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Diet during Pregnancy is Implicated in the Regulation of Hypothalamic RNA Methylation and Risk of Obesity in Offspring

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Diet during pregnancy is implicated in the regulation of hypothalamic RNA methylation and risk of obesity in offspring

Short title: Parental diet regulates m6A and offspring obesity

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Conflict of Interest

There is no conflict of interest to disclose.

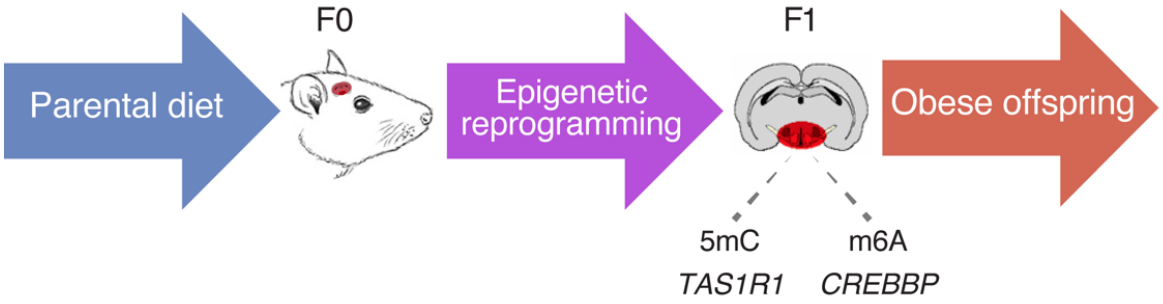
Abstract

Scope: Early life nutrition has long-lasting influence in adults through key mediators that modulate epigenetic states, although the determinants involved that underlie this response remain controversial. Because of the similarities between metabolic, physiological and endocrine changes and those occurring in human type 2 diabetes we studied the interaction of diet during pregnancy regulating RNA adenosine methylation (m6A) and the transcriptome in *Psammomys obesus*.

Methods and Results: Breeding pairs were randomly allocated standard diet (total digestible energy 18 MJ/kg) or low-fat diet (15 MJ/kg). Offspring were weaned on to the low-fat diet at 4 weeks of age and given *ad libitum* access, resulting in 2 experimental groups: (1); male offspring of animals fed a low-fat diet and weaned on to the low-fat diet and (2); male offspring of animals fed a standard diet and weaned on to the low-fat diet. Hypothalamic RNA was used to assess m6A by immunoprecipitation. Parental low-fat diet alters the metabolic phenotype in offspring. An association between parental diet and hypothalamic m6A was observed in regulating the expression of *FTO* and *METTL3* in the offspring.

Conclusions: We propose the regulatory capacity is now broadened for the first time to include m6A in developmental programming and obesity phenotype.

Graphical Abstract



Summary and hypothetical model illustrating asymmetric DNA and RNA methylation serves precise epigenetic control over the expression of genes implic in metabolic disease. In this study, we show parental nutrition is implicated in th control of hypothalamic m6A and the expression of an important obesity gene, CREBBP. The experimental results extend work previously done in the same mo showing obesity genes have DNA methylation changes with parental diet.

Keywords

Obesity, hypothalamus, animal model(s), gene-environment interactions, RNA methylation,

Psammomys obesus

Abbreviations

m6A – N6-methyladenosine

RNA IP – RNA Immunoprecipitation

GWAS – Genome wide association study

T2D – type 2 diabetes

Introduction

The first glimpse into early life origins of human disease came with the seminal papers from Barker and colleagues published in *The Lancet,* these epidemiological studies of infant and adult mortality illustrate the striking parallels that led to the concept of the fetal origins of human health and disease [1, 2]. The fact that undernutrition during gestation was an important but uncharacterized determinant of the origins of adult cardiac and metabolic disease was consistent with the postulate that fetal programming shaped disease later in life, this was later referred to "Barker's hypothesis" with subsequent studies strengthening the concept of predictive adult disease. Subsequent observational studies have determined that fetal nutrition is also tightly linked with the risk of cardiovascular disease and type 2 diabetes [3]. These studies were also important because they offered a resolution to a conundrum, how could early life programming influence postnatal metabolic disease? We now understand that metabolic disease has intrauterine origins which is now referred to as "developmental origins of health and disease" or DOHaD [4]. Since then, the developmental programming field has grown tremendously, becoming an integral component of the way we view the thrifty phenotype [5].

While early life factors such as under-nutrition [6], maternal obesity and gestational diabetes [7] are different, they share common long term outcomes with respect to the metabolic health of the offspring. This is strikingly reminiscent of maternal life experiences that involve hypothalamic appetite systems and offspring predisposition to disease risk as a result of fetal programming [8]. Subject to developmental programming, the hypothalamus regulates food intake and energy balance which is influenced by maternal nutrition. Indeed, fetal

programming of adult disease involves hypothalamic appetite signaling pathways subject to maternal undernutrition [9] or overfeeding [10].

In order to study obesity which reflects the polygenic nature of the human population, we investigated *Psammomys obesus* (Israeli sand rat). *P. obesus* is an outbred rodent adapted to a low energy diet, of which a proportion becomes obese and develops associated diseases when fed a regular laboratory diet, while others remain lean and healthy [11, 12]. Given the predisposition to obesity is not driven by a single gene, *P. obesus* is an ideal animal model for studying critical gene and environment interactions relevant to multifactorial chronic disease.

