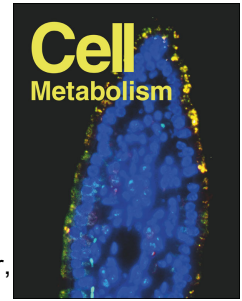


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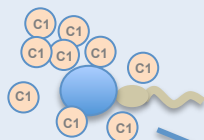
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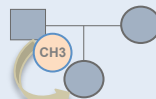
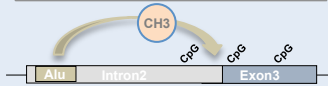
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Multiple factors trigger *POMC*-methylation



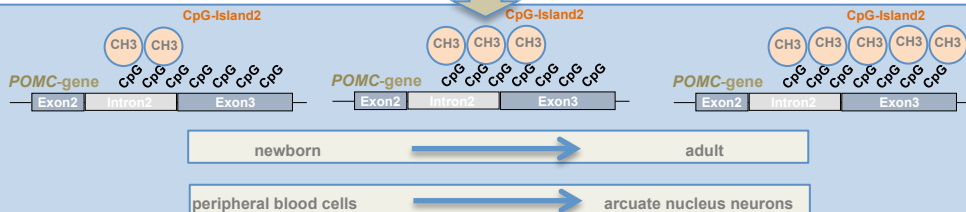
Alu element-driven stochastic methylation



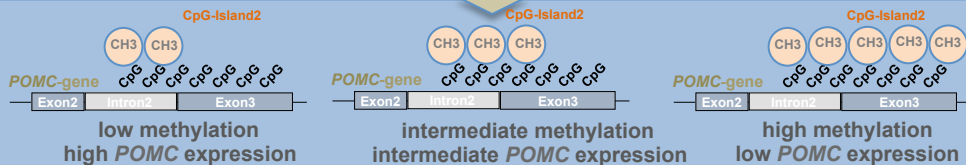
C1-metabolites at conception

interindividual variability of *POMC*-methylation

paternal transmission



intraindividual stability of *POMC*-methylation



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 body mass index (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 MSH (melanocyte-stimulating-hormone) 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 body mass index (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 less than 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 single nucleotide polymorphisms (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.

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). Hypermethylation 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 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 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 intron2-exon3 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 intron2 (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 9 *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-standard deviation score (SDS) (Supplemental Figure S1). Thereafter we analyzed this region in obese versus normal weight adult individuals. We again found variable methylation at the border of intron2-exon3 and significant hypermethylation at positions +1, +2, +3 and +5 (Figure 1 B). 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 1 C).

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 (Supplemental Figure S1) and the methylation status was analyzed by bisulfite pyrosequencing (see Experimental Procedures). Again we observed variable methylation at the intron2-exon3 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 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 (PBC) - 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 which 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 2 C) 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 (EWAS) have identified methylation variants (meQTL) associated with SNPs 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 9 VMR-CpGs (Supplemental Figure S2 A, B). In addition, although the African and European cohorts show significant differences in their SNP characteristics (Supplemental Figure S2 A, B) average *POMC* methylation in both cohorts was essentially identical (Supplemental Figure S2 C). 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 analyzed *POMC* methylation in sperm and blood (PBC) DNA of 17 German men. Interestingly, *POMC* VMR methylation in sperm was significantly lower than that in

blood (Supplemental Figure S3 A), consistent with a loss of methylation during germ line differentiation as has been shown for imprinted regions (Monk, 2015). PBC methylation is however correlated with BMI in the same individuals ($r=0.54$, $p=0.025$) (Supplemental Figure S3 B). 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 the weight change during pregnancy was correlated with *POMC* methylation level determined in the child's PBC-DNA (Supplemental Figure S3).

POMC methylation is influenced by one-carbon (C1) metabolism

Establishment of DNA methylation at human MEs has shown to be sensitive to C1-metabolites at the time of conception. One-carbon 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 1A), 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 S-adenosylhomocysteine (SAH) and positive correlations with betaine and the ratio of S-adenosylmethionine (SAM) to S-adenosylhomocysteine (SAM:SAH) were found at all CpGs -2 to +7 (Table 1A, B and 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 suggest 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 mouse *A^{vy}* locus. Namely, it is i) triggered by a transposable element; ii) sensitive to nutritional modification by C1-metabolites during early embryonic development; iii) in part transmitted via one parental germline; iv) stable over the life course; and v) positively 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 completely 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 micro-RNAs (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) and the joint Gambian Government/MRC Unit. 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 section (Supplemental Table S1).

