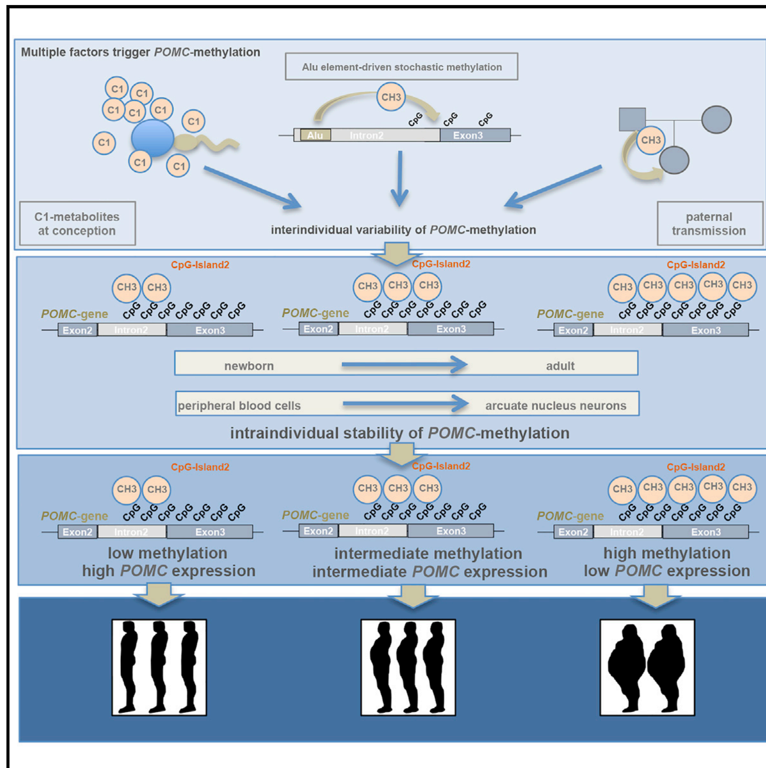
Short Article

Cell Metabolism

Interindividual Variation in DNA Methylation at a Putative *POMC* Metastable Epiallele Is Associated with Obesity

Graphical Abstract



Highlights

- A *POMC* hypermethylation variant leads to increased risk for obesity development
- Maternal C1 metabolism at conception affects *POMC* methylation in the offspring
- Paternal *POMC* methylation correlates with methylation in the offspring
- This *POMC* region fulfills criteria for a putative metastable epiallele

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In Brief

Kühnen et al. explore the epigenetic regulation of body weight/BMI and identify a *POMC* DNA hypermethylation variant in obese individuals. This putative metastable epiallele is present after birth and leads to an increased individual risk of developing obesity later in life.

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Interindividual Variation in DNA Methylation at a Putative *POMC* Metastable Epiallele Is Associated with Obesity

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SUMMARY

The estimated heritability of human BMI is close to 75%, but identified genetic variants explain only a small fraction of interindividual body-weight variation. Inherited epigenetic variants identified in mouse models named "metastable epialleles" could in principle explain this "missing heritability." We provide evidence that methylation in a variably methylated region (VMR) in the pro-opiomelanocortin gene (*POMC*), particularly in postmortem human laser-microdissected melanocyte-stimulating hormone (MSH)-positive neurons, is strongly associated with individual BMI. Using cohorts from different ethnic backgrounds, including a Gambian cohort, we found evidence suggesting that methylation of the *POMC* VMR is established in the early embryo and that offspring methylation correlates with the paternal somatic methylation pattern. Furthermore, it is associated with levels of maternal one-carbon metabolites at conception and stable during postnatal life. Together, these data suggest that the *POMC* VMR may be a human metastable epiallele that influences body-weight regulation.

INTRODUCTION

Family and twin studies indicate that adiposity (assessed as BMI) is highly heritable. A recent meta-analysis estimated BMI heritability up to 75% in twins and 46% in families (Elks et al., 2012). However, despite enormous efforts (Speliotes et al., 2010), including whole-genome sequencing (Yang et al., 2015), the genetic variants identified thus far together explain <30% of individual BMI and body-weight variation (Locke et al., 2015). The last few years have seen efforts to explain this "missing heritability" by exploring the relation between epigenetic modifications and body-weight regulation (Eichler et al., 2010). However, the molecular nature and ontogeny have remained elusive (Waterland, 2014). The most comprehensive search for obesity-related DNA methylation changes in humans based on the Illumina 450K array recently revealed *HIF3A* methylation variants associated with BMI (Dick et al., 2014; Murphy and Mill, 2014). However, as these were linked with SNPs in *cis*, they may represent secondary epigenetic differences that are driven by genetic variation.

