RPGRIP1L and *FTO* – genes implicated in the effects of *FTO* intronic sequence variants on food intake – also affect adipogenesis and adipocyte biology.

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Abstract

***RPGRIP1L* and *FTO* – genes implicated in the effects of *FTO* intronic sequence variants on food intake – also affect adipogenesis and adipocyte biology.**

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Single nucleotides in the first intron of *FTO* convey effects on adiposity by mechanisms that remain unclear, but appear to include modulation of expression of *FTO* itself, as well as other genes (e.g. *RPGRIP1L*, *IRX3*) in the vicinity of *FTO*. This locus affects food intake, the browning of white adipose tissue and risk of type 2 diabetes (independent of its effects on body weight). *FTO* and *RPGRIP1L* expression are decreased in fibroblasts and iPSC-derived human neurons of individuals segregating for obesity risk alleles of *FTO* at rs8050136 and rs1421085. These alleles exhibit decreased binding of isoform p110 of the CUX1 transcription factor. This isoform activates transcription of both *FTO* and *RPGRIP1L*. The *FTO* locus conveys effects on adiposity via hyperphagia, in part, by regulating *FTO* and *RPGRIP1L* expression in the hypothalamus. We examined whether *FTO* and *RPGRIP1L* also modify adipogenesis and adipose tissue lipid storage. Such effects would influence systemic consequences of the hyperphagia driven by the actions of the genes in the hypothalamus.

Given the role in energy homeostasis of genes encoding elements of the primary cilium, we hypothesized that mice hypomorphic for *Rpgrip1l* would display increased adiposity. In confirmation, we find that *Rpgrip1l*$^{+/-}$ mice are hyperphagic and obese, and display diminished suppression of food intake in response to leptin administration. These findings suggest that

RPGRIP1L may be partly or exclusively responsible for the obesity susceptibility signal at the *FTO* intronic locus.

We describe effects of Rpgrip1l in adipocytes which may contribute to the adiposity phenotype observed in these animals, and possibly humans. Loss of *Rpgrip1l* in 3T3-L1 preadipocytes increased the number of cells capable of differentiating into mature adipocytes. Knockout of *Rpgrip1l* in mature adipocytes (using Adipoq-Cre) did not increase adiposity in mice fed chow or high fat diet. Neither did we observe any effects of *Rpgrip1l* knockdown in mature 3T3-L1 adipocytes *in vitro*. Thus, to the extent that Rpgrip1l affects cell-autonomous adipose tissue function, it appears to do so by effects conveyed in preadipocytes, a cell type in which the primary cilium – as a mediator of developmental signals – may have functional importance. We propose that decreased *RPGRIP1L* expression in preadipocytes in humans segregating for *FTO*-associated obesity risk alleles increases the potential storage capacity of adipose tissue. Such capacity would influence the metabolic consequences of positive energy balance due to the action of these alleles within the brain.

Fto expression is upregulated during adipogenesis in murine and human cells *in vitro*, and is more highly expressed in isolated mouse adipocytes than in preadipocytes. Here we demonstrate that FTO is required for the maintenance of adipocyte lipid filling and endocrine function in murine 3T3-L1 cells and human adipose tissue-derived stromal cells. RNAseq analysis indicates that this effect on adipocyte programming is conveyed in part by modulation of C/ebpβ- and C/ebpδ-regulated transcription, consistent with reports that Fto acts a transcriptional coactivator. $Fto^{-/-}$ mice have normal fat mass in early life, but spontaneously lose adipose tissue as they age. We propose that Fto is required to maintain adipocyte viability, a function critical to the prevention of ectopic lipid accumulation in obese states. Such

accumulation – both total and in specific anatomic regions – has adverse metabolic consequences.

In addition to the developmental effects on adiposity mediated by RPGRIP1L, and the effects conveyed on adipocyte function related to FTO, the *FTO* locus could also impact systemic energy homeostasis by modifying production of humoral signals that are integrated centrally to regulate energy balance. We explored molecular modifiers of adipocyte production of leptin identified by GWAS that may modify obesity risk. The *FTO* locus was associated with circulating leptin concentration, but this association was abrogated when corrected for BMI, indicating that this locus does not contribute to adiposity by dysregulating leptin production. Our *in vitro* findings are consistent in this regard, as knockdown of *Rpgrip1l* and *Fto* in 3T3-L1 cells did not affect leptin production per adipocyte. These results, however, are not inconsistent with a role for *FTO* in maintenance of adipocyte viability.

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This work is dedicated to my devoted and loving parents, Paul and Barbara Martin,

and to my extraordinary husband, Joseph Carli.

Thank you for your enduring support and dedication.

Chapter 1 Introduction

Identification of the obesity-associated *FTO* locus

Almost simultaneously in 2007, three independent groups reported studies which took advantage of the decreased cost and increased density of single nucleotide polymorphism (SNP) genotyping to identify an association between adiposity and risk of overweight/obesity in Europeans and a group of highly related SNPs within the first intron of a gene called *FTO* on the long arm of chromosome 16 at position 12.2 (SNPs: rs9939609 (Frayling, Timpson et al. 2007, Scuteri, Sanna et al. 2007), rs1421085 and rs17817449 (Dina, Meyre et al. 2007)). The first among these groups originally identified an association for this region with type 2 diabetes (T2D) while performing case control studies in a UK population (Frayling, Timpson et al. 2007). Following adjustment for BMI, the association was completely abrogated, indicating that the effect of this genetic region on T2D status is mediated predominantly by its effect on adiposity, a potent enabler of T2D by effects on insulin resistance and intermediary metabolism. The group then followed up their analysis of this association using thirteen cohorts comprising approximately 30,000 white European adults and children. There was no association of the obesity risk alleles with birth weight. However, at the earliest timepoint in childhood examined, i.e. at 7 years old, each copy of the *FTO* risk allele for increased adiposity was associated with an increase of 0.08 BMI Z-score units (odds ratio (OR) of obesity at 11 years = 1.35; 95% CI = 1.14 - 1.61 and OR of overweight = 1.27; 95% CI = 1.16 - 1.39), reflecting an elevated body weight that was observed at all ages studied, with a per-allele effect of 0.09 Z-score units, or approximately 1.2 kg body weight in adults (OR for obesity = 1.31, 95% CI = 1.23 to 1.39; for overweight, OR = 1.18, 95% CI = 1.13 to 1.24), a modest effect size that has been replicated in other populations to yield a signal at least one hundred orders of magnitude greater in statistical

significance than most other known variants associated with body weight (Locke, Kahali et al. 2015). In all adult cohorts characterized, there was no difference in the magnitude of the effect between men and women. The effect on body weight is conveyed entirely by fat mass, with no effect on lean mass, as measured by dual-energy x-ray absorptiometry (DEXA). Notably, there was no association between height and risk allele status. Increased food intake (with preference for higher caloric density), rather than decreased energy expenditure, is the primary cause of this increased body weight in individuals segregating for *FTO* risk alleles (Cecil, Tavendale et al. 2008, Speakman, Rance et al. 2008, Wardle, Carnell et al. 2008, Tanofsky-Kraff, Han et al. 2009).

The SNPs originally genotyped are in linkage disequilibrium (LD) with other SNPs spanning 47 kilobases. This LD block encompasses portions of the first two introns and the intervening exon (exon 2) of *FTO* (Dina, Meyre et al. 2007, Frayling, Timpson et al. 2007). None of the adiposity-associated SNPs suggested themselves as functional variants and sequencing of the region in a subset of subjects with extreme obesity did not yield evidence to suggest modified FTO protein sequence or splicing of the *FTO* transcript (Frayling, Timpson et al. 2007). Both Frayling *et al*. and Dina *et al*. found *FTO* to be ubiquitously expressed in human tissues, with particularly high levels of expression in the cerebral cortex, parietal lobe and hypothalamus (Dina, Meyre et al. 2007, Frayling, Timpson et al. 2007). At the time, no structural domains could be identified in FTO to shed light on its possible effect on adiposity. More recent approaches utilizing fine mapping and studies of individuals of African descent, which have smaller regions of LD, have reduced the interval associated with obesity to include only SNPs within the first intron of *FTO* (Hassanein, Lyon et al. 2010, Peters, North et al. 2013, Claussnitzer, Dankel et al. 2015).

Search for genes that convey effects on adiposity with regard to allelic variation in the first intron of the *FTO* locus

Figure 1.1 *The obesity-associated FTO locus and surrounding genes*

The region shaded red contains SNPs associated with adiposity by GWAS in 2007 (Dina, Meyre et al. 2007, Frayling, Timpson et al. 2007, Scuteri, Sanna et al. 2007).

Deletion of *Fto* was first described in the Fused toes mouse, the eponymous model generated by insertional mutagenesis that caused the loss of a 1.6 megabase region encompassing six genes on mouse Chr 8 (van der Hoeven, Schimmang et al. 1994, Peters, Ausmeier et al. 2002); three genes of the *IroquoisB* (*IrxB*) cluster (*Irx3*, *Irx5* and *Irx6*) as well as novel genes designated *Ft1*, now *Fts* (Lesche, Peetz et al. 1997), Fantom/*Ftm*, now *Rpgrip1l* (Vierkotten, Dildrop et al. 2007) and Fatso/*Fto* (Peters, Ausmeier et al. 1999). Originally named due to its large size (~350 kb), *FTO* was renamed "Fat Mass and Obesity Associated" (Frayling, Timpson et al. 2007) to avoid pejorative implications with regard to the gene's association with human adiposity. The homozygous Fused toes mouse dies around E10. Associated anomalies include loss of left-right asymmetry, pericardial enlargement and severe craniofacial malformation, with almost complete loss of the telencephalon and mesencephalon. Heterozygous mice survive but display syndactyly as well as thymic hyperplasia (van der Hoeven, Schimmang et al. 1994).

These animals have not been described as obese but their body composition has not been completely characterized.

GWAS have been utilized to identify numerous variants that are associated with human disease, the vast majority of which are noncoding, and are customarily identified by the gene closest to the SNP that conveys the strongest signal. Some of these SNPs alter the function or expression of the genes in which they were identified. For example, rs1061170, located within complement factor H (*CFH*) encodes a coding variant that leads to age-related macular degeneration (Day, Willis et al. 1988, Klein, Zeiss et al. 2005). Several SNPs in distinct populations have been identified in the enhancer region of the human lactase gene (LCT) which regulate its expression, leading to hypolactasia, or lactose intolerance (Tishkoff, Reed et al. 2007). When many GWAS-implicated loci are interrogated carefully however, the causal SNPs are not necessarily those closest to the genes they regulate. For example, low-density lipoprotein cholesterol- and myocardial infarction-related SNP rs12740374 modulates hepatic VLDL secretion by regulating expression of Sortilin (*SORT1*), a gene ~25 kb away (Musunuru, Strong et al. 2010). Obesity, a complex human trait, has been associated with the *FTO* locus as well as many others, including SNP rs17782313, near the gene encoding the melanocortin 4 receptor (*MC4R*). This risk allele is associated with decreased *MC4R* expression, a known regulator of food intake in rodents and humans (Loos, Lindgren et al. 2008, Tang, Jin et al. 2017).

Several groups have assessed the possible roles of genes in the vicinity of *FTO* in conveying the effects of some, or all, of its intronic alleles with adiposity. Expression quantitative trait loci (eQTL) mapping has been used for this purpose. In eQTL analyses, SNP genotypes are related to tissue-specific transcript levels. An early eQTL study examining expression in lymphocytes of *FTO* as well as surrounding genes identified an association

between rs8050136, an adiposity-associated SNP in the first intron of *FTO*, and expression of Retinoblastoma-like 2 (*RBL2*), approximately 270 kb away from *FTO* (Jowett, Curran et al. 2010). *RBL2* is a member of the Retinoblastoma family of tumor suppressor genes (Sun, Bagella et al. 2007) and protein levels are downregulated in the mitotic clonal expansion phase of adipogenesis in 3T3-L1 cells, suggesting a role for Rbl2 in the maintenance of the preadipocyte state (Richon, Lyle et al. 1997). Jowett *et al*. did not observe any association between rs8050136 and *FTO* expression *per se* (Jowett, Curran et al. 2010). Two other groups have used eQTL mapping to identify a role for Iroquois (IRX) homeobox genes in the development of obesity related to the *FTO* locus. One demonstrated an association between 11 SNPs in and around the *FTO* locus and an increase in *IRX3* expression in human cerebellum (Smemo, Tena et al. 2014) The other found increased expression of *IRX3* and *IRX5* in primary subcutaneous preadipocytes - following a short period of differentiation - from patients segregating for the *FTO* risk allele at rs1421085 (Claussnitzer, Dankel et al. 2015). Neither group found evidence of a relationship in these tissue/cell types between risk allele status and expression of *FTO per se*.

While there is little evidence supporting an effect of RBL2 on body weight in humans, a compelling case has been made for the involvement of *IRX3* and *IRX5* in the development of obesity associated with risk alleles in intron 1 of *FTO*. Profiles of genomic interactions in mouse brains exhibit long-range physical interactions between the *Irx3* promoter and the obesity-associated interval in *FTO*. The obesity-associated allele of SNP s9930506 was associated with an increase in *IRX3* expression in human cerebellum. *Irx3* knockout mice are hypermetabolic and have lower total body weights and less fat mass; these animals are resistant to weight gain when fed a high fat diet (Smemo, Tena et al. 2014). Tissue-specific *Irx3* knockout models have implicated both the hypothalamus and adipose tissue as regulators of this phenotype. A

hypothalamus-specific model of dominant-negative *Irx3* recapitulates the whole-body knockout with regard to body weight and fractional fat mass. Additionally, these hypothalamic *Irx3* dominant negative mutants exhibit increased adipose tissue "browning", which contributes to increased energy expenditure by uncoupled oxidative phosphorylation (Smemo, Tena et al. 2014). However, an adipose tissue-specific *Irx3* knockout model also phenocopies the whole-body knockout (Claussnitzer, Dankel et al. 2015). Claussnitzer *et al*. have demonstrated a cell-autonomous role for *IRX3* and *IRX5* in adipocyte browning by showing that the loss of these genes induces thermogenesis in primary human preadipocytes differentiated *in vitro* and that overexpression of these genes inhibits thermogenesis. Importantly, they also implicated rs1421085 as the causal SNP, located within an ARID5B repressor binding location. The protective T allele binds ARID5B whereas the C risk allele does not, accounting for the elevated *IRX3* and *IRX5* expression seen in primary preadipocytes of those individuals segregating for the risk allele. Targeted genome editing of rs1421085 from C to T in risk allele carriers decreased *IRX3* and *IRX5* expression and restored thermogenesis in preadipocytes. T to C editing in preadipocytes from protective allele carriers resulted in elevated *IRX3* and *IRX5* expression. This suggests that expression of *IRX3* and *IRX5* is decreased in individuals segregating for the protective allele, resulting in increased thermogenesis. Notably, however, the demonstrated effect of *Irx3* on body mass in mice is due to alterations in energy expenditure without an effect on food intake, whereas, in humans, the primary effect of the *FTO* locus is conveyed by increased food intake (Speakman 2015).

The strength of the statistical signal associating the *FTO* locus to adiposity is sufficiently large to suggest that there may be multiple causative variants in the region, and/or that there may

be effects on multiple genes in many different tissues. Exploration of this locus should not, therefore, be restricted to any single gene in this region.

Our group has identified two SNPs in the first intron of *FTO* that modify transcriptional regulation of *FTO* and Retinitis pigmentosa GTPase regulator-interacting protein-1 like (*RPGRIP1L*), a gene ~100 bp 5' of *FTO* (Stratigopoulos, Padilla et al. 2008, Stratigopoulos, LeDuc et al. 2011, Stratigopoulos, Burnett et al. 2016). *In silico* analysis of the locus identified rs17817449 and rs8050136 as Cut-like homeobox 1 (CUX1) binding sites. CUX1 was originally described as a CCAAT-displacement protein and is expressed as a full-length isoform, p200, which acts as a transcriptional repressor. Proteolytic cleavage by cathepsin L generates a smaller isoform, p110, that can act as a transcriptional activator or repressor (Sansregret and Nepveu 2008). Chromatin immunoprecipitation of DNA from human fibroblasts followed by pyrosequencing identified differential binding of CUX1 to the rs8050136 allele, with elevated CUX1 binding to the protective C allele, and decreased binding to the risk (increased adiposity) A allele. *CUX1* knockdown decreased expression of both *FTO* and *RPGRIP1L* in human fibroblasts. Electrophoretic mobility shift assays (EMSAs) demonstrated that this preferential protective allele binding and transcriptional activation was specific to the proteolytically processed p110 isoform of CUX1 that acts as a transcriptional activator. The inhibitory CUX1 isoform p200 preferentially binds the risk allele, and a reporter assay utilizing the minimal promoter regions of both *FTO* and *RPGRIP1L* demonstrated that CUX1 p200 decreased expression of *FTO*, without affecting *RPGRIP1L* expression. These findings were confirmed in murine hypothalamic N41 cells where overexpression of Cux1 p110 elevated both *Fto* and *Rpgrip1l* expression and overexpression of Cux1 p200 decreased *Fto* expression alone (Stratigopoulos, Padilla et al. 2008, Stratigopoulos, LeDuc et al. 2011).

Another SNP in the *FTO* locus, rs1421085, was associated with obesity in individuals of African descent (Peters, North et al. 2013) and is also predicted to be a CUX1 binding site (Stratigopoulos, Burnett et al. 2016). EMSA studies of this SNP revealed that, similar to rs8050136, transcriptional inhibitor CUX1 p200 bound preferentially to the obesity-risk C allele of rs1421085 and transcriptional activator CUX1 p110 bound preferentially to the obesity-protective T allele. Promoter probing of this SNP by luciferase transcriptional assay demonstrated that CUX1 p110 activates transcription of *FTO* and *RPGRIP1L* while CUX1 p200 inhibits *FTO* expression alone. These findings suggest that individuals segregating for CUX1-related *FTO* risk alleles would exhibit decreased *FTO* and *RPGRIP1L* expression, whereas those with the protective alleles would display elevated *FTO* expression in tissues mediating the effects of these alleles on adiposity.

Fto and *Rpgrip1l* expression is decreased in the arcuate nucleus (ARC) and ventromedial nucleus (VMH) of the hypothalamus as well as mesenteric adipose tissue upon fasting in C57BL/6 mice, and in *Lep*$^{ob/ob}$ hyperphagic mice. Leptin treatment restored *Fto* and *Rpgrip1l* expression in the hypothalamus, suggesting that decreased expression of these genes is associated with an increased drive to eat (Gerken, Girard et al. 2007, Stratigopoulos, Padilla et al. 2008, Poritsanos, Lew et al. 2011, Stratigopoulos, LeDuc et al. 2011). Individuals segregating for *FTO* risk alleles may have decreased *FTO* and *RPGRIP1L* expression in the hypothalamus or other brain regions which may influence ingestive behaviors.

Investigating a role for Rpgrip1l in obesity

RPGRIP1L encodes a protein which localizes to the transition zone of the primary cilium, a sensory organelle present on almost all non-dividing cells (Vierkotten, Dildrop et al. 2007).

Rpgrip1l assembles two modules of the transition zone of the primary cilium, the Meckel Syndrome (MKS) module and the nephronophthisis (NPHP) module, and facilitates their assembly. These modules then function as a "ciliary gate" modulating the compartmentalization of ciliary proteins (Williams, Li et al. 2011, Jensen, Li et al. 2015), thereby regulating the signaling faculties of the primary cilium.

It has been previously reported that the expression patterns of *RPGRIP1L* and *FTO* in human tissues are quite similar (Frayling, Timpson et al. 2007), and work by our group, detailed above, has highlighted the impact of the obesity-associated *FTO* locus on these two vicinal genes. **Chapter 2** reports experiments demonstrating increased adiposity in mice systemically hypomorphic for Rpgrip1l. These mice are hyperphagic, in part, as a result of impaired leptin sensitivity in the hypothalamus. In **Chapter 3** we endeavored to determine if there are any "peripheral" cellular processes and metabolic signals contributing to this hyperphagia and adiposity. We knocked down *Rpgrip1l* in 3T3-L1 preadipocytes prior to adipogenesis and found increased proliferation and differentiation in these cells, suggesting that Rpgrip1l restrains adipogenesis. We did not find evidence of an effect on peripheral signals such as leptin or adiponectin due to *Rpgrip1l* knockdown that could contribute to the hyperphagia observed in Rpgrip1l hypomorphic animals. However, we anticipate that the increased adipogenesis we observed after *Rpgrip1l* knockdown could contribute to the establishment of increased adiposity during development.

Investigating a role for Fto in adiposity

FTO was identified as a member of the ALKB homolog family of non-heme dioxygenases (Fe(II)- and α-ketoglutarate dependent dioxygenases) by sequence analysis

(Gerken, Girard et al. 2007, Sanchez-Pulido and Andrade-Navarro 2007). FTO was also demonstrated to exhibit RNA N6-methyladenosine (m6A) and 3-methyluridine (m3U) demethylation activity *in vivo* (Jia, Fu et al. 2011). M6A constitutes an abundant mRNA modification and has been proposed to play a role in the regulation of mRNA stability, splicing and translation. M6A RNA modifications are increased around stop codons and 3'UTRs, as well as within long exons (Dominissini, Moshitch-Moshkovitz et al. 2012, Meyer, Saletore et al. 2012, Fu, Dominissini et al. 2014).

Classical gain- and loss-of-function experiments in rodents of have been used to assess the possible role of the Fto molecule *per se* in the regulation of body weight. Several *Fto* knockout mouse models have been generated (Fischer, Koch et al. 2009, Gao, Shin et al. 2010, McMurray, Church et al. 2013) which recapitulate many aspects of the human malformation syndrome caused by congenital absence of *FTO* (described below), including growth retardation and postnatal lethality. Some mice do survive into adulthood and exhibit decreased adiposity in adulthood and protection from high fat diet (HFD)-induced obesity (Fischer, Koch et al. 2009, Ronkainen, Huusko et al. 2015). A milder Fto loss-of-function model was described using an ENU mutagenized mouse with a dominant negative isoform of Fto (Church, Lee et al. 2009). Mice that were either heterozygous or homozygous for this mutation had decreased adiposity in adulthood with no change in lean body mass when fed chow and were also protected from HFD-induced obesity. Additionally, they did not exhibit any of the growth impairment or lethality phenotypes that characterize *Fto* knockout models. Congenital overexpression of *Fto* in mice results in a dose-dependent increase in body weight and adiposity, due to an increase in food intake (Church, Moir et al. 2010). Lean mass is also increased in these mice.

The decreases in adiposity seen in *Fto*$^{-/-}$ mice have been reported in adulthood (~20 weeks old) (Fischer, Koch et al. 2009, Ronkainen, Huusko et al. 2015). However, we (Stratigopoulos, Burnett et al. 2016) and others (Gao, Shin et al. 2010, McMurray, Church et al. 2013) have found that at earlier timepoints (≤15 weeks), adiposity is actually increased as a percentage of body weight in *Fto*$^{-/-}$ mice. And, consistent with reports by Fischer *et al*. and Ronkainen *et al*., we also observed dramatic loss of adipose tissue as these mice aged (G. Stratigopoulos, personal communication). We anticipate that this early increase in adiposity is due to the primary role of Fto in regulating energy balance centrally, and that the loss in adiposity seen later in life is due to a defect in adipose tissue structure and function. Mice lacking Fto display decreased overall adiposity late in life, exhibit smaller adipocytes and eventually lose almost all adipose tissue (Fischer, Koch et al. 2009). Conversely, *Fto*-overexpressing mice have larger adipocytes (Church, Moir et al. 2010). These phenotypes suggest a role for Fto in enabling adipose tissue expansion, possibly in the context of diet-induced obesity. Despite decreased body weight, mice with the dominant negative Fto mutation exhibit increased fasting glucose, triglyceride, cholesterol and high-density lipoprotein (HDL) levels, possibly reflecting a lipodystrophic metabolic profile (Church, Lee et al. 2009, Unger and Scherer 2010, Virtue and Vidal-Puig 2010).

FTO **mutations in humans cause an extreme malformation syndrome**

eQTL studies in humans, which thus far have failed to identify an effect of the FTO adiposity-associated genetic locus on *FTO* or *RPGRIP1L* expression, may be limited in assessing the timing and cell-specificity of the effects of the risk alleles; not all tissues and cell types can be probed in a sufficiently large number of subjects or at the appropriate developmental

timepoints to ascertain whether a relationship exists (Pastinen and Hudson 2004). Several groups have sequenced *FTO* in lean and obese individuals as an alternative approach to this question; to determine whether *FTO per se* is responsible for the association with obesity. Missense mutations have been identified with similar frequency in both groups, suggesting that any relationship between the *FTO* gene product *per se* and obesity is limited (Meyre, Proulx et al. 2010, Deliard, Panossian et al. 2013, Zheng, Hong et al. 2013). Among these mutations, however, only a small number of variants were located within the catalytic domain (R322Q and R316Q) or within the so-called "substrate recognition lid" (R96H). Heterozygous mutations in these domains were found in almost equal numbers in both lean (3) and obese (2) individuals in this western European cohort. The small number of individuals with these mutations makes it difficult to draw any conclusions regarding effects on adiposity. Similar findings regarding the number of missense mutations in *FTO* identified in lean and obese populations have also been described in Chinese (Zheng, Hong et al. 2013) and African American populations (Deliard, Panossian et al. 2013), but none of these mutations have been confirmed to be deleterious. Case studies of individuals homozygous for loss-of-function mutations in the catalytic domain have been described with severe developmental phenotypes and failure to thrive (Boissel, Reish et al. 2009, Daoud, Zhang et al. 2016, Rohena, Lawson et al. 2016). A large, consanguineous, Palestinian family included nine affected (R316Q) individuals all of whom displayed growth retardation as well as impaired brain development and function including postnatal microcephaly as well as hypertonicity and hydrocephalus, lissencephaly and/or seizures (Boissel, Reish et al. 2009). Additionally, cardiovascular defects such as ventricular septal and atrial ventricular defects, patent ductus arteriosus and hypertrophic cardiomyopathy were also observed. The R316Q mutation impairs the catalytic activity of FTO, as demonstrated by decreased

decarboxylation of 2-oxoglutarate to succinate. A Canadian patient of Tunisian origin homozygous for FTO S319F presented with similar phenotypes (overall growth retardation, brain malformation and cardiac defects) (Daoud, Zhang et al. 2016). This mutation also impairs FTO's catalytic activity, evident by impaired 2-oxoglutarate conversion to succinate as well as demethylation of 3-methylthymine. No obvious structural differences were apparent by circular dichroism spectroscopy of the recombinant mutant protein. Two other individuals have presented with homozygous mutations in the catalytic domain of FTO (R322Q). Both were offspring of a Yemeni consanguineous union and both exhibited failure to thrive and developmental delay as well as characteristic facial features (Rohena, Lawson et al. 2016). In most of these instances, however, the affected individuals died prior to 6 years of age, in some instances due to severe, chronic infections, indicating that FTO is a critical component of structural development as well as systemic homeostasis. Information about the parents of these individuals, which, in cases not involving *de novo* mutations would be obligate heterozygotes for these mutations, is limited. The death of individuals homozygous for mutations in *FTO* at such a young age, in addition to their extreme malformations, prevents observations of the effect of FTO on body weight throughout life.