The effects of maternal over-nutrition on hypothalamic programming can regulate pathways that involve epigenetic changes, and the best characterized modification to DNA is 5-methylcytosine [13]. In the first study of its kind, we have recently shown in *P. obesus* that parental diet during early life regulates the expression of genes associated with DNA methylation in offspring [14]. For example, taste sensory receptor type 1 member 1 (T1R1) is a G protein-coupled receptor that is encoded by the *Tas1r1* gene [15, 16].T1R1 complexes with T3R3 to form the umami taste receptor on the tongue [17]. The expression of *Tas1r1* is tightly regulated by DNA methylation. Low fat parental diet reduces *Tas1r1* methylation and is linked with increased gene expression and risk of obesity in offspring [14]. This and other studies suggest that diet-induced metabolic changes can be transmitted from parent to offspring by mechanisms under epigenetic control [18]. The inheritance of diet-induced metabolic changes happens in germ cells *in utero* during critical developmental windows that not only impact offspring germline but also increase the risk of metabolic disease. For example, parental prediabetes is inherited in the germline transgenerationally and mediated

by DNA methylation [19]. While these studies implicate the significance of the parental methylome influencing future generations there no studies to our knowledge that have shown a role for RNA methylation in acquired metabolic disease [20].

Methylation of the adenosine base at the nitrogen-6 position (N6-methyladenosine or m6A) is an abundant modification found in mammalian mRNA. The fat mass and obesity-associated risk gene, *FTO*, encodes m6A demethylase and is strongly implicated in epigenetic regulation [21, 22]. Furthermore, *FTO* gene mutations are associated with increased risk for obesity and type 2 diabetes, supporting the notion the m6A determinant may regulate the expression of genes important in metabolic disease [23]. In the hypothalamus, *FTO* gene expression is significantly upregulated in rodents after food deprivation and implicated in the control of energy homeostasis. Whereas *FTO* is involved in demethylation (eraser), the *METTL3* gene encodes an m6A methylase (writer) that ensures post-transcriptional control of RNA. Emerging evidence suggests *METTL3* mediated m6A regulates transcriptional silencing of long non-coding RNA molecules such as *XIST* [24]. This asymmetric regulation of m6A by *FTO* and *METTL3* may serve to coordinate transcriptional control. Clearly, this explains only part of the complexity of RNA metabolism and energy balance.

We have recently shown the expression of hypothalamic genes controlling appetite and energy balance as well as nutrient sensing are subject to regulation by DNA methylation [14], yet a role for m6A and *in utero* programming remains to be elucidated. In this work, we investigate the mechanism by which parental diet affects offspring metabolism with respect to m6A and risk of obesity.

Materials and methods

P. obesus experimental design

Breeding pairs fed a low-fat or standard diet during gestation and lactation used in this study were previously described [14]. All animals were housed in a temperature-controlled room (22±1°C) with a 12–12h light-dark cycle (light 0600–1800 h). Breeding pairs were randomly allocated to one of two diets: (A) Standard diet (20% kcal/fat, 64% kcal/carbohydrate, 16% kcal/ protein; total digestible energy 18 MJ kg − 1; n = 20 breeding pairs) or (B) Low-fat diet (10% kcal/fat, 74% kcal/carbohydrate, 16% kcal/protein; total digestible energy 15 MJ kg − 1; n = 30 breeding pairs). Proximate composition of closed formula diets is shown as g/kg diet in **Table 1**. The animals had *ad libitum* access to food and water. Offspring were weaned on to the low fat diet at 4 weeks of age and given *ad libitum* access, resulting in 2 experimental groups: (1); male offspring of animals fed a low-fat diet and weaned on to the low-fat diet and (2); male offspring of animals fed a standard diet and weaned on to the low-fat diet, as previously detailed [14]. Animals were maintained in accordance with the Code of Practice of the National Health and Medical Research Council of Australia, and all procedures were carried out subject to the approval of the Deakin University Animal Ethics Committee.

mRNA sequencing

Total RNA was extracted from P. obesus hypothalami (low-fat (n=4) and standard (n=4) parental diet) using TRIzol reagent (Life Technologies) and RNeasy Kit (Qiagen, cat#74106), following homogenisation with a Polytron homogenizer (Bio-Gen PRO200) using the manufacturer's recommendations. Libraries were prepared according to the Illumina mRNA-seq library preparation kit (cat. #RS-100-0801). Briefly, 2 µg of total RNA was taken for

mRNA isolation using poly-T oligo-attached magnetic beads. Poly-A mRNA was fragmented with the presence of divalent cations under elevated temperatures. The mRNA fragments were reverse-transcribed using SuperScript II (Invitrogen) with random primers. The second strand was synthesized with the addition of RNase H and DNA Pol I. Double-stranded complementary DNA (dscDNA) was end-repaired, followed by 3′ adenylation and ligation to universal adapters. The ligated cDNA library was separated on a 2.0% agarose gel. Library cDNA fragments in the range of 200 bp ± 10 bp were isolated by gel purification (Qiagen) and amplified with 15 cycles of PCR. Column-purified libraries were quantified fluorometrically and visualized by MultiNA for quality assurance. Libraries were diluted to 10 nM and stored at -20°C prior to cluster generation at a concentration of 6 pM. Short-read sequences of 76 base pairs were generated using Illumina Pipeline 1.4.

Assembly of the *P. obesus* transcriptome

Sequences from all 8 samples were pooled together. The FASTX-toolkit version 0.0.13 fastq quality trimmer (http://hannonlab.cshl.edu/fastx_toolkit/) was used to eliminate bases with a quality score less than 30 from the 3′ end. Trimmed reads shorter than 30 bp were removed from further analysis. Trinity (r2012-06-08) was used to assemble the remaining sequences using default settings [25]. Homology of genes to the mouse transcriptome (Ensemble Version GRCm38.71) was determined using the most statistically significant BLASTN alignment [26].