The most compelling data supporting epigenetic regulation of body weight/BMI is derived from mouse models identifying changes in DNA methylation and gene expression (Dalgaard et al., 2016; Radford et al., 2014). For example, cloned (Tamashiro et al., 2002) and isogenic inbred (Koza et al., 2006) mouse strains are divergent in their body weight early in life despite sharing the same genomes and environment.

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At the molecular level these inherited, stable epigenetic variants were initially shown for fur color and body weight in the agouti viable yellow (A^{vy}) mouse (Morgan et al., 1999). In the A^{vy} mutant strain, epigenetic variants are triggered by a transposable element (intracisternal A particle [IAP]). Methylation of this retrotransposon in the early embryo leads to stable, non-tissue-specific epigenetic variation among isogenic A^{vy} mice that influences fur color and metabolic phenotypes. Accordingly, A^{vy} was dubbed a "metastable epiallele" (ME) (Rakyan et al., 2002). This concept of a retro-element triggered ME was confirmed in the murine $Axin^{Fu}$ mutation, at which interindividual variation in methylation is associated with tail kinking (Rakyan et al., 2002). It was shown that methylation at MEs occurs stochastically but can in part be modified by maternal intake of nutrients related to one-carbon (C1) metabolism (Waterland et al., 2006; Waterland and Jirtle, 2003). In addition, the methylation state can in part be transmitted via a parental germline, either maternal (A^{vy}) (Morgan et al., 1999) or paternal ($Axin^{Fu}$) (Rakyan et al., 2003). Thus, epigenetic differences at metastable epialleles represent a potential molecular mechanism to explain stable inheritance that is not linked to genetic variants.

Here, we pursued a candidate gene approach to identify BMI-associated DNA methylation differences. We focused our search on the *POMC* gene, because individuals homozygous for variants in the coding region of *POMC* (giving rise to the melanocyte-stimulating hormone [MSH] peptides that mediate the anorectic functions of leptin) develop early-onset severe obesity (Krude et al., 1998). Since heterozygous carriers are obese to a lesser extent (Farooqi et al., 2006; Krude et al., 2003), *POMC* represents a good candidate for investigating the potential effects of methylation differences on gene expression and obesity/BMI.

We previously identified a *POMC* variably methylated region (VMR) located at the intron 2/exon 3 border (Figure 1A), which is more frequently hypermethylated in peripheral blood cells (PBCs) of obese children (Kuehnen et al., 2012). Hypermethylation of the VMR seems to be triggered by adjacent Alu elements in intron 2 (Figure 1A), since the homologous CpGs in species without those Alu elements (mouse and lemur) are not methylated. We have shown that hypermethylation in this VMR decreases P300 enhancer binding and *POMC* transcription and is present before the onset of obesity (Kuehnen et al., 2012), suggesting a functional effect of the methylation variant on *POMC* gene expression. Here, we extend our previous study to look at *POMC* methylation in PBCs and MSH neurons in adults.

RESULTS

POMC Hypermethylation in Obese Adults in Peripheral Blood Cells and MSH Neurons

Initially, we performed bisulfite pyrosequencing at each of nine *POMC*-VMR-CpG-sites to reproduce our previous finding of hypermethylation in obese compared to normal-weight children (Kuehnen et al., 2012). As in the previous study, we found a significant positive correlation of methylation across CpG sites −2 to +5 with individual BMI SD score (SDS) (Figure S1). Thereafter, we analyzed this region in obese versus normal-weight adult individuals. We again found variable methylation at the border of intron 2/exon 3 and significant hypermethylation at positions +1, +2, +3, and +5 (Figure 1B). Moreover, we found a significant positive correlation of average methylation across CpG sites −2 to +5 with BMI ($r = 0.18$, $p = 0.006$) (Figure 1C).

We next performed laser microdissection of MSH-positive arcuate nucleus neurons from postmortem human brain. *POMC* gene expression in those neurons is most crucial for individual body-weight regulation. Laser-microdissected MSH-positive neurons were collected from 41 obese and normal-weight individuals (Figure S1), and the methylation status was analyzed by bisulfite pyrosequencing (see Experimental Procedures). Again, we observed variable methylation at the intron 2/exon 3 border, and *POMC* methylation across sites −2 to +5 of the MSH neurons was positively correlated with individual BMI ($r = 0.34$, $p = 0.025$) (Figure 1D). Together with our previous finding that hypermethylation at positions −2 to +5 decreases *POMC* gene expression (Kuehnen et al., 2012), it is likely that hypermethylation of the *POMC* gene in MSH neurons from obese individuals results in a lower expression of *POMC* gene product, which might inhibit normal satiety responses and promote obesity. This correlation would imply that a 10% increase in methylation is associated with 2.8 kg/m^2 increase in BMI ($p = 0.025$) (Figure 1D), a much stronger effect than observed for top genome-wide association study (GWAS) SNPs associated with obesity, such as at *FTO* (0.39 kg/m^2 per allele) (Loos, 2012; Murphy and Mill, 2014).

