differences are due to the labile activation state of the A^{vy} allele during conceptus development. The increased appetite and obesity of the yellower mice most likely arises from ectopic expression of ASIP outside the hair follicle, especially in the hypothalamus [5], where it antagonizes the melanocortin 4 receptor (MC4R) [8], and in the pancreas [9].

Analysis of the A^{vy} allele has demonstrated an inserted retrotransposon (an intracisternal A particle or IAP) not present in the wild type *A* allele that is located upstream (in pseudoxon 1a) of the customary (exon 2) transcriptional start site [2, 10]. This viral insertion contains a 5' long-term repeat (LTR) region, which acts as a cryptic promoter capable of taking over the transcriptional controls that typically confine the production of ASIP to the hair follicles [1, 4, 10–12]. However, the expression driven by the 5' LTR is highly variable so that the majority of mice are epigenetic mosaics. Thus, if the fur of A^{vy}/a mice is yellow, the promoter in the majority of its cells is hypomethylated and active, while if the mice are brown it is widely hypermethylated and inactive. Mottled mice result from mosaicism, reflecting relative size and number of clonal patches of tissue where the A^{vy} allele is either active or inactive. However, when A^{vy}/a males on a C57Bl6J background are bred to C57Bl6J (*a/a*) black females, the resulting F1 A^{vy}/a offspring can range from completely yellow to mottled to fully brown, whatever the coat color pattern of the breeder male [2]. Thus, the reestablishment of the IAP methylation state in F1 offspring born to A^{vy}/a males appears to be stochastic. On the other hand, if the A^{vy} allele passes through the female germ line, rather than through the male, the coat color and associated IAP methylation patterns of the A^{vy}/a offspring, though still variable, are biased towards those of their mother, prompting the conclusion that some epigenetic information governing the methylation state of the IAP is able to pass through the female germ line and be transmitted to the F1 progeny.

Another intriguing aspect of the A^{vy} allele is that its expression status in the offspring can be influenced by the diet of the mother while she is pregnant. For example, a diet enriched in

various methyl donors, when provided to dams carrying A^{vy}/a conceptuses, boosts the average methylation state of the IAP and provides an increased proportion of offspring towards the browner end of the color coat spectrum, which have an accompanying decreased risk for adult-onset disease [1, 3, 4, 13]. Diets contaminated with chemicals that are weak estrogens have also been reported to be capable of shifting the coat colors and IAP methylation patterns within litters born to a/a dams after they had been bred to A^{vy}/a males [14–16], although these results have been questioned [17, 18]. Despite the controversial nature of the latter studies, these findings overall are consistent with many others in the nutrition field [19–21] that indicate maternal uterine environment can modulate epigenetic marks established early in development that are the forerunner of events manifested after the animal is born and matures into an adult.

Post-weaning diets that are either deficient or enriched in compounds that are anticipated to promote methylation have been observed to have a variety of effects on adult health [22–25] and have suggested that there might be malleability in the methylation status of select genes, including ones implicated in cancer [14–16, 26, 27]. However, as far as we are aware, such diets have not been examined to determine whether they can alter the coat color and adult health of the (A^{vy}/a) mouse. Here we have asked whether or not a methyl-enriched diet can ameliorate the weight gain and high blood glucose status of yellow A^{vy}/a mice and even reverse their coat colors towards brown. Additionally, we sought to determine whether administration of any of these three diets could alter the average DNA methylation status of the A^{vy}/a gene, as has been previously reported to occur with uterine exposure of conceptuses to the methyl-supplemented diet [1, 3, 4, 13, 16]. If so, it would suggest that the methylation status, and hence expression, of genes with important roles in governing the health of adults might be changed by appropriate adjustments in nutrition.