RNA isolation and qRT-PCR

RNA was DNase-treated to remove genomic DNA contaminants and then cDNA was generated from total RNA using Maxima H Minus First Strand cDNA Synthesis kit (Thermo

Scientific). Real Time primers were designed using Primer-Blast (NCBI). Gene expression levels were measured using Luminaris HiGreen Low ROX qPCR Master Mix (2x) (Thermo Scientific) on a PikoReal 96 Real-Time PCR System (Thermo Scientific), and normalised to cDNA concentration determined using the Quant-iT™ OliGreen® ssDNA Assay Kit (Molecular Probes). Gene expression were assessed by qRT-PCR and Ct values were adjusted to cyclophilin endogenous reference gene [27]. RT-PCR primer sequences are described in **Table 2**. Statistical analyses for RT-PCR data was performed using GraphPad Prism version 7 for Mac OS X, GraphPad Software (La Jolla, CA, USA), http://www.graphpad. com. To compare mean phenotypic values between groups, independent samples t-test was used.

m6A RNA methylation quantification

Overall methylated m6A content was measured in 200-ng aliquots of total RNA using an m6A RNA methylation quantification kit (ab185912; Abcam).

m6A RNA immunoprecipitation

m6A enrichment was performed using EpiMark N6-Methyladenosine Enrichment Kit (E1610S New England BioLabs). 5 µg of total hypothalamic RNA was chemically fragmented with Mg2+ into ~100-nt-long fragments (E6150S: New England BioLabs). Fragmented RNA was concentrated using Agencourt RNAclean XP beads (A63987; Beckman Coulter) and resuspended in 20 µl nuclease-free water and subjected to immunoprecipitation. For m6A immunoprecipitation (IP), 25 µl of protein-G magnetic beads were washed and resuspended in 200 µl of reaction buffer (150 mM NaCl, 0.1% NP-40, 10 mM Tris-HCl, pH 7.5), and rotated with 2 µl of affinity purified anti-m6A polyclonal

antibody (EpiMark kit) for 1 hr at 4°C. Following 2 washes with reaction buffer, RNA (18 µl) and m6A control RNA (1ul +/- control RNA; 1:1000 dilution) was added to the antibody-bead mixture and incubated for 2 hr at 4°C. 2 µl of leftover RNA was used as INPUT for downstream analysis. After incubation with antibody-bead mixture, unbound RNA was collected and stored at -20°C. The bound RNA was then washed twice in 200 µl of reaction buffer, twice in low-salt reaction buffer (50 mM NaCl, 0.1% NP-40, 10 mM Tris-HCl, pH 7.5), and twice in high-salt reaction buffer (500 mM NaCl, 0.1% NP-40, 10 mM Tris-HCl, pH 7.5), and eluted in 30 µl RLT buffer (Qiagen). To purify the bound RNA and Input RNA, MyOne Silane Dynabeads (Life Technologies) were used following the standard EpiMark kit clean and concentrate protocol. RNA was eluted from the beads in 30 µl of nuclease-free water and was stored at -20°C for RT-qPCR analysis. Quantitative PCR was performed, and percentage input corrected (% Input (bound RNA) - % Input (unbound RNA)) was calculated for the m6A immunoprecipitation. Results are expressed for the target sequences compared between low fat and standard parental diet.

Prediction of m6a mRNA methylation sites

Sequence-based RNA adenosine methylation site tool (SRAMP) was used to predict sites of m6A methylation using mature RNA mode [28]. Gene sequences were assigned a combined score for adenosine site methylation and a rank generated across species (*Homo sapiens*, *Mus musculus* and *P. obesus*). Predicted N6-methyladenosine sites with threshold specificity >95% were used to aid in the design of amplimers used for gene targets using m6A RNA immunoprecipitation (Table 2).

Results

Low-fat parental diet increases weight and insulin resistance in *P. obesus* offspring

To establish a model of *in utero* transmission of metabolic disease in *P. obesus*, parental nutrition consisted of either the standard diet (20% of energy from fat) or a low fat diet (10% of energy from fat) *ad libitum* [14]. Offspring at 4 weeks of age received a low fat diet for 12 weeks as shown in **Figure 1A** and presented recently [14]. At 16 weeks of age, offspring were culled, and tissues collected and weighed (n = 26). As expected, offspring of parents fed the low-fat diet were 20% heavier than those from parents on standard diet (**Figure 1B**, p=2.86e-9). While blood glucose concentrations were similar for both groups at 8 weeks of age; blood glucose levels significantly increased by 56% in offspring of parents fed the low-fat diet (p=0.003) (**Figure 1C**). Plasma insulin levels were three times higher in the low-fat diet group at 16 weeks of age (p<0.001) (**Figure 1D**). Furthermore, offspring of parents fed the low-fat diet had increased body fat (by 49%) and leg muscle weight was reduced by 8% (**Figure 1E**), while no changes in liver and pancreas mass were observed. These results are consistent with the idea that low-fat parental diet confers weight gain and insulin resistance in offspring [14].