Understanding the physiological and cellular functions of Fto in central regulation of metabolic homeostasis

Expression of *FTO* in both humans and rodent models is ubiquitous, with the highest levels consistently being observed in the brain (Frayling, Timpson et al. 2007, Gerken, Girard et al. 2007, Fredriksson, Hagglund et al. 2008, Stratigopoulos, Padilla et al. 2008, Boissel, Reish et al. 2009) where it is widely expressed but restricted to neurons (Fredriksson, Hagglund et al.

2008, McTaggart, Lee et al. 2011). Both transcript and protein levels are enriched in areas of the brain that regulate feeding behavior, specifically, the ARC, as well as the paraventricular (PVH) and dorsomedial nuclei (DMH) of the hypothalamus (Gerken, Girard et al. 2007, Fredriksson, Hagglund et al. 2008, Stratigopoulos, Padilla et al. 2008, Olszewski, Radomska et al. 2011, Poritsanos, Lew et al. 2011). Additionally, the ventral tegmental area (VTA) and substantia nigra (SN) of the midbrain express *Fto* (Hess, Hess et al. 2013). Notably, *Fto* is expressed in POMC-, TH- and oxytocin-producing neurons, which are key regulators of food intake (Fischer, Koch et al. 2009, Olszewski, Fredriksson et al. 2009, Tung, Ayuso et al. 2010, McTaggart, Lee et al. 2011, Olszewski, Fredriksson et al. 2011, Hess, Hess et al. 2013).

A neuron-specific *Fto* knockout mouse model, utilizing Nestin-Cre, recapitulates the growth retardation effects seen in the whole-body knockouts, with decreased body length and lower lean body mass; however, these mice exhibit an increase in food intake when normalized for lean body mass (Gao, Shin et al. 2010). This increase in food intake is consistent with evidence that hypothalamic *Fto* expression is decreased following fasting and restored upon leptin treatment, suggesting a drive to eat that is mediated by reduced neuronal levels of Fto (Stratigopoulos, LeDuc et al. 2011). In rats, overexpression of *Fto* in the ARC by stereotactic injection of AAV decreased food intake, and *Fto* knockdown by AAV shRNA increased food intake. Overexpression in the PVN also decreased food intake, whereas *Fto* knockdown in this region had no effect (Tung, Ayuso et al. 2010). These central effects of Fto are likely the primary regulators of body weight in $Fto^{-/-}$ animals, although Fto likely plays additional roles in peripheral tissues such as adipose, modifying metabolic homeostasis.

Identification of a primary role for Fto in adipocyte development

While *FTO* is primarily expressed in the brain, mRNA expression has also been observed in adipose tissue, suggesting a role in adipocyte function and/or development (Frayling, Timpson et al. 2007, Stratigopoulos, Padilla et al. 2008, Willer, Speliotes et al. 2009). A number of groups have attempted to relate adipose tissue *FTO* expression to adipose depot location, measures of body mass, as well as risk allele genotype (Kloting, Schleinitz et al. 2008, Wahlen, Sjolin et al. 2008, Grunnet, Nilsson et al. 2009, Zabena, Gonzalez-Sanchez et al. 2009, Lappalainen, Kolehmainen et al. 2010, Samaras, Botelho et al. 2010, Terra, Auguet et al. 2010, Susleyici-Duman, Zengin et al. 2011, Bravard, Veilleux et al. 2013). There is no clear consensus that *FTO* is expressed most highly in any particular adipose depot in humans (Kloting, Schleinitz et al. 2008, Wahlen, Sjolin et al. 2008, Zabena, Gonzalez-Sanchez et al. 2009, Samaras, Botelho et al. 2010, Terra, Auguet et al. 2010, Susleyici-Duman, Zengin et al. 2011, Bravard, Veilleux et al. 2013). Similarly, the relationship between adipose tissue *FTO* expression and BMI or obesity is unclear, with some groups finding an inverse correlation (Kloting, Schleinitz et al. 2008, Terra, Auguet et al. 2010, Susleyici-Duman, Zengin et al. 2011) while others report a direct relationship (Wahlen, Sjolin et al. 2008, Grunnet, Nilsson et al. 2009, Zabena, Gonzalez-Sanchez et al. 2009, Lappalainen, Kolehmainen et al. 2010, Tews, Fischer-Posovszky et al. 2011). Of the populations studied, none has exhibited a relationship between *FTO* expression in whole adipose tissue and *FTO* risk allele genotype (Kloting, Schleinitz et al. 2008, Wahlen, Sjolin et al. 2008, Grunnet, Nilsson et al. 2009, Zabena, Gonzalez-Sanchez et al. 2009, Lappalainen, Kolehmainen et al. 2010). The lack of a correlation of *FTO* expression with numbers of risk alleles, however, does not preclude a role for this gene in adipose tissue or adipocytes *per se*. Adipose depots consist of adipocytes in addition to immune cells (Weisberg, McCann et al. 2003, Wu, Ghosh et al. 2007,

Elgazar-Carmon, Rudich et al. 2008, Duffaut, Galitzky et al. 2009, Liu, Divoux et al. 2009, Winer, Winer et al. 2011, Wu, Molofsky et al. 2011), and adipocyte precursors (Ng, Poznanski et al. 1971). A correlation between *FTO* expression and risk allele genotype in any one of these cell types may be obscured in measurements of whole tissue. Additionally, any role for FTO during adipose tissue development may not be apparent in mature tissue of weight-stable individuals.

Another approach has been to analyze expression of *FTO* throughout the course of adipogenesis in isolated preadipocytes or commercially available preadipocyte cell lines. Studies performed in primary human preadipocytes and murine 3T3-L1 cells described in **Chapter 4** demonstrate an increase in the expression of *FTO* during the process of adipogenesis. Additionally, isolated murine adipocytes exhibit higher *Fto* expression (>3-fold) than the preadipocyte-containing stromal vascular fraction.

In order to more directly investigate Fto's role in adipocyte development and function, a number of groups, including our own, have performed gain- and loss-of-function studies in isolated primary preadipocytes and preadipocyte cell lines, described in **Chapter 4**. Such studies have provided convincing evidence for a role of Fto in adipocyte development and function. The loss of *Fto* impairs development of adipocytes *in vitro*, while *Fto* overexpression increases adipocyte differentiation (Zhao, Yang et al. 2014, Merkestein, Laber et al. 2015, Wang, Zhu et al. 2015, Zhang, Zhang et al. 2015, Chen, Zhou et al. 2016, Jiao, Zhang et al. 2016). We find that the loss of *Fto* decreases the number of preadipocytes that are able to undergo adipogenesis and limits the lipid handling and endocrine functions of mature adipocytes. In particular, Fto's m6A demethylase activity is necessary for adipogenesis, as overexpression of catalytically inactive Fto (H231A/D233A/ H307A) was incapable of rescuing the inhibitory effect of *Fto* knockdown, whereas wild type Fto did so (Zhao, Yang et al. 2014). Fto (R96Q), which cannot demethylate

m6A (Han, Niu et al. 2010), acts as a dominant negative, inhibiting adipogenesis to an extent similar to *Fto* knockdown (Zhang, Zhang et al. 2015). Knockdown of the m6A methyltransferase *Mettl3* in preadipocytes increased subsequent adipocyte differentiation, whereas *Mettl3* overexpression inhibited adipogenesis (Zhao, Yang et al. 2014). When *Mettl3* levels were manipulated in mature porcine adipocytes, overexpression inhibited triglyceride levels via decreased expression of Fatty Acid Synthase (*Fas*) (Wang, Zhu et al. 2015). Additionally, chemical regulators of m6A methylation affect adipogenesis similarly to endogenous regulators of m6A methylation. Cycloleucine, which inhibits m6A methyltransferase, increased triglyceride accumulation in porcine adipocytes and betaine, a methyl donor, decreased triglyceride accumulation, further highlighting the relationship between m6A status of the transcriptome and adipogenesis (Wang, Zhu et al. 2015), although it remains unclear what transcripts may be conveying these effects.

The mechanism(s) by which Fto affects adipogenesis remain incompletely characterized. Merkestein and colleagues have demonstrated an effect of Fto on mitotic clonal expansion early in the adipogenesis program in murine MEFs and primary preadipocytes, where the loss of *Fto* inhibits cell division, but does not affect cell survival. Conversely, *Fto* overexpression increases cell division (Merkestein, Laber et al. 2015). Chen *et al*. have confirmed this effect of Fto on cell division in porcine intramuscular preadipocytes (Chen, Zhou et al. 2016), and Jiao *et al*. found the same effect in 3T3-L1 cells (Jiao, Zhang et al. 2016). It remains unclear whether Fto's effect on adipogenesis is simply due to its regulation of total cell number or whether there is a developmental (maturational) component altered by Fto as well. Pparg activation by Rosiglitazone restores adipogenesis in 3T3-L1 cells expressing a dominant negative Fto (R96Q), providing evidence for a developmental role for Fto beyond regulating proliferation (Zhang,

Zhang et al. 2015). In studies reported in this thesis, I provide evidence that C/ebpβ and C/ebpδ-mediated transcription is disrupted in adipocytes following *Fto* knockdown, as is *Pparg2* expression, impairing critical components of the adipogenic program.

As systemic congenital *Fto*$^{-/-}$ models do develop adipose tissue, and at early time points even exhibit greater adiposity than wild type animals, it is clear that a loss of Fto does not induce lipodystrophy, as might be inferred from the *in vitro* studies described above. There must be other signals *in vivo* driving adipogenesis that have not been recapitulated in cell culture models. Alternatively, Fto may affect developmental adipogenesis and obesogenic adipogenesis observed in mature organisms distinctly. The upregulation in *FTO* expression in mature adipocytes differentiating *in vitro*, consistent with the dramatically elevated *FTO* expression in isolated mature adipocytes compared to isolated adipocyte precursors, suggests that FTO's role may be most critical in mature adipocytes, enabling them to carry out their critical lipogenic and lipolytic as well as endocrine functions. Knockdown of *Fto* in mature adipocytes inhibited expression of adipocyte effectors and endocrine signals, such as *Pparg2*, *Fapb4*, *Glut4*, *Lpl* and *Adipoq*.

Adipose tissue-intrinsic effects on body weight and metabolic homeostasis

The ability of animals to regulate food intake so that it matches energy expenditure is remarkable in both its accuracy and flexibility. Landmark studies in the middle of the 20th century demonstrated that the hypothalamus plays an important role in this coordination. Lesioning in the region of the VMH caused rats to become obese due to pronounced hyperphagia (Hetherington and Ranson 1940, Hervey 1959), whereas lesioning of the lateral hypothalamus (LHA) induced anorexia to the point of death (Anand and Brobeck 1951). Conversely, electrical

stimulation of the VMH caused aphagia and stimulation of the LHA induced hyperphagia (Hoebel and Teitelbaum 1962).

The afferent signals regulating these hypothalamic responses were unknown at the time and Gordon Kennedy proposed that the adipose tissue of these rats was producing a factor which reported its lipid content to the hypothalamus, a theory which has been termed the *Lipostatic Model* (Kennedy 1953). G. Romaine Hervey postulated that this signal must be a circulating factor. Rats parabiosed to each other, so that there is blood exchange between the two, with one of the pair lesioned in the VMH caused the non-lesioned rat to die of starvation. Hervey interpreted these results to suggest that there was a satiety factor being produced by the hyperphagic VMH-lesioned animal that circulated to the contralateral animal through the blood. The VMH-lesioned animal was no longer sensitive to this satiety factor, while the non-lesioned animal retained sensitivity and became hypophagic (Hervey 1959).

Doug Coleman in the 1960s characterized two interesting spontaneous mutations in mice at the Jackson Laboratory, *ob* (*obesity*) and *db* (*diabetes*). He noted the similarities between parabiosed *ob*/*ob* and *db*/*db* mice and the VMH-lesioned rats parabiosed to non-lesioned rats described by Hervey (Hervey 1959). The *ob*/*ob* mice became aphagic to the point of death after parabiosis with *db*/*db* mice, which remained obese, like the VMH-lesioned rats (Coleman 1973). Coleman concluded that the *ob*/*ob* mice were deficient in a satiety factor to which the *db*/*db* mice were insensitive. In the years since this discovery, relentless effort to characterize this satiety factor and its sensor resulted in the identification of the *ob* and *db* genes as leptin and the leptin receptor, respectively (Zhang, Proenca et al. 1994, Tartaglia, Dembski et al. 1995, Chen, Charlat et al. 1996, Chua, Chung et al. 1996).

Leptin, a circulating 16 kDa polypeptide, is as predicted by Kennedy, produced by adipocytes in proportion to their triglyceride content (Zhang, Proenca et al. 1994, Halaas, Gajiwala et al. 1995, Maffei, Halaas et al. 1995). It reports this information to the hypothalamus, where it modulates both energy intake and expenditure. Leptin stimulates the leptin receptor, expressed in pro-opiomelanocortin (POMC) expressing neurons of the ARC, signaling through the JAK2/STAT3 pathway. Leptin activates these glutamatergic neurons to express POMC, producing a cleavage product, α melanocyte-stimulating hormone (αMSH), which activates the melanocortin 4 receptor (MC4R) on second order neurons in the PVH. These second order neurons decrease food intake and increase energy expenditure. Leptin receptors are also expressed on neuropeptide Y (NPY) neurons in the ARC. NPY neurons are gamma-aminobutyric acid (GABA)ergic neurons that produce agouti related protein (AgRP), an inverse agonist of MC4R that inhibits MC4R neurons, leading to an orexigenic response. Leptin inhibits NPY neurons, relieving their inhibition of downstream MC4R neurons and blunting their orexigenic effect. These interactions form the basis of the central regulation of metabolism by the hypothalamus (reviewed in (Zhang and Leibel 2017)).

Other brain regions, including the nucleus of the solitary tract (NTS) of the brainstem and the lateral parabrachial nucleus (PBN) of the hindbrain convey the signals generated by neurons in the hypothalamus and are sensitive to leptin in their own regard. Additionally, leptin regulates the dopaminergic reward system that is overlaid on the feeding circuitry by acting on neurons of the ventral tegmental area (VTA) which innervates the nucleus of accumbens (NAc) of the ventral striatum and the central nucleus of the amygdala (CeA). Lepr expressing neurons in the aforementioned LHA innervate VTA neurons, regulating dopamine production (Morton, Meek et al. 2014, Zhang and Leibel 2017).

Leptin is a critical determinant of food intake and energy expenditure, and therefore, body weight overall. Circulating leptin concentrations are positively correlated with total body adiposity (Rosenbaum, Nicolson et al. 1996). Moreover, larger adipocytes produce more leptin than smaller adipocytes, to the extent that adipose tissue from an obese individual produces more leptin per gram than adipose tissue from a lean individual (Lonnqvist, Nordfors et al. 1997, Zhang and Leibel 2017). Variations in leptin production by adipose tissue could conceivably impact overall body weight (Kilpeläinen, Carli et al. 2016).

It is unclear if adipose tissue itself is a determinant (in a cell-autonomous manner) of depot size beyond its effect on leptin production and systemic energy balance. Lipectomy studies provide strong evidence that the total amount of adiposity among individuals is accurately defended. In rodents, total body adiposity was restored within a few months of lipectomy, with either partial regrowth of the lipectomized adipose depot and/or compensatory growth of other adipose depots (Schemmel, Mickelsen et al. 1971, Faust, Johnson et al. 1977, Larson and Anderson 1978, Faust, Johnson et al. 1984, Michel and Cabanac 1999). Regrowth of the lipectomized epididymal adipose depot was usually limited, whereas lipectomized subcutaneous adipose depots were generally fully restored. Liposuction in humans provokes equal regeneration and/or compensation of adipose tissue (Kral 1975, Hernandez, Kittelson et al. 2011, Seretis, Goulis et al. 2015). A model of inducible lipoatrophy, the FAT-ATTAC mouse, also demonstrated regeneration of adipose tissue after virtual ablation of adipose tissue (Pajvani, Trujillo et al. 2005). These results demonstrate a clearly established and zealously defended level of adiposity in mature individuals.

White adipocytes derive from mesenchymal stem cells that form adipocyte precursors before committing to becoming preadipocytes (Rosen and Spiegelman 2014). In humans,

adipose tissue grows by hyperphagia within the first year or two of life, followed by a reduction in adipocyte size. The number of adipocytes does not increase until children are approximately 10 years of age in lean individuals. In obese children, hyperplasia begins earlier than in non-obese children (Knittle, Timmers et al. 1979). Subcutaneous white adipose tissue is formed prenatally in humans and mice (Poissonnet, Burdi et al. 1984, Wang, Tao et al. 2013). The visceral gonadal depot develops shortly after birth (Siegel, Hildebolt et al. 2007, Spalding, Arner et al. 2008, De Lucia Rolfe, Modi et al. 2013, Wang, Tao et al. 2013, Kim, Lun et al. 2014). Peter Arner's group has pioneered the use of ^{14}C testing assays of genomic DNA to establish the time of generation and ages of adipocytes as well as neurons. Atmospheric ^{14}C was generated during aboveground nuclear bomb tests in the mid-20th century and levels quickly equilibrated around the globe. Since then, diffusion from the atmosphere has caused an exponential decrease in atmospheric ^{14}C levels. Incorporation of ^{14}C into vegetation, and the subsequent consumption of such plant matter, results in DNA labeling of human cells as they are born with a ^{14}C level matching the concentration found in the environment at that time (Spalding, Arner et al. 2008). This method was used to confirm that adult adipocyte number is established during childhood and adolescence, and remains relatively constant throughout adulthood. These cells turn over at a rate of approximately 10% per year, a rate that has been supported using a prospective measurement of cell birth rate utilizing stable isotope tracers (Guillermier, Fazeli et al. 2017).

Developmental sufficiency of energy balance has important consequences for subsequent adiposity. In humans, maternal nutrition has been well established to affect the adiposity of offspring later in life. This was described in studies performed on young men of the Dutch military who had been born following the Dutch "Winter Hunger" near the end of World War II. The Winter Hunger was a famine caused by the German blockade of food and fuel shipments to

the German-occupied region of the Netherlands in 1944-1945 (Ravelli, Stein et al. 1976). Infants that had been exposed to famine during the first half of gestation grew into adults with elevated obesity rates, whereas those exposed to famine in the second half of pregnancy, the time during which adipose tissue is developing, displayed decreased obesity later in life. Rodent models show similar effects. Rats reared in small litters received more milk from their mothers and when weaned, display greater food intake and grow to larger body weights than rats reared in large litters with limited access to milk (Widdowson and Kennedy 1962, Oscai and McGarr 1978). Findings by Spalding and colleagues using ^{14}C labeling of adipocytes, indicate that adiposity level is established during childhood and is set by adolescence. Obese adolescents had larger number of adipocytes than lean adolescents, a difference that was maintained throughout adulthood (Spalding, Arner et al. 2008). Differences in food intake are the most likely determinants of such a disparity, but modifiers of adipose tissue expansion may play a secondary role in determining the size and anatomic location of the adipose depots.

Lipectomy studies have not been conducted in early life and such studies could shed light on the role adipose tissue *per se* plays in setting the level of adiposity. Adipose transplant studies provide some evidence that adipocytes/adipose tissue have cell-autonomous capacities that could influence fat cell size and number. When adipose tissue of C57BL/6 mice segregating for the *Lep*^{ob} mutation and transplanted into lean C57BL/6 mice, the transplant adopted the phenotype of the lean host (smaller adipocytes), indicating that the primary determinant of adipocyte size is the cell's environment (Ashwell, Meade et al. 1977). However, adipose tissue from these mice differed only in their ability to produce leptin. Adipose tissue explants from 5-day old obesity-susceptible mice (NH strain) and obesity-resistant mice (DBA/2 strain) were transplanted onto the ears of NH-DBA/2 hybrid mice. After 90 days, the transplants from the obesity-susceptible

strains were significantly larger than the transplants from the resistant strains. This difference is independent of effects of food intake, as the different transplants grew in the same environment in a single mouse (Liebelt 1963). These data suggest that there are adipose/adipocyte determinants of adipose depot size beyond that determined by effects on food intake.

Adipose tissue development and expansion are carefully regulated. An informative model has been generated by the Scherer group, which allows for inducible labeling of mature adipocytes. Pulse-chase experiments using these "AdipoTracker" mice provide distinction between mature adipocytes labeled during the pulse, and the birth of nascent adipocytes during the chase, which are unlabeled. This model indicates that during diet-induced obesity, adipogenesis (hyperplasia) occurs following adipocyte hypertrophy. This hyperplasia occurs primarily in the epididymal adipose depot in male mice, and has been shown to be concurrent with the death of hypertrophic adipocytes (Strissel, Stancheva et al. 2007). Hyperplasia follows hypertrophic adipose tissue growth in human obesity as well (Hirsch and Batchelor 1976). Subsequent weight loss reduces the size but not the number of adipocytes present in adipose tissue (Salans, Knittle et al. 1968). Another elegant pulse-chase labeling model illustrated hyperplasia in epididymal adipose tissue of male mice fed a high fat diet. Mature adipocytes were pulse labeled green, with all remaining adipocyte precursor cells retaining a red label. A chase period of high fat diet feeding induced rapid proliferation of red labeled adipocyte precursor cells in the epididymal adipose depot (Jeffery, Church et al. 2015). Using this same model, it was demonstrated that in female mice, hyperplasia is primarily observed in the subcutaneous depot, and this subcutaneous hyperplasia is inhibited in ovariectomized animals. Furthermore, in male mice, estrogen injections induced adipocyte hyperplasia in the subcutaneous depot, indicating that the expansion of this depot is hormonally influenced (Jeffery,

Wing et al. 2016). In humans, these depot-specific differences are also apparent and well characterized in terms of their metabolic consequences. Males tend to accumulate adipose tissue viscerally and women subcutaneously in the abdominal, gluteal and femoral regions, colloquially identified as apple- or pear-shaped distributions (Vague 1956, Karastergiou, Smith et al. 2012, Palmer and Clegg 2015). This unequal distribution plays out physiologically to the detriment of men, where increased visceral adipose tissue leads to T2D, dyslipidemia, fatty liver and cardiovascular disease (Gesta, Tseng et al. 2007). There is little evidence that *FTO* risk alleles affect adipose tissue distribution with regard to waist-hip-ratio (WHR) a common measure signifying differential distribution of adipose tissue in the visceral or subcutaneous depots (Vasan, Fall et al. 2013). The *FTO* locus was associated with elevated adiposity to the same extent in both males and females, suggesting that sex hormones play a minimal role in the association (Frayling, Timpson et al. 2007). However, it remains possible that there are anatomic regional differences in the *FTO*-mediated effects on adipocyte lipid handling function, further contributing to the effects of *FTO* genotypes on insulin sensitivity and T2D risk.

Experiments described in **Chapter 3** detail that the loss of *Rpgrip1l* in 3T3-L1 preadipocytes increases the number of cells able to differentiate into mature adipocytes. *Rpgrip1l* knockdown in 3T3-L1 preadipocytes increases the number of cells that are able to undergo adipogenesis. We anticipate that Rpgrip1l, a key protein regulating the ability of primary cilia to accurately sense the extracellular environment, may regulate adipogenesis, and in turn, the development of the adipose depot, especially in the context of positive energy balance. Individuals segregating for *FTO* obesity risk alleles could exhibit hypomorphism of RPGRIP1L due to differential CUX1 binding and therefore enlarge the adipose anlagen, setting a permissive stage for elevated body weight throughout adulthood.

Effects of the *FTO* locus on metabolic status beyond fat mass *per se*

While the effect of the *FTO* locus on adiposity is clear, there is growing evidence to suggest that individuals segregating for the *FTO* risk allele are more susceptible to T2D, even when their level of adiposity is taken into account (Hertel, Johansson et al. 2011, Rees, Islam et al. 2011, Li, Kilpelainen et al. 2012, Fall, Hagg et al. 2013, Yang, Liu et al. 2017) consistent with the original association between the FTO locus and T2D (Frayling, Timpson et al. 2007). Both increased adiposity and impairments of adipose tissue expansion are associated with reduced insulin sensitivity and disordered glucose metabolism (Moitra, Mason et al. 1998, Pajvani, Trujillo et al. 2005, Arner and Spalding 2010). Stable isotope tracing in mice suggests that adipogenesis decreases as animals age, and is associated with impairments in insulin sensitivity (Kim, Lun et al. 2014). The same holds true as humans age (Guillermier, Fazeli et al. 2017). The "adipose tissue expandability hypothesis" as stated by Scherer, Vidal-Puig, and colleagues suggests that each individual has a genetically and environmentally determined limit on the amount that adipose tissue can expand. Beyond this, the organ cannot serve as a lipid sink and lipid accumulates ectopically, inducing lipotoxicity and leading to the disorders associated with the so-called "metabolic syndrome": diabetes, atherosclerosis, and fatty liver (Unger and Scherer 2010, Virtue and Vidal-Puig 2010).