Non-tissue Specificity of POMC Methylation

We analyzed the extent of systemic (cross-tissue) variation in *POMC* methylation. A comparison of *POMC* methylation of neurons within the arcuate nucleus with methylation in PBCs in a subset of 14 postmortem-studied individuals revealed a strong positive correlation ($r = 0.63$, $p = 0.014$) (Figure 2A), suggesting that cells from different germ layers—ectoderm (brain) and mesoderm (PBCs)—maintain a similar methylation level. We further confirmed this by analyzing an independent second set of tissue samples from different individuals (see Experimental Procedures). Again, we found high intra-individual correlation of methylation in ectoderm (brain) and mesoderm (kidney) samples ($r = 0.7$, $p = 0.002$) (Figure 2B). These data argue that methylation across the *POMC* VMR may be established very early during development, before separation of germ layers at gastrulation.

Longitudinal Stability of POMC Methylation

To further test the longitudinal stability of interindividual variation in *POMC* methylation, we analyzed the methylation in DNA extracted from newborn screening cards (a blood collection that is routinely performed after birth between day 3 and 10 postpartum) and compared this with the *POMC* methylation status of the same individual in a second blood collection performed later in life ($n = 52$; mean time span, 11.69 years; range, 3–24 years). We observed a strong correlation of methylation within individuals across this time period (Figure 2C), indicating that the *POMC* methylation pattern is stable over time and not strongly influenced by postnatal environment.

Impact of Genetic Variation in *cis* on POMC Methylation

Several epigenome-wide association studies (EWASs) have identified methylation variants (meQTL) associated with SNPs

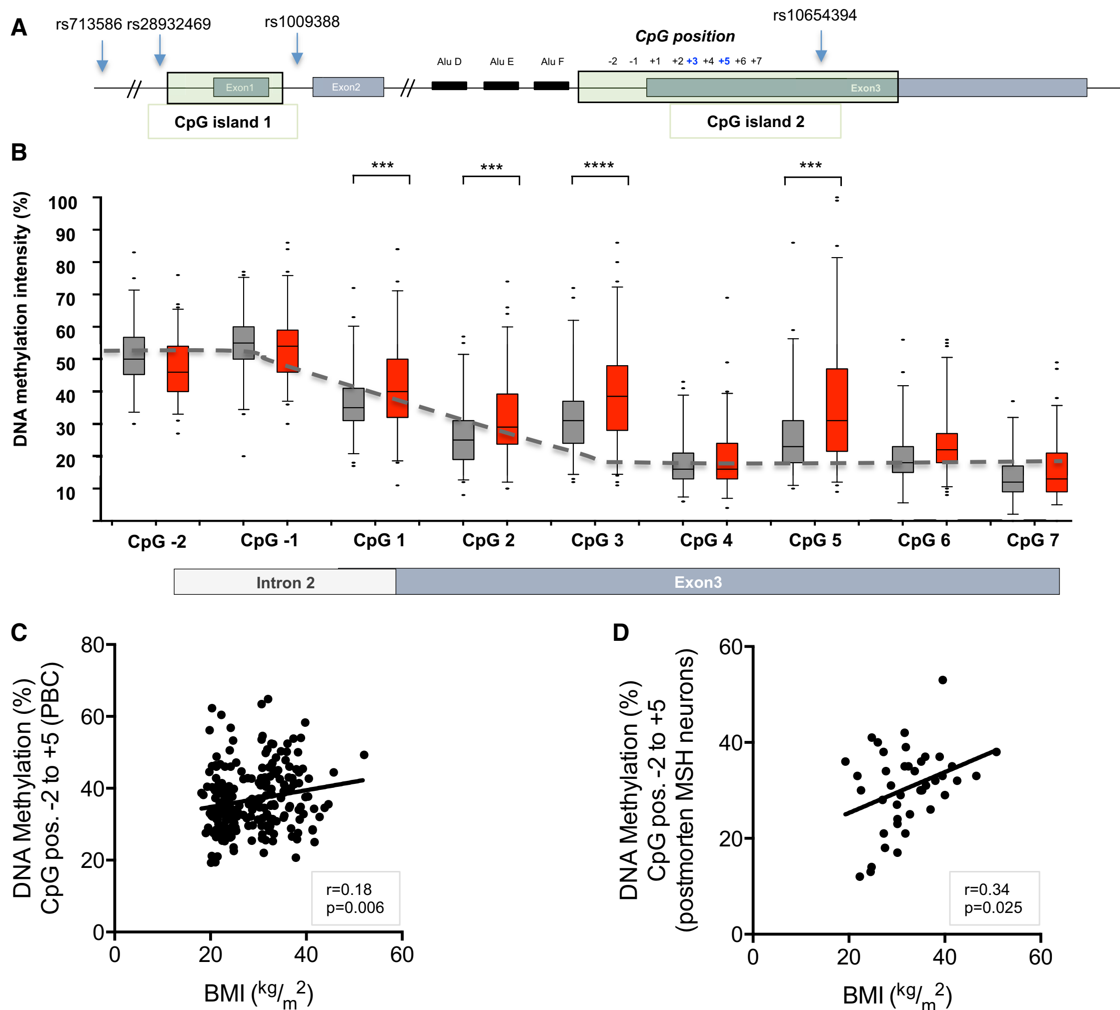

Figure 1. *POMC* Gene Structure and Pyrosequencing Results in an Adult Cohort

(A) The *POMC* gene structure indicating the location of CpG islands (green), the Alu elements (black boxes) and CpGs −2 to +7, which were analyzed by pyrosequencing.