Parental low-fat diet regulates *FTO* and *METTL3* gene expression

Strongly conserved but of unknown function in fetal programming, m6A is involved in the regulation of RNA and remains uncharacterized in *P. obesus* [20]. To assess gene expression changes in offspring we assembled the *P. obesus* RNA sequence and compared this with the assembled mouse sequence (BLASTN Evalue < 1e-100, sequence abundance > 1 Read Per Million). We identified changes in the expression of genes that encode for the N6-adenosine methyltransferase "m6A writer" (*METTL3*, methyltransferase Like 3) and the gene that encodes the oxidative RNA demethylase "m6A eraser" (*FTO*, Alpha-Ketoglutarate

Dependent Dioxygenase). Amplimers were designed to assess their expression using qRT-PCR in the offspring of parents exposed low-fat and standard diets. At 16 weeks of age, offspring of parents fed the low-fat diet show significant increases in *METTL3* (18%) and *FTO* (28%) gene expression (**Figure 2A**). Since *FTO* controls demethylation we measured overall m6A levels. In the hypothalamus overall m6A content was reduced by 20% in offspring from low-fat parental diet (**Figure 2B**). These results show increased *FTO* gene expression inversely correlates with m6A levels in the hypothalamus.

Identification of m6A methylation targets

Because overall m6A content is reduced in offspring from low-fat parental diet, we sought to identify whether obesity related genes might be involved. To do this, we assessed possible m6A modification sites from mRNA sequences of 304 genes implicated in obesity [29] using the RNA adenosine methylation site predictor SRAMP. The reference transcriptomes of *Homo sapiens*, *Mus musculus*, as well as the newly assembled *P. obesus* hypothalamus transcriptome were combined using a rank sum, and the top results were filtered on relevance to transcriptional control and hypothalamic function (**Figure 2C**). These results reveal regulatory genes important in obesity are putative targets for m6A modification such as *CREBBP*.

Low-fat parental diet increases m6A and CREBBP RNA expression in offspring

CREB-binding protein or CREBBP is a ubiquitously expressed transcriptional coactivator involved in response to nutritional status and energy balance in the hypothalamus. If the overall loss of m6A in the hypothalamus regulates gene expression of ubiquitous transcriptional coactivators, we postulated CREBBP could be subject to m6A modification by

low-fat parental diet. Six m6A methylation motifs spanning 753 bp were identified on the CREBBP gene (**Figure 3A**). The m6A motif with the highest prediction score (site start at 190 bp) was examined by RNA-immunoprecipitation (RNA-IP) enrichment technique. This assay allows us to identify m6A modification by using an antibody that specifically recognizes N6-methyladenosine on soluble RNA and we assessed purified nucleic acid by qPCR. To test the feasibility of this approach we assessed m6A modification of CREBBP and show significant enrichment using the m6A antibody (five times higher) in the hypothalamus of offspring derived from low-fat parental diet when compared to parents on a standard diet (**Figure 3B**). To exclude the possibility that m6A enrichment was non-specific we assessed other putative gene targets such as *NRF1* and *TAS1R1* that were recently identified as gene targets for 5mC [14]. We observed no significant difference in offspring m6A enrichment for *NRF1* and *TAS1R1* between low-fat and standard parental diets. Antibody specificity was also assessed using m6A control RNA. As shown in Figure 3B, soluble RNA was significantly enriched for m6A on the *GLUC* gene (positive m6A control). Because our m6A analysis specifically identified *CREBBP,* we hypothesized gene expression was also subject to regulation. CREBBP gene expression in the hypothalamus of offspring derived from low-fat parental diet was significantly increased (p=0.002) (**Figure 3C**). The results implicate m6A modification and CREBBP gene regulation subject to parental diet and increased risk of obesity in offspring.

Discussion

While there is strong evidence for early-life nutrition regulating the modification of DNA, the role of N6-methyladenosine modification of RNA remains to be elucidated. Our experimental findings are novel for two reasons. First, to our knowledge there is no epigenetic evidence for

m6A involvement in the developmental programming of obesity. Secondly, precise control over m6A modulation is mediated by the fat mass and obesity-associated gene, *FTO*, which is implicated in mRNA transport, degradation and translation [20]. A common variant of *FTO* is robustly associated with increased body mass risk in childhood and adult obesity [23].

Recent studies linked *FTO* and m6A demethylation with pre-adipocyte differentiation [30]. During adipogenesis m6A is inversely correlated with gene expression. Furthermore, *FTO* is implicated in the regulation of mRNA splicing with the participation of splicing regulatory (SR) factors, *Srsf1* and *Srsf2*. Knockdown of *FTO* affects alternative splicing patterns of adipogenesis-related gene Runt-related transcription factor 1 (*Runx1t1*). *FTO* regulates activity of the dopaminergic midbrain circuitry, with higher m6A in *FTO* knockout mice [31]. Analysis of overall m6A modification of mRNAs using an RNA-IP technique similar to the protocol used in this study showed the midbrain and striatum had increased m6A decoration of mRNAs important for dopaminergic signalling in *FTO*-deficient mice. The functional importance of *FTO* also extends to brain-food responses. Causing marked consequences to hunger the *FTO* risk allele is associated with ghrelin hormone levels and ingestive behaviour. This link between FTO, ghrelin, and impaired brain food-cue responsivity is connected by dynamic m6A modification. Nowhere is this complexity more evident than in the developmental programming of obesity. Our study shows for the first time that parental diet during early life modulated hypothalamic m6A of mRNAs important for transcriptional control in the offspring.