2. Materials and Methods

2.1 Animals

The NIH guidelines for the care and use of laboratory animals were followed, and all experiments were approved by the University of Missouri Animal Care and Use Committee (ACUC, Protocol #7753). Female C57Bl6J a/a females on a phytoestrogen-free AIN93G (AIN) control diet were bred to A^{vy}/a males that were also maintained on this same diet. This breeding scheme provides A^{vy}/a F₁ mice with coat colors ranging from all brown, Y₀, to all yellow, Y₅, with intermediate mottled patterns (Y₁–Y₄) between the two extremes [17, 28]. At the time the F₁ offspring were weaned at 21 days post-partum, predominantly yellow (Y₄–Y₅) and brown (Y₀–Y₂) pups were selected and each housed with a same sex partner of the opposite color, i.e. yellow with brown, for the duration of the experiment. This design is similar to that used by other groups employing this animal model [1, 3, 4, 14–16]. At four weeks of age, the mice were randomly chosen to be placed on one of the three diets: AIN, NIH-31 (NIH), or the NIH methyl-supplemented (NIHMe) diet (Table S1). The latter two diets have previously been used in experiments to test effects of methyl supplementation [1, 3, 4, 13–17], and thus, we sought to use them as controls for the current study. Ten brown and ten yellow mice of each sex were assigned to each diet. Food and water were provided on *ad libitum* basis, and the animals housed in polypropylene cages and provided filtered

water in glass water bottles to reduce exposure to the estrogenic compound, bisphenol A. The room temperature was 21–23.5°C and humidity range was 40–45%. The AIN diet (Table S1) is a refined, soy-free diet with supplemental corn oil that is more energy rich than either the NIH or NIHMe diet. Mice were maintained on their assigned diets for the duration of the experiments.

2.2 Measurement of body weight and blood glucose concentrations in $A^{vy/a}$ mice

To determine if the diets altered body weight or blood glucose concentrations, both measurements were taken once a week at 1 pm, in the middle of the light cycle, from non-fasted 4 to 12 weeks of age. This time of day was chosen, as laboratory mice, particularly those in a C57Bl6J background, eat primarily during the dark cycle [29]. We opted to test non-fasted animals to minimize any metabolic disruptions that repeated fasting might cause, especially in obese yellow-coat color animals where fasting may increase their risk for hepatic lipodosis. Samples were then drawn every two weeks until the mice were 32 weeks of age, at which point mice were culled and final assessments performed. Blood was sampled in live animals from the saphenous vein, and blood glucose concentration was determined by using the Bayer Contour Glucometer (Bayer, Tarrytown, NY).

2.3 DNA methylation analysis of A^{vy} promoter site

Livers ($n=3$ for each A^{vy} coat color, brown and yellow, and sex, i.e. 36 mice) were collected when the animals were euthanized at 32 weeks of age, and stored at -80°C . This organ was selected based on the vital metabolic role of the liver. Moreover, prior rodent studies have demonstrated that dietary methyl donors can significantly affect hepatic DNA methylation patterns [24, 30]. The three yellow and brown males and the three yellow and brown females from each diet group were selected randomly for analysis. Genomic DNA from the samples was isolated with the DNeasy blood and tissue kit (Qiagen, Valencia, CA). The integrity and quantity of the DNA were assessed by electrophoresis in 1% agarose gels and by fluorometric analysis (Qubit fluorometer, Life Technologies, Grand Island, NY), respectively. The DNA (1 μg per sample) was then subjected to bisulfite modification with the EZ DNA methylation kit (Zymo Research, Irvine, CA) and PCR amplification with Platinum Taq (Life Technologies). The primer sequences (Forward primer, IAPF3-5' ATTTTGTAGGAAAAGAGAGTAAGAAGTAAG 3' and reverse primer, IAPR4-5' TAATTCCTAAAAATTTCAACTAATAACTCC 3' and the PCR condition for the $A^{vy/a}$ IAP promoter region were based on those used in other studies with this animal model [3, 14–16]. This primer pair leads to a 336 bp product with 9 CpG sites (Fig. S1). Gel-purified (Wizard® SV gel and PCR clean-up kit, Promega, Madison, WI) amplicons were TA-cloned into pGEM T easy vector (Promega). The plasmids in *E. coli* colonies were directly amplified by TempliPhi DNA amplification kits (GE Healthcare, Buckinghamshire, UK), and submitted for sequencing (Macrogen USA, Rockville, MD). The methylation status in each of the nine targeted CpG sites [3, 14–16] was analyzed by using a BiQ Analyzer [31]. For each sample, at least 12 clones were analyzed to determine the DNA methylation status within the liver.