(B) Bisulfite-pyrosequencing analysis of DNA methylation in peripheral blood cells from 103 normal-weight (gray) and 125 obese (red) adult individuals. The most significant differences were observed at CpG positions +3 and +5 (corresponding to nucleotide number chr2:25.384.590 and chr2:25.384.569 [UCSC human genome browser hg19]). The gray dotted line shows the variation in mean methylation values from the intron (CpG positions −2 and −1) to the exon at CpG positions +4 to +7. Error bars represent SD.

(C and D) Linear regression based on the mean value of CpG positions −2 to +5 and BMI in the German adult cohort (n = 228) (C) and MSH laser-microdissected neurons from postmortem human brain samples (n = 41) (D).

in *cis* (Dick et al., 2014), implying that they may simply represent secondary epigenetic differences resulting from genetic variation (Murphy and Mill, 2014). We searched for meQTL at *POMC* by sequencing the entire region of the *POMC* gene, including the promoter and 3′ UTR, in two cohorts of normal-weight children of different genetic (European and West African) origin (see Experimental Procedures). None of the genetic variants present, including rs713586 (the only known BMI-associated *POMC* SNP identified in a GWAS meta-analysis; Speliotes et al., 2010), was associated with methylation across the nine VMR-CpGs (Figures S2A and S2B). In addition, although the African and European cohorts show significant differences in their SNP characteristics (Figures S2A and S2B), average *POMC* methylation in both cohorts was essentially identical (Figure S2C). It is therefore unlikely that the *POMC* methylation variant is driven by a genetic polymorphism.

Paternal Impact on POMC Methylation

To further analyze a potential genetic effect on the *POMC* methylation variant, we investigated the inheritance of methylation status at the *POMC* VMR in 47 family trios of obese children and their parents. Notably, methylation of a child's *POMC* VMR correlated only with its father's *POMC* methylation level (Figure 3A) and not with that of its mother (Figure 3B). These data suggest a partial transmission of the *POMC* VMR methylation state through the paternal germline that is not driven by genetic variation. To investigate the underlying mechanism explaining this correlation between paternal and offspring methylation, we

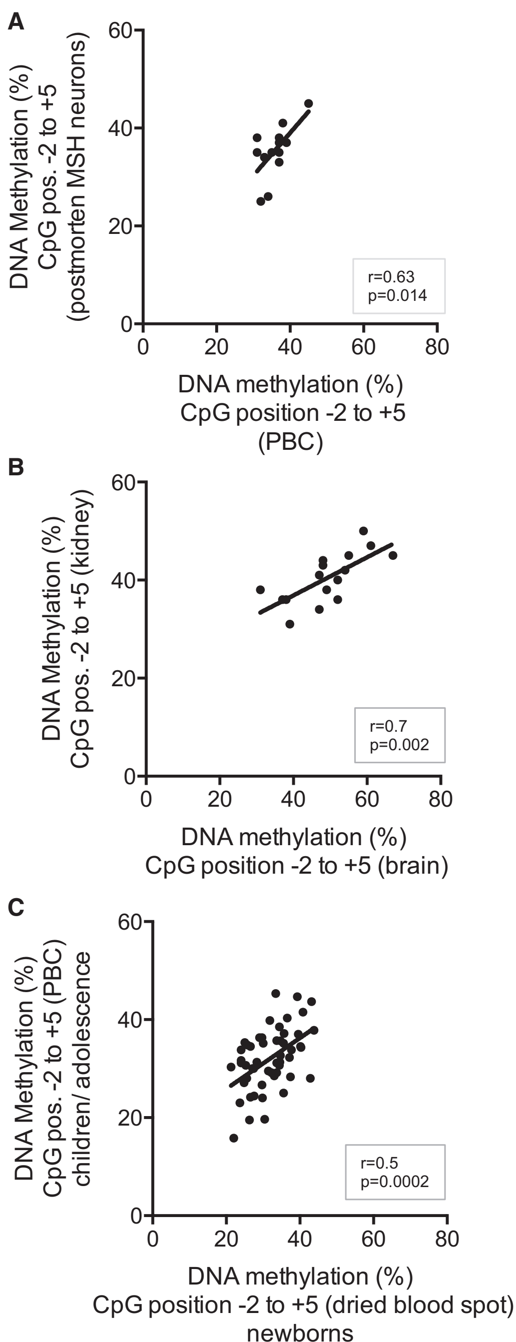

Figure 2. Cross-Tissue Correlations in Intra-individual *POMC* Methylation and Stability over Time

(A) *POMC* DNA methylation at CpG positions −2 to +5 correlates significantly between laser-microdissected MSH neurons and laser-microdissected blood cells (n = 14).