Nutrient sensing of metabolic status and homeostasis is controlled centrally by the hypothalamus. Strongly conserved in mammals, CREBBP serves to acetylate histone and non-histone proteins. Regulation of gene expression in the hypothalamus is primarily attained

at the transcriptional level by CREBBP. Indeed, the hypothalamus interprets extracellular cues to control energy balance, for example, dietary restriction induces CREBBP and is associated with protective effects against proteotoxicity. *CREBBP* expression in the hypothalamus decreases with age and diabetes and is induced by dietary restriction [32]. The functional importance of CREBBP in diabetes pathogenesis was shown in a GWAS of almost 35,000 cases with T2D [33]. As for the precise role of CREBBP in obesity this remains poorly understood. In different mice strains hypothalamic expression of CREBBP is correlated with lifespan and serving a key role in responses to nutritional status and energy balance. For example, specific CREBBP deletion in the hypothalamus causes a dramatic obesity phenotype associated with impaired glucose homeostasis and increased food intake. These mice are significantly overweight with impaired leptin and insulin signaling [34]. Despite great advances, how acetyltransferease regulators such as CREBBP precisely control core pathways in metabolism signalling remains an important challenge to understand in disease. This is clearly complicated by the fact that acetyltransferases like CREBBP share greater than 90% sequence identity with p300 and this paralogue of CREBBP serves a primary role in regulating gene transcription [35]. Not surprisingly, CREBBP and p300 homozygous knockout mice are embryonic lethal whereas heterozygous deficient mice have lipodystrophy and are resistant to diet-induced obesity [36]. While the conventional view of lipodystrophy is normally associated with insulin resistance, CREBBP-deficient mice were completely protected from high-fat diet weight-gain, more insulin sensitive and glucose tolerant, probably a result of reduced hepatic gluconeogenesis [37].

An important distinction from obesity phenotypes that are derived from monogenetic animal models, *P. obesus* is a polygenic rodent that develops metabolic syndrome by dietary

modification alone that closely resembles human type 2 diabetes [38]. In this study, we observe parental diet is associated with hypothalamic RNA methylation and reduced FTO expression in offspring. The *FTO* gene contains the most penetrant predictive polymorphisms for obesity [39]. Furthermore, *FTO* is an important regulator of metabolism and energy utilization. Genetic deletion of *FTO* in mice reduces adiposity and diet-induced obesity [40]. These lean mice show increased energy expenditure and systemic sympathetic activation. The challenge now is to understand the molecular basis of protection from obesity.

In this study, we show expression of genes that encode the m6A writer METTL3 and m6A eraser FTO are increased in offspring of parents fed the low fat-diet. It is probable that parental low-fat diet that we describe is responsible for reduced m6A levels in offspring. This is because overall m6A content assessed by N6-methyladenosine antibody is significantly higher in offspring of parents fed standard diet. We propose parental low-fat diet increases hypothalamic CREBBP RNA modification by m6A. This is because, irrespective of reduced overall m6A content in the hypothalamus, CREBBP RNA is characterized by a dramatic increase in N6-methyladenosine in offspring derived from parental low-fat diet. The experimental results also extend the functional importance of hypothalamic 5-methylcytosine (5mC) on DNA recently described for the taste sensory receptor gene *Tas1r1*. Using methyl-CG capture technique combined with massive parallel sequencing we have shown the expression of *Tas1r1* is inversely correlated with 5mC. Furthermore, close inspection of methylation variability in the hypothalamus confirmed low fat parental diet reduces DNA methylation on the *Tas1r1* which is linked with increased risk of obesity in offspring [14]. We propose the regulatory capacity of important genes in the hypothalamus is now broadened and described for the first time to include m6A in developmental programming

and obesity phenotype. While the principles of DNA and RNA modification are considered consistent with the idea of transcriptional control in offspring this has not yet been critically tested completely in parents. It is probable that diet restriction can target transcriptional control of hypothalamic genes by altering methylation states of DNA and RNA. A key challenge is explaining how the regulatory events in utero lead to the developmental consequences associated with obesity in offspring. What is clear from our data is that hypothalamic regulation is complex. It is a complex order of distinguishable methylation events that modify RNA and DNA nucleobases. These specific instructions regulate hypothalamic gene expression. For example, *TAS1R1* is decorated by DNA methylation independent of m6A whereas *CREBBP* is mediated by m6A independent of DNA methylation. The fact that parental diet can alter the expression of genes implicated in obesity offers an interesting example of simultaneous regulation of seemingly disconnected signaling pathways. This asymmetric mode of epigenetic modification regulates gene expression and fetal programming associated with increased risk of obesity (Figure 4).

The timing of hypothalamus development determines the window of vulnerability to environmental stimulation including malnutrition. In rodents, the perinatal period of life corresponds largely to the period of brain maturation and neuronal differentiation. The development of these systems occurs primarily during the last week of gestation and accelerates during early postnatal life, still being active after weaning. During this dynamic period, developmental programming of hypothalamic neuronal circuits located in the arcuate nucleus controlling appetite and energy homeostasis are subject to maternal nutrition that involve metabolic factors and environmental changes. For example, DNA methylation in white adipose tissue is an effective determinant regulating leptin transcription of adult

offspring from high-fat fed dams [41]. While DNA methylation is known to repress gene transcription, this determinant does not strictly function alone. Indeed, distinct modifications such as lysine acetylation and methylation of histones are also sensitive to diet, changes in energy balance and metabolic intermediates.

Little is known about nucleobase modification, specificity and the dynamic macromolecular complexes regulating gene transcription in the hypothalamus, so an emerging issue is how m6A and 5mC patterns are written and erased with diet and how it pertains to the developmental origins of health and disease. For example, while a link has been established between the epigenetic determinants m6A and 5mC with gene regulation the potential complexity of parental nutrition influencing offspring disease risk remains a curious challenge to understand. Nowhere is this more evident than in the appetite signals in the hypothalamus. This understanding may have applications in biomarker discovery and identifying candidates involved in the regulatory crosstalk between DNA and RNA modification.