2.4 Statistics

The body weight and serum glucose concentration data were analyzed by using a repeated measurement design with PROC GLIMMIX and SAS version 9.2 software analyses (SAS Institute, Cary, NC). In order to determine the potential effects of the variables (detailed below) on body weight and serum glucose concentrations, the data were grouped into three age categories: weaning to onset of puberty (4 to 9 weeks of age), young adulthood (10 to 14 weeks of age), and adulthood (15 to 32 weeks of age). The values shown in Figs. 2 & 3 and in Fig. S1 are mean values $\pm$ S.E.M. for the mouse weights and blood glucose concentrations averaged across each of the three growth periods. In addition, the weights of the animals at the time they were euthanized, i.e. final weights, were also analyzed. For all dependent variables, sources of variation that were considered in the analyses included, coat color, sex, and the interaction of coat color $\times$ sex, and the repeated measures of time (age) and all possible interactions of time with coat color and sex for both variables. When main effects were detected, the difference between treatment groups was determined by using the Fisher Least Significant Difference analysis. All data are expressed as least squares means $\pm$ SEM, and $P < 0.05$ was considered significant.

For the individual and average CpG methylation values within the IAP site of the *A* locus, the ANOVA provided the effects of diet, color, and sex, and all possible interactions of diet, color and sex, as a 3 by 2 by 2 factorial design (diet, color, and sex). This approach could be used because the central limit theorem (CLT) indicates that, if the average of a number of values is taken, then that average value becomes normally distributed as N goes to ∞ . The convergence to normality is very rapid from 8 to 10 values, and since the individual and average methylation is the average of > 8 values, the CLT assumes normality. Mean differences were determined by using Fisher's Least Significant Differences (LSD) for all of the main effects and possible interactions (diet, color and sex).

Linear regression correlation analyses were performed to determine if there were any relationships between individual and average CpG island methylation states and phenotypic effects (body weight and serum glucose concentrations). These analyses were also performed for all possible combinations of diet, color and sex for those animals examined in both the DNA methylation and phenotypic studies.

3.0 Results

3.1 Offspring Coat Color

Ten animals of each sex and coat color for each diet, i.e. 120 mice, were assessed for changes in coat color from weaning until the mice were 32 weeks of age, when the study was terminated. No shifts in coat color status were observed in response to any of the three post-weaning diets, including NIHMe in our experiments. Fig. 1A is a typical example of a slightly mottled to almost all yellow $A^{vy/a}$ weanling mouse before it was placed on the NIHMe diet, while Fig. 1B is the same mouse subsequently fed the diet for 29 weeks.

3.2 Body Weight

For statistical purposes, the weekly body weight data were grouped into three time periods: puberty (weaning to onset of sexual maturity; 4 to 9 weeks of age), young adulthood (10 to 14 weeks of age), and adulthood (15 to 32 weeks of age) for the three diets: AIN, NIH31 (NIH), and NIH31 with methyl supplements (NIHMe) (Table S1). Comparisons below represent those between diet groups for the same coat color and across coat color for groups that had been fed the same diet. For further details on body weight gain over time, Figs. S2 and S3 provide mean body weight plotted over the course of the experiments for males and females, respectively. Although we attempted to randomize the mice into the dietary groups, it was important to determine that there were not preexisting differences in body weight, as these could influence outcomes. The P-value for the three-way interaction (diet $\times$ sex $\times$ color) at this time was 0.30, i.e. not significant. However, pairwise comparisons revealed that the yellow coat color females to be placed on the AIN diet were slightly heavier than the yellow coat color females to be placed on the NIH diet ($P = 0.05$). None of the other pairwise comparisons were significant at this initiating time point.

3.21 Males—The male A^{vy}/a mice, whether yellow or brown, gained weight faster on the control AIN diet than they did on either the control NIH diet or the experimental NIHMe diets ($P < 0.05$). The trend was evident at puberty and the differences significant in young adult and adults relative to male mice on the NIH diet (Fig. 2A; Table S2, P value range 0.0005 to 0.05) and was still seen at the time animals were sacrificed at 32 weeks ($P < 0.05$, Figs. S2 & S4). At sacrifice, yellow coat males fed both AIN and NIH weighed more than their brown counterparts on the same diets (AIN: yellow males 58.7 ± 1.1 g; brown males: 52.3 ± 1.8 g, $P < 0.05$; NIH: yellow males 52.0 ± 3.0 g, brown males 46.0 ± 2.6 g, $P < 0.05$, Fig. S4). No differences in weight were observed between yellow and brown males fed NIHMe.