(B) Intra-individual correlation between the *POMC* DNA methylation (%) at CpG positions −2 to +5 of postmortem human brain and kidney tissues.

(C) Longitudinal stability of DNA methylation in the same individuals measured perinatally and in adolescence (n = 52).

analyzed *POMC* methylation in sperm and blood (PBC) DNA of 17 German men. Interestingly, *POMC* VMR methylation was significantly lower in sperm than in blood (Figure S3A), consistent with a loss of methylation during germline differentiation as has been shown for imprinted regions (Monk, 2015). However, PBC methylation is correlated with BMI in the same individuals (r = 0.54, p = 0.025) (Figure S3B). These data suggest that paternal transmission of the epigenetic state at the *POMC* VMR occurs by some mechanism other than DNA methylation.

POMC Methylation Is Not Influenced by Maternal Body-Weight Changes during Pregnancy

To further search for the origin of *POMC* VMR methylation we assessed the potential influence of the early in utero environment. We analyzed 33 European mother-child pairs and tested for a correlation of maternal body weight during pregnancy with the child's *POMC* methylation level. Neither maternal body weight at conception nor weight change during pregnancy was correlated with the *POMC* methylation level determined in the child's PBC DNA (Figure S3).

POMC Methylation Is Influenced by One-Carbon Metabolism

Establishment of DNA methylation at human MEs has shown to be sensitive to C1 metabolites at the time of conception. C1 metabolism is central because it regulates the supply of methyl groups required for DNA methylation. Betaine and 5-methyltetrahydrofolate are methyl donors for the methylation of methionine, converting it to S-adenosylmethionine (SAM). In transmethylation reactions, SAM donates a methyl group and is transformed into S-adenosylhomocysteine (SAH), which is then metabolized into homocysteine. To analyze the impact of these C1 metabolites on *POMC* methylation, we investigated a West African cohort of 144 mother-child pairs from a rural area of The Gambia, in which data on maternal periconceptional C1 metabolites were available (Dominguez-Salas et al., 2014). In this region, the alternation of rainy and dry seasons leads to large changes in the environment affecting maternal nutrition and the supply of C1-metabolites (Dominguez-Salas et al., 2013). We found lower DNA methylation at the *POMC* VMR in children conceived in the dry season compared to those conceived in the rainy season (Table 1), consistent with previous observations at known MEs (Dominguez-Salas et al., 2014; Silver et al., 2015). Testing for a direct influence of maternal serum C1-metabolites, a robust and significant negative correlation for SAH and positive correlations with betaine and the ratio of SAM to SAH were found at all CpGs from −2 to +7 (Tables 1 and 2; Experimental Procedures).

DISCUSSION

Our findings in different obese cohorts and postmortem studies indicate that methylation of the human *POMC* VMR is correlated with individual body weight. The finding of reduced *POMC* gene expression in the presence of the hypermethylated variant (Kuehnen et al., 2012), together with our observation that the same association is observed in MSH-positive arcuate nucleus neurons, suggests that hypermethylation at the *POMC* VMR may be functionally related to an individual's body weight.

In addition, the findings in the present study imply that the *POMC* VMR shares a set of common characteristics with the

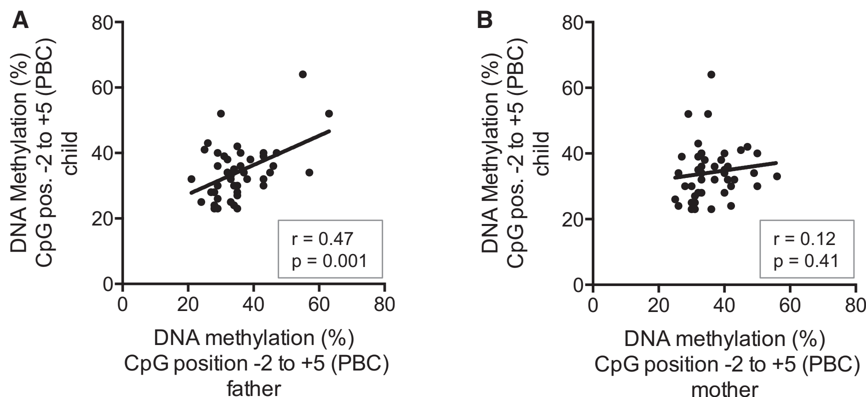

Figure 3. *POMC* Pyrosequencing Analysis in Family Trios with One Obese Child

Peripheral blood DNA samples from 47 trio families, each with one obese child, were analyzed by pyrosequencing.