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Authors Contributions

I.K. T.C., B.S. performed the investigation, I.K. and A.K. performed the validation experiments and data visualization, M.Z., A.K., I.K., were involved in data curation, software and methodology, M.Z., A.K., I.K., were involved in provision of resources and methodology, K.W., P.Z., A.E-O. were involved in conceptualization, supervision, project

administration and funding acquisition. A.E-O. involved in original draft. All authors were involved in review and approve the manuscript.

Duality of interest

Authors declare none

Data availability

Illumina mRNA sequencing data described here are publicly available at the NCBI Short Read Archive under accession number SRP06513.

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Figure 1

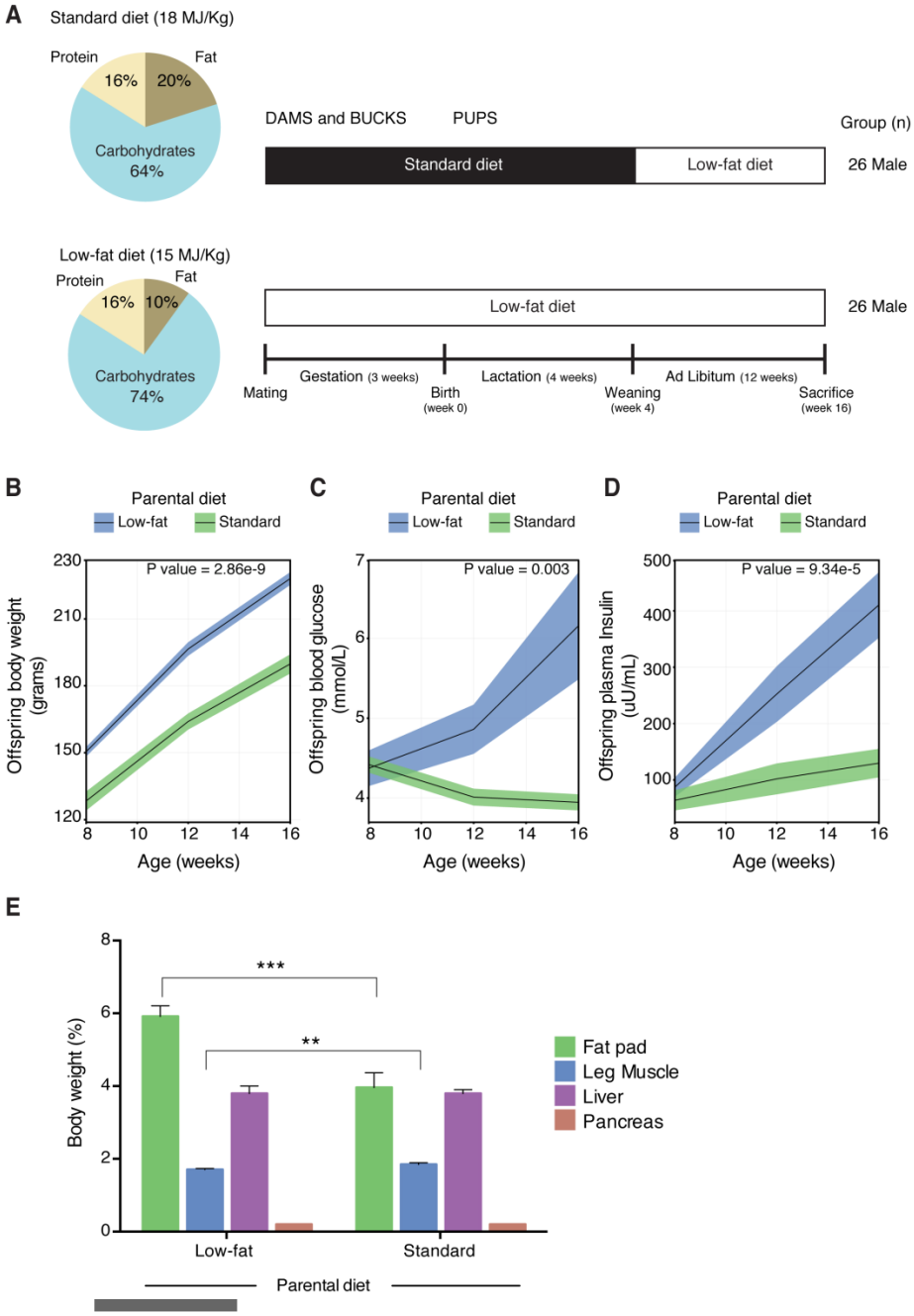

Figure 1. Parental low-fat diet alters metabolic phenotype of *P. obesus* offspring.

(**A**) Design of dietary plan. Male animals from the following groups were investigated: parents and offspring fed low-fat diet (low-fat); parents fed standard diet and offspring fed low-fat diet (standard), n =26. (**B**) Effects of parental dietary manipulation on body weight (BW), glucose **(C)** and plasma insulin levels **(D)** of offspring fed a low-fat and standard parental diet at 8,12, and 16-weeks of age. Bold line represents mean and coloured shading is S.E.M. **(E)** Fat pad, leg muscle, liver and pancreas mass as a percentage of body weight (BW) of

offspring fed a low-fat and standard parental diet. Error bars correspond to S.E.M., ** p-value <0.01, *** p-value <0.001. Student T-test used for statistical analysis.