3.22 Females—As with the males, diet, particularly AIN, had a major effect on relative weight gain of both brown and yellow coat color females (Figs. 2 & S3). For example, yellow mice on the AIN diet already weighed more than their counterparts on the NIH diet at puberty (23.3 ± 2.0 g *versus* 16.6 ± 2.0 g, $P < 0.0001$, Fig. 2B, Table S2). The increased weight gain on the AIN diet relative to the NIH and NIHMe diets was clearly evident among young adult and adults of both coat colors (P value range < 0.0001 to 0.01) and the differences were retained until sacrifice at 32 weeks (P value range < 0.0001 to 0.01, Fig. S4). For reasons that are unclear, brown adult females fed the methyl-enriched NIHMe diet weighed more than brown adult females on NIH (40.1 ± 2.3 g *versus* 32.1 ± 2.0 g, respectively, $P = 0.04$); whereas, there was no difference in weight between yellow females on these two diets ($P = 1.0$). Brown females on all the diets were leaner than their yellow counterparts by the time they reached young adulthood, and these differences continued into adulthood (P value range < 0.0001 to 0.04 for young adults; < 0.0001 to 0.04 for adults).

3.3 Serum Glucose Concentrations

The weekly data were grouped into the same three age groups as detailed above.

3.31 Males—While there were no differences in serum glucose concentrations based on diet or coat color in males at puberty, significant differences had begun to emerge at young adulthood (Fig. 3a, Table S3). Specifically, young adult and adult brown males on AIN were able to maintain blood glucose levels similar to those they displayed during puberty and fell in the normal range for mice on a C57Bl6J background [32], whereas values for brown males on the NIH and NIHMe diets had risen significantly by young adulthood (P value range = 0.002 to 0.005). Unlike AIN, the NIH and NIHMe diets were clearly ineffective in opposing the rise in serum glucose that accompanied maturity. Serum blood glucose concentrations among yellow males on all three diets did not differ either at puberty or during young adulthood, but, by adulthood, the AIN diet again supported lower serum glucose values than the other two (P value range = 0.01 to 0.03).

Young adult and adult brown males on the AIN diet were able to maintain lower blood glucose concentrations than their yellow counterparts (young adults, P value = 0.003 and adults, P value = 0.01). These coat color distinctions relative to blood glucose concentrations were also evident on the NIH diet in early-adult females (P = 0.03), and were significant for NIHMe and approached significance on the NIH diets in adulthood (P = 0.03 and 0.06, respectively, Fig. 3A, Table S3).

3.32 Females—There were no differences in serum glucose concentrations based on diet or coat color in females at puberty, young adulthood, or adulthood (P value range = 0.2150 to 0.9860; Fig. 3B, Table S3). All females, whatever their coat color and the diet they were fed, had values within the normal range.

3.4 DNA methylation analysis within the 5' LTR promoter site of the A^{vy} allele

To determine whether any of the three diets affected the methylation status of the cryptic promoter site, we chose to examine the same nine CpG sites within the IAP that have been most frequently analyzed by others when studying the relationship between methylation state and coat color and especially effects of maternal diet during pregnancy on these parameters (Fig. S1) [1, 3, 4, 13, 16]. Analyses were performed on 36 different liver samples (three diets, two coat colors; three males and three females for each group). Twelve clones were sequenced for each liver sample, i.e. 288 total sequences were analyzed. The percentage of methylation within the 5' LTR promoter site of the A^{vy} allele for each liver sample was then calculated from the number of methylated CpG sites relative to the total CpG sites. As no differences were observed in methylation status between males and females on any of the diets, data for both sexes were combined. In other words, each of the values shown in Fig. 4 represents the means for six mice for each coat color and diet.

As observed consistently by others conducting such analyses, brown coated mice on all three of the diets had a greater degree of methylation at each individual CpG site than the yellow mice (Fig. 4). Moreover, the average percentage methylation within the entire cryptic promoter region was higher for brown than yellow mice. Despite the lack of sexually dimorphic differences, there were significant diet effects. Notably, feeding the AIN diet led to a lower extent of methylation at all nine of the CpG sites and in average total methylation at all sites in brown coat color animals compared to brown morphs fed the NIH (control)

diet ($P = 0.003$, Fig. 4). Surprisingly, the NIHMe diet provided values for CpG site methylation intermediate between those noted for the AIN and NIH diets. For the brown coat color animals, these values approached but did not reach significance compared to their counterparts on the NIH diet ($P = 0.07$, Fig. 4). When yellow mice were fed NIHMe, methylation patterns were not significantly different from those observed on the AIN and NIH diets ($P = 0.6$ for both diet comparisons). These findings clearly indicate that enrichment of methyl donors in the NIHMe diet failed to enhance methylation within the IAP relative to the control NIH diet.