(A) Correlation between paternal and offspring DNA methylation at CpG positions −2 to +5.

(B) Correlation between maternal and offspring DNA methylation CpG positions −2 to +5.

mouse A^{vy} locus. Namely, it is (1) triggered by a transposable element, (2) sensitive to nutritional modification by C1 metabolites during early embryonic development, (3) in part transmitted via one parental germline, (4) stable over the life course, and (5) correlated with body weight. In addition, our data argue against an influence of maternal body weight during pregnancy and of genetic variation at the *POMC* gene locus. In this respect, the observed *POMC* VMR methylation differs fundamentally from other identified methylation variants that are associated with obesity, which result either from non-inherited environmental influences or represent secondary epigenetic differences resulting primarily from genetic variation (Murphy and Mill, 2014).

Together, these complementary data across multiple populations argue that the variation of the *POMC* VMR resembles a metastable epiallele, like those described in the mouse A^{vy} locus. As already shown for the A^{vy} locus, methylation of the *POMC* VMR appears to be established before the separation of the germ layers during the very early stages of genome re-methylation, resulting in significant correlation of methylation levels across somatic cell types derived from different germ layers. We cannot, however, exclude the possibility that an environmental exposure in adulthood may influence methylation patterns in multiple tissues. Additionally, early embryonic establishment of *POMC* VMR methylation appears to be influenced by the availability of C1 metabolites in the maternal circulation around the time of conception. Our data also point to the influence of some other factor transmitted from the father to the offspring, although the mechanism is unknown. One possibility is the transmission of sperm microRNAs (Gapp et al., 2014; Wagner et al., 2008). Our interpretation of these various observations is that *POMC* methylation is determined combinatorially by stochastic epigenetic events during early embryonic development (which are influenced by maternal nutrition) and by non-genetic paternal transmission, although further studies in independent samples will be required to confirm that the *POMC* VMR is acting as a metastable epiallele.

Importantly, our data indicating early embryonic establishment and long-term stability of methylation at the *POMC* VMR help inform causality. In particular, together with our findings showing that methylation at this locus is correlated with individual BMI, these data suggest that individual epigenetic variation at the *POMC* VMR is a cause rather than a consequence of obesity. Moreover, if stochastic establishment of DNA methylation at the *POMC* VMR occurs before the embryo cleavage that results in monozygotic (MZ) twins, this could lead to a shared *POMC* VMR epigenotype in monozygotic twins, independent of their genetic identity (Waterland et al., 2010). This may offer a partial explanation for the missing heritability of BMI (Llewellyn et al., 2013).

EXPERIMENTAL PROCEDURES

All procedures and measurements were approved by the Ethics Committee of the Charité Universitätsmedizin Berlin (EA2/131/11, EA1/019/13 and EA2/116/10), the Universitätsklinikum Essen (05-2954), the joint Gambian Government/MRC Unit, and the Gambia Ethics Committee (L2013.25) and according to the declaration of Helsinki. The patients and/or their parents/guardians gave informed consent.

POMC Genotyping Analysis

The genomic *POMC* region was analyzed in DNA samples from a European cohort (Berlin, Charité Universitätsmedizin Berlin) and from the Gambian cohort (infants) by traditional Sanger sequencing on an ABI Sequencer (Applied Biosystems 3130xl, Genetic Analyzer). For oligo sequences and further details, please see Supplemental Information (Table S1).

DNA Methylation Analysis

DNA (500 ng) for German cohorts was extracted according to standard protocols (Promega) from PBCs. For Gambians, DNA was extracted from venous blood using a standard salting-out method (Miller et al., 1988). The samples were converted with sodium-bisulfite (EpiTect-Kit, QIAGEN). For further details of methylation analysis and DNA extraction, see Supplemental Experimental Procedures.

Study Cohorts

Adult Case-Control Cohort

The control group consists of 103 normal-weight adults with a mean age of 48.2 ± 11.74 years and a mean BMI of 22.5 ± 1.65 kg/m^2 (32 males, 71 females). The obese group includes 125 individuals with a mean age of 54.2 ± 8.35 years and a mean BMI of 36.03 ± 5.61 kg/m^2 (50 males, 75 females). The samples were part of the MesyBepo follow-up study (Bobbert et al., 2013).

Family Trios

A total of 47 family trios each with one obese child (mean age 13.23 ± 2.34 years, mean BMI 30.2 ± 4.34 kg/m^2) were analyzed. The fathers had a mean age of 44.53 ± 8.06 years and a mean BMI of 28.6 ± 5.73 kg/m^2. The mothers had a mean age of 41.11 ± 4.11 years and a mean BMI of 28.37 ± 8.33 kg/m^2.