DNA methylation analysis:

DNA (500 ng) for German cohorts was extracted according to standard protocols (Promega) from peripheral blood cells. 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 information.

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 $22.5 \pm 1.65 \text{ kg/m}^2$ (32 male, 71 female). The obese group includes 125 individuals with a mean age of 54.2 ± 8.35 years and a mean

BMI of $36.03 \pm 5.61 \text{ kg/m}^2$ (50 male, 75 female). The samples were part of the MesyBepo follow-up study (Bobbert et al., 2013).

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

European healthy children (Berlin, Germany) (genetic variant analysis):

84 normal weight children were analyzed with a mean BMI $18 \pm 0.61 \text{ kg/m}^2$ and a mean age of 8.9 ± 2.01 years (female: 44, male: 40), recruited in the outpatient clinic of the Department of Pediatric Endocrinology, Charité Universitätsmedizin Berlin.

European normal weight and obese children and mother child pairs (Berlin, Germany) (for POMC methylation analysis):

76 normal weight (37 female, 39 male, average age: 5.7 ± 5.5 , average BMI: 17.3 ± 3) and 83 obese patients (43 female, 40 male, average age: 13.7 ± 1.9 , average BMI: 30.83 ± 4.2) were recruited in the outpatient clinic of the Department of Pediatric Endocrinology of the Charité Universitätsmedizin Berlin. From 33 obese patients (20 female, 13 male, average age: 13.4, average BMI: 30.6) data were available about the body weight course of the mother during pregnancy. None of the mothers had gestational diabetes.

Gambian mother-infant pairs (West Kiang, The Gambia): Samples originate from an observational prospective study (registered at <http://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., 2014; Dominguez-Salas et al., 2013). For details see supplemental information.

Human tissue samples (brain, kidney): Postmortem samples of 16 Vietnamese motor vehicle accident victims (Waterland et al., 2010) were collected at the human tissue bank (ILSbio, LLC, Chestertown, MD, USA).

Longitudinal analysis of DNA methylation

The *POMC* DNA methylation pattern has been analyzed longitudinally from the same individual ($n=51$; female $n=24$, male 27, BMI $27.92 \pm 6.8 \text{ kg/m}^2$). The first sample was extracted from the newborn screening card (routinely performed blood collection between

day 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 (Berlin):

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 female, 29 male, average age 67 ± 12.76 years, average BMI = $32.3 \pm 6.8 \text{ kg/m}^2$). For technical details especially the laser-micro dissection and immunostaining see supplemental information.

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 position -2 to +7 was statistically analyzed by t-Test – either this 9 CpG positions in total, CpG position -2 to +5 or each CpG position separately. All the 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 position -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 about the Gambian C1-analysis see supplemental information. The error bars represent standard deviation (SD).

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Author contributions

P.K., H.K. designed the study. P.K. and D.H. performed functional experiments. L.W. performed the analysis of the 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.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.

Competing financial interests

The authors declare no competing interests.

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Figures and Table

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 (grey) and 125 obese (red) adult individuals. The most significant differences were observed at CpG position +3 and +5 (corresponding to nucleotide number

chr2:25.384.590 and chr2:25.384.569 (UCSC human genome browser hg19)). The grey 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. The error bars represent standard deviation (SD). (C) Linear regression based on the mean value of CpG position -2 to +5 and BMI in the German adult cohort (n=228) and (D) in MSH laser-microdissected neurons from postmortem human brain samples (n=41).

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 position -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).

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.

Table 1:

Correlations between maternal biomarker status around conception and offspring DNA methylation.

(A) Environmental impact on peripheral blood DNA methylation in 144 children from The Gambia who were conceived during either the dry or 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; se, standard error; z, z-value (B) 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 Supplementary Material).