3.5 Correlation between DNA methylation within the cryptic promoter and the $A^{vy/a}$ phenotype

3.51 Final Adult Body Weight—When data for all diets and all coat colors, i.e. for 36 mice, were combined, there was a negative correlation between average CpG methylation and body weight ($R = -0.69$, $P < 0.0001$, Fig. S5, Table S4). This same correlation held when the data for the AIN and NIH diets were analyzed separately (AIN: $R = -0.81$, $P = 0.0015$; NIH: $R = -0.79$, and $P = 0.0022$) but failed to hold for the NIHMe diet ($R = -0.36$, $P = 0.25$). When brown coat color animals from all diet groups were considered, there was a strong negative correlation between body weight and CpG methylation at 32 weeks ($R = -0.71$, $P = 0.0009$), but it failed to hold with yellow coat color animals ($R = -0.34$, $P = 0.17$) (Fig. S5, Table S4). When the interaction of diet and coat color was considered in analyzing the correlation between average CpG methylation and body weight of those animals screened in the DNA methylation assay, only one combination, that of brown coat color animals on the AIN diet exhibited a significant correlation between average CpG methylation and body weight ($R = -0.97$, $P = 0.001$, Fig. 5), but there was no significant correlation for brown coat color animals on either the NIH (Fig. S6) or NIHMe diet (Fig. S7). This correlation suggests that as average CpG methylation of the IAP site was reduced on the AIN diet, the more likely the animal was to be heavier even in the absence of any overt changes in coat color. When all diets and coat colors were considered, with the sexes separated, there remained a strong correlation for average CpG methylation and body weight for both males and females (Males: $R = -0.65$, $P = 0.004$; Females: $R = -0.73$, $P = 0.0006$).

3.52 Final Adult Serum Glucose Concentrations—The most significant correlation, albeit a weak one, between average CpG methylation and serum glucose concentrations occurred when all diet groups, coat colors, and sexes were considered (-0.36 , $P = 0.03$; Table S4). It was most pronounced in males ($R = -0.49$, $P = 0.04$), and absent in females, ($R = -0.34$, $P = 0.17$), but, even within males, the correlation did not hold true for all dietary groups. Thus, yellow coat color males on the NIH diet exhibited a positive correlation ($R = 1.0$, $P = 0.05$) between CpG methylation and serum glucose values.

4.0 Discussion

This study had three main goals. The first was to determine whether a diet rich in methyl donors (NIHMe) was able to reverse the yellow coat color phenotype of $A^{vy/a}$ mice. Second, we sought to establish whether or not other aspects of the phenotype of the viable yellow mouse strain, namely the early tendency of the yellower mice to become obese and of

yellow males, in particular, to become hyperglycemic as they matured into adulthood, could be modulated by diet, including one that was of higher caloric density than the other two. A third goal was to assess whether diet, and especially the methyl-rich NIHMe diet, could alter CpG site methylation within the 5' LTR promoter site of the A^{vy} gene. The rationale for the experiment was that a high methyl diet supplied to pregnant a/a mice carrying $A^{vy/a}$ conceptuses biases coat color of the pups by increasing the extent of CpG site methylation in the regulatory regions of the A^{vy} allele [1, 3, 4, 13] and hence leads to potentially better health outcomes in offspring [33–38]. This increase in methylation within the cryptic promoter of the allele is presumed to reduce the promiscuous, ectopic production of ASIP both within the hair follicles, where melanization is affected, and at ectopic sites, such as the hypothalamus and pancreas, where ASIP may influence metabolism of fats and carbohydrates [8, 9, 39].

With regard to goal 1, our studies indicated that coat color and its mosaic patterning could not be influenced by feeding the methyl-enriched, NIHMe diet to post-weaning $A^{vy/a}$ mice. Nor did coat patterning appear to be altered by either of the other two diets. One caveat here is that we chose not to shave the animals at the time of weaning when the consumption of the experimental diets began and, thus, cannot conclusively rule out whether or not re-emerging dorsal hair had altered melanization patterns relative those prior to weaning. However, we consider this to be unlikely and favor the conclusion that color pattern is fixed by the time $A^{vy/a}$ mice are weaned (Fig. 1).