European Healthy Children Analyzed for Genetic Variant

Eighty-four normal-weight children with a mean BMI of 18 ± 0.61 kg/m^2 and a mean age of 8.9 ± 2.01 years (44 females, 40 males), recruited in the outpatient clinic of the Department of Pediatric Endocrinology, Charité Universitätsmedizin Berlin, were analyzed.

Table 1. Correlations between Maternal Biomarker Status around Conception and Offspring DNA Methylation

Covariate	Coef	SE	z value	p value	[95% CI]	
Season (dry)	−0.152	0.07	−2.14	0.034	−0.291	−0.012
Folate	0.070	0.09	0.79	0.432	−0.106	0.247
B12	0.017	0.09	0.18	0.854	−0.164	0.197
B2	−0.108	0.15	−0.73	0.467	−0.402	0.185
B6	−0.071	0.10	−0.71	0.482	−0.270	0.128
Choline	0.095	0.12	0.78	0.437	−0.146	0.336
Betaine	0.275	0.10	2.80	0.006	0.081	0.470
Methionine	0.211	0.19	1.13	0.262	−0.160	0.583
Cysteine	0.158	0.30	0.53	0.594	−0.429	0.746
Homocysteine	−0.187	0.11	−1.67	0.098	−0.410	0.035
DMG	0.002	0.06	0.03	0.975	−0.125	0.129
SAM	−0.135	0.22	−0.62	0.537	−0.568	0.297
SAH	−0.353	0.10	−3.57	0.001	−0.549	−0.157
SAM:SAH ratio	0.340	0.10	3.34	0.001	0.138	0.541
Betaine:DMG ratio	0.093	0.06	1.59	0.114	−0.022	0.208
BMI	−0.006	0.01	−0.53	0.599	−0.029	0.017

Environmental impact on peripheral blood DNA methylation in 144 children from The Gambia who were conceived during either the dry season or the rainy season. DNA methylation was analyzed against maternal periconceptional methyl-donor biomarker status (Dominguez-Salas et al., 2014). Season of conception, maternal betaine, SAH, and the SAM:SAH ratio predict offspring mean methylation across the *POMC* VMR. CI, confidence interval; Coef, coefficient; CpG, CpG site within *POMC* locus; DMG, dimethylglycine; p, p value; SAM, s-adenosylmethionine; SAH, s-adenosylhomocysteine; z, z value.

Table 2. Correlation between Each Analyzed CpG Position and the Maternal Serum Concentration of SAH, SAM:SAH, and Betaine during Conception

Predictor	CpG	Coef	SE	z	p	[95% CI]	
SAH	−2	−0.29	0.10	−2.99	0.003	−0.47	−0.10
	−1	−0.33	0.11	−2.95	0.004	−0.55	−0.11
	1	−0.41	0.11	−3.58	< 0.001	−0.63	−0.18
	2	−0.32	0.14	−2.30	0.023	−0.60	−0.05
	3	−0.34	0.12	−2.76	0.007	−0.59	−0.10
	4	−0.32	0.12	−2.63	0.009	−0.57	−0.08
	5	−0.59	0.16	−3.58	< 0.001	−0.91	−0.26
	6	−0.40	0.12	−3.34	0.001	−0.64	−0.16
	7	−0.32	0.16	−2.00	0.048	−0.64	0.01
SAM:SAH	−2	0.21	0.10	2.11	0.036	0.01	0.40
	−1	0.23	0.11	2.08	0.039	0.01	0.46
	1	0.33	0.12	2.81	0.006	0.10	0.56
	2	0.24	0.14	1.66	0.099	−0.05	0.52
	3	0.28	0.13	2.23	0.028	0.03	0.53
	4	0.23	0.12	1.83	0.070	−0.02	0.47
	5	0.58	0.17	3.50	0.001	0.25	0.91
	6	0.35	0.12	2.92	0.004	0.11	0.59
	7	0.25	0.16	1.58	0.117	−0.06	0.57
Betaine	−2	0.04	0.09	0.46	0.647	−0.14	0.23
	−1	0.07	0.11	0.64	0.525	−0.14	0.28
	1	0.10	0.11	0.89	0.378	−0.12	0.32
	2	0.34	0.13	2.63	0.010	0.08	0.59
	3	0.32	0.12	2.69	0.008	0.08	0.56
	4	0.35	0.11	3.03	0.003	0.12	0.57
	5	0.28	0.16	1.72	0.088	−0.04	0.60
	6	0.37	0.11	3.23	0.002	0.14	0.60
	7	0.16	0.15	1.09	0.278	−0.13	0.46

Summary of SAM, SAM:SAH ratio, and betaine-dependent DNA methylation at individual *POMC* CpG positions −2 to +7. Associations between SAH, SAM:SAH, and methylation show no significant differential effect with CpG site. Methylation associated with betaine varies by CpG site (see Gambian Cohort Analyses Section in Supplemental Information). CI, confidence interval; Coef, coefficient; CpG, CpG site within *POMC* locus; p, p value; SAM, s-adenosylmethionine; SAH, s-adenosylhomocysteine; z, z value.