We propose that Fto may play a role in adipose tissue expandability, which may contribute to the T2D risk associated with the *FTO* locus, independent of its association with BMI. The late-onset loss of adipose tissue in $Fto^{-/-}$ mice (Church, Lee et al. 2009, Fischer, Koch et al. 2009, Ronkainen, Huusko et al. 2015) suggests that there is a limit in these animals on the extent of adipose depot expansion, consistent with the decreased adipogenesis we have observed

in vitro following *Fto* knockdown in 3T3-L1 cells. This decrease in *FTO* expression may be partially recapitulated in adipose tissue of individuals segregating for *FTO* obesity risk alleles, where differential CUX1 binding may lead to the repression of *FTO* expression (Stratigopoulos, Padilla et al. 2008, Stratigopoulos, LeDuc et al. 2011, Stratigopoulos, Burnett et al. 2016).

Investigating genetic modifiers of adipocyte function with regard to leptin secretion

As previously mentioned, the amount of leptin produced per unit of body mass is somewhat variable among individuals. In an effort to better understand the molecular basis for such differences, we have identified loci associated with circulating leptin concentrations adjusted for BMI or body fat. Efforts detailed in **Chapter 5** were made to interrogate the causal genes at specific loci by molecular vetting of candidate genes using adipose tissue expression studies in mice. These candidates were knocked down in adipose tissue explants to evaluate their effects on leptin production and secretion. The *FTO* locus was initially identified as a regulator of circulating leptin concentrations but this effect was abrogated when leptin was normalized to BMI. This underscores our results from *in vitro* studies of *Fto* and *Rpgrip1l* knockdown, where leptin secretion was not changed when normalized to the number of adipocytes, suggesting that insomuch as FTO and RPGRIP1L are responsible for the adiposity effects of the *FTO* locus, they do not alter food intake by disrupting leptin production.

Peripheral roles for RPGRIP1L and FTO may partially account for the adiposity and metabolic dysfunction associated with the *FTO* locus.

Here we present evidence that Fto and Rpgrip1l play opposing roles in the differentiation of adipocytes. We suggest that RPGRIP1L plays a role early in the development of adipocytes, when primary cilia are displayed, responding to tissue environmental signals such as Shh, Wnts and Pdgfs to determine whether and how cells differentiate. We suggest that a decrease of *RPGRIP1L* expression, due to segregation of obesity-risk alleles within the *FTO* locus, could increase the cellular anlagen of adipose tissue mass during development by triggering proliferation and differentiation of adipocyte precursors, setting a permissive stage for elevated body weight throughout adulthood. The dramatic upregulation of *FTO* expression during adipogenesis suggests that FTO functions primarily in mature adipocytes. Our data indicate that FTO is required for the maintenance of the adipocyte state. In individuals segregating for *FTO* risk alleles, which decrease *FTO* expression, lipid handling function of the adipose tissue of mature individuals could be impaired via decreased glucose and fatty acid uptake and decreased lipogenesis. This diminished lipid storage capacity would predispose to ectopic fat deposition and the susceptibility to T2D associated with the *FTO* locus, independent of its effect on body weight.

Chapter 2 Hypomorphism for *RPGRIP1L*, a ciliary gene vicinal to the *FTO* locus, causes increased adiposity in mice.

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Author Contributions

J.F.M.C. and D.R.O. contributed equally to this work. G.S., J.F.M.C and R.L.L. conceived and designed the study. G.S. and R.L.L. wrote the manuscript. G.S. and J.F.M.C. performed the experiments and analyzed the data. C.A.L. gave technical support and conceptual advice.

The following has been abridged to emphasize the potentially adipocyte-specific role of Rpgrip1l from: Cell Metab 19(5): 767-779 (Stratigopoulos, Martin Carli et al. 2014).

http://www.cell.com/cell-metabolism/abstract/S1550-4131(14)00173-9.

Abstract

Common polymorphisms in the first intron of *FTO* are associated with increased body weight in adults. Previous studies have suggested that a CUX1-regulatory element within the implicated *FTO* region controls expression of *FTO* and the nearby ciliary gene, *RPGRIP1L*. Given the role of ciliary genes in energy homeostasis, we hypothesized that mice hypomorphic

for *Rpgrip1l* would display increased adiposity. We find that *Rpgrip1l*$^{+/-}$ mice are hyperphagic and fatter and display diminished suppression of food intake in response to leptin administration. In the hypothalamus of *Rpgrip1l*$^{+/-}$ mice, and in human fibroblasts with hypomorphic mutations in *RPGRIP1L*, the number of AcIII-positive cilia is diminished, accompanied by impaired convening of the leptin receptor to the vicinity of the cilium, and diminished pStat3 in response to leptin. These findings suggest that RPGRIP1L may be partly or exclusively responsible for the obesity susceptibility signal at the *FTO* locus.

Introduction

Common single-nucleotide Common polymorphisms in the first intron of *FTO* are associated with increased body weight in adults. Previous studies have suggested that a CUX1-regulatory element within the implicated *FTO* region controls expression of *FTO* and the nearby ciliary gene, *RPGRIP1L*. Given the role of ciliary genes in energy homeostasis, we hypothesized that mice hypomorphic for *Rpgrip1l* would display increased adiposity. We find that *Rpgrip1l*$^{+/-}$ mice are hyperphagic and fatter and display diminished suppression of food intake in response to leptin administration. In the hypothalamus of *Rpgrip1l*$^{+/-}$ mice, and in human fibroblasts with hypomorphic mutations in *RPGRIP1L*, the number of AcIII-positive cilia is diminished, accompanied by impaired convening of the leptin receptor to the vicinity of the cilium, and diminished pStat3 in response to leptin. These findings suggest that *RPGRIP1L* may be partly or exclusively responsible for the obesity susceptibility signal at the *FTO* locus.

polymorphisms (SNPs) within an ~47 Kb interval located in the first intron of the Fat Mass and Obesity-Associated (*FTO*) gene are associated with a dose-dependent body weight difference in humans (Frayling, Timpson et al. 2007, Scuteri, Sanna et al. 2007, Meyre, Delplanque et al. 2009). *FTO* encodes a nuclear protein that is orthologous to proteins of the AlkB family of deoxygenases and appears to function as a DNA or RNA demethylase (Han, Huang et al. 2010). In mice and rats, manipulations of *Fto* gene expression have produced changes in body weight, but not in a consistent direction (Stratigopoulos, Padilla et al. 2008, Church, Moir et al. 2010, Tung, Ayuso et al. 2010, Stratigopoulos, LeDuc et al. 2011, Wang, Yang et al. 2011), and humans heterozygous for null alleles of *FTO* show no consistent effects on body weight (Meyre, Proulx et al. 2010). Retinitis Pigmentosa GTPase Regulator-Interacting Protein-1 Like (*RPGRIP1L*) is located >100 bp 5′ in the opposite transcriptional orientation of

FTO (**Figure 1.1**), and encodes a protein localized at the transition zone of the primary cilium (Liu, Zhang et al. 2011, Williams, Li et al. 2011). Functional derangements of the primary cilium in Bardet-Biedl and Alström syndromes are associated with obesity (Baker and Beales 2009). We have previously demonstrated that fasting reduces hypothalamic expression of *Rpgrip1l* in mice which is restored by exogenous leptin administration (Stratigopoulos, LeDuc et al. 2011). The first intron of *FTO* contains a binding site for the CUX1 p110 isoform—capable of long-range regulation of transcription (Vadnais, Awan et al. 2013)—that increases promoter activity and expression of *RPGRIP1L in vitro*, and the obesity-risk allele of intronic SNP rs8050136 (Scuteri, Sanna et al. 2007) located in the CUX1 binding site lowers the affinity of the CUX1 p110 isoform for DNA (Stratigopoulos, LeDuc et al. 2011). The long isoform of the leptin receptor (Lepr-b) localizes to the vicinity of the primary cilium in cultured neuronal cells, and *in vitro* knockdown of *Rpgrip1l* reduces Lepr-b localization in the vicinity of the cilium and decreases leptin signaling (Stratigopoulos, LeDuc et al. 2011). Thus, it is possible that some or all of the association with obesity of the intron 1 SNPs of *FTO* is actually conveyed by reduced expression of *RPGRIP1L*. In mice, homozygosity for a null allele of *Rpgrip1l* is embryonically lethal (Vierkotten, Dildrop et al. 2007), but based on the allele-dosage effect of the *FTO* intronic SNPs on human adiposity, and our identification of a CUX1-regulatory element in *FTO* intron 1 that controls *Rpgrip1l* expression, we hypothesized that mice heterozygous for a null allele of *Rpgrip1l* would be fatter than wild-type animals. Here we show that these animals are indeed fatter, and report the details of their metabolic and behavioral phenotypes.

Results

Deletion of *Rpgrip1l* in mice, as well as biallelic *RPGRIP1L* mutations in Joubert patients, is linked with severe brain and craniofacial abnormalities (Arts, Doherty et al. 2007, Delous, Baala et al. 2007, Vierkotten, Dildrop et al. 2007). In addition, *Rpgrip1l*-deleted embryos display polydactyly (Vierkotten, Dildrop et al. 2007), which is also characteristic of mutations in cilia-related proteins related to human syndromic obesity (e.g., (Zaghloul and Katsanis 2009)). Nevertheless, *RPGRIP1L* mutations have not been directly linked to obesity in mice or humans. Deletion of *Rpgrip1l* is embryonic lethal in mice, and patients with homozygous or compound heterozygous loss-of-function mutations in *RPGRIP1L* show severe renal and skeletal developmental defects that may mask a role of RPGRIP1L in energy homeostasis. We obtained mice segregating for a *Rpgrip1l* allele in which LacZ is knocked into intron 4 (**Figure 2.1B**). LacZ is downstream of the Engrailed-2 exon-2 splice acceptor, resulting in a functionally null *Rpgrip1l* allele. Interbreeding of heterozygous mice failed to produce viable mice homozygous for the knockin allele, in agreement with the embryonic lethality previously reported (Vierkotten, Dildrop et al. 2007). *Rpgrip1l* mRNA and protein levels in the hypothalamus of heterozygous mice were decreased by ~50%, and *Rpgrip1l* mRNA was also decreased by ~50% in subcutaneous fat (**Figure 2.1C**), demonstrating that these mice are systemically heterozygous for *Rpgrip1l* (*Rpgrip1l*$^{+/-}$). Conversely, *Fto* mRNA remained unchanged in the hypothalamus and subcutaneous fat of *Rpgrip1l*$^{+/-}$ mice, and mRNA levels of nearby genes *Chd9*, *Rbl2*, *Aktip*, and *Irx3-6* in the hypothalami of *Rpgrip1l*$^{+/-}$ mice were equivalent to control mice. *Rpgrip1l*$^{+/-}$ C57BL6/J mice generated viable *Rpgrip1l*$^{+/-}$ mice at expected Mendelian ratios. By 10 weeks of age, male and female *Rpgrip1l*$^{+/-}$ mice fed regular chow (9% of calories as fat) ad libitum were ~10% heavier than +/+ animals (**Figure 2.2A** and

Figure 2.3A). Thereafter, we concentrated on male *Rpgrip1l*$^{+/-}$ mice. At 18 weeks of age, male *Rpgrip1l*$^{+/-}$ mice fed regular chow were ~12% heavier due to a ~40% difference in fat mass (**Figure 2.2B**–**2.2D**). After 1 week of ad libitum feeding of a high-fat diet (HFD; 65% of calories as fat), 19-week old male *Rpgrip1l*$^{+/-}$ mice were ~15% heavier (~4 g) and had ~80% more body fat (~2.5 g) and 6% more lean mass (~1.5 g) than *Rpgrip1l*$^{+/+}$ littermates (**Figure 2.2E**–**2.2G**). Energy intake was significantly increased (~23%) in *Rpgrip1l*$^{+/-}$ mice consuming the HFD during the 12 hr light phase compared with *Rpgrip1l*$^{+/+}$ littermates (Figure S1B). No statistically significant differences in energy expenditure or physical activity were detected between *Rpgrip1l*$^{+/-}$ and *Rpgrip1l*$^{+/+}$ mice when ingesting the HFD (**Figure 2.3C** and **2.3D**). After 18 weeks of ad libitum feeding of regular chow followed by 7 days of the HFD, male *Rpgrip1l*$^{+/-}$ mice had ~82% more subcutaneous fat, ~65% more perigonadal and perirenal fat, and ~50% more mesenteric fat than +/+ littermates (**Figure 2.2H**). The ~2-fold increase in serum leptin concentrations of male *Rpgrip1l*$^{+/-}$ mice correlated with their increased adiposity (**Figure 2.3E** and **2.3F**). No notable differences by genotype where observed in blood glucose concentrations of fed or fasted *Rpgrip1l*$^{+/-}$ and *Rpgrip1l*$^{+/+}$ mice (**Figure 2.3G**).

Rpgrip1l$^{+/-}$ male mice fed regular chow showed increased food intake as early as 4 weeks old (**Figure 2.2I**). At this age, both groups weighed the same, had the same amount of fat mass, and displayed the same activity levels, whereas energy expenditure was ~25% lower in *Rpgrip1l*$^{+/-}$ mice (**Figures 2.2J**, **2.3H**, and **2.3I**). By 5 weeks of age, after being switched to the HFD for 1 week, *Rpgrip1l*$^{+/-}$ mice ate more and displayed ~20% lower energy expenditure compared to the control group (**Figures 2.2K** and **2.3J**). At this age, body weight, body composition, and physical activity levels were indistinguishable between *Rpgrip1l*$^{+/-}$ and control mice (**Figures 2.2J** and **2.3K**).

Other research from our group has demonstrated that *Rpgrip1l*$^{+/-}$ mice were less sensitive to leptin injection, evident by decreased pStat3 staining, resulting in increased food intake (**Figure 2.2K**). Using skin-derived primary fibroblasts, exogenously expressed LEPR-b was shown to localize to the area surrounding the base of the primary cilium upon leptin treatment, and co-immunoprecipitated with RPGRIP1L. This LEPR trafficking did not occur in fibroblasts derived from patients with mutations in *RPGRIP1L*, and pSTAT3 signaling was decreased in these cells. The same Lepr-b mislocalization and impaired pStat3 signaling were observed in cells of the arcuate nucleus of the hypothalamus (ARC) in *Rpgrip1l*$^{+/-}$ mice.

Discussion

We have previously shown that the protective C allele of rs8050136, one of the *FTO* intronic SNPs associated with increased BMI (Scuteri, Sanna et al. 2007), favors binding of the short isoform of Cut-like homeobox 1 (CUX1 P110) that acts as a transcriptional activator of *RPGRIP1L* and *FTO* (Stratigopoulos, LeDuc et al. 2011). As in the case of mouse embryos lacking functional Rpgrip1l, loss of Fto in zebrafish results in multiple developmental defects that are due in part to ciliary structural defects accompanied by dysregulated β-catenin-dependent Wnt signaling (Vierkotten, Dildrop et al. 2007, Huang, Szymanska et al. 2011, Osborn, Roccasecca et al. 2014). CUX1 p110 controls the expression of genes that participate in the β-catenin-dependent Wnt pathway (Cadieux, Kedinger et al. 2009). Moreover, CUX1 P110 is a positive regulator of leptin signaling, whereas *FTO* knockdown results in diminished leptin signaling in vitro (Stratigopoulos, LeDuc et al. 2011). Therefore, it is conceivable that individuals segregating for the obesity-risk A allele at rs8050136 have lower hypothalamic *RPGRIP1L* and *FTO* expression levels due to lower DNA binding affinity of P110 at that cognate CUX1-binding site. Consequently, due to pre- and/or postnatal consequences of RPGRIP1L hypomorphism, these individuals would have diminished leptin signaling resulting in increased food intake and adiposity, thus providing a mechanism that links the allelic variation at *FTO* intron 1 with increased food intake and adiposity.

The possibility that SNPs associated with adiposity are embedded in regulatory elements of nearby genes that extend within the first intron of *FTO* is supported by the finding that the promoter of the homeobox gene *IRX3* interacts with the obesity-associated *FTO* interval through long-range DNA looping (Smemo, Tena et al. 2014). This recent report has suggested that the intronic region of *FTO* implicated in obesity interacts at a distance (of ~0.5 Mbp) with *IRX3* to

increase adiposity primarily by effects on energy expenditure. The susceptibility allele of *FTO* has been proposed to increase *IRX3* expression resulting in lower energy expenditure due to diminished activation of brown adipose tissue and limited "browning" of white adipose tissue (Smemo, Tena et al. 2014). However, in humans, the implicated *FTO* allele conveys its impact on adiposity, proportional to allele dose, primarily by effects on energy intake (Cecil, Tavendale et al. 2008, Speakman, Rance et al. 2008, Wardle, Carnell et al. 2008, den Hoed, Westerterp-Plantenga et al. 2009, Haupt, Thamer et al. 2009, Tanofsky-Kraff, Han et al. 2009, Wardle, Llewellyn et al. 2009, Rutters, Lemmens et al. 2010). *IRX3* expression was not affected in proportion to dosage of the *FTO* obesity risk allele in human brain, and an intermediate adiposity phenotype in mice segregating for a single *Irx3* null allele was not reported (Smemo, Tena et al. 2014). It is certainly possible, of course, that intronic *FTO* has effects on the expression of several genes (Jowett, Curran et al. 2010). Our data support a quantitatively important role for *RPGRIP1L* in conveying the orexigenic effects of intronic sequence variants in *FTO*.

We anticipate that there may be peripheral effects of Rpgrip1l that contribute to the adiposity phenotype described here. Effects on the adipogenic capacity of preadipocytes or the endocrine or lipid-storage functioning of mature adipocytes attributable to Rpgrip1l could conceivably influence the metabolic homeostasis and resultant adiposity of these animals. These possibilities are described in **Chapter 3** of this thesis.

Experimental Procedures

Mouse Strains

Mice heterozygous for the *Rpgrip1l* floxed-LacZ allele (*Rpgrip1l*$^{tm1a(EUCOMM)Wtsi}$; referred to here as *Rpgrip1l*$^{+/-}$) were derived from targeted embryonic stem cells on a C57BL/6NTac

background, purchased from the International Knockout Mouse Consortium (http://www.mousephenotype.org/martsearch_ikmc_project/), and maintained on a C57BL/6J background. Targeting was confirmed by the International Knockout Mouse Consortium. Mice were housed and handled according to Columbia University animal welfare guidelines. All procedures were approved by the Columbia University Institutional Animal Care and Use Committee (IACUC).

Diet and Dietary Treatment

Female *Rpgrip1l*$^{+/-}$ (n = 6) and control (*Rpgrip1l*$^{+/+}$) littermates (n = 5) were fed regular chow (9% Kcal from fat; Picolab 5058; Purina Mills, USA) until 12 weeks of age. Male *Rpgrip1l*$^{+/-}$ (n = 10) and control (*Rpgrip1l*$^{+/+}$) littermates (n = 6) were fed regular chow ad libitum until 18 weeks of age and switched to a HFD (65% of calories as fat; catalog number D12492; Open Source Diets) for 1 week. An additional five *Rpgrip1l*$^{+/-}$ and seven control (*Rpgrip1l*$^{+/+}$) male mice were fed regular chow ad libitum until 13 weeks of age and their body weights measured at 8, 12, and 13 weeks of age. A second experimental group of male *Rpgrip1l*$^{+/-}$ (n = 6) and control (*Rpgrip1l*$^{+/+}$) mice (n = 6) were fed regular chow ad libitum until 4 weeks of age and then switched to the HFD for 1 week. After 2 days of ad libitum ingestion of the HFD, mice were fasted overnight for 10 hr in order to assure that both groups were identical with regard to metabolic and feeding status. Food was reintroduced, and serial intraperitoneal saline injections were performed in all mice to accustom them to handling. Nine hours after refeeding, mice were administered leptin (4 μg/g). This higher than customary (2 μg/g) dose was used in order to accentuate distinctions in behavioral responses between +/+ and +/− animals. Room temperature was constant at 23°C on a 12 hr light/12 hr dark cycle (lights were turned off at 7 p.m.), and mice had ad libitum access to food and water.

Indirect Calorimetry

Mice were weighed and their body composition determined before individually caged in a LabMaster-CaloSys-Calorimetry System (TSE Systems). Calorimetry was performed on the 18-week-old *Rpgrip1l*$^{+/-}$ (n = 10) and control (*Rpgrip1l*$^{+/+}$; n = 6) experimental groups for ~72 hr while fed regular chow ad libitum, and then for another 3 days after the groups were switched to the HFD ad libitum. Calorimetry was also performed on the 3-week-old *Rpgrip1l*$^{+/-}$ (n = 6) and control (*Rpgrip1l*$^{+/+}$; n = 6) groups for 1 week while being fed regular chow ad libitum, and then for another week after the groups were switched to the HFD ad libitum. Forty hours after they were switched to the HFD, mice were fasted overnight for 10 hr and administered saline intraperitoneally at 2, 4, and 6 hr after a 10 hr overnight fast, followed by intraperitoneal leptin administration (4 μg/g) at the start of the dark cycle. During this period, food intake and energy expenditure were automatically recorded every 26 min by the calorimeter. Mice were allowed to acclimate during the first ~24 hr. Energy intake was calculated by multiplying cumulative food intake for a 24 hr period by the metabolizable energy present in the HFD (5.24 Kcal/g) and regular chow (3.56 Kcal/g).

Body Mass and Composition Measurements

Mice were weighed on an electronic scale. Body composition was determined by TD-NMR using a Minispec Analyst AD lean fat analyzer (Bruker Optics, Silberstreifen Germany). The TD-NMR was calibrated using mouse carcasses that ranged from 5 to 70 g in mass (Halldorsdottir et al., 2009).

Isolation of Total RNA and Nuclear Protein Extracts

Total RNA was extracted and DNase treated using the RNeasy Lipid Tissue Mini Kit (QIAGEN, Gaithersburg, MD) according to the manufacturer's instructions. Nuclear protein

extracts were isolated in the presence of Halt Protease and Phosphatase Inhibitor cocktail (Pierce, Rockford, IL) using the NE-PER Nuclear and Cytoplasmic Extraction Reagents (Pierce) according to the manufacturer's instructions. Total protein levels were measured using a Bradford Assay (Pierce).

cDNA Synthesis

cDNA synthesis from 1 μg of total RNA was performed utilizing the Transcriptor First Strand cDNA Synthesis Kit (Roche, Indianapolis, IN) with Oligo (dT)20 and Random Hexamer primer mix according to the manufacturer's instructions.

qPCR

For expression analysis, a set of male mice were kept singly caged, fed regular chow till 18 weeks of age, and thereafter fed either the HFD for 7 days (four *Rpgrip1l*$^{+/-}$ and four *Rpgrip1l*$^{+/+}$ males) or fed the HFD for 6 days and fasted for 24 hr (four *Rpgrip1l*$^{+/-}$ and four *Rpgrip1l*$^{+/+}$ males). Murine whole brains were placed on a dissection block, and the hypothalamus was dissected from the rostral border of the optic chiasm to the rostral border of the mammillary body. *Fto* and *Rpgrip1l* mRNA levels were measured by quantitative PCR as described elsewhere (Stratigopoulos et al., 2011). Similarly, quantitative PCR was performed to validate mRNA levels of Cdh9 (F-CATGGCTCAAACTCAGCTGCAA, R-GCAGCTTAGTGTATGTCCCAG), Rbl2 (F-GGCCTGGAGCAGCTACCGCAG, R-GATACATAGTTTCCTTCAGCGGT), Aktip (F-GCAGCGCAGTCAACAAACGGC, R-CAGAGCGGTAAGATGGCTGTAC), Irx3 (F-CAATGTGCTTTCATCAGTGTACG, R-GGATGCTGGACGCCAGGGCTGT), Irx5 (F-GCACGGATGAGCTCGGCCGCTC, R-GGGTGATATCCCAAGGAACCTG), and Irx6 (F-CTCAGTATGAGTTCAAGGATGCTG, R-

CTCCCTTGTGGCATTCTTCCTG) in the hypothalamus of *Rpgrip1l*$^{+/-}$ and *Rpgrip1l*$^{+/+}$ littermates. mRNA levels were normalized as described elsewhere (Stratigopoulos et al., 2011).

Antibodies

Anti-RPGRIP1L (1:200; human and mouse; provided by Daniel Doherty) was used for determining Rpgrip1l protein levels in *Rpgrip1l*$^{+/-}$ mice by western blotting. Phospho-Stat3 (Tyr705) antibody purchased from Cell Signaling Technology (1:1,000; catalog number 9131, Danvers, MA) was also utilized for western blotting. β-tubulin (1:2,000; catalog number 05-661; Millipore, Bedford MA) was used as an internal control.

Analysis

Data are expressed as mean ± SE. Statistical analysis was performed using Student's t test (StatView 5.0, SAS Institute Inc.). Levels of statistical significance were set at two-tailed p alpha < 0.05. All error bars represent SEM.