A second goal of our experiments was to determine whether diet could alter the disease trajectory of $A^{vy/a}$ mice, which tend to become prematurely obese and show symptoms of metabolic disease. Our hypothesis was that weight gain and disease progression in the yellow mice, in particular, might be slowed on the NIHMe diet relative to the NIH control diet but accelerated by the more calorie-dense AIN diet. However, neither of these expectations was fulfilled. On all three diets, yellow coat color females, gained more weight over time than their brown coat colored counterparts (Fig. 2B) and there was no evidence that the NIHMe diet slowed weight gain of the yellow mice of either sex relative to the isocaloric NIH control diet. A further caveat here is that, at the time of the studies, we did not have access to an automated metabolic cage system to determine whether food intake and possibly palatability of the diets, as well as energy expenditure, differed across treatments and between sexes. Nonetheless, we have no reason to believe that the NIHMe diet in any way opposed the tendency of yellow mice to become obese. Of course weight gain of mice might be influenced by the initial weights of the animals when they were placed on a particular diet. However, as detailed in Results, even though mice were randomized for diet assignments, yellow coat color females placed on the AIN diet had an average body weight slightly greater ($P = 0.05$) than yellow coat color females placed on the NIH diet. This initial difference may have influenced the weight trajectories of these two groups and affected the ultimate differences in body weight when the experiment was terminated (Fig. 2b). Importantly, however, there was no difference in initial weights of AIN and NIHMe yellow coat color females ($P = 0.19$). Even so, by early adulthood, AIN yellow coat color females were already heavier than yellow females on the NIHMe diet, and these differences persisted through adulthood (Fig. 2b). As this and the other pairwise

comparisons (including the brown coat color groups discussed below) were not significant at the time the animals were first placed on the diet the body weight differences that subsequently emerged over the course of the study were most likely due to the effects of the diets rather than to initial basal weight of the mice.

Not only did NIHMe not ameliorate the progressive weight gain of yellow mice relative to controls on the NIH diet, it did not prevent the progressive hyperglycemia experienced by yellow male mice (Fig. 3A). Yellow males on both the NIH and NIHMe diets averaged approximately 250 mg glucose/dL of serum as adults (Fig. 2A), values that reflect a pre-diabetic state [40]. Brown males on the lower calorie NIH and NIHMe diets also became hyperglycemic with age, but those on the AIN diet did not, a surprising outcome, as this diet has the higher caloric density of the three (Table S1) and promoted the greatest weight gain for both sexes (Fig. 1A). In contrast to the males, serum glucose concentrations of $A^{vy/a}$ females remained within the normal range [41] and, as observed by others [39], were relatively unaffected by diet and the weight gain associated with age (Fig. 3B). These findings are consistent with previous studies that suggest $A^{vy/a}$ females are more resistant to hyperglycemia than males regardless of the post-weaning diet, although the factors underpinning this sexual dimorphism are uncertain [39, 42–44]. It should be emphasized that our glucose measurements were performed in non-fasted mice and that the amount and timing of food consumption might have influenced the results. A prior study, for example, reported that yellow coat color animals have a tendency to be hyperphagic relative to brown coat color siblings [45]. Nonetheless, the greater obesity of the yellow mice in that study was still attributed to more efficient caloric utilization rather than to increased food consumption.

Goal 3 was to determine whether diet could influence CpG site methylation within the A^{vy} AIP. Liver was chosen as a source of DNA rather than the usual tail tip [2, 13, 16, 46, 47], because of its central role in controlling lipid and carbohydrate metabolism. Cost restrictions limited our ability to assess other tissues, but the methylation patterns were generally quite similar to those observed by others on tail DNA. As large scale de-methylation followed by extensive but selective re-methylation is more a feature of development *in utero* rather than after birth [48–50] and the fact that none of the diets, including NIHMe, caused a significant change in either coat color or apparent disease trajectory of the yellow mice, no major alterations in DNA methylation patterns were anticipated. However, contrary to these expectations, it became clear that diet did have a pronounced effect. Methylation at each of the nine CpG sites, for example, was markedly decreased from an average of about 82 % to 44 % in brown $A^{vy/a}$ mice, by consumption of the higher caloric AIN diet relative to the lower caloric NIH diet, while brown mice on the NIHMe diet tended towards an intermediate degree of IAP methylation (Fig. 4). Indeed, body weight of the brown mice was negatively correlated with extent of CpG methylation within the IAP (Fig. S5 and Table S4), especially for those consuming the AIN diet (Fig. 5). This data set was not only unanticipated, but represented a disconnect between coat color and extent of CpG methylation. Thus, the methylation patterns within the cryptic promoter of the $A^{vy/a}$ gene in the liver of the brown-coated mice, whatever their sex, seem to be malleable and influenced by post-weaning diet, especially one of higher caloric density. Thus, there appears to be a