European Normal-Weight and Obese Children and Mother-Child Pairs Analyzed for POMC Methylation

Seventy-six normal-weight patients (37 females, 39 males; average age, 5.7 ± 5.5 years; average BMI, 17.3 ± 3 kg/m^2) and 83 obese patients (43 females, 40 males; average age, 13.7 ± 1.9 years; average BMI, 30.83 ± 4.2 kg/m^2) were recruited in the outpatient clinic of the Department of Pediatric Endocrinology of the Charité Universitätsmedizin Berlin. From 33 obese patients (20 females, 13 males; average age, 13.4 years; average BMI, 30.6 kg/m^2), data on the body-weight course of the mother during pregnancy were available. None of the mothers had gestational diabetes.

Gambian Mother-Infant Pairs

Samples originate from an observational prospective study (registered at https://www.clinicaltrials.gov, reference NCT01811641) in The Gambia, West Africa, assessing season of conception and maternal nutritional status on infant epigenetic outcomes (Dominguez-Salas et al., 2013, 2014). For details, see Supplemental Experimental Procedures.

Human Tissue Samples

Postmortem samples of 16 Vietnamese motor vehicle accident victims (Waterland et al., 2010) were collected at the human tissue bank (ILSbio).

Longitudinal Analysis of DNA Methylation

The *POMC* DNA methylation pattern has been analyzed longitudinally from the same individual (n = 51 [24 females, 27 males]; BMI, 27.92 ± 6.8 kg/m^2). The first sample was extracted from the newborn screening card (routinely performed blood collection between days 3 and 10 in Germany). The second sample originated from a blood collection at an age of 11.69 ± 5.5 years.

Postmortem Human Brain Tissue Samples

Hypothalamic samples of 41 individuals were obtained from autopsies performed according to Berlin law (Sektionsgesetz, Gesetz- und Verordnungsblatt für Berlin, 1996; 52 32, 237–239) from the Department of Neuropathology, Charité Universitätsmedizin Berlin. Subjects were selected with no history of neurodegenerative diseases or cancer (12 females, 29 males; average age, 67 ± 12.76 years; average BMI, 32.3 ± 6.8 kg/m^2). For technical details, especially on laser microdissection and immunostaining, see Supplemental Experimental Procedures.

Sperm-PBC Sample Pairs

Sperm and PBC DNA pairs from the same male obese (n = 7; mean age, 36.4 years; BMI, 31.9 kg/m^2) and non-obese (n = 10; mean age, 36.5 years; BMI, 23.01 kg/m^2) individual were obtained from the Center of Reproductive Medicine and Andrology, Münster, Germany (Prof. Gromoll). DNA was extracted as described previously (Laurentino et al., 2015).

Statistical Analysis

The DNA methylation at CpG positions −2 to +7 (nine CpG positions in total, CpG positions −2 to +5, or each CpG position separately) was statistically analyzed by Student's t test. All results were adjusted for age and sex. The linear regression between BMI and DNA methylation at position −2 to +5 was tested as an exploratory analysis. The DNA methylation mean value of

CpG positions −2 to +5 was analyzed against the BMI after Bonferroni correction for multiple testing (p = 0.0005). The logistic regression was calculated with a BMI above 30 kg/m^2 as a binary outcome to obtain the risk for becoming obese. The calculations were performed with PASW (SPSS 21). For further details, especially on the Gambian C1 analysis, see Supplemental Experimental Procedures. Error bars represent SD.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, three figures, and two tables and can be found with this article online at http://dx.doi.org/10.1016/j.cmet.2016.08.001.

AUTHOR CONTRIBUTIONS

P.K. and H.K. designed the study. P.K. and D.H. performed functional experiments. L.W. performed the analysis of DNA extracted from newborn screening cards. A.H. and J.H. provided samples from family trios. B.J.H., A.M.P., M.S., P.D.-S., and S.E.M. provided samples and information for the Gambian mother-infant cohort. R.A.W. provided the samples from human postmortem kidney and brain tissue. A.G. was involved in results-discussion. J.S. provided samples from normal-weight and obese adults. A.J.F. performed the statistical analysis of the Gambian cohort. F.L.H. provided postmortem brain tissue for laser microdissection. C.G. provided the facility for laser microdissection. J.G. provided sperm and blood DNA sample pairs. S.W. provided the samples from the outpatient clinic of the Institute for Experimental Pediatric Endocrinology, Charité, Universitätsmedizin Berlin. P.K., H.K., B.J.H, A.P., M.S., and R.A.W. wrote the manuscript with the contributions of all other co-authors.

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