Acknowledgements

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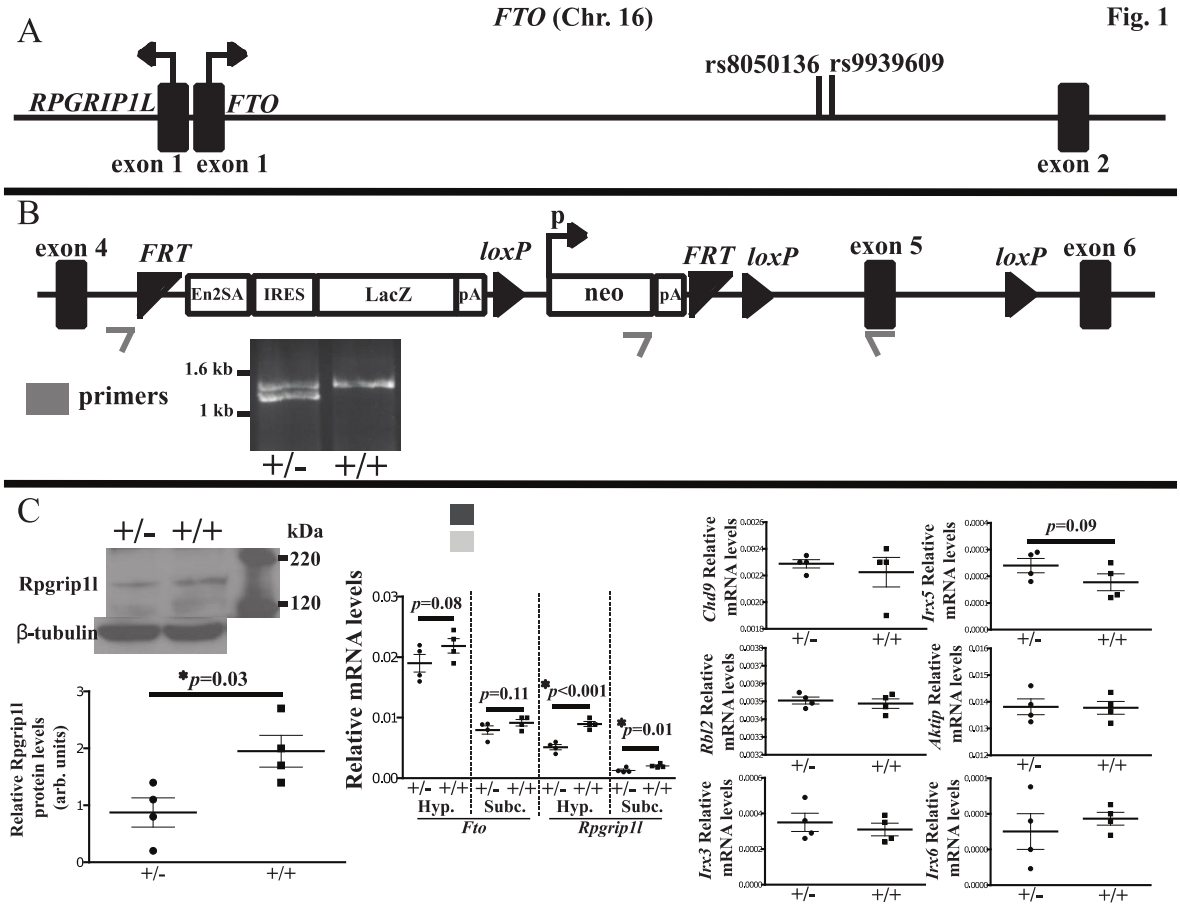
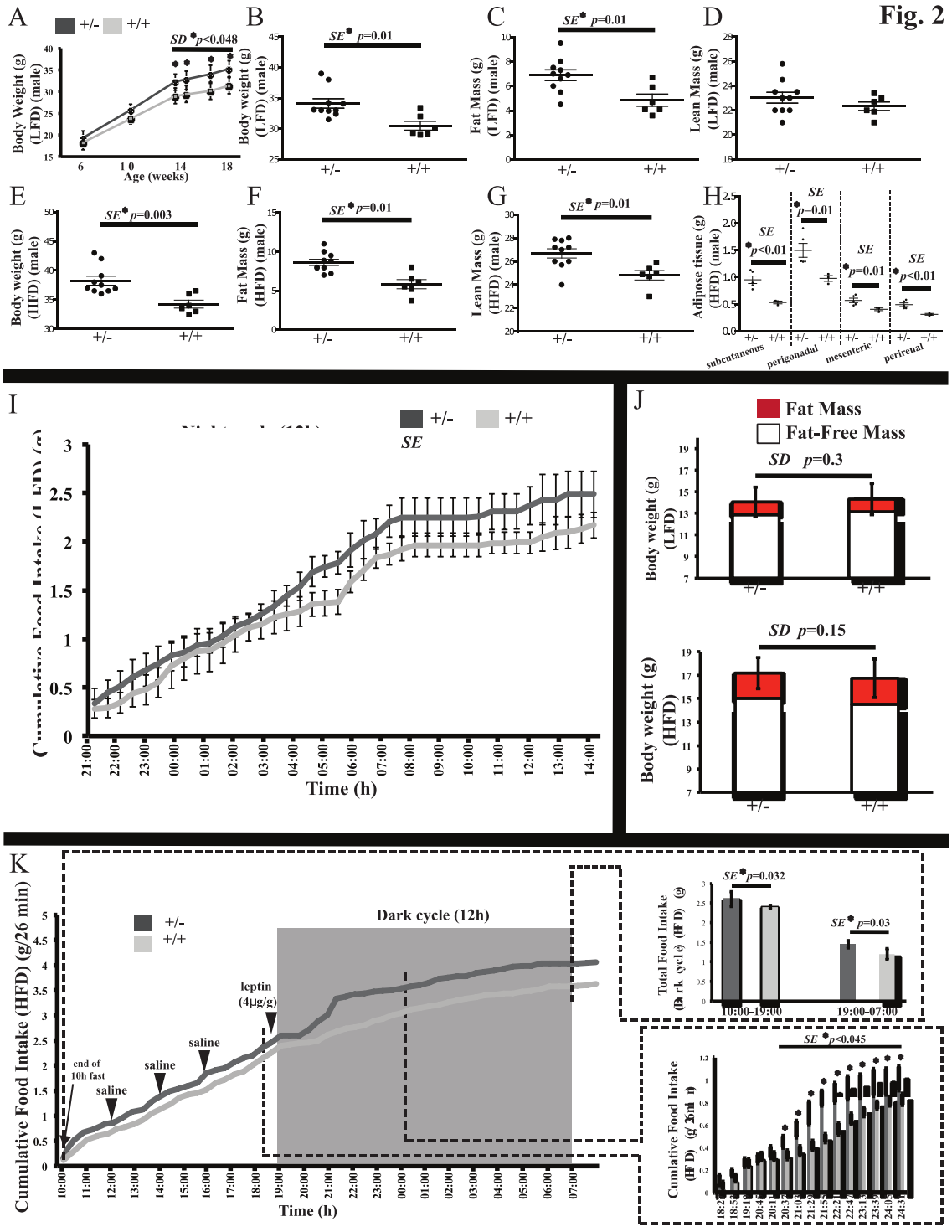
Figure 2.1 *Targeted Disruption of the Rpgrip1l Locus*



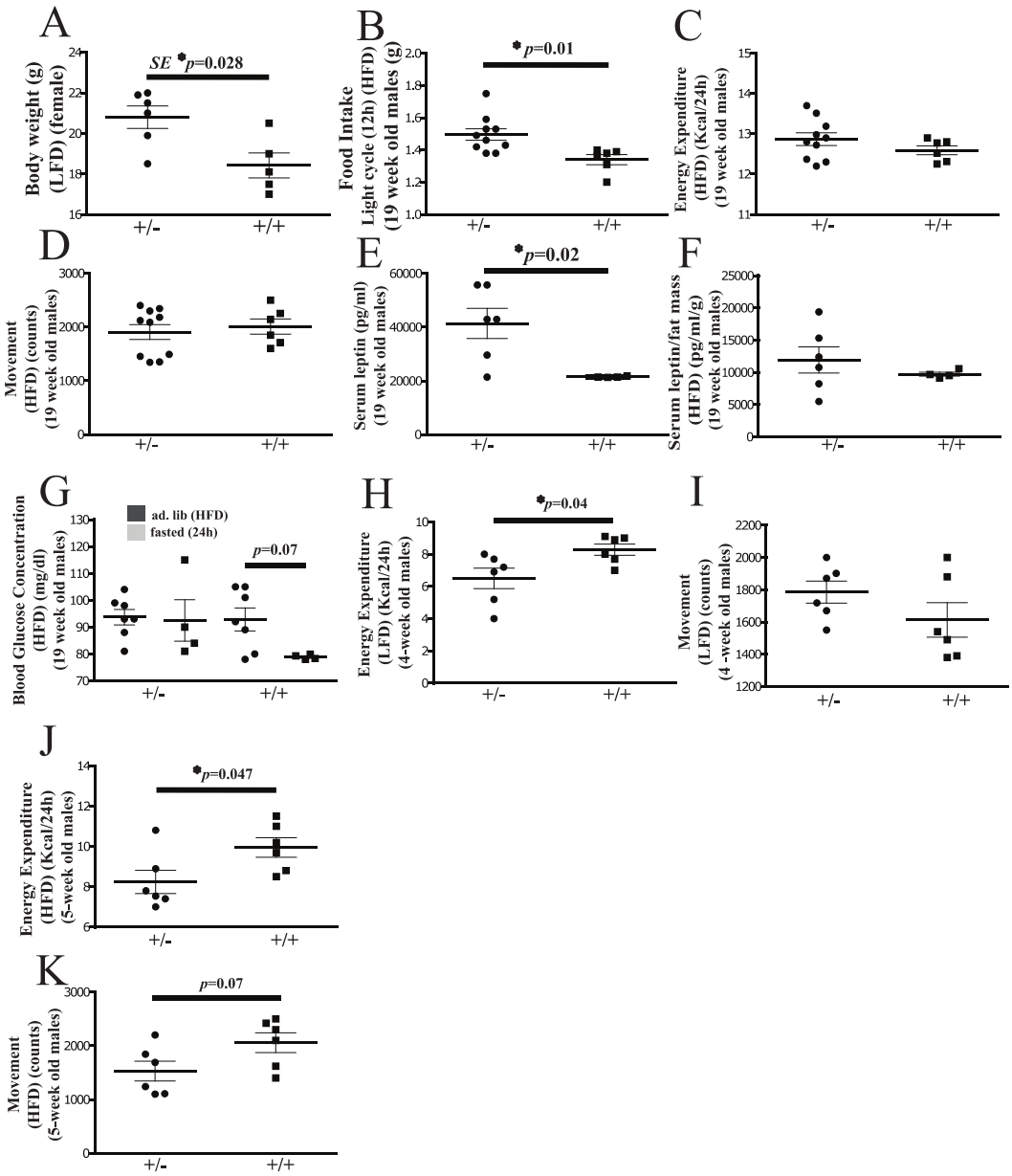
(**A**) Genomic organization of the human *FTO/RPGRIP1L* interval on chromosome 16. SNPs rs8050136 and rs9939609 are associated with increased BMI. Figure not drawn to scale. (**B**) Targeted disruption of the murine *Rpgrip1l* allele. A cassette comprised of the promoterless LacZ gene and the promoter-driven Neomycin resistance (neo) gene separated by a *loxP* site and flanked by FRT sites. The LacZ gene is flanked by the Engrailed-2 exon-2 splice acceptor (En2SA) and an internal ribosome entry site (IRES) designed to integrate the *LacZ* cDNA with *Rpgrip1l* exon 4 resulting in premature transcriptional termination of the *Rpgrip1l* mRNA. Figure not drawn to scale. (**C**) *Fto* and *Rpgrip1l* transcript levels and Rpgrip1l protein levels assessed by western blotting and RT-PCR, respectively, in the hypothalamus of $Rpgrip1l^{+/-}$ (+/−) and $Rpgrip1l^{+/+}$ (+/+) mice. *Fto* and *Rpgrip1l* mRNA levels were also assessed in subcutaneous (Subc) fat. Transcript levels of nearby genes *Chd9*, *Rbl2*, *Aktip*, *Irx3*, *Irx5*, and *Irx6* were unaltered in the the hypothalamus of $Rpgrip1l^{+/-}$ and $Rpgrip1l^{+/+}$ mice. Error bars represent SEM.

Figure 2.2 *Positive Energy Balance in Mice Heterozygous for a Null Allele of Rpgrip1l*



(**A**) Total body weight increased in male *Rpgrip1l*$^{+/-}$ versus *Rpgrip1l*$^{+/+}$ mice fed regular chow (LFD) over an 18-week period. Total body weight (**B** and **E**), fat mass (**C** and **F**), and lean mass (**D** and **G**) increased in 18-week-old male *Rpgrip1l*$^{+/-}$ versus *Rpgrip1l*$^{+/+}$mice fed LFD and a high-fat diet (HFD = 65% of calories as fat) respectively. (**H**) Increase in weight of various adipose tissue depots of *Rpgrip1l*$^{+/-}$ compared with *Rpgrip1l*$^{+/+}$ mice after being fed regular chow for 18 weeks followed by the HFD for 7 days. Food intake (**I** and **K**) and body composition (**J**) of male *Rpgrip1l*$^{+/-}$ and *Rpgrip1l*$^{+/+}$ mice fed LFD up to 4 weeks of age and subsequently fed HFD for 1 week. Error bars represent SEM.

Figure 2.3 *Energy expenditure, food intake, blood glucose and serum leptin concentrations in* *Rpgrip1l*$^{+/-}$ *and Rpgrip1l*$^{+/+}$ *mice*



(**A**) Total body weight is higher in 10-week old female *Rpgrip1l*$^{+/-}$ compared with *Rpgrip1l*$^{+/+}$ mice fed regular chow (LFD). (**B**) Energy intake during the light cycle of 19-week old male *Rpgrip1l*$^{+/-}$ and *Rpgrip1l*$^{+/+}$ mice having been fed a high fat diet (HFD = 65% of calories as fat) for 3 days. Energy expenditure (**C**) and total movement counts (**D**) of 19-week old male *Rpgrip1l*$^{+/-}$ and *Rpgrip1l*$^{+/+}$ mice having been fed HFD for 3 days. Serum leptin concentration (**E**) and serum leptin adjusted for fat mass (**F**) of *Rpgrip1l*$^{+/-}$ and *Rpgrip1l*$^{+/+}$ mice having been fed the high-fat diet for 7 days before they were sacrificed. (**G**) Blood glucose concentrations of 19-week old male mice fed HFD for 7 days, or fed HFD for 6 days and subsequently fasted for 24h. Energy expenditure (**H**, **J**) and total movement counts (**I**, **K**) of 4-week old and 5-week old male *Rpgrip1l*$^{+/-}$ and *Rpgrip1l*$^{+/+}$ mice fed regular chow (LFD) or HFD for one week, respectively. Error bars represent SEM.

Chapter 3 The role of *Rpgrip1l* (a component of the primary cilium) in adipocyte development

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Author Contributions

J.F.M.C and R.L.L. conceived and designed the study and wrote the manuscript. J.F.M.C. performed the experiments and analyzed the data. C.A.L., Y.Z. and G.S. gave technical support and conceptual advice.

Abstract

Single nucleotide polymorphisms (SNPs) in the first intron of *FTO* have been strongly associated with a modest increase in adiposity and a 1.2 and 1.3-fold risk of overweight or obesity, respectively. We have previously demonstrated that two of these SNPs, rs8050136 and rs1421085, are binding sites for the transcriptional regulator CUX1, with the activating isoform (p110) preferentially binding to the protective alleles of these SNPs, thereby increasing expression of both *FTO* and *RPGRIP1L*. *FTO* encodes an RNA m6A demethylase; *RPGRIP1L* encodes a protein critical to the formation and function of the primary cilium. *Rpgrip1l*$^{+/-}$ mice exhibit increased adiposity, in part due to hyperphagia. Here, we describe effects of Rpgrip1l in adipocytes which may contribute to the adiposity phenotype observed in these animals, and possibly humans. Loss of *Rpgrip1l* in 3T3-L1 preadipocytes increased the number of cells capable of differentiating into mature adipocytes. Knockout of *Rpgrip1l* in mature adipocytes

(using Adipoq-Cre) did not increase adiposity in mice fed chow or high fat diet. Neither did we observe any effects of *Rpgrip1l* knockdown in mature 3T3-L1 adipocytes. Thus, to the extent that Rpgrip1l affects cell-autonomous adipose tissue function, it may do so by effects conveyed in preadipocytes, a cell type in which the primary cilium may have functional importance. We propose that decreased *RPGRIP1L* expression in preadipocytes in humans segregating for obesity risk alleles may increase the potential storage capacity of adipose tissue for the consequences of positive energy balance.

Introduction

The strongest genetic signal associated with increased adiposity in large groups of humans is located within the first intron of *FTO* (16q12.2). Originally identified by GWAS in 2007, SNPs within a 47-kb region of linkage disequilibrium are associated with a ~0.36 kg/m^2 increase in BMI (per risk allele), corresponding to a ~1.2 kg increase in body weight in adults (Dina, Meyre et al. 2007, Frayling, Timpson et al. 2007, Scuteri, Sanna et al. 2007). The genetic mechanism(s) for this association are unclear; the primary proximal phenotypic effect is increased food intake (Berentzen, Kring et al. 2008, Cecil, Tavendale et al. 2008, Speakman, Rance et al. 2008, Timpson, Emmett et al. 2008). Studies of loss- and gain-of-function manipulations of *Fto per se* in mice have found conflicting results on adiposity (Church, Lee et al. 2009, Fischer, Koch et al. 2009, Gao, Shin et al. 2010, McMurray, Church et al. 2013). The effects of the obesity-associated intronic variants on vicinal genes have implicated *IRX3* and *IRX5*, by mechanisms that include decreased energy expenditure due to impaired "browning" of adipose tissue, which drives thermogenesis due to oxidative phosphorylation uncoupling (Smemo, Tena et al. 2014, Claussnitzer, Dankel et al. 2015).

RPGRIP1L, the transcriptional start for which is ~100 bases 5' of *FTO*, in the opposite orientation, has been studied by us for its roles in hypothalamic control of energy homeostasis. We have previously demonstrated that risk alleles rs8050136 and rs1421085 within intron 1 of *FTO* are binding sites for isoforms of the transcription factor CUX1 and thereby regulate the expression of both *FTO* and *RPGRIP1L* (Stratigopoulos, Padilla et al. 2008, Stratigopoulos, LeDuc et al. 2011, Stratigopoulos, Burnett et al. 2016). Rpgrip1l is a component of the transition zone of the primary cilium (Vierkotten, Dildrop et al. 2007). The primary cilium, functioning as a cellular antenna, plays a key role in coordinating a wide range of signal transduction systems,

including those related to body weight homeostasis (Mariman, Vink et al. 2016, Vaisse, Reiter et al. 2017). Mice heterozygous for a null *Rpgrip1l* allele are hyperphagic and obese (Stratigopoulos, Martin Carli et al. 2014, Stratigopoulos, Burnett et al. 2016). Rpgrip1l functions to congregate and assemble two modules of the transition zone of the primary cilium: the Meckel Syndrome (MKS) module and the nephronophthisis (NPHP) module. The transition zone then functions as a "ciliary gate" regulating the compartmentalization of ciliary proteins, restraining them to the ciliary axoneme (Williams, Li et al. 2011, Jensen, Li et al. 2015).

The increased adiposity we observed in *Rpgrip1l*$^{+/-}$ mice was associated with reduced hypothalamic sensitivity to leptin effects on Stat3 signaling and food intake. We hypothesized that there might be additional primary effects of *Rpgrip1l* hypomorphism affecting adipogenesis and/or the regulation of adipocyte secretion of molecules such as leptin. We found that loss of *Rpgrip1l* in 3T3-L1 preadipocytes enhanced the differentiation capacity of these cells. *In vivo*, this effect could contribute to the obesity of *Rpgrip1l*$^{+/-}$ mice by increasing the compliant capacity of fat depots. We generated adipose-specific *Rpgrip1l* knockout mice to assess this possibility.

Methods

Cell culture and gene knockdown

3T3-L1 preadipocytes were purchased from ATCC and maintained in culture without achieving confluence. Growth medium consisted of DMEM (with 25mM glucose, GlutaMAX™, and sodium pyruvate) supplemented with 10% newborn calf serum (both Thermo Fisher). To amplify the magnitude of the increased adipogenesis seen in the *Rpgrip1l* knockdown condition, we limited differentiation by treating cells with differentiation media containing DMEM plus insulin (from Bovine Pancreas 1 μg/ml), dexamethasone (0.25 μM) and isobutylmethylxanthine (IBMX; 0.5mM; all Sigma-Aldrich) supplemented with 10% fetal bovine serum (Thermo Fisher) as soon as cells reached confluence. Two days later, differentiation media was replaced with maintenance media (DMEM containing only 1 μg/ml insulin and 10% FBS). Maintenance media was replaced every two to three days and cells filled with lipid by days 6-12; at which point we refer to them as "mature adipocytes". We observed lot-to-lot variability in the differentiation capacity of 3T3-L1 cells, with a diminished, but still appreciable, increase in adipogenesis after *Rpgrip1l* knockdown in better differentiating lots. Experiments reported here were performed on a lot with moderate differentiation potential to more effectively interrogate the phenotype.

Using either Lipofectamine 2000 or 3000 (Thermo Fisher) 3T3-L1 cells were transfected prior to differentiation with a mix of 3 Stealth siRNAs (Thermo Fisher) targeted either to *Rpgrip1l* or non-targeted controls. Mature 3T3-L1 adipocytes were electroporated with the same non-targeted control or *Rpgrip1l* siRNAs using a Bio-Rad GenePulser II (Jiang, Zhou et al. 2003, Okada, Mori et al. 2003).

RNA isolation and quantification

RNA was isolated using the RNeasy lipid tissue kit including DNase treatment of columns (Qiagen) and reverse transcribed using Transcriptor First Strand cDNA Synthesis Kit (Roche) using both OligoDT and random hexamer primers. Quantitative PCR (qPCR) was performed on a Lightcycler 480 using SYBR Green I Master (Roche). Expression was determined by the Lightcycler 480 software (Roche) using the 2nd derivative max calculation based on a standard curve. Transcript levels were normalized to *36B4* when comparing a single cell type or tissue, or to the mean of *Actb*, *Gapdh* and *Ppia* when multiple tissues or cell types were compared. Primers were designed using Primer 3: http://bioinfo.ut.ee/primer3/ to span exon-exon junctions.

36B4 (*Rplp0*): F:ACCTCCTTCTTCCAGGCTTTGG, R:CGAAGGAGAAGGGGGAGATGTT;

ActB: F:CGGGCTGTATTCCCCTCCAT, R:GGGCCTCGTCACCCACATAG;

Gapdh: F:CTGGAGAAACCTGCCAAGTATGATG, R:GAGACAACCTGGTCCTCAGTGTAGC;

Ppia: F:CTTCGAGCTGTTTGCAGACAAAGTT, R:GGAGGAACCCTTATAGCCAAATCCT;

Rpgrip1l ex4F:CCAAACAGCAGCTCCAAGTCCAGGG,

ex5R:GAGCGTGGGTTGTACAGTTTCTGCTTC;

Rpgrip1l: ex21F:TGCCTCAGGAAGTGTGGCTTC,

ex22R:TCATCACTGTCTGAAGCTGATCTGTC;

Glut4: F:AGCTGTGCTTGGCTCCCTTC, R:CCCAGCCACGTTGCATTGTA;

Fabp4: F:TTGGTCACCATCCGGTCAGA, R:TCCACCACCAGCTTGTCACC;

Plin1: F:GTGTACAGGGTGCCAGCAA, R:CTCTGCAGGCCAACTCATTG;

Lep: F:CGAGGAATCGTTCTGCAAATCC, R:GCCAGGTTAAGTGCAGCTATCACA;

Adipoq: F:CAGGCCGTGATGGCAGAGAT, R:GTGGCCCTTCAGCTCCTGTC;

Pparg2: F:TTTGAAAGAAGCGGTGAACCA, R:CGAAGTTGGTGGGCCAGAAT

Cebpa: F:GCCATGCCGGGAGAACTCTA, R:GGGCTCTGGAGGTGACTGCT

Lpl: F:CCACAGCAGCAAGACCTTCG, R:TACAGGGCGGCCACAAGTTT

Shh: F:GGAGCAGACCGGCTGATGAC, R:TCGGTCACTCGCAGCTTCAC;

Ptch1: F:ATCTCGAGACCAACGTGGAG, R:GCCTCTTCTCCTATCTTCTGACG;

Gata2: F:ATCTCGACTCGCAGGGCAAC, R:AGTGTGGTCGGCACATCTGG;

Gata3: F:CAGGGCTACGGTGCAGAGGT, R:GGTGGCTGCTCAGGGCTTT;

Gli1: F:TGGTACCATGAGCCCTTCTT, R:GTGGTACACAGGGCTGGACT;

Gli3: F:CCTTACCGTGGGACTGTGTT, R:ATGGAAGGCAGGGAAAAGAT;

Lef1: F:CCAATATGAACAGCGACCCG, R:GGAGTTGACATCTGACGGGA;

Ctnnb1: F:CTTTTCCCAGTCCTTCACGC, R:ATGCCCTCATCTAGCGTCTC

Protein quantification

Leptin and Adiponectin ELISAs were performed using kits from R&D Systems. Cell culture supernatants from mature adipocytes were removed 3 days after media had been changed. Samples (from 12 well plates) were diluted 1:20 for Leptin ELISA and 1:1,000 for Adiponectin ELISA.