link between the extent of methylation of the IAP and the metabolic status of the animal. Importantly, however, CpG site methylation was not significantly enhanced in yellow mice on any of the diets, including NIHMe, and remained extremely low under all treatment regimens. While it would have been optimal to test the DNA methylation pattern of all 10 animals for each coat color and sex, such an analysis was cost-prohibitive. Therefore, we randomly selected three representatives of each sex and coat color and diet to test in this screening method to minimize any bias. Another limitation of our study, however, was that, because of improper sample storage for RNA isolation, we were unable to compare A^{vy} gene expression across the various treatment groups to determine if transcript concentrations were correlated with the DNA methylation status of the IAP CpG sites. Even so, the DNA methylation studies are consistent with a prior study with viable yellow mice [51] that indicated that the methylation status of the A^{vy} allele can be influenced by diet. Our experiment, thus, suggests that post-weaning diet might sway interpretation of epigenetic and phenotypic studies in which pseudoagouti (brown) and yellow coat color A^{vy}/a mice are compared, particularly if the methylation analyses on the A^{vy} IAP site are performed on liver or other metabolically sensitive tissues or organs.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations used

ACUC	Animal Care and Use Committee
ASIP	agouti signaling protein
IAP	intracisternal A particle
MSH	melanocyte stimulating hormone
LTR	long term repeat
NIH	National Institutes of Health
NIHMe	methyl supplemented NIH31 diet

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Fig. 1. Photographic comparison of a Y₄–Y₅ yellow coat color mice before (A) and after (B) being placed on the NIHMe diet for 29 weeks.

Fig. 2.

Average body weights for juvenile, early adult, and adult yellow and brown coat color $A^{vy/a}$ males and females on the different post-weaning diets. A) Male weights; B) Female weights. Data are reported as the average $\pm$ SEM. Two comparisons are represented in the graph: 1) Comparing different coat color animals fed the same diet and 2) Comparing same coat color animals within diets. Letter superscripts, such as a,b , demarcate differences between diets (AIN, NIH, or NIHMe) for animals of the same coat color groups (yellow or brown) ($P < 0.05$).

0.05); * represent differences between $A^{vy/a}$ mice possessing different coat colors (yellow or brown) but within the same diet groups (AIN, NIH, or NIHMe) ($P < 0.05$).

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Fig. 3.

Average serum glucose concentrations for juvenile, early adult, and adult yellow and brown coat color A^{vy}/a males and females on the different post-weaning diets. A) Males; B) Females. Data are reported as the average $\pm$ SEM. Two comparisons are represented in the graph: 1) Comparing different coat color animals fed the same diet and 2) Comparing same coat color animals within diets. Letter superscripts, such as $a^{a,b}$, demarcate differences between diets (AIN, NIH, or NIHMe) for animals of the same coat color groups (yellow or

brown) ($P < 0.05$); * represent differences between $A^{vy/a}$ mice possessing different coat colors (yellow or brown) but within the same diet groups (AIN, NIH, or NIHMe) ($P < 0.05$).

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Fig. 4.

DNA methylation analysis within the AIP region A^{vy} cryptic promoter of brown and yellow coat color male and female ($n = 6$ for each diet and coat color) on the different post-weaning diets. A) Percent CpG methylation for the 9 CpG sites; B) Mean total CpG methylation for the IAP site in the A^{vy} locus. * $P = 0.01$, ** $P = 0.003$, and *** $P < 0.0001$.

Fig. 5.

Type II regression analysis for brown coat color A^{vy}/a mice on the AIN diet comparing A^{vy} CpG methylation versus body weight. Stars (*) represent individual animals. $R^2 = 0.9421$, $R = -0.97$, and P value = 0.0013. There are six data points to reflect the three males and three females whose DNA methylation status was analyzed.