Figure 2

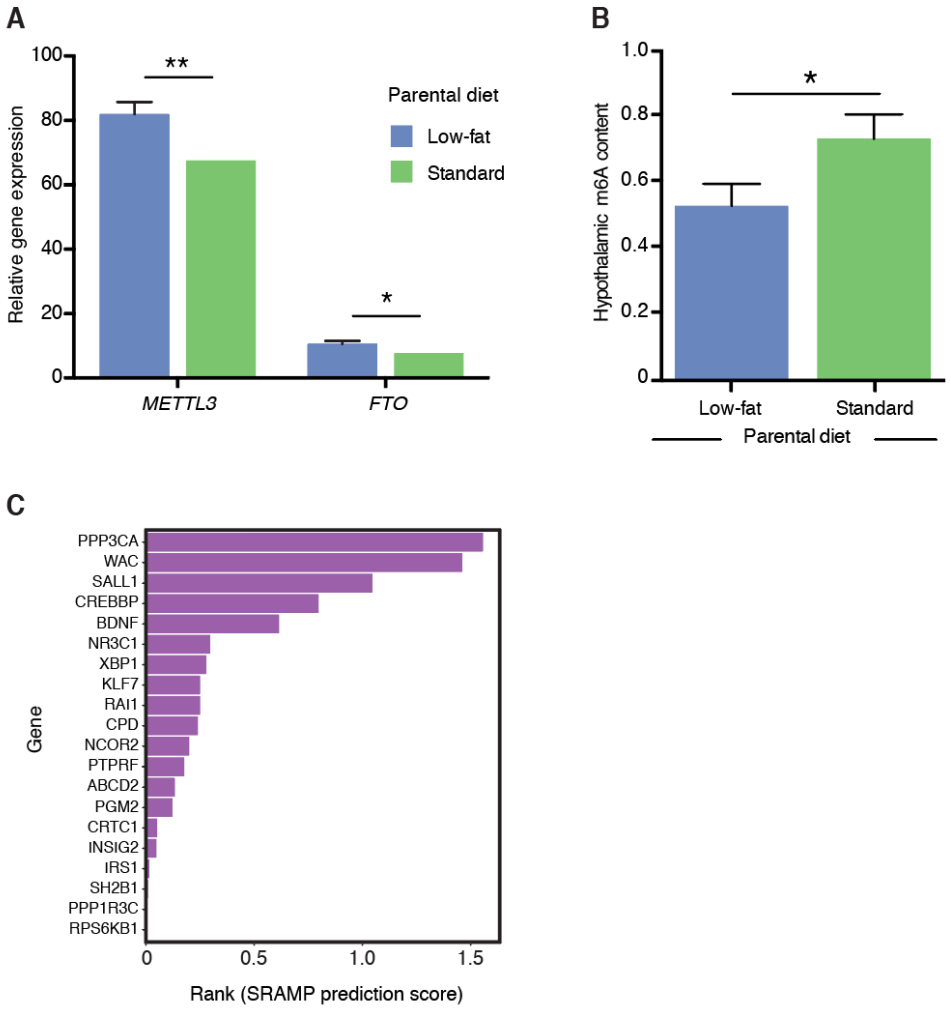

Figure 2. **Parental low-fat diet intake regulates m6A modifying enzymes and m6a levels in *P. obesus.*** (**A**) Relative gene expression of m6A modifying enzymes *METTL3* "writer" and *FTO* "eraser" in hypothalamic RNA isolated from offspring of parents fed a low-fat and standard diet (control). **(B)** Overall m6A levels in total RNA isolated from hypothalamus from offspring exposed to low-fat and standard parental diet, m6A levels were determined as a ratio of all adenosine residues in RNA. (**C**) The sum of the rank of highest m6A prediction score using SRAMP (sequence-based RNA adenosine methylation site predictor) for each obesity gene conserved across *Homo sapiens*, *Mus musculus*, and *P. obesus*. Top 20 obesity genes are shown. *CREBBP* is a strong candidate for m6A across all species. Error bars correspond to s.e.m, *p-value <0.05, ** p-value <0.01.

Student T-test used for statistical analysis.