Lipid staining

Oil Red O staining was performed on mature adipocytes fixed with 4% paraformaldehyde for 10 minutes. A stock solution of 25mg Oil Red O (Sigma) in 50ml isopropanol was freshly mixed with water at a 3:2 ORO:H_2O ratio. This solution was filtered and cells were stained for 30 mins. Following a PBS wash, cells were imaged and Oil Red O was extracted in 300µl of isopropanol with 4% IGEPAL CA-630 for 5 mins (Sigma). 100µl were taken to measure absorbance at 490nm (Church, Berry et al. 2014). The Nile Red staining protocol was adapted

from Smyth *et al* (Smyth and Wharton 1992). In brief, mature adipocytes were trypsinized, fixed for 10 minutes with 4% paraformaldehyde and stained with Nile Red at a final concentration of 500ng/µl (Thermo-Fisher). We added equal volumes of 8.0-12.9 µm counting beads ($2.5x10^4$ per condition; Spherotech) prior to acquisition by flow cytometry using the PE-TR channel to measure Nile Red fluorescence.

Mice

Mice were housed and handled according to guidelines established by Columbia University and protocols were approved by Columbia University Institutional Animal Care and Use Committee (IACUC). Room temperature was maintained at 23°C ±1°C with a 12 hr light (beginning at 7AM)/12 hr dark cycle. Animals had *ad libitum* access to food and water. Mice were fed either regular chow (Purina #5058) or high fat diet (HFD; 60% of calories from fat; Research Diets #D12492i) as indicated. Mice were weighed weekly and body weight composition was obtained by TD-NMR with a Minispec Analyst AD analyzer (Bruker Optics).

Mice segregating for a *Rpgrip1l* "knockout-first" allele containing an IRES:lacZ trapping cassette and a floxed promoter-driven neo cassette (*Rpgrip1l*$^{tm1a(EUCOMM)Wtsi}$; International Knockout Mouse Consortium; (Skarnes, Rosen et al. 2011, Stratigopoulos, Martin Carli et al. 2014)) were crossed with Flp deleter mice (C57BL/6-Tg(CAG-Flpe)2Arte; Taconic) segregating for the Flp recombinase allele under the control of the *β-actin* promoter to excise the lacZ and neo cassettes and restore gene activity (*Rpgrip1l*$^{tm1c(EUCOMM)Wtsi}$ referred to here as "*Rpgrip1l*$^{fl/fl}$") while retaining LoxP sites surrounding exon 5. *Rpgrip1l*$^{fl/fl}$ mice were then crossed to Adipoq-Cre mice (B6;FVB-Tg(Adipoq-cre)1Evdr/J; Jackson) to generate *Rpgrip1l*$^{tm1d(EUCOMM)Wtsi}$ (*Rpgrip1l*$^{+/fl}$;Adipoq-Cre) mice and their offspring were intercrossed to obtain *Rpgrip1l*$^{fl/fl}$;Adipoq-Cre, *Rpgrip1l*$^{+/fl}$;Adipoq-Cre and *Rpgrip1l*$^{+/+}$;Adipoq-Cre mice. Exon 5 of

Rpgrip1l was deleted in only the adipose tissue of *Rpgrip1l*$^{fl/fl}$;Adipoq-Cre mice, causing a frameshift mutation triggering nonsense mediated decay of any remaining transcript (Skarnes, Rosen et al. 2011). We also generated *Rpgrip1l*$^{fl/fl}$;Adipoq-Cre and *Rpgrip1l*$^{fl/fl}$ littermates by crossing *Rpgrip1l*$^{fl/fl}$;Adipoq-Cre$^{Cre/+}$ and *Rpgrip1l*$^{fl/fl}$ mice.

We compared either *Rpgrip1l*$^{fl/fl}$;Adipoq-Cre or *Rpgrip1l*$^{fl/+}$;Adipoq-Cre mice to *Rpgrip1l*$^{+/+}$;Adipoq-Cre mice for the chow feeding study. *Rpgrip1l*$^{fl/fl}$;Adipoq-Cre mice were compared to *Rpgrip1l*$^{fl/fl}$ mice without Adipoq-Cre in the HFD feeding study. Genotyping was performed as in (Stratigopoulos, Martin Carli et al. 2014) without the internal *Neo* primer. Using *Rpgrip1l* forward primer (GTCTTGGACAGATCTTGGTCCAGTCTCC; upstream of the LoxP site and exon 5) and reverse primer (GTGGGTTGTACAGTTTCTGCTTCATCCAC; located within exon 5), we generated a band of 1407 bases indicating the wild type *Rpgrip1l* allele. A band approximately 200 bases larger was detected in mice with the intervening LoxP site.

Results

Rpgrip1l knockdown enhances adipogenesis

To assess possible cell autonomous effects of systemic inactivation of *Rpgrip1l* on adipose tissue development, we knocked down *Rpgrip1l* (**Fig. 3.1A**) in 3T3-L1 preadipocytes using siRNA. Knockdown persisted throughout differentiation, and in mature adipocytes was still decreased by 60%. Lipid accumulation per well was increased in the *Rpgrip1l*-knockdown condition (Oil Red O; **Fig. 3.1B**&**C**). A partial differentiation protocol was employed, limiting the time that 3T3-L1 cells spent at confluence prior to differentiation, increased the magnitude of the difference between the *Rpgrip1l* and control knockdown. To identify whether this increase in lipid accumulation was due to increased efficiency of differentiation, the amount of lipid per cell, or both, we performed flow cytometry on Nile Red stained cells. The observed increase in lipid was attributable mainly to an increase in the percentage of cells containing lipid (**Fig. 3.1D**), although there was a trend toward an increase in the amount of lipid per adipocyte (**Fig. 3.1E**; p=0.12) in *Rpgrip1l* knockdown cells. Additionally, we observed an increase in the total number of live cells following *Rpgrip1l* knockdown, suggesting that Rpgrip1l limits cell survival and/or proliferation (**Fig. 3.1F**). *Rpgrip1l*-knockdown adipocytes exhibited increased expression of genes involved in glucose uptake (*Glut4*), lipid uptake (*Fabp4*, *Lpl*) and storage (*Plin1*), as well as adipokines (*Lep*, *Adipoq*). We also observed increased expression of developmental regulators of adipogenesis, *Pparg2* & *Cebpa* (**Fig. 3.1G**), indicating that differentiation itself as well as functional regulators of adipocyte biology are enhanced by *Rpgrip1l* knockdown.

Rpgrip1l has been previously reported to modulate the sonic hedgehog (Shh) signaling pathway (Vierkotten, Dildrop et al. 2007), a known inhibitor of adipogenesis (Spinella-Jaegle, Rawadi et al. 2001, Suh, Gao et al. 2006, Fontaine, Cousin et al. 2008). We observed decreased

expression of Shh target genes in *Rpgrip1l*-knockdown mature adipocytes; however, we also observed decreased expression of *Shh* itself (**Fig. 3.1H**), indicating that Rpgrip1l may not directly regulate Shh signaling. The effect of *Rpgrip1l* knockdown on Shh targets may be a consequence of increased adipogenesis *per se* with a secondary decrease in *Shh* expression (James, Leucht et al. 2010). Consistent with this inference is the fact that after *Rpgrip1l* knockdown, neither *Shh* or Shh target genes are changed in either confluent preadipocytes (**Fig. 3.1I**) or preadipocytes that have been treated with differentiation media for two days (**Fig. 3.1J**).

To examine whether suppression of *Rpgrip1l* was sufficient to induce adipogenesis on its own, we knocked down *Rpgrip1l* expression in preconfluent 3T3-L1 preadipocytes and measured adipogenesis in cells 9 days after they reached confluence. *Rpgrip1l* knockdown persisted (-26%; **Fig. 3.2A**) for this length of time, but we did not see any evidence of adipogenesis in the si*Rpgrip1l* condition (Oil Red O: **Fig. 3.2B&C**; Nile Red staining: **Fig. 3.2D**; qPCR of adipocyte markers: **Fig. 3.2E**) in the absence of chemically induced differentiation.

Rpgrip1l knockdown does not affect production or secretion by adipocytes of factors regulating food intake

Rpgrip1l hypomorphic mice are obese, due primarily to hyperphagia resulting from impaired hypothalamic leptin sensitivity (Stratigopoulos, Martin Carli et al. 2014). We measured leptin (**Fig. 3.3A**) and adiponectin (**Fig. 3.3B**) secretion (by ELISA) by mature 3T3-L1 adipocytes transfected with anti-*Rpgrip1l* siRNA prior to differentiation. Although we observed increased secretion of both of these hormones in the *Rpgrip1l* knockdown condition, when normalized to the number of adipocytes in each condition (measured by Nile Red staining detected by flow cytometry; **Fig. 3.3C**), there was no change in the production of these hormones per cell (**Fig. 3.3D**&**E**).

Adipocyte-specific knockout of *Rpgrip1l* does not alter body weight in mice.

To determine whether the increased lipid filling capacity observed in *Rpgrip1l*-knockdown adipocytes contributed to the adiposity phenotype previously observed in systemic, congenital *Rpgrip1l* heterozygotes, we generated adipocyte-specific *Rpgrip1l* knockout mice (*Rpgrip1l*$^{fl/fl}$;Adipoq-Cre) which we compared to either to *Rpgrip1l*$^{+/+}$;Adipoq-Cre or *Rpgrip1l*$^{fl/fl}$ controls.

Rpgrip1l transcript was decreased in subcutaneous and perigonadal adipose depots of male (**Fig. 3.4A**; SCAT: -69%, PGAT: -67%) *Rpgrip1l*$^{fl/fl}$;Adipoq-Cre mice compared to *Rpgrip1l*$^{fl/fl}$ controls. Additionally, *Rpgrip1l* expression was decreased or trended toward a decrease in perirenal and mesenteric adipose depots, respectively. *Rpgrip1l* expression was decreased similarly in female mice (data not shown). Adipose tissue contains diverse cell types, so we separated the stromal vascular and adipocyte fractions in male SCAT. We detected *Cre* transcript only in the adipocyte fraction (**Fig. 3.4B**); and this fraction had a greater decrease in *Rpgrip1l* expression (SCAT: -83%, PGAT: -63%) than whole tissue (SCAT: -67%, PGAT: -28%) (**Fig. 3.4C**). There was no change in *Rpgrip1l* expression in the preadipocyte-containing stromal vascular fraction.

To ensure that the decrease in expression of *Rpgrip1l* was due to excision of the *Rpgrip1l* allele, rather than to an off-target effect of the Adipoq-Cre allele, we quantified expression of *Rpgrip1l* in PGAT of male *Rpgrip1l*$^{fl/fl}$;Adipoq-Cre, *Rpgrip1l*$^{fl/+}$;Adipoq-Cre and *Rpgrip1l*$^{+/+}$;Adipoq-Cre mice and found that the homozygous and heterozygous *Rpgrip1l*-floxed animals exhibited dose-response related decreases in *Rpgrip1l* expression (**Fig. 3.5A**). Additionally, as the aforementioned measurements utilized primers detecting the exon 4-5 junction of *Rpgrip1l*, which is disrupted by Cre recombinase activity, we verified that levels of

Rpgrip1l transcript downstream of the Cre recombination site were also decreased in *Rpgrip1l*$^{fl/fl}$;Adipoq-Cre animals compared to *Rpgrip1l*$^{+/+}$;Adipoq-Cre controls (**Fig. 3.5B**). The Adipoq-Cre mouse used to generate this loss-of-function allele of *Rpgrip1l* is highly specific to adipose tissue (Eguchi, Wang et al. 2011, Lee, Russell et al. 2013), consistent with our observation that in non-adipose tissues there was no difference in *Rpgrip1l* expression in *Rpgrip1l*$^{fl/fl}$;Adipoq-Cre animals compared with *Rpgrip1l*$^{+/+}$;Adipoq-Cre controls (**Fig. 3.5C**).

We monitored the body weight of these animals until they reached 23 weeks of age. During this period, the *Rpgrip1l*$^{fl/fl}$;Adipoq-Cre and *Rpgrip1l*$^{+/fl}$;Adipoq-Cre mice did not exhibit any difference in weight gain compared to their *Rpgrip1l*$^{+/+}$;Adipoq-Cre littermates (**Fig. 3.6A**). At 12 weeks, we analyzed body composition, and did not detect any differences in either fat mass (**Fig. 3.6B**) or lean mass (**Fig. 3.6C**). We considered the possibility that *Rpgrip1l* may not affect body weight unless the animals are over-fed. We fed another cohort of mice a high fat (60% calories as fat) diet for 17 weeks. We did not observe any differences in body weight, body composition, plasma glucose or leptin concentrations between R*pgrip1l*$^{fl/fl}$;Adipoq-Cre mice and R*pgrip1l*$^{fl/fl}$ controls (**Fig. 3.7A-E**). The regression lines relating circulating leptin concentration to fat mass were not different (**Fig. 3.7F**), indicating that hypomorphism for R*pgrip1l* in adipocytes did not affect production/release of leptin.

Knockdown of *Rpgrip1l* in mature 3T3-L1 adipocytes does not alter lipid content.

As the Adipoq-Cre driver is not turned on until after well after preadipocytes have started to undergo adipogenesis, it is possible that the *Rpgrip1l*$^{fl/fl}$;Adipoq-Cre model did not alter *Rpgrip1l* expression until after its physiologically relevant effects on adipogenesis had occurred. We attempted to model this possibility by knocking down (-54%) *Rpgrip1l* in fully mature adipocytes (**Fig. 3.8A**) and observed no difference in lipid content quantified by Oil Red O (**Fig.**

3.8B&**C**) or Nile Red staining (**Fig. 3.8D**&**E**) in the *Rpgrip1l*-knockdown condition. Additionally, we did not see any differences in mRNA expression of adipocyte developmental (*Pparg* and *Cebpa*) and functional genes (*Glut4*, *Fabp4*, *Plin1*, *Lpl*, *Lep*, *Adipoq*) after *Rpgrip1l* knockdown in mature adipocytes (**Fig. 3.8F**).

Discussion

We have shown previously that *RPGRIP1L* expression is decreased in iPSC-derived neurons of humans segregating for obesity-risk alleles in intron 1 of *FTO* (Stratigopoulos, Burnett et al. 2016). Two of the obesity-associated SNPs (rs8050136 and rs1421085) in this interval are binding sites for the transcriptional regulator CUX1. Specifically, the risk alleles of these two SNPs exhibit decreased *in vitro* binding of an activating isoform of CUX1 (p110), decreasing expression of both *RPGRIP1L* and *FTO* by their minimal promoters in N2a murine neuroblastoma cells (Stratigopoulos, LeDuc et al. 2011, Stratigopoulos, Burnett et al. 2016). *Rpgrip1l*$^{+/-}$ mice are hyperphagic and obese (Stratigopoulos, Martin Carli et al. 2014). Here, we tested whether a decrease in *Rpgrip1l* expression had any direct, cell-autonomous effect on adipocyte development and function that might contribute to the increased adiposity of *Rpgrip1l*$^{+/-}$ mice.

We identified an effect of *Rpgrip1l* knockdown on adipogenesis in 3T3-L1 cells, to enhance differentiation and proliferation via a combination of reciprocal effects on expression of pro- and anti-adipogenic factors. However, congenital adipocyte-specific loss of *Rpgrip1l* did not alter body weight or adiposity in mice. The absence of an effect under these latter circumstances suggests that the function of *Rpgrip1l* in adipogenesis precedes the expression of the Adipoq-Cre recombinase in this model (discussed further below). Consistent with this possibility, knockdown of *Rpgrip1l* in mature adipocytes *in vitro* does not cause detectable change in lipid content or expression of adipocyte developmental or functional genes.

The primary cilium, developing from the mother centriole in quiescent cells, is an organelle present in almost all mammalian cell types which coordinates cellular development and environmental interactions (Satir and Christensen 2007). The role of RPGRIP1L in the assembly

of the primary cilium's transition zone qualifies it to regulate differentiation of a wide variety of cell types (Williams, Li et al. 2011, Jensen, Li et al. 2015) as the transition zone participates in compartmentalization of specific signal transduction proteins such as Shh, Wnt and Pdgfrα signaling components within the ciliary membrane (Goetz and Anderson 2010, Li, Jensen et al. 2016). Mice homozygous for a null allele of *Rpgrip1l* die embryonically with craniofacial and neural tube defects as well as deranged left-right asymmetry (Vierkotten, Dildrop et al. 2007). Humans homozygous for null alleles of *RPGRIP1L* present with either Joubert syndrome, a developmental disorder with mid-hindbrain malformation, as well as renal and hepatic defects (Arts, Doherty et al. 2007), or Meckel syndrome, an autosomal recessive lethal syndrome also characterized by CNS malformations that typically include occipital encephalocele (Delous, Baala et al. 2007). Obesity has not been described in patients with these syndromes. However, the resultant severe systemic consequences are sufficiently severe to mask effects on adiposity. It may be informative to evaluate adiposity in the obligate heterozygous parents of these individuals.

"Ciliopathies" comprise a genetically heterogeneous class of disorders characterized by mutations in genes encoding over 40 proteins that contribute to the establishment and/or function of the primary cilium (Mariman, Vink et al. 2016). Phenotypes shared among members of this class of disorders include renal and retinal dysfunction as well as developmental malformations such as polydactyly and defects of the CNS. Some ciliopathies (i.e. Bardet-Biedl Syndrome and Alström Syndrome) also present with co-morbid obesity (Mariman, Vink et al. 2016, Vaisse, Reiter et al. 2017). Hyperphagia – presumably of hypothalamic origin – is a primary contributor to the obesity of these individuals (Sherafat-Kazemzadeh, Ivey et al. 2013); primary

developmental changes in adipose tissue development and/or function have also been implicated (Marion, Mockel et al. 2012).

Bardet-Biedl Syndrome (BBS) is one such ciliopathy, caused by mutations in at least 20 genes, several of which encode proteins that form a complex known as the BBSome (Nachury, Loktev et al. 2007). This complex regulates intraflagellar trafficking of ciliary structural components as well as receptors conveying signals mediating food intake (e.g. Sstr3 and Mchr1) (Berbari, Lewis et al. 2008, Jin, White et al. 2010). Loss of BBSome components decreases leptin sensitivity in mice, possibly due to impaired leptin receptor (LepR) trafficking to the trans-Golgi network (Rahmouni, Fath et al. 2008, Seo, Guo et al. 2009). We have shown that congenital absence of *Rpgrip1l* also causes decreased leptin sensitivity, due to impaired LepR trafficking in relation to the primary cilium (Stratigopoulos, Martin Carli et al. 2014). Knockout of *Rpgrip1l* in *POMC*- or *LepR*-expressing neurons partially phenocopies the increased body weight seen in the *Rpgrip1l* hypomorph (Stratigopoulos, Burnett et al. 2016). However, it is possible that decreased leptin sensitivity in mice null for *Bbs* genes is secondary to their increased adiposity, as leptin concentrations and signaling are normal in *Bbs4*$^{-/-}$ mice prior to the onset of obesity (Berbari, Pasek et al. 2013).

Knockdown of BBSome proteins increases adipogenesis in human mesenchymal stem cells and murine 3T3-F442A cells (Marion, Mockel et al. 2012, Aksanov, Green et al. 2014), suggesting that disruption of the BBSome could convey primary effects on adipogenesis in addition to CNS-mediated effects on food intake. Adipose tissue develops in a temporally coordinated manner, with the subcutaneous depot developing prenatally in both humans and mice, followed by the visceral gonadal depot developing shortly after birth (Poissonnet, Burdi et

al. 1984, Siegel, Hildebolt et al. 2007, Spalding, Arner et al. 2008, De Lucia Rolfe, Modi et al. 2013, Wang, Tao et al. 2013, Kim, Lun et al. 2014).

White adipocytes derive from mesenchymal stem cells that form adipocyte precursors before committing to becoming preadipocytes (Rosen and Spiegelman 2014). Adipocyte number is established during childhood and adolescence, and adipose tissue expands initially by adipocyte filling (hypertrophy) followed by recruitment, proliferation and differentiation of preadipocytes (hyperplasia). Adipose tissue cellularity then remains relatively constant throughout adulthood. Obese individuals have larger numbers of adipocytes from an early age (Knittle, Timmers et al. 1979, Spalding, Arner et al. 2008). In mice, diet-induced expansion of adipose tissue occurs initially by hypertrophy, followed by hyperplasia. In mice, adipocyte cell death is linked with hyperplasia of new adipocytes, and in males, this occurs primarily in the epididymal depot (Strissel, Stancheva et al. 2007, Wang, Tao et al. 2013, Kim, Lun et al. 2014). Diet-induced adipose tissue expansion in humans also begins with hypertrophy followed by hyperplasia (Hirsch and Batchelor 1976). Increased age is associated with diminished capacity for adipocyte turnover and this reduction in adipose tissue storage capacity can lead, in turn, to insulin resistance due to ectopic lipid deposition in liver and muscle (Heilbronn, Smith et al. 2004, Kim, Lun et al. 2014, Guillermier, Fazeli et al. 2017). Developmental and “obesogenic” adipogenesis, or that induced by positive energy balance, appear to be regulated distinctly with the latter dependent on the PI3K-Akt2 pathway (Jeffery, Church et al. 2015). It is unclear whether Rpgrip1l differentially inhibits these two pathways.

Are there cell autonomous contributions by adipocytes to the establishment of body weight? or does CNS regulation of energy intake or energy expenditure entirely drive adiposity? Liebelt *et al*. described transplantation of adipose tissue from 5-day old "obese" (NH strain) or

"lean" (DBA/2 strain) onto the ears of hybrid NH-DBA/2 mice. After 90 days, the NH grafts were significantly larger (~30%) than the DBA/2 grafts (Liebelt 1963), indicating that there may be adipose-intrinsic control of adipose depot size. It is reasonable to expect that adipose tissue-cell autonomous limitations on hyperplasia might contribute to an upper limit of somatic fat gain. In *Akt2*$^{-/-}$;*Lep*$^{ob/ob}$ mice in which adipocyte progenitor proliferation is impaired, fat gain is limited (Jeffery, Church et al. 2015). Conversely, *Lep*$^{ob/ob}$ mice which constitutively overexpress adiponectin develop massive obesity with extreme adipose hyperplasia compared to their *Lep*$^{ob/ob}$ littermates (Kim, van de Wall et al. 2007), suggesting that promoting the development of increased numbers of adipocytes can facilitate the storage of body fat. None of these experiments has focused explicitly on the developmental biology of the preadipocyte, however, which would determine whether differentiation potential of the preadipocyte itself is responsible for the consequences of these genetic manipulation on body fat. These critical questions highlight the necessity of access to genetic tools with which preadipocyte development can be manipulated. Preadipocyte genes such as *Pparg2*, *Pdgfra* and *Prx1* are attractive candidates for promoters as Cre drivers; however, none of these genes are specific to preadipocytes, confounding efforts to isolate preadipocyte-autonomous roles in the control of body fat stores (Berry, Jeffery et al. 2014, Krueger, Costa et al. 2014).

The molecular physiology governing how restrictive or expansive adipose tissue geometry affects overall adiposity is not completely understood. The brain is the ultimate regulator of food intake, but adipose tissue that expands readily might prevent inhibitory feedback, leading to facilitated accommodation of increased food intake (Ravussin, Leibel et al. 2014, Zhang and Leibel 2017). If the *Rpgrip1l* knockdown-induced effect on adipogenesis does contribute to the whole-body adiposity of *Rpgrip1*$^{+/-}$ mice, we hypothesized that one way that

adipocytes *per se* might influence food intake is via dysregulated production of appetite-regulating factors, such as leptin and/or adiponectin. We observed increased expression of both of these hormones in 3T3-L1 adipocytes after *Rpgrip1l* knockdown prior to differentiation, but when normalized to the increased number of adipocytes observed, there was no change in production on a per cell basis.

As a driver of Hh signaling (Vierkotten, Dildrop et al. 2007), Rpgrip1l might be expected to limit adipogenesis, as Hh has previously been demonstrated to inhibit adipogenesis in both rodent and human mesenchymal cells (Spinella-Jaegle, Rawadi et al. 2001, Suh, Gao et al. 2006, Fontaine, Cousin et al. 2008). Hedgehog signaling maintains 3T3-L1 cells in a preadipocyte state, and is decreased as they differentiate into adipocytes (Suh, Gao et al. 2006). Inhibition of this pathway alone, however, is not sufficient to induce adipogenesis in 3T3-L1 preadipocytes (Cousin, Dani et al. 2006), a finding consistent with what we have observed in *Rpgrip1l*-KD preadipocytes cultured without differentiation factors. Additionally, while we did observe decreased expression of Shh targets in mature adipocytes after *Rpgrip1l* knockdown, *Shh* itself was decreased. The reduction of Shh target gene expression may be a secondary effect of the increased adipogenesis seen in these cells, as Shh production is decreased after adipogenesis (James, Leucht et al. 2010).

Other signaling molecules known to regulate adipogenesis – such as PdgfRα, Igf1R and Wnt effectors – localize to the ciliary membrane upon growth arrest and ciliogenesis/ciliary elongation (Schneider, Clement et al. 2005, Marion, Stoetzel et al. 2009, Zhu, Shi et al. 2009, May-Simera and Kelley 2012, Dalbay, Thorpe et al. 2015, Sun, Berry et al. 2017). There is extensive crosstalk among pathways mediated by these molecules (Christensen, Morthorst et al. 2017), and evidence that Rpgrip1l may participate in some of this coordination (Mahuzier,

Gaude et al. 2012, Gerhardt, Lier et al. 2013). Recently, an ortholog of Rpgrip1l in *C. elegans*, Mks5, has been shown to establish a "ciliary zone of exclusion" which limits phosphatidylinositol 4,5-bisphosphate (PIP_2) localization in the ciliary membrane, thereby forming a "lipid gate" restricting access of membrane-bound proteins to the ciliary compartment. Additionally, Mks5 mutants exhibited dysregulated intraflagellar transport (IFT) velocity (Jensen, Li et al. 2015). Collectively, these findings suggest a critical role for Rpgrip1l in cell fate commitment.