Table 1

A

covariate	coef.	se	z	p	[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

B

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
SAM:SAH	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
	-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
betaine	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
	-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	

Figure 1

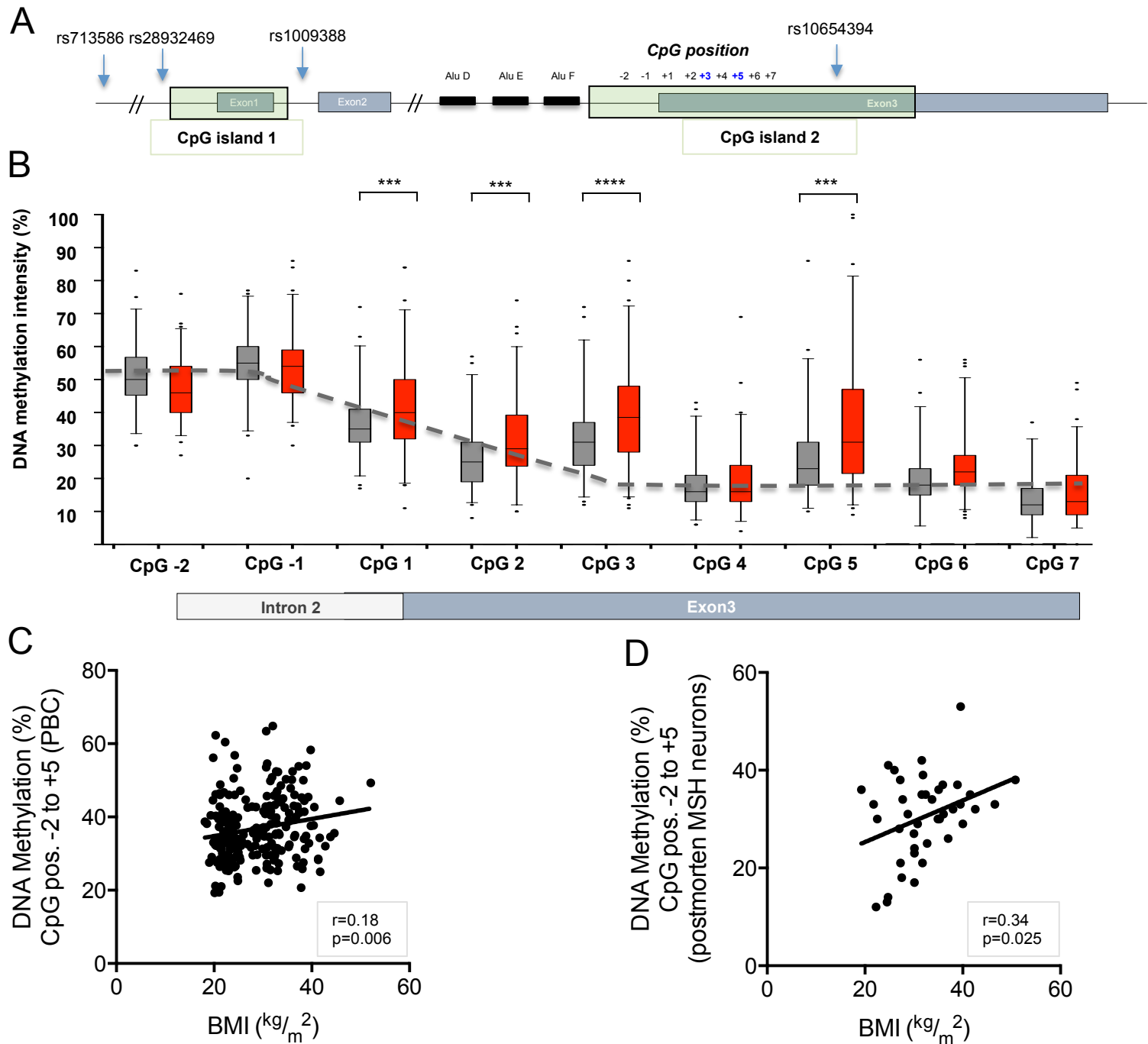
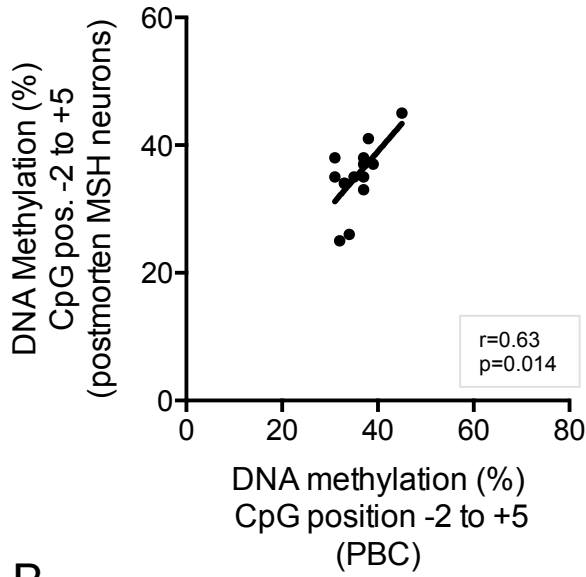
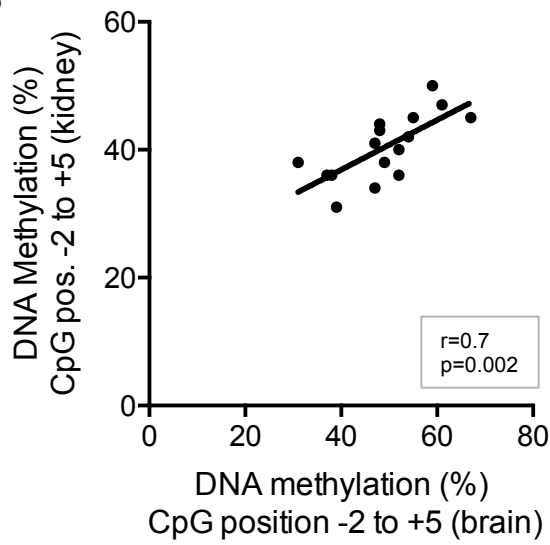