Figure 3

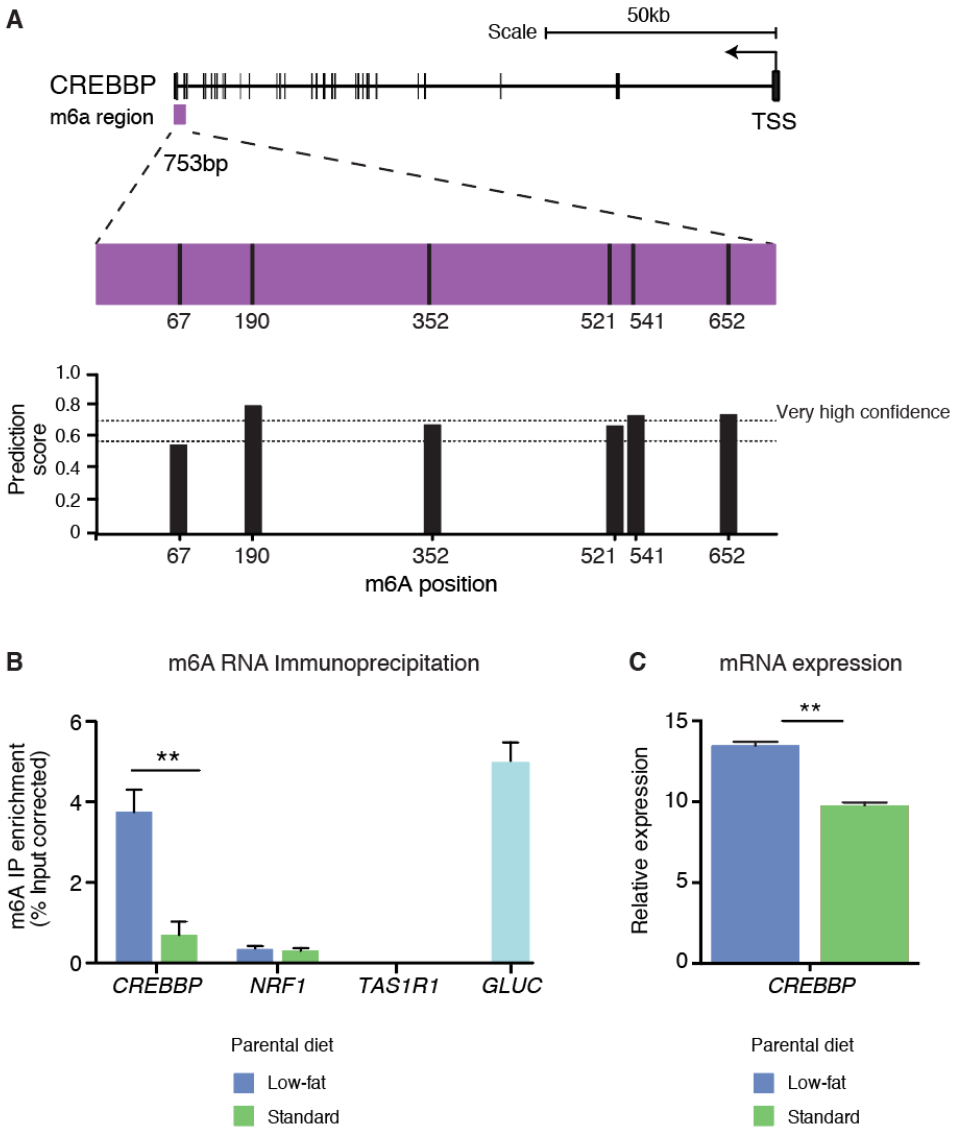

Figure 3 Elevated *CREBBP* m6A in offspring derived from parental low-fat diet.

(A) Schematic representation of *CREBBP* gene (mm10), showing the predicted m6A motif (purple) common to *Homo sapiens*, *Mus musculus*, and *P. obesus*. Graph shows prediction score by base position and confidence, motif starting at 190bp on *CREBBP* was examined by m6A RNA immunoprecipitation assay. **(B)** RNA immunoprecipitation coupled with qPCR shows m6A methylation at motifs predicted by SRAMP on *CREBBP* and *NRF1* obesity associated genes. *TAS1R1* gene was used as m6A negative control. Data from m6A IP methylation control (*GLUC* gene) is also shown **(C)** Relative gene expression of *CREBBP* in offspring fed a

low-fat and standard diet. Error bars correspond to S.E.M., * p-value < 0.05, ** p-value <0.01. Student T-test used for statistical analysis.

Figure 4

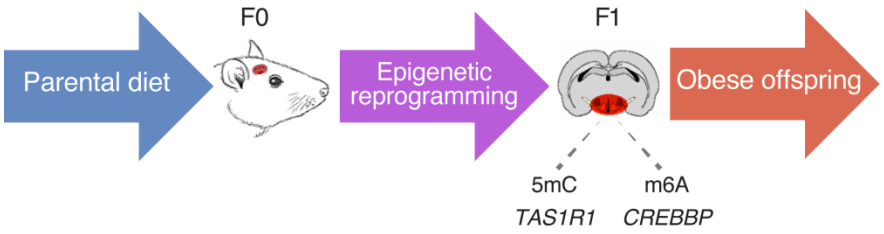

Figure 4. Summary and hypothetical model illustrating asymmetric DNA and RNA methylation serves precise epigenetic control over the expression of genes implicated in metabolic disease. In this study we showed parental nutrition controls overall hypothalamic m6A and the expression of an important obesity gene, *CREBBP*. The experimental results extend work previously done in the same model showing the TAS1R1 gene is subject to DNA methylation changes with parental diet. Paternal diet restriction affects hypothalamic appetite signals that coordinate epigenetic consequences attained by methylation of DNA and RNA that underpin the expression of genes implicated with obesity.

Table 1

Composition of closed formula diet	**Standard diet**	**Low-fat diet**
Protein (g/kg)	**19.8**	**20**
Fat (g/kg)	**10**	**5**
Crude fibre (g/kg)	**3.4**	**5**
Energy (Mj/kg)	**18**	**15**

Table 2

Primers	Sequence	Assay
GLUC Forward Primer	CGACATTCCTGAGATTCCTGG	m6a postive control
GLUC Reverse Primer	TTGAGCAGGTCAGAACACTG	
CLUC Forward Primer	GCTTCAACATCACCGTCATTG	m6a negative control
CLUC Reverse Primer	CACAGAGGCCAGAGATCATTC	
CREBBP Forward Primer	CACAAGGACCTGGAGGCTAC	m6a enrichment
CREBBP Reverse Primer	ACCCAATCTGCTGCTTCATC	
TAS1R1 Forward Primer	CTCCTGAATGGCTTGGGTTA	m6a enrichment
TAS1R1 Reverse Primer	CCGAGCTCTCTTGAGTCCTG	
NRF1 Forward Primer	CCAGAGGGTAACTTGCAGGA	m6a enrichment
NRF1 Reverse Primer	CATAACCATTGGGCATCTCC	
CREBBP Forward Primer	CACAGAACCAGTTTCCATCATCCAGT	qRT-PCR
CREBBP Reverse Primer	CATGTTCAGAGGGTTAGGGAGAGCA	