Cilia are present on confluent, non-dividing human preadipocytes *in vitro*, and dexamethasone treatment elicits ciliary elongation in the first few days of differentiation, concurrent with inhibition of Hedgehog signaling. Ciliary length then decreases or the cilium disappears as cells become terminally differentiated, although it remains unclear whether ciliary length per se alters signaling (Marion, Stoetzel et al. 2009, Dalbay, Thorpe et al. 2015, Forcioli-Conti, Lacas-Gervais et al. 2015). In the absence of RPGRIP1L, cilia may elongate more readily in response to dexamethasone or may lessen the requirement for dexamethasone. Cilia might also be maintained into later stages of the differentiation program, increasing sensitivity to pro-adipogenic factors and enhancing the strength of signal transduction in response to these factors, or inhibiting the response to anti-adipogenic factors. Reduction of ciliary proteins such as Ift88, Kif3a and Alms1, which can be associated with decreased ciliary length, impairs adipogenesis (Zhu, Shi et al. 2009, Huang-Doran and Semple 2010, Heydet, Chen et al. 2013, Dalbay, Thorpe et al. 2015). However, impaired ciliogenesis in human white preadipocytes with *BBS10* and *BBS12* knockdown is associated with increased adipogenesis, underscoring the complicated nature of the effect of cilia on adipogenesis (Marion, Stoetzel et al. 2009).

The timing of ciliary elongation and disassembly may be critical to understanding why we did not observe effects on adiposity in the *Rpgrip1l*$^{fl/fl}$;Adipoq-Cre animals fed either chow or high fat diet. The expression of adiponectin, and therefore Cre recombinase in these mice, is not expressed in preadipocytes, and is only turned on late in adipogenesis (Scherer, Williams et al. 1995). Hence, the Adipoq-Cre driver may not have been expressed early enough in adipogenesis to affect ciliary functioning in preadipocytes. Consistent with this possibility, knockdown of *Rpgrip1l* in fully mature 3T3-L1 adipocytes did not affect lipid content or expression of adipocyte genes, whereas knockdown of *Rpgrip1l* in preadipocytes increased the differentiation capacity of 3T3-L1 cells.

Here, we propose a role for Rpgrip1l in the developing adipocyte that may contribute permissively to the increased body adiposity observed in *Rpgrip1l*$^{+/-}$ mice. This could ultimately play a role in the effect of the *FTO* locus in body weight regulation, where *RPGRIP1L* expression may be decreased due to the loss of CUX1 p110 binding of the risk allele. Loss of *Rpgrip1l* in mature adipocytes, as we modeled in the *Rpgrip1l*$^{fl/fl}$;Adipoq-Cre mice, may be too late in the ontogeny of adipocyte development to have any consequential biological effects. The increased adipogenesis seen in the early *Rpgrip1l* knockdown condition in 3T3 L1 cells could implicate *Rpgrip1l* hypomorphism in adipose tissue – by facilitating preadipocyte production and differentiation – in the increased adiposity of *Rpgrip1l*$^{+/-}$ mice. A larger depot fat capacity would permit greater fat storage before suppressive consequences on food intake might be invoked (Ravussin, Leibel et al. 2014).

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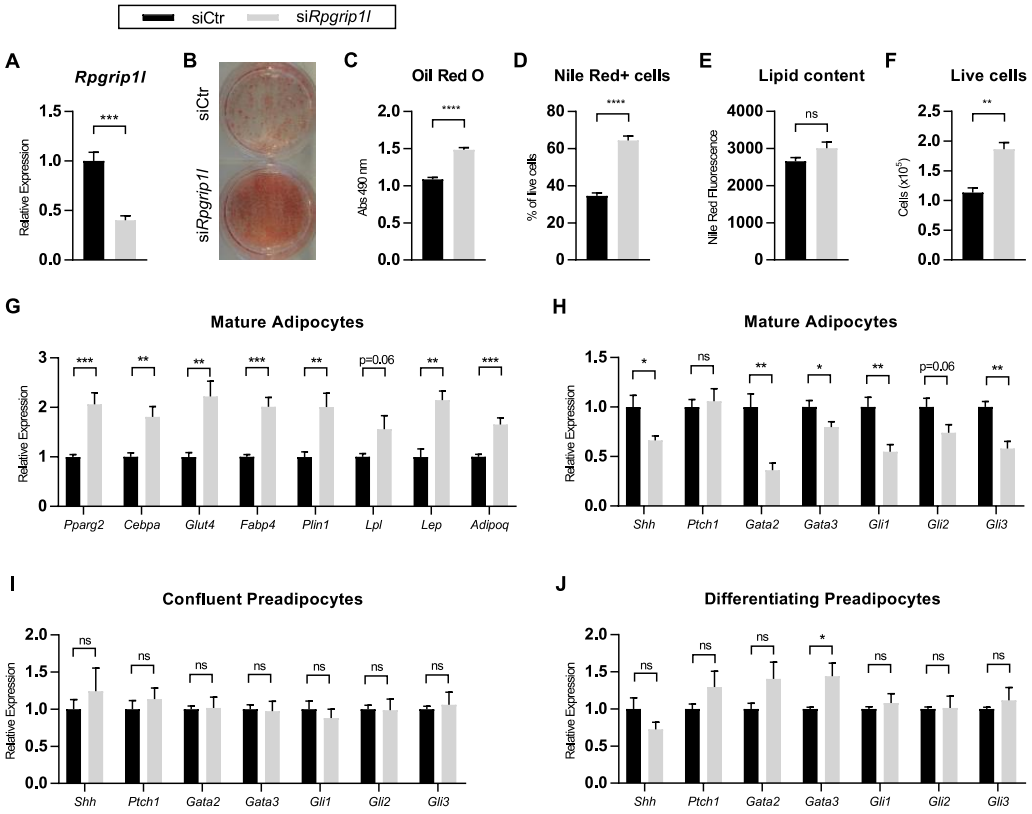
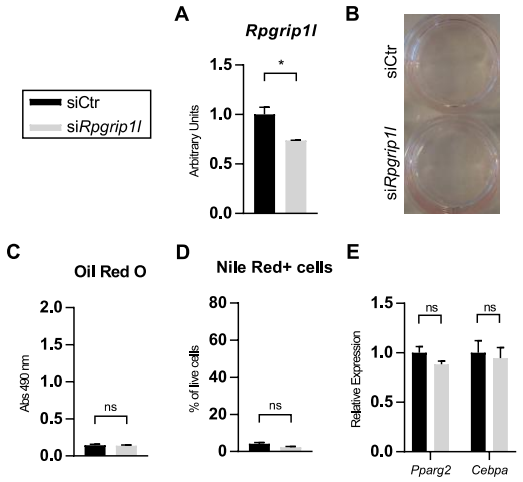
Figures

Figure 3.1 *Rpgrip1l knockdown increases adipogenesis of 3T3-L1 adipocytes.*



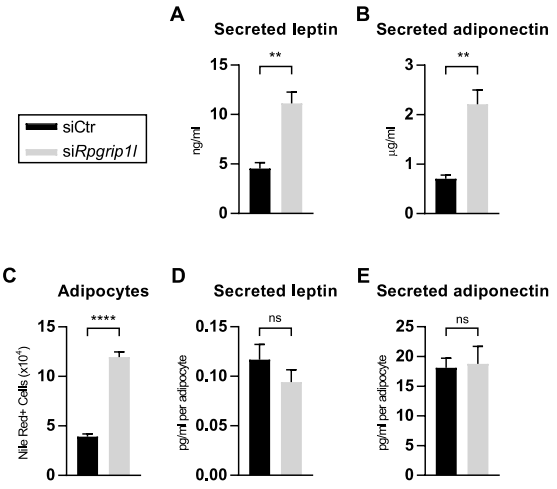
mRNA expression of *Rpgrip1l* in mature adipocytes after siRNA-induced knockdown prior to differentiation (**A**; siCtr: black bars, si*Rpgrip1l*: light gray bars). Representative image (**B**) and quantification (**C**) of Oil Red O staining of mature adipocytes treated with siRNA against *Rpgrip1l* prior to differentiation. Percentage of cells positive for Nile Red staining for intracellular lipid acquired by flow cytometry (**D**) and median fluorescence intensity of Nile Red + cells (**E**). Total number of live cells normalized to number of flow cytometry counting beads (**F**). mRNA expression (**G**) of developmental (*Pparg* & *Cebpa*) and functional genes (*Glut4*, *Fabp4*, *Plin1*, *Lpl*, *Adipoq* and *Lep*) in mature adipocytes when *Rpgrip1l* was knocked down prior to differentiation. Expression of *Shh* and target genes of the Shh pathway in mature adipocytes (**H**), confluent preadipocytes (**I**) and differentiating preadipocytes (**J**; treated for 2 days with differentiation media). mRNA expression has been normalized to *36B4*. n=4-6/condition. * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$, unpaired t-test.

Figure 3.2 *Rpgrip1l knockdown is not sufficient to induce adipogenesis of 3T3-L1 adipocytes in the absence of differentiation factors.*



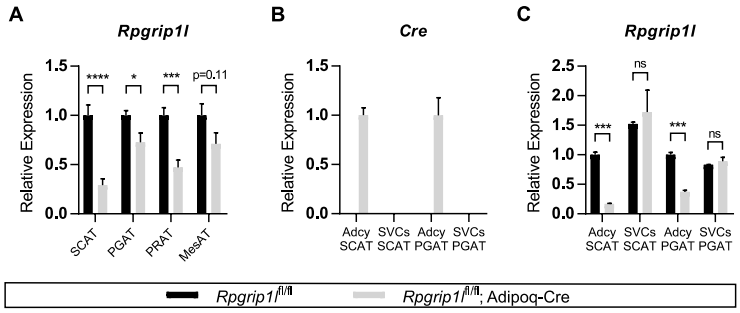
mRNA expression of *Rpgrip1l* (**A**; siCtr: black bars, si*Rpgrip1l*: light gray bars) 9 days after confluence of 3T3-L1 cells treated with siRNA against *Rpgrip1l* prior to confluence. Representative image (**B**) and quantification (**C**) of Oil Red O staining. Percentage of cells positive for Nile Red staining for intracellular lipid acquired by flow cytometry (**D**). mRNA expression (**E**) of developmental genes (*Pparg* & *Cebpa*). mRNA expression has been normalized to *36B4*. n=4/condition. * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$, unpaired t-test.

Figure 3.3 *Rpgrip1l knockdown does not alter production of leptin or adiponectin by 3T3-L1 adipocytes.*



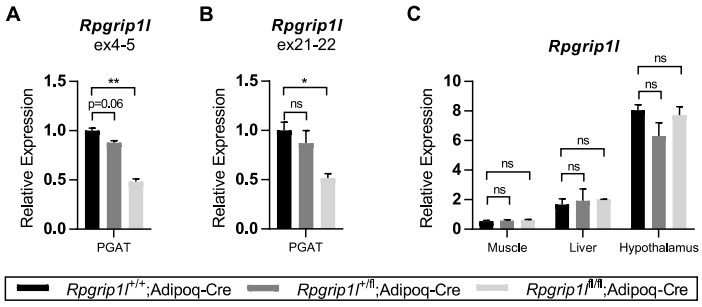
Secretion of leptin (**A**) and adiponectin (**B**) into culture media by mature adipocytes 3 days after media change (siCtr: black bars, si*Rpgrip1l*: light gray bars). Leptin (**D**) and adiponectin (**E**) levels in media normalized to number of adipocytes, measured by Nile Red stained cells acquired by flow cytometry. Adipocyte number was normalized to counting beads (**C**). n=4/condition. * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$, unpaired t-test.

Figure 3.4 *Rpgrip1l knockout is specific to adipocytes in Rpgrip1l$^{fl/fl}$;Adipoq-Cre mice.*



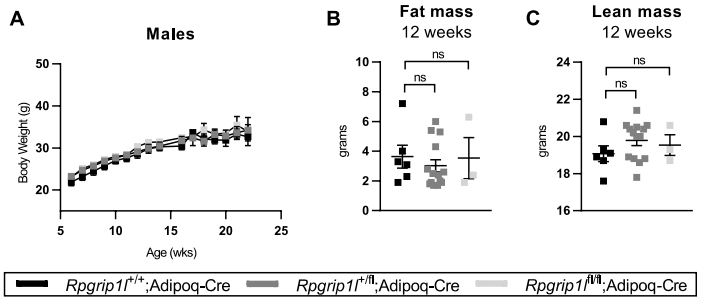
qPCR analysis of *Rpgrip1l* mRNA expression (**A**) in subcutaneous (SCAT), perigonadal (PGAT), perirenal (PRAT) and mesenteric (MesAT) adipose depots of male *Rpgrip1l*$^{fl/fl}$;Adipoq-Cre mice (light gray bars) compared to *Rpgrip1l*$^{fl/fl}$ controls (black bars). SCAT and PGAT depots were separated by collagenase digestion into adipocytes and cells from the stromal vascular fraction (SVCs) prior to qPCR analysis for *Cre* (**B**) and *Rpgrip1l* (**C**) expression in 25 week old male *Rpgrip1l*$^{fl/fl}$;Adipoq-Cre mice (n=3) and *Rpgrip1l*$^{fl/fl}$ controls (n=2) fed high fat diet. Transcript levels have been normalized to *36B4*. N=6 per condition. * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$, unpaired t-test.

Figure 3.5 *Rpgrip1l knockout is specific to adipose tissue of Rpgrip1l*$^{fl/fl}$*;Adipoq-Cre mice.*



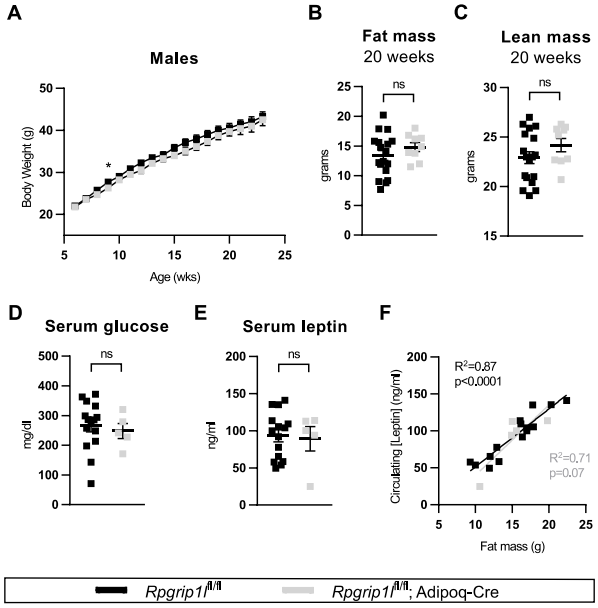
mRNA expression of *Rpgrip1l* using primers for exons 4-5 (**A**) and 21-22 (**B**) in PGAT from male *Rpgrip1l*$^{fl/fl}$;Adipoq-Cre (light gray bars) and *Rpgrip1l*$^{+/fl}$;Adipoq-Cre mice (dark gray bars) compared to *Rpgrip1l*$^{+/+}$;Adipoq-Cre controls (black bars). (**C**) *Rpgrip1l* mRNA expression in non-adipose tissues (muscle, liver and hypothalamus) in 1 month old male *Rpgrip1l*$^{fl/fl}$;Adipoq-Cre, *Rpgrip1l*$^{+/fl}$;Adipoq-Cre and *Rpgrip1l*$^{+/+}$;Adipoq-Cre animals. N=2 per condition. Transcript levels have been normalized to the arithmetic mean of *bAct*, *Gapdh* and *Ppia*. * $p<0.05$, ** $p<0.01$, unpaired t-test.

Figure 3.6 *Adipocyte-specific loss of Rpgrip1l function does not affect body weight or fat mass in chow-fed mice.*



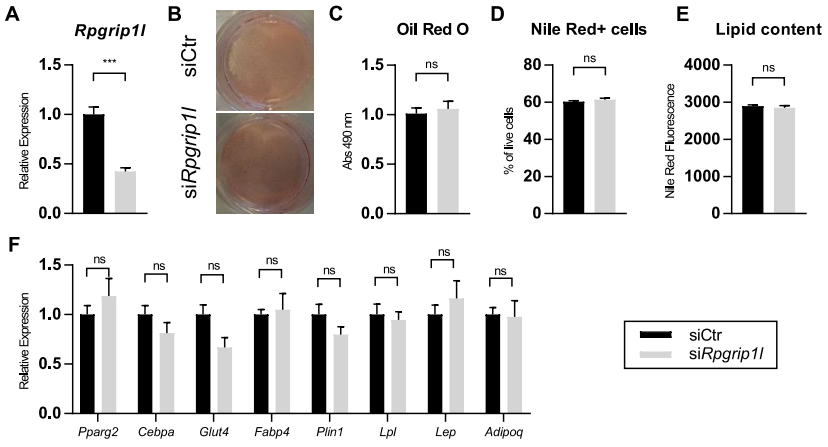
Weekly body weight measurements of male (**A**) *Rpgrip1l*$^{fl/fl}$;Adipoq-Cre (light gray bars; n=3), *Rpgrip1l*$^{+/fl}$;Adipoq-Cre mice (dark gray bars; n=14) and *Rpgrip1l*$^{+/+}$;Adipoq-Cre controls (black bars; n=6) fed chow. Fat mass (**B**) and lean mass (**C**) were measured by NMR at 12 weeks of age. Unpaired t-test.

Figure 3.7 *Adipocyte-specific loss of Rpgrip1l function does not affect body weight or fat mass in high fat diet-fed mice.*



Weekly body weight measurements of male (**A**) *Rpgrip1l*$^{fl/fl}$;Adipoq-Cre mice (black squares; n=13) and *Rpgrip1l*$^{fl/fl}$ controls (light gray squares; n=26) fed high fat diet (HFD = 60% of calories) starting at 6 weeks. Fat mass (**B**) and lean mass (**C**) were measured by NMR at 12 weeks of age in a subset of these (*Rpgrip1l*$^{fl/fl}$;Adipoq-Cre n=9; *Rpgrip1l*$^{fl/fl}$ n=18). Serum glucose (**D**) and leptin (**E**) concentrations. Correlation of serum leptin concentration with fat mass (**F**; *Rpgrip1l*$^{fl/fl}$;Adipoq-Cre n=5; *Rpgrip1l*$^{fl/fl}$ n=15) * $p<0.05$, unpaired t-test.

Figure 3.8 *Knockdown of Rpgrip1l in 3T3-L1 mature adipocytes does not affect lipid storage.*



mRNA expression of *Rpgrip1l* (**A**) 3 days after knockdown in mature adipocytes (siCtr: black bars, si*Rpgrip1l*: light gray bars). Representative image (**B**) and quantification (**C**) of Oil Red O staining of mature *Rpgrip1l*-knockdown adipocytes. Percentage of cells positive for Nile Red staining for intracellular lipid acquired by flow cytometry (**D**) and median fluorescence intensity (**E**) of Nile Red + cells. mRNA expression (**F**) of developmental (*Pparg* & *Cebpa*) and functional genes (*Glut4*, *Fabp4*, *Plin1*, *Lpl*, *Lep* & *Adipoq*) in mature adipocytes. Expression has been normalized to *36B4*. n=4/condition. *** $p<0.001$, unpaired t-test.

Chapter 4 The role of FTO in adipogenesis and adipocyte lipid balance.

Jayne F. Martin Carli, Charles A. LeDuc, Yiying Zhang, George Stratigopoulos and Rudolph L. Leibel

Author Contributions

J.F.M.C and R.L.L. conceived and designed the study and wrote the manuscript. J.F.M.C. performed the experiments and analyzed the data. C.A.L., Y.Z. and G.S. gave technical support and conceptual advice.

Abstract

Single nucleotides in the first intron of *FTO* convey effects on adiposity by mechanisms that remain unclear, but appear to include modulation of expression of *FTO* itself, as well as other genes (e.g. *RPGRIP1L*, *IRX3*) in the vicinity of *FTO*. This locus also affects T2D risk independent of its effects on body weight. *FTO* expression is decreased in fibroblasts and iPSC-derived human neurons of individuals segregating for obesity risk alleles of *FTO* at rs8050136 and rs1421085. These alleles exhibit decreased binding of the CUX1 activator isoform p110. Here we demonstrate that *Fto* expression is upregulated during adipogenesis, and is required for the maintenance of adipocyte lipid filling and endocrine function in murine 3T3-L1 cells and human adipose tissue-derived stromal cells. RNAseq analysis indicates that this effect on adipocyte programming is conveyed in part by modulation of C/ebpβ- and C/ebpδ-regulated transcription, consistent with reports that Fto acts a transcriptional coactivator. $Fto^{-/-}$ mice have normal fat mass in early life, but spontaneously lose adipose tissue as they age. We propose that

Fto is required to maintain adipocyte viability, a function critical to the prevention of ectopic lipid accumulation in obese states. Such accumulation – both total and in specific anatomic regions – has adverse metabolic consequences.

Introduction

Multiple genes in the region surrounding *FTO* may convey the obesity risk associated with this locus

Single nucleotide polymorphisms (SNPs) in a region of strong LD within the first intron of the *Fat Mass and Obesity Associated* (*FTO*) gene are very highly associated with adiposity; each minor allele is associated with respective increases of BMI of ~0.36 kg/m^2 (or body weight of ~1.2 kg in adults (Dina, Meyre et al. 2007, Frayling, Timpson et al. 2007, Scuteri, Sanna et al. 2007). The increased body weight in individuals segregating for the *FTO* risk allele rs9939609 is attributable primarily to increased food intake (with preference for higher caloric density) rather than decreased energy expenditure (Cecil, Tavendale et al. 2008, Speakman, Rance et al. 2008, Wardle, Carnell et al. 2008, Tanofsky-Kraff, Han et al. 2009).

The *Fto* gene was first identified in the "fused toes mouse" which was deleted for a 1.6 megabase region on Chr 8, encompassing six genes, including *Fto*. Originally named Fatso for its large intronic regions (Peters, Ausmeier et al. 1999), the gene was renamed "Fat Mass and Obesity Associated" after the discovery of the association with adiposity in human subjects (Frayling, Timpson et al. 2007). The mechanism by which this association is conveyed is still unclear. Neighboring *IRX3* has been implicated on the basis of long-range genetic interactions between the *IRX3* promoter and non-coding sequences in the first intron of *FTO*, which may regulate *IRX3* expression. *Irx3* knockout mice are protected from diet-induced obesity by an increase in basal metabolic rate putatively due to increased thermogenesis driven by adipose tissue "browning" (Smemo, Tena et al. 2014). Additionally, a SNP (rs1421085) within the intron disrupts binding of the ARID5B repressor, increasing *IRX3* expression (Claussnitzer, Dankel et

al. 2015). Increased *IRX3* expression in human primary preadipocytes impairs browning, driving them toward a white adipocyte phenotype.

The statistical strength of the association with adiposity and the large number of SNPs within the obesity-associated locus in the first intron of *FTO*, suggests that there may be multiple causal variants contributing to the observed effect. For example, we have demonstrated that two SNPs in the obesity-associated locus (rs8050136 and rs1421085) differentially bind the transcription factor CUX1. The protective alleles of these SNPs preferentially bind the activating isoform of CUX1, p110, proteolytically processed by Cathepsin L, increasing expression of *FTO* and neighboring *RPGRIP1L* (Stratigopoulos, Padilla et al. 2008, Stratigopoulos, LeDuc et al. 2011, Stratigopoulos, Burnett et al. 2016).

FTO affects somatic growth and adiposity.

Mice congenitally null for *Fto* exhibit a complex phenotype that includes postnatal lethality (Fischer, Koch et al. 2009, Gao, Shin et al. 2010, McMurray, Church et al. 2013). Those mice that survive have impaired linear growth and in adulthood (~20 weeks), these mice are protected from diet-induced obesity and develop lipoatrophy (Church, Lee et al. 2009, Fischer, Koch et al. 2009, Ronkainen, Huusko et al. 2015). Conversely, *Fto*-overexpressing mice display increased body weight and adiposity (Church, Moir et al. 2010). As indicated in **Chapter 1**, we (Stratigopoulos, Burnett et al. 2016) and others (Gao, Shin et al. 2010, McMurray, Church et al. 2013) have found that at earlier timepoints (~15 weeks), adiposity is actually increased as a percentage of body weight in $Fto^{-/-}$ mice. And, consistent with reports by Fischer *et al*. and Ronkainen *et al*. (Fischer, Koch et al. 2009, Ronkainen, Huusko et al. 2015), we observed dramatic loss of adipose tissue as these mice aged (G. Stratigopoulos, personal communication).

We suggest that this early increase in adiposity is due to the primary role of Fto in regulating energy balance centrally, and that the loss in adiposity seen later in life is due to a defect in adipose tissue structure and function. Mice lacking Fto have smaller adipocytes (Fischer, Koch et al. 2009) and *Fto*-overexpressing mice have larger adipocytes than controls (Church, Moir et al. 2010). These phenotypes suggest a role for Fto in enabling facultative adipose tissue expansion, possibly in the context of diet-induced obesity. Despite decreased body weight and adiposity, 24 week old mice segregating for a dominant-negative *Fto* mutation exhibit increased circulating concentrations of fasting glucose, triglyceride, cholesterol and high-density lipoprotein (HDL) levels, consistent with a lipodystrophic metabolic profile (Church, Lee et al. 2009, Unger and Scherer 2010, Virtue and Vidal-Puig 2010).