Figure 2

A



B



C

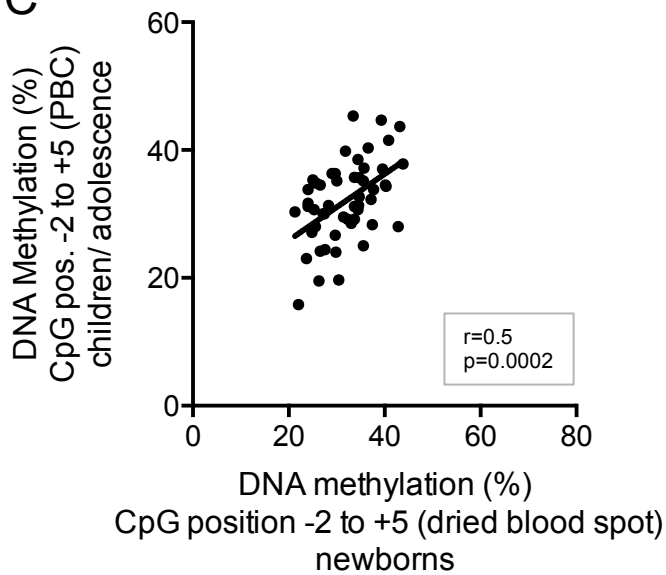
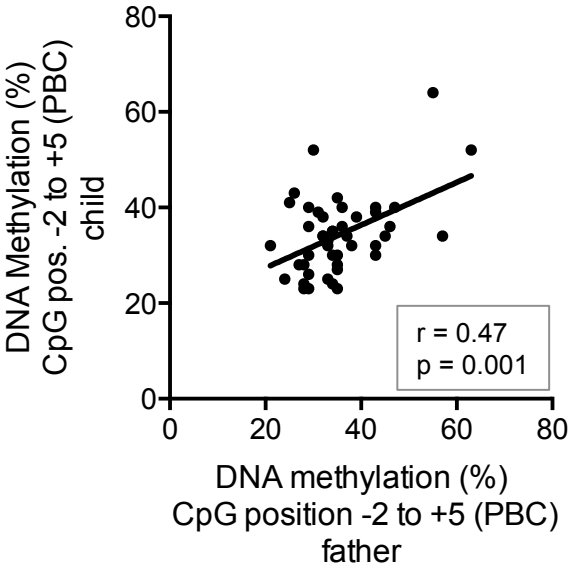


Figure 3

A



B