Humans homozygous for null mutations of *FTO* display characteristic brain (microcephaly, hydrocephalus and lissencephaly) and cardiac malformations (ventricular septal and atrial ventricular defects, patent ductus arteriosus) and hypertrophic cardiomyopathy that result in growth retardation and death within the first few years of life (Boissel, Reish et al. 2009, Daoud, Zhang et al. 2016, Rohena, Lawson et al. 2016). If the obesity-associated region on chromosome 16 is conveyed the FTO protein itself, partial function of this protein must be preserved, as these extreme malformations are not evident in individuals segregating for the risk alleles located in intron 1 of *FTO*. Additionally, *FTO* risk alleles are not associated with any change in height, compared to humans with *FTO* mutations and *Fto* loss-of-function mouse models that exhibit dramatic growth retardation (Frayling, Timpson et al. 2007).

The effects on adiposity cannot be accurately studied in individuals with *FTO* loss-of-function mutations, as the developmental defects and their medical consequences mask potential effects on adiposity. Heterozygotes for a variety of *FTO* mutations have no obvious phenotypic

characteristics (Meyre, Proulx et al. 2010, Deliard, Panossian et al. 2013, Zheng, Hong et al. 2013). The number of these individuals with variants predicted or proven to have a deleterious effect on FTO function is extremely small, however, and studies attempting to associate these variants with adiposity are likely underpowered to make conclusions regarding metabolic homeostasis.

Fto is a putative m6A RNA demethylase

FTO is a member of the ALKB homolog family of non-heme dioxygenases (Fe(II)- and α-ketoglutarate dependent dioxygenases) (Gerken, Girard et al. 2007, Sanchez-Pulido and Andrade-Navarro 2007), and exhibits RNA N6-methyladenosine (m6A) and 3-methyluridine (m3U) demethylation activity *in vivo* (Jia, Fu et al. 2011). m6A is an abundant mRNA modification and is thought to play a role in the regulation of mRNA stability, splicing and translation. These modifications are increased around stop codons and 3'UTRs, as well as along long exons (Dominissini, Moshitch-Moshkovitz et al. 2012, Meyer, Saletore et al. 2012, Fu, Dominissini et al. 2014).

FTO plays a role in maintaining adipocyte lipid handling and endocrine functions

Although expressed most highly in the brain, *Fto* is also expressed in adipose tissue (Gerken, Girard et al. 2007, Gao, Shin et al. 2010) where its role remains unclear. While eQTL studies examining the expression of *FTO* in individuals segregating for risk or protective alleles have not shown different levels of expression, sample sizes were small and in some cases, whole adipose tissue was examined, potentially masking effects in single cell types such as adipocytes or preadipocytes (Kloting, Schleinitz et al. 2008, Grunnet, Nilsson et al. 2009, Zabena, Gonzalez-Sanchez et al. 2009, Claussnitzer, Dankel et al. 2015). In mice, *Fto* expression is

decreased in mesenteric adipose tissue upon fasting (Stratigopoulos, Padilla et al. 2008) and increased in subcutaneous and visceral adipose tissue upon acute exposure to high fat diets (Wang, Shie et al. 2015), indicating that nutritional status might confound efforts to attribute expression of *FTO* to risk genotype.

Fto has recently been implicated in the differentiation of adipocytes (Zhao, Yang et al. 2014, Merkestein, Laber et al. 2015, Zhang, Zhang et al. 2015). In particular, Fto's demethylase function is required for adipogenesis, and may be involved in regulating mRNA splicing (Zhao, Yang et al. 2014, Zhang, Zhang et al. 2015). Whether this effect on adipogenesis contributes to the adiposity or metabolic phenotypes described in *Fto*$^{-/-}$ mice is unclear. The effects on m6A RNA methylation, purported to be regulated by Fto, are incompletely characterized with regard to adipogenesis. One possible mediator of Fto's demethylase function in adipocytes is an alternatively spliced version of *Runt related transcription factor 1* (*Runx1t1*) lacking exon 6. Inclusion of exon 6 may be due to enhanced binding of m6A-methylated RNA by serine/arginine rich splicing factor 2 (SRSF2) in 3T3-L1 cells lacking Fto (increased methylation) (Zhao, Yang et al. 2014, Merkestein, Laber et al. 2015). Exon 6 skipping, possibly due to the lack of SRSF2 binding sites in cells with functional Fto demethylase activity, generates a short isoform that is pro-adipogenic in 3T3-L1 cells and mouse embryonic fibroblasts *in vitro* (Zhao, Yang et al. 2014, Merkestein, Laber et al. 2015).

We assessed the possibility that Fto may be involved in adipocyte differentiation by developmentally-timed manipulation of *Fto* gene expression in 3T3-L1 preadipocytes. We also analyzed the effect of *FTO* knockdown on adipogenesis in human adipose tissue-derived preadipocytes. We found that *Fto* expression was required for adipogenesis; and using RNAseq we identified molecular targets of Fto that might mediate this effect. While we identified C/ebpβ

and C/ebpδ expression and function as primary targets of Fto, we did not find evidence that these transcripts are differentially methylated or exhibit altered stability or translational efficiency following *Fto* knockdown. Surprisingly, *Fto* knockdown enhanced Akt signaling despite the net inhibition of adipogenesis.

Methods

Cell culture and transfection

3T3-L1 preadipocytes were purchased from ATCC and maintained in non-confluent cultures. Growth medium consisted of DMEM (with 25mM glucose, GlutaMAX™, and sodium pyruvate) supplemented with 10% newborn calf serum (both Thermo Fisher). Differentiation was achieved by treating cells two days after they reached confluence with differentiation media containing DMEM plus insulin (from Bovine Pancreas 1 μg/ml), dexamethasone (0.25 μM) and isobutylmethylxanthine (IBMX; 0.5mM; all Sigma-Aldrich) supplemented with 10% fetal bovine serum (Thermo Fisher). Two days later, differentiation media was replaced with DMEM containing only 1 μg/ml insulin and 10% FBS. Insulin media was replaced every two to three days and cells were filled with lipid and considered mature adipocytes by days 8-12.

Human ASCs were generously provided by the Boston NORC and were cultured and differentiated as previously described (Lee and Fried 2014). Briefly, cells were maintained in αMEM supplemented with 10% FBS (all Thermo Fisher) to avoid confluence. To differentiate, cells were allowed to reach confluence and 2 days later were treated with a chemically defined, serum free media (DMEM/F12 containing HEPES and Antibiotic-Antimycotic (Thermo Fisher), $NaHCO_3$, d-Biotin, Pantothenate, Dexamethasone, human insulin, Rosiglitazone, IBMX, T3 and Transferrin (Sigma Aldrich)). Differentiation media was replaced regularly and switched to

maintenance media after 3-7 days (differentiation media without Rosiglitazone, IBMX, T3 and Transferrin). Cells were filled with lipid and assayed between 10-14 days after initial treatment with differentiation factors.

Transfections were performed prior to differentiation using a mix of 3 Stealth siRNAs targeted against *Fto* or 3 nontargeted controls with Lipofectamine 2000 or 3000 (all Thermo Fisher). *Fto*/*Ptprv* and *Fto*/*Spon2* double knockdowns were performed by adding a single Stealth siRNA against *Ptprv* or *Spon2* to the mix of 3 anti-*Fto* siRNAs (all Thermo Fisher). Knockdown of *Fto* in mature adipocytes was achieved by electroporation (Jiang, Zhou et al. 2003, Okada, Mori et al. 2003, Kilpeläinen, Carli et al. 2016) using the same siRNAs after 3T3-L1 cells had been differentiated.

RNA expression studies, RNAseq and m6A-RIP

Total RNA was isolated using RNeasy Lipid Tissue Mini Kit and columns were treated with DNase (both Qiagen). cDNA was reverse transcribed with Transcriptor First Strand cDNA Synthesis Kit (Roche) using both OligoDT and random hexamer primers. Quantitative real time PCR (qPCR) was performed on a Lightcycler 480 using SYBR Green I Master (Roche). Analysis was performed by the LightCycler 480 software (Roche) using the 2nd derivative max calculation based on a standard curve. qPCR primers were designed using Primer 3: http://bioinfo.ut.ee/primer3/ to span exon-exon junctions and are provided in Supplementary Table 1.

Sample integrity for RNA utilized for RNAseq was assessed with an Agilent 2100 Bioanalyzer. All samples had RIN numbers greater than 8.0. mRNA was isolated using poly-A pulldown and reverse transcribed to generate cDNA (Wang, Gerstein et al. 2009). cDNA was sequenced using single-ended sequencing on a HiSeq2000 according to manufacturer's

recommendations (Illumina). The pass filter reads were mapped to mouse reference genome mm9 using TopHat (version 2.0.4). TopHat infers novel exon-exon junctions and combines them with junctions from known mRNA sequences as the reference annotation (Trapnell, Pachter et al. 2009). For each read, up to 3 mismatches and 10 multiple hits were allowed during mapping.

RNA Immunoprecipitation was performed based on previously published protocols (Meyer, Saletore et al. 2012, Dominissini, Moshitch-Moshkovitz et al. 2013). Briefly, RNA was isolated (as described above, including DNase treatment) from 2-day post-confluent 3T3-L1 preadipocytes following *Fto* knockdown and a 5µg aliquot was treated with RiboMinus (Thermo Fisher) to decrease rRNA content. 300ng of heat-denatured RNA was pulled down with rabbit anti-m6A antibody (Synaptic Systems) coupled to Protein A/G Dynabeads in the presence of an RNase inhibitor SUPERase•In (both Thermo Fisher). Eluted RNA was isolated by QIAzol:chloroform extraction and column purification (as described above, without DNase treatment). The resulting RNA and a reserved aliquot containing 10% of the input were reverse transcribed and analyzed by qPCR. mRNA expression was calculated using the Delta Delta C(T) method and normalized to *36B4* transcript levels (Livak and Schmittgen 2001).

Transcript stability was measured following treatment with Actinomycin D (ActD). Cells were treated with 1 µg/ml (stock: 1 mg/ml in DMSO; Thermo Fisher) for the indicated time periods prior to isolation of RNA for analysis by qPCR. Transcript levels were normalized by the amount of 18S transcript present, which remained constant throughout all 8 hours of ActD treatment.

Lipid Staining

Mature adipocytes were stained with Oil Red O for lipid content by first fixing cells with 4% paraformaldehyde for 10 minutes. A freshly mixed solution of 3:2 Oil Red O stock (25mg

Oil Red O in 50ml isopropanol; Sigma) was prepared and filtered prior to staining for 30 mins. Cells were then washed with PBS and imaged. For quantification, Oil Red O was extracted in 300µl of isopropanol with 4% IGEPAL CA-630 for 5 mins and 100µl were used to measure absorbance at 490nm (Church, Berry et al. 2014).

Nile Red staining was performed based on the protocol developed by Smyth *et. al.* (Smyth and Wharton 1992). Mature adipocytes were trypsinized and treated with 4% paraformaldehyde for 10 mins. Nile Red (stock: 1mg/ml in DMSO; Thermo Fisher) was then added for a final concentration of 500ng/µl and cells were acquired within 30 mins by flow cytometry using the PE-TR channel to measure Nile Red fluorescence. Prior to acquisition, equal volumes of 8.0-12.9 µm counting beads (Spherotech) were added to quantify cell numbers per condition. Live cells were gated to include all events except debris (very low FSC & SSC). The Nile Red gate was set on Nile Red-negative preadipocytes.

Protein isolation and quantification

Whole cell lysates were collected in RIPA buffer in the presence of Halt protease-phosphatase inhibitor and quantified by BCA assay (all Thermo Fisher). Following denaturing gel electrophoresis, proteins were transferred to nitrocellulose membranes and probed with the following primary antibodies: βAct (Abcam), Fto (Santa Cruz), αTub, C/ebpβ, pAkt, Akt, pAkt1, Akt1, pAkt2, pFoxo1, Foxo1, and Adiponectin (Cell Signaling). Fluorescent secondary IRDye 680LT and 800CW antibodies were from LI-COR. Blots were imaged using LI-COR's Odyssey Classic and bands were quantified with Image Studio software (LI-COR).

Leptin and Adiponectin ELISAs were performed using kits from R&D Systems. Cell culture supernatants from mature adipocytes were removed 3 days after media had been changed.

Samples (from 12 well plates) were diluted 1:20 for Leptin ELISA and 1:1,000 for Adiponectin ELISA.

Glucose uptake was measured using the Glucose Uptake Colorimetric Assay Kit (Sigma) according to the manufacturer's instructions. Briefly, following *Fto* knockdown, 2-day post-confluent 3T3-L1 preadipocytes were serum starved overnight and then glucose starved for 40 mins. Cells were treated with or without insulin (1µM) for 20 mins and then treated with or without 2-deoxyglucose (2-DG) for 20 mins. Lysed cells were assayed for 2-DG concentration using the colorimetric assay.

Mice

Mice were group housed by gender and maintained at an ambient temperature of 22 °C–24 °C with a 12-h dark–light cycle (lights on at 0700, h) in a pathogen-free barrier facility. The protocol was approved by the Columbia University Institutional Animal Care and Use Committee. *Fto* expression was evaluated in isolated adipocytes and preadipocyte-containing stromal vascular cells from perigonadal and subcutaneous adipose tissue from 4-month-old C57BL/6 mice (derived from Jackson, Stock number 000664) fed chow (Purina PicoLab 5058). Adipose depots were excised and digested with collagenase (Roche) for 45min-1hr prior to separation by centrifugation. The SVC pellet was treated with erythrocyte lysis buffer (0.154 M NH_4Cl, 10 mM $KHCO_3$ and 0.1mM EDTA) for 3 minutes. RNA was isolated, cDNA was generated and qPCR was performed as described above. mRNA expression was normalized to the arithmetic mean of *36B4*, *Actb*, *Gapdh* and *Ppia*.

Results

Fto knockdown impairs adipogenesis in mouse and human cells

To determine whether *Fto* participates in adipogenesis – and by that process in energy storage capacity – we treated 3T3-L1 preadipocytes with siRNA targeted to *Fto* prior to *in vitro* adipogenesis. mRNA transcript and protein levels of *Fto* were decreased during the entire differentiation timecourse in the *Fto* knockdown condition (**Fig. 4.1A**, **Sup. Fig. 4.1A**&**B**). Knockdown of *Fto* impaired the ability of 3T3-L1 cells to develop into mature adipocytes, as demonstrated by Oil Red O staining (**Fig. 41B**, **Sup. Fig. 4.1D**). Quantitative RT-PCR (qPCR) analysis of preadipocytes showed decreased expression of *Cebpd*, and a modest but statistically non-significant decrease in *Cebpb* (**Fig. 4.1C**). Expression of adipocyte developmental and functional genes, *Pparg*, *Cebpa*, *Fabp4*, *Glut4*, *Plin1*, *Lpl*, *Adipoq* and *Lep* was decreased in mature adipocytes (**Fig. 4.1D, Sup. Fig. 4.1C**). The total amount of leptin secreted into the culture media was decreased as well (**Sup. Fig. 4.1E**); however, when normalized to the number of adipocytes per well/condition (**Sup. Fig. 4.1F**), there was no difference in amount of leptin secreted per cell (**Sup. Fig. 4.1G**), suggesting that Fto does not directly regulate leptin synthesis or release on a per adipocyte basis (Kilpeläinen, Carli et al. 2016). The decrease in lipid content and adipocyte gene expression in bulk culture could have been caused by a decrease in the number of cells that could fully develop into adipocytes or by a limitation causing cells to arrest at a less mature stage. We analyzed Nile Red staining for neutral lipids by flow cytometry and found that both factors contributed to the decrease in lipid per well. There were both fewer Nile Red positive cells as a percentage of total cells analyzed (**Fig. 4.1E**), and of the cells that were Nile Red positive, there was less lipid in each cell, indicated by the median fluorescence intensity of the Nile Red+ adipocyte population (**Sup. Fig. 4.1H**). As previously reported

(Merkestein, Laber et al. 2015), we also saw a decrease in the total number of live cells (**Sup. Fig. 4.1I**).

To confirm the observed effects of Fto on adipogenesis in 3T3-L1 we manipulated *FTO* expression in primary human adipose tissue-derived stromal cells (ASCs) (Lee and Fried 2014). Using the same experimental paradigm (*FTO* knockdown prior to differentiation) with cells from four different individuals (males and females), we found that knockdown of *FTO* expression (**Fig. 4.1F**, **Sup. Fig. 4.1J**&**K**) impaired adipogenesis. We observed the same decrease in Oil Red O (**Fig. 4.1G**, **Sup. Fig. 4.1L**) staining and percentage of Nile Red positive adipocytes (**Fig. 4.1J**), although there was no difference in Nile Red fluorescence in mature cells (**Sup. Fig. 4.1M**) in the FTO knockdown condition. In preadipocytes, expression of both *CEBPB* and *CEBPD* was decreased (**Fig. 4.1H**), and in mature adipocytes, expression of adipocyte developmental and functional genes *PPARG*, *CEBPA*, *FABP4*, *GLUT4*, *PLIN1*, *LEP* and *ADIPOQ* (**Fig. 4.1I**) was decreased in the *FTO* knockdown condition compared to non-targeted siRNA controls.

Previous reports have suggested that Fto may affect splicing of exon 6 of *Runx1t1* as a mechanism for alterations in adipogenesis (Zhao, Yang et al. 2014, Merkestein, Laber et al. 2015). We measured long (containing exon 6) and short (missing exon 6) splice variants of *Runx1t1* by qPCR and were unable to detect any significant difference in total amounts or the ratio of the isoforms between control and *Fto* knockdown conditions in either the preadipocyte or mature adipocyte stage in 3T3-L1 cells (**Sup. Fig. 4.1N**-**P**). It has also been suggested that Fto inhibits the "browning" or "beiging" of white adipose tissue, decreasing thermogenesis driven by the uncoupling of oxidative phosphorylation (Tews, Fischer-Posovszky et al. 2013, Ronkainen, Mondini et al. 2016). We did not detect an increase in brown adipocyte markers *Ucp1*, *Cidea*,

Pgc1a and *Cox7a* following *Fto* knockdown. We observed a decrease in expression of these genes, likely due to the blunting of adipogenesis (**Sup. Fig. 4.1Q**).

Preliminary evidence suggests that C/ebpδ binds to rs8050136 in the first intron of *FTO* (data not shown). Because of the decrease in *CEBPD* expression following *FTO* knockdown in preadipocytes, we evaluated whether *FTO* knockdown affected the expression of genes near the *FTO* locus (*CHD9*, *RBL2*, *AKTIP*, *RPGRIP1L*, *IRX3*, *IRX5*, *CRNDE*; **Fig. 4.2A**&**B**), in 3T3-L1 and human ASC preadipocytes. *RBL2* expression was decreased modestly after *FTO* knockdown in both mouse and human cells. *Rpgrip1l* expression was decreased more dramatically, but only in murine 3T3-L1 preadipocytes (**Fig. 4.2C**&**D**).

RNAseq analysis identifies Fto-targeted genes as candidates for regulation of adipogenesis

To identify a mechanism for Fto's adipogenic functions in 3T3-L1 cells, we analyzed the gene expression profile following *Fto* knockdown by RNAseq. We chose to examine 2-day post-confluent preadipocytes prior to the induction of differentiation in order to focus on primary targets of Fto, minimizing secondary effects produced by an altered adipogenic program. There were 20 genes significantly ($p<0.05$) up- or downregulated following Bonferroni correction (See **Fig. 4.3D**). We identified four additional genes (*Clca1*, *Rhob*, *Tusc5* and *Klf2*) with differential expression that was not as statistically significant (uncorrected $p<1x10^{-5}$; Bonferroni corrected $p<0.23$) but with effect sizes of ≥2-fold or ≤0.5-fold (**Fig. 4.3A**). One other notable change was the decrease in *Pten* expression ($p=0.1$ following Bonferroni correction, 0.8-fold). We then confirmed the effect of *Fto* knockdown on these genes by measuring transcript levels in *Fto*-knockdown preadipocytes from two independent experiments by qPCR. We verified the effect of *Fto* on 20 of 24 genes identified by RNAseq (**Fig. 4.3D**).

We also examined the effects of *FTO* knockdown on human homologs of the most highly regulated targets by RNAseq in 3T3-L1 preadipocytes (>3-fold increase or <0.3-fold decrease after *Fto* knockdown in 3T3-L1 cells by qPCR confirmation: *PTPRVP*, *TMED5* and *SPON2*) as well as genes known to be associated with adipogenesis (*GREM2*, *KLF2* and *WNT4* (Banerjee, Feinberg et al. 2003, Wu, Srinivasan et al. 2005, Nishizuka, Koyanagi et al. 2008, Wu, Tang et al. 2015)) and *PTEN*, an inhibitor of insulin signaling (Sasaoka, Wada et al. 2006). These studies were conducted in human ASC-derived preadipocytes (**Fig. 4.3E**) and mature adipocytes (**Fig. 4.3F**) to identify molecules that might enable FTO to regulate adipogenesis.

Upregulation in adipocytes of *SPON2* (spondin 2), a gene not previously associated with adipogenesis, and downregulation of *PTEN* (phosphatase and tensin homolog) in preadipocytes, were consistent with the results of the RNAseq and qPCR analyses in 3T3-L1 preadipocytes. We were unable to detect mRNA expression of *PTPRVP* (protein tyrosine phosphatase, receptor type V, pseudogene), consistent with a report indicating that it is a pseudogene in humans (Cousin, Courseaux et al. 2004). *Ptprv* in murine cells, however, is dramatically upregulated in conditions of p53-regulated G1 cell cycle arrest, and loss of this gene restores cell cycle progression in a manner similar to loss of p53 (Doumont, Martoriati et al. 2005, Doumont, Martoriati et al. 2005). Expression of other p53-induced cell cycle arrest effectors, *GADD45A*, *MDM2* and *CDKN1A*, was upregulated in human ASC preadipocytes (**Fig. 4.3G**) and mature adipocytes (**Fig. 4.3H**).

We analyzed our 3T3-L1 preadipocyte *Fto* knockdown RNAseq dataset using Enrichr (http://amp.pharm.mssm.edu/Enrichr/; (Chen, Tan et al. 2013, Kuleshov, Jones et al. 2016)), a publicly available tool for enrichment analysis. We analyzed genes that were up- or down-regulated by more than 10% with $p<0.05$ (without correction; 2,455 genes). The most striking results came from the ChEA 2016 analysis, which queries a database of chromatin

immunoprecipitation (ChIP) datasets for transcription factor targets. The *Fto*-knockdown condition was highly enriched for C/ebpβ and C/ebpδ targets identified in ChIP studies performed in 3T3-L1 cells, with Enrichr's calculated combined scores of 85.74 (adjusted p=1.12E-25; 410/2000 overlap (*Fto*-affected transcripts/all C/ebpβ-regulated transcripts) and 80.53 (adjusted p=6.34E-23; 358/1735 overlap), respectively. This result is consistent with the decreased expression of *Cebpd* (57%) demonstrated in **Fig. 4.1**. We observed a non-statistically significant (14%) decrease in *Cebpb* expression in 3T3-L1 *Fto*-knockdowns, but consistent, significant downregulation of both *CEBPB* and *CEBPD* in *FTO*-knockdown human ASCs. Peak expression of *Cebpb* (after 2 hrs) and *Cebpd* (after 1 hr) immediately after the addition of differentiation media in 3T3-L1 preadipocytes displayed the same pattern (**Fig. 4.3B**&**C**).

Differentially-expressed Fto targets identified by RNAseq are not likely to be direct targets of Fto's demethylation function

As noted, Fto acts as a demethylase of m6A (Gerken, Girard et al. 2007, Jia, Fu et al. 2011). We looked for changes in RNA methylation of the abovementioned candidate genes (*Cebpb*, *Cebpd*, *Ptprv*, *Tmed5*, *Spon2*, *Grem2*, *Klf2*, *Wnt4* and *Pten*). We immunoprecipitated RNA using an anti-m6A antibody and then quantified transcript levels by qPCR to assess whether any of these transcripts is a direct target of Fto's demethylase function. When normalized to the amount of transcript found in input samples prior to m6A immunoprecipitation, there were no changes in m6A status in any of the transcripts evaluated (**Fig. 4.4A**&**B**). This finding suggests that these transcripts are secondary targets of an upstream target of Fto's m6A demethylase function or are direct targets of Fto via a mechanism independent of m6A demethylation, such as transcriptional activation. A recent report indicates

that the preferred target of Fto's demethylase function is actually N6,2′-O-dimethyladenosine (m6Am), a subset of m6A found only at the 5' end of mRNA transcripts (Mauer, Luo et al. 2016). Loss of m6Am demethylation may be masked by precipitating transcripts with an antibody specific for all m6A modifications, indicating that ours are not conclusive negative results regarding the possible direct demethylation action of Fto on these transcripts.

Demethylation of m6A and, more specifically, m6Am, by Fto has been associated with altered transcript stability (Wang, Lu et al. 2014, Mauer, Luo et al. 2016). Thus, if *Cebpb*, *Cebpd*, *Ptprv*, *Spon2* and/or *Pten* are direct targets of Fto despite their unchanged m6A pattern, the increased transcript levels observed may be the result of increased stability. To test this possibility, we inhibited nascent transcription with Actinomycin D (ActD) following knockdown of *Fto* in 3T3-L1 preadipocytes (**Fig. 4.4B**-**F**, **H**-**L**). We did not observe any change in transcript stability of *Cebpb* (**Fig. 4.4B**&**H**), *Cebpd* (**Fig. 4.4C**&**I**), *Ptprv* (**Fig. 4.4D**&**J**) *Spon2* (**Fig. 4.4E**&**K**) or *Pten* (**Fig. 4.4F**&**L**) that might account for the altered expression levels observed following *Fto* knockdown. Methylation of m6A residues may also contribute to changes in translation from the RNA transcript (Meyer, Patil et al. 2015). We anticipated that this reaction might contribute to the altered expression of C/ebpβ target genes observed by RNAseq despite the absence of a decrease in *Cebpb* transcript levels, as it is possible that *Cebpb* was not being properly translated or was being translated from alternative start sites. Western blotting for C/ebpβ protein in 3T3-L1 preadipocytes showed slightly (but not statistically significant) decreased levels of all isoforms in the *Fto*-knockdown condition compared to controls (**Fig. 4.4N**&**O**), consistent with the mRNA expression data described. These data indicate that *Cebpb*, *Cebpd*, *Ptprv*, *Spon2* and *Pten* may not be direct targets of Fto's demethylase function, even though they are critically regulated by Fto.

Ptprv and Spon2 contribute to Fto's role in adipogenesis in 3T3-L1 cells

We evaluated the importance of candidate genes identified as Fto targets by RNAseq to the adipogenesis phenotypes we observed. Simultaneous knockdown of both *Fto* and *Ptprv* (**Fig. 4.5A**&**B**) restored adipogenesis lost when *Fto* alone was knocked down. *Pparg* expression (**Fig. 4.5C**) and Oil Red O staining (**Fig. 4.5D**; **Sup. Fig. 4.2A**) were increased in these cells. *Fto*/*Ptprv* double knockdown also increased the percentage of Nile Red staining (**Fig. 4.5F**) and fluorescence of Nile Red+ cells (**Sup. Fig. 4.2B**) compared to *Fto* knockdown alone. Elevated expression of *Spon2* after *Fto* knockdown (**Fig. 4.5E**) is attributable to Ptprv, as *Fto*/*Ptprv* knockdown restored *Spon2* expression to control levels. *Fto*/*Ptprv* knockdown did not increase *Cebpb* (**Fig. 4.5H**) or *Cebpd* (**Fig. 4.5I**) expression, however, indicating that Ptprv is either downstream of Cebpb and Cebpd, or functioning in a different pathway from these critical regulators of adipogenesis. Knockdown of *Fto* in addition to *Spon2* also restored adipogenesis but to a lesser degree (**Sup. Fig. 4.3A-G**), and like *Fto*/*Ptprv* knockdown, did not increase *Cebpb* (**Sup. Fig. 4.3H**) or *Cebpd* (**Sup. Fig. 4.3I**) expression. As *Spon2* and *Pten* (discussed below) were the only genes differentially regulated in both 3T3-L1 and human ASC preadipocytes following *Fto*/*FTO* knockdown, we propose that they are critical effectors of Fto's function.

Fto regulates Akt signaling in adipocytes

The decrease in *Pten* expression associated with *Fto* knockdown – identified by RNAseq and illustrated in **Fig. 4.3** – was unexpected, as *Pten* is a well characterized tumor suppressor (Song, Salmena et al. 2012), and its decreased expression after *Fto* knockdown would be predicted to increase cell proliferation, in contrast to the decreased cell proliferation that we

observed. Pten also inhibits insulin signaling, however, by dephosphorylating phosphatidylinositol (3,4,5)-trisphosphate (PIP_3) to PtdIns(4,5)P2 (PIP_2), thereby inhibiting Akt phosphorylation (Nakashima, Sharma et al. 2000, Sasaoka, Wada et al. 2006). A previous report (Pitman, Fong et al. 2012) indicated that *Fto* knockdown increases Akt phosphorylation of Ser473 in 3T3-L1 cells. This finding is consistent with the decreased *Pten* expression we observed following *Fto* knockdown. We also observed increased phosphorylation of Akt at Ser473 in 3T3-L1 preadipocytes after 4hr serum starvation and after serum starvation plus exposure to 1µg/ml insulin for 15 mins (**Fig. 4.6A**). This effect was seen in both the Akt1 and Akt2 isoform (**Fig. 4.6B**&**C**, **Sup. Fig. 4.4A**&**B**), and elevated pAkt/Akt ratios were sustained until the cells became mature adipocytes (**Fig. 4.6D**, **Sup. Fig. 4.4C**). Foxo1 is also phosphorylated via the Akt signaling pathway (Guo, Rena et al. 1999, Jackson, Kreisberg et al. 2000), and pFoxo1/Foxo1 ratios were elevated in 3T3-L1 adipocytes in response to insulin (**Fig. 4.6F**, **Sup. Fig. 4.4D**).

Insulin regulates adiponectin production via a PI3K-dependent mechanism (Blumer, van Roomen et al. 2008). The increased pAkt signaling we observed following *Fto* knockdown was associated with an increase in adiponectin protein levels (**Fig. 4.6G**, **Sup. Fig. 4.4E**). In *Fto*-knockdown 3T3-L1 adipocytes, adiponectin was secreted into the media in almost the same quantity as in the non-targeted control knockdown condition (**Fig. 4.6H**), despite the knockdown cells exhibiting roughly half the adipogenesis as controls.

Increased Akt signaling in the *Fto* knockdown condition was not associated with an increase in glucose uptake in either the serum/glucose-starved or insulin treated state. Uptake of 2-deoxyglucose, a non-metabolizable glucose analog, into 3T3-L1 preadipocytes was decreased in the *Fto* knockdown condition (**Fig. 4.6I**). This result is consistent with the decrease in *Glut4*

expression described above (**Fig. 4.1D**). Additionally, expression of the non-insulin sensitive glucose transporter, *Glut2* (*Slc2a1*), was decreased by *Fto* knockdown in both 3T3-L1 preadipocytes and mature adipocytes (**Fig. 4.6J**). *Fto*/*Ptprv* knockdown did not restore *Pten* expression (**Sup. Fig. 4.2C**), suggesting that *Pten* is not regulated by Fto by the same mechanism as *Ptprv* and *Spon2*.

Fto knockdown in mature adipocytes leads to impaired adipocyte lipid handling and endocrine function.

We and others have demonstrated an impairment in the ability of cells to undergo adipogenesis when *Fto* expression is decreased prior to differentiation (Zhao, Yang et al. 2014, Merkestein, Laber et al. 2015). *Fto* expression increases as 3T3-L1 and human ASC preadipocytes differentiate into mature adipocytes (**Fig. 4.1A**&**F**). We collagenase digested perigonadal and subcutaneous adipose tissue of 4-month-old male C57BL/6 mice to isolate mature adipocytes and preadipocyte-containing stromal vascular cells (SVCs). *Fto* expression in mature adipocytes was approximately 3-fold higher than that in the SVCs (**Fig. 4.7A**) suggesting that Fto may play a more substantial role in mature, lipid filled cells. We assessed knockdown of *Fto* in mature (8-10 days post differentiation) 3T3-L1 adipocytes (**Fig. 4.7B**) to understand whether Fto affects adipocyte function after cells have undergone differentiation. Expression of developmental (*Cebpd*, *Pparg2* and *Cebpa*) and functional (*Fabp4*, *Glut4*, *Plin1*, *Lpl* and *Adipoq*) adipocyte genes were decreased in the *Fto* knockdown condition, with no change in *Cebpb* expression (**Fig. 4.7C**); however, there was no detectable difference in lipid content by staining with Oil Red O (**Fig. 4.7D**&**E**). We did not observe any change in total cell number (**Fig. 4.7F**) or the percentage (**Fig. 4.7G**) or number (**Fig. 4.7H**) of Nile Red positive cells

reported by flow cytometry. There was a modest decrease, however, in the amount of Nile Red staining per cell (**Fig. 4.7I**) and a decrease in the amount of adiponectin (**Fig. 4.7J**&**K**) secreted into the cell culture media per adipocyte. There was no difference in the amount of leptin expressed (**Fig. 4.7C**) or secreted per cell (**Fig. 4.7L**&**M**). There was no difference in Akt phosphorylation in the *Fto* knockdown condition following insulin treatment (Data not shown). This finding is consistent with the lack of effect of *Fto* knockdown in mature adipocytes on *Pten* (**Fig. 4.7N**) expression.

Discussion

The molecular bases for the strong association of sequence variants in intron one of *FTO* with human adiposity remain unclear. *FTO* itself, and nearby *RPGRIP1L*, *IRX3*, and *IRX5*, have all been implicated by mechanisms relating to food intake, adipocyte differentiation and energy expenditure. Here, we provide evidence that FTO plays a permissive role in *in vitro* adipogenesis in both murine 3T3-L1 cells and human adipose-derived stromal cells.

In humans, the effects of intronic alleles of *FTO* are conveyed primarily by effects on food intake (Cecil, Tavendale et al. 2008, Speakman, Rance et al. 2008, Wardle, Carnell et al. 2008, Tanofsky-Kraff, Han et al. 2009), though not necessarily by the FTO molecule itself (Smemo, Tena et al. 2014, Stratigopoulos, Martin Carli et al. 2014, Claussnitzer, Dankel et al. 2015). Adipose tissue mass is determined by complex interactions of cell autonomous, metabolic and behavioral processes. Adipose tissue may affect metabolic and behavioral phenotypes by hormonal secretions (leptin, adiponectin, TNF-α), effects on circulating metabolites (free fatty acids), and possibly by direct neural interactions with the CNS (via sympathetic innervation of white adipose tissue (Bartness, Liu et al. 2014)). Thus, genetic effects on adipose tissue development and/or function can have consequences for adiposity by both simple anatomic and indirect systemic effects.

Previous studies have not implicated FTO as a primary regulator of adipogenesis in human cells (Tews, Fischer-Posovszky et al. 2013). However, the cells used for these experiments, SGBS preadipocytes, were derived from a patient with X-linked Simpson-Golabi-Behmel (SGBS) syndrome (Fischer-Posovszky, Newell et al. 2008). SGBS is characterized as an overgrowth syndrome caused by mutations in the gene encoding glypican-3, which regulates signaling in response to growth factors such as Wnts, Hedgehogs, FGFs and BMPs (Filmus,

Capurro et al. 2008, Tenorio, Arias et al. 2014). Our data, and those of others (Merkestein, Laber et al. 2015, Chen, Zhou et al. 2016, Jiao, Zhang et al. 2016), indicate that Fto positively regulates adipocyte proliferation. The proliferative decrease in the *FTO* knockdown condition might have been circumvented in SGBS cells which are hyper-responsive to growth signals.

An *in vivo* adipose tissue-specific role for *Fto* remains unclear, although the effect of *Fto* knockdown in *in vitro* models is striking, and suggests an adipocyte-autonomous effect of Fto. *Fto* knockout mice exhibit decreased body weight and adiposity as well as protection from diet-induced obesity late in life (Church, Lee et al. 2009, Fischer, Koch et al. 2009, Ronkainen, Huusko et al. 2015). However, there is no deficiency of fat tissue in young animals; there is, in fact, an increase in adiposity relative to body mass (G. Stratigopoulos, personal communication (Gao, Shin et al. 2010, McMurray, Church et al. 2013)). Conversely, overexpression of *Fto* increases adiposity which is accentuated by high fat diet feeding (Church, Moir et al. 2010). Growth retardation and postnatal lethality occurs with loss of *Fto* in mice (Fischer, Koch et al. 2009, Gao, Shin et al. 2010, McMurray, Church et al. 2013), consistent with the human polymalformation syndrome seen in individuals homozygous for loss-of-function mutations in *FTO* (Boissel, Reish et al. 2009, Daoud, Zhang et al. 2016, Rohena, Lawson et al. 2016). We suggest that this early increase in adiposity is due to the hyperphagia resulting from a reduction of *Fto* expression centrally. The decrease in total body weight, due to decreased lean mass, of *Fto* knockout animals is recapitulated in a neuron-specific *Fto* knockout model (utilizing Nestin-Cre), although these latter mice exhibit a secondary increase in percent body fat, indicative of positive energy balance (Gao, Shin et al. 2010). Additionally, the downregulation in *Rpgrip1l* expression in preadipocytes due to loss of *Fto*, which only occurs in murine (3T3-L1) cells and not in human ASCs, could – during embryonic development – create the cellular anlagen for

subsequent increased adiposity. While the effect of decreased *Fto* expression *in vitro* (inhibited adipogenesis) supercedes the effect of decreased *Rpgrip1l* expression (enhanced adipogenesis), there are likely to be many factors to which preadipocytes may be sensitive to *in vivo* that were not represented in cell culture that may alter this balance between the effects of these co-regulated genes.

We suggest that the late loss of fat mass in $Fto^{-/-}$ mice is a form of lipodystrophy, with impaired lipid handling and endocrine function in adipocytes. *Fto* expression is increased during adipogenesis, and this correlation could point to its primary function in mature adipocytes. We tested the role of Fto in mature adipocytes by knocking it down in mature 3T3-L1 cells after differentiation. We observed decreased expression of developmental genes (*Cebpd, Pparg* and *Cebpa*) glucose (*Glut4*) and fatty acid and lipid uptake (*Fabp4* and *Lpl*) genes in addition to decreased lipid content. Adiponectin expression and secretion were also decreased. Although we did not observe a significant decrease in leptin produced per cell, 3T3-L1 adipocytes in culture express low levels of leptin (Slieker, Sloop et al. 1998), making them unreliable reporters of leptin production *in vivo*. Adiponectin itself may also impact food intake, although overexpression of adiponectin in mice has had divergent effects on body weight and food intake (Qi, Takahashi et al. 2004, Kim, van de Wall et al. 2007, Kubota, Yano et al. 2007). Mice segregating for a dominant negative mutation of *Fto*, while protected from diet-induced obesity, exhibit metabolic defects consistent with a lipodystrophic phenotype; namely increased circulating glucose, triglycerides and cholesterol (Church, Lee et al. 2009). Growing evidence, including a recent meta-analysis, has suggested that there may be some contribution of the *FTO* locus to diabetes beyond its effect on adiposity (Hertel, Johansson et al. 2011, Li, Kilpelainen et al. 2012, Fall, Hagg et al. 2013, Yang, Liu et al. 2017). With risk alleles of rs8050136 and

rs1421085 decreasing expression of *FTO* by limiting binding of the activator form of CUX1, p110, adipocytes might be limited in their ability to properly store lipid, leading to ectopic lipid accumulation and adverse metabolic consequences (Unger and Scherer 2010, Virtue and Vidal-Puig 2010).

Fto has been shown to be decreased upon fasting in mesenteric adipose tissue, likely due to the decreased enzymatic activity of Cathepsin L, the proteolytic enzyme responsible for processing full length Cux1 p200 into p110 (Stratigopoulos, LeDuc et al. 2011). We anticipate that adipocytes in mesenteric adipose tissue might be especially responsive to the presence of *FTO* risk alleles, thereby reducing *FTO* expression. This decreased expression of *FTO* might allow normal to increased lipid handling in subcutaneous adipose depots, while rendering adipocytes of the mesenteric depot incapable of performing their lipid handling duties, which are already functionally impaired, compared to other depots (Karastergiou, Smith et al. 2012). These effects could explain the association of the *FTO* obesity locus to type 2 diabetes, beyond that attributable to elevated body weight.

We performed RNAseq in preadipocytes to identify primary regulators of Fto's effect on adipogenesis and found that C/ebpβ and C/ebpδ-mediated transcription, required for adipogenesis, is dramatically altered. Fto has been identified as an RNA demethylase of m6A modifications (Gerken, Girard et al. 2007, Jia, Fu et al. 2011) and the locations and functions of m6A modifications have been increasingly well described (Dominissini, Moshitch-Moshkovitz et al. 2012, Meyer, Saletore et al. 2012, Dominissini, Moshitch-Moshkovitz et al. 2013, Meyer, Patil et al. 2015). Although the demethylase function of Fto is clearly required for its effects on adipogenesis (Zhao, Yang et al. 2014, Merkestein, Laber et al. 2015, Wang, Zhu et al. 2015, Zhang, Zhang et al. 2015), we did not identify any changes by m6A-RIP in the m6A methylation

status of candidates most highly regulated by *Fto* knockdown. It is possible we were unable to detect differences in m6Am, which Fto preferentially demethylates, among a large number of m6A moieties (Mauer, Luo et al. 2016). Additionally, we did not observe any effects on Fto-regulated targets which would be predicted by the purported mechanisms of m6A demethylation, such as changes in transcript stability or translational differences. We did not test for variations in splicing, but the most promising candidates, *Cebpb* and *Cebpd*, are monoexonic genes that lack splice variants. Also, we were unable to recapitulate previous results indicating that *Runx1t1* is differentially spliced in the *Fto* knockdown condition (Zhao, Yang et al. 2014, Merkestein and Sellayah 2015). Our results are consistent with the report by Wu *et. al.* (Wu, Saunders et al. 2010) identifying Fto as a transcriptional coactivator that facilitates C/ebpβ transactivation of the unmethylated C/ebp-response element (CEBPRE). Additionally, Fto activates C/ebpβ-driven transcription from methyl-inhibited CEBPREs. Whether Fto demethylates CpGs in CEBPREs directly or m6A modifications of transcripts which modify CEBPRE methylation remains unclear.

We also identified *Ptprv* and *Spon2* as effectors of Fto's effect on proliferation. Although the human homolog of murine *Ptprv*, *PTPRVP*, is a pseudogene (Cousin, Courseaux et al. 2004), and undetectable by qPCR, other effectors of cell cycle arrest – *GADD45A*, *MDM2* and *CDKN1A* – are upregulated in human ASCs following *FTO* knockdown. C/ebpβ has been demonstrated to inhibit expression of the tumor suppressor p53 (Ewing, Zhu et al. 2008), which drives expression of *Ptprv* (Doumont, Martoriati et al. 2005) suggesting that the upregulation of *Ptprv* after *Fto* knockdown is an effect of impaired C/ebpβ function. *SPON2*, a gene encoding an extracellular matrix protein (Feinstein, Borrell et al. 1999) is upregulated in human ASCs upon FTO knockdown. Spon2 *upregulation* is due to elevated *Ptprv* expression in 3T3-L1 cells

following *Fto* knockdown, suggesting that a similar pathway is being regulated by FTO in human cells. We suggest that this pathway may contribute to the severe malformations evident in humans with *FTO* loss-of-function mutations, and in the runting of $Fto^{-/-}$ mice.

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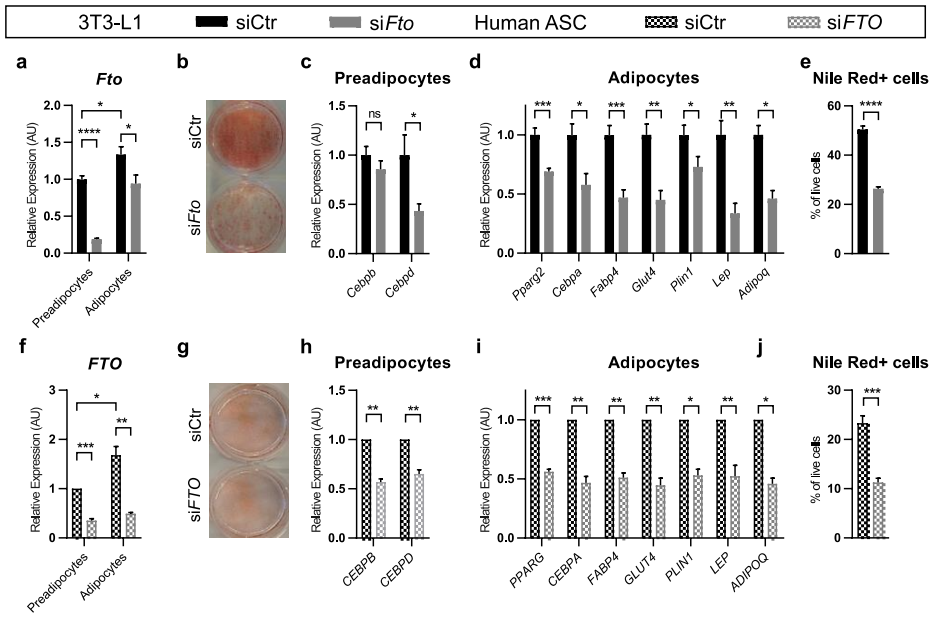
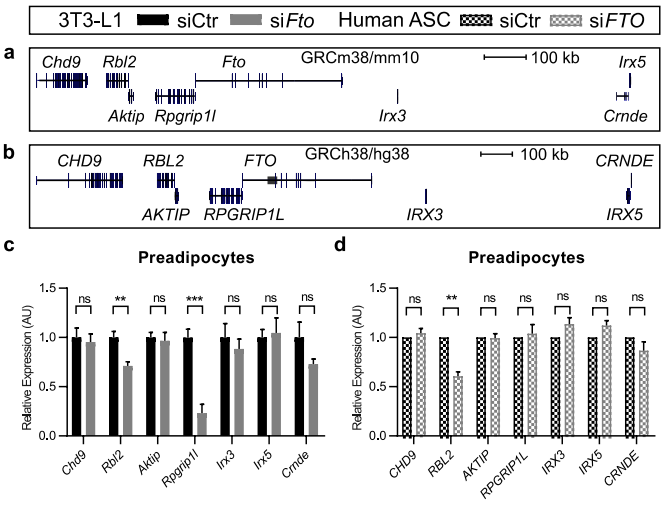
Figures

Figure 4.1 *Knockdown of Fto/FTO prior to differentiation impairs adipogenesis in 3T3-L1 adipocytes and human adipose tissue-derived stromal cells (ASCs).*



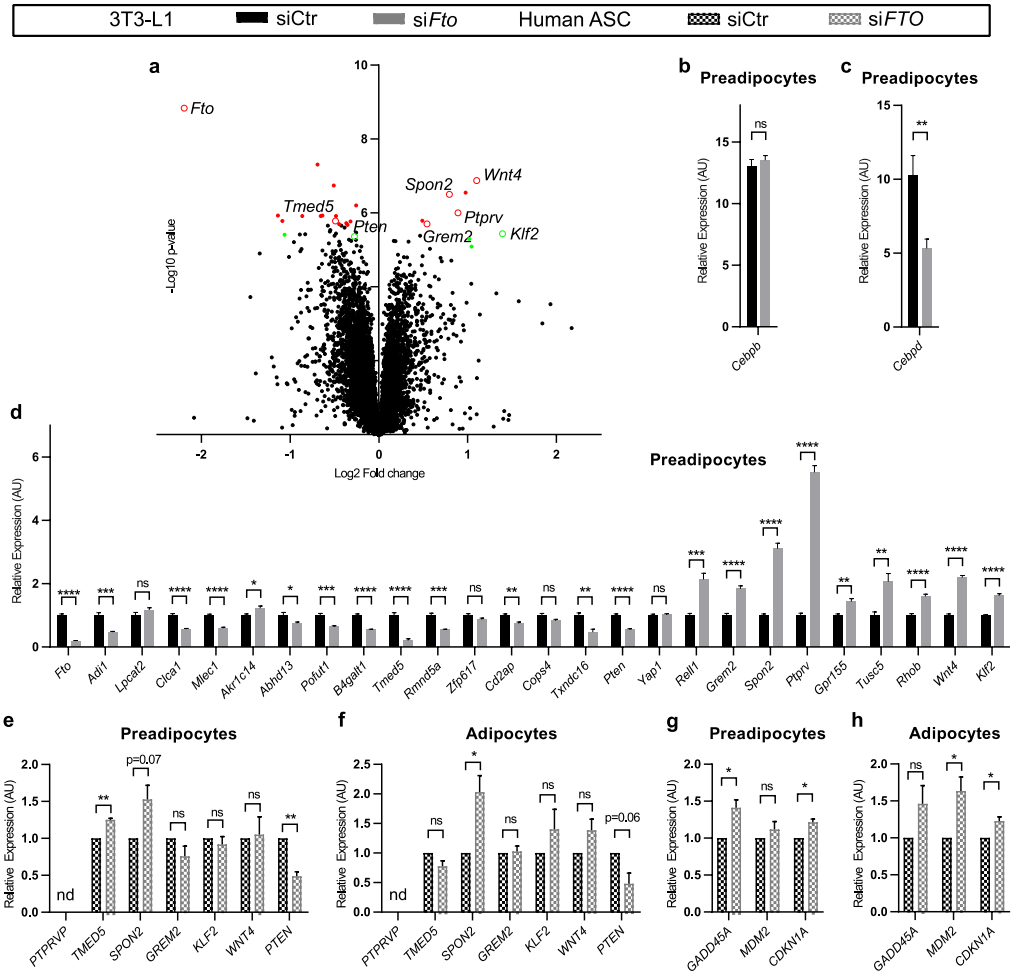
Results in upper panels (**A**-**E**) are from murine 3T3-L1 cells treated prior to confluence with siRNA targeted against *Fto* (si*Fto*; dark gray bars) vs non-targeted controls (siCtr; black bars). Results in lower panels (**F**-**J**) are from human ASCs treated at confluence with siRNA targeted against *FTO* (si*FTO*; dark gray checkered bars) vs non-targeted controls (siCtr; black checkered bars). (**A**&**F**) qPCR analysis of *Fto/FTO* transcript levels in preadipocytes (2 days post-confluence) and mature adipocytes (3T3-L1: 2-day post-confluent cells differentiated with insulin, dexamethasone and 3-Isobutyl-1-methylxanthine (IBMX) for 2 days, followed by maintenance for 5-9 days with insulin only; human ASCs: 2-day post-confluent cells incubated with differentiation cocktail for 7 days, followed by maintenance media for 3-7 days (Lee and Fried 2014)) after treatment with siRNA targeted against *Fto/FTO*. (**B**&**G**) Representative images of Oil Red O staining of adipocytes for neutral lipid. mRNA transcript levels of adipocyte developmental and functional genes (**C**&**H**) *Cebpb/CEBPB* and *Cebpd/CEBPD* in preadipocytes and (**D**&**I**) *Pparg/PPARG*, *Cebpa/CEBPA*, *Fabp4/FABP4*, *Glut4/GLUT4*, *Plin1/PLIN1*, *Lep/LEP*, and *AdipoQ/ADIPOQ* in mature adipocytes. (**E**&**J**) Percentage of live cells positive for Nile Red staining for intracellular lipid analyzed by flow cytometry. mRNA expression has been normalized to *Rplp0 or RPLP0*. For human ASCs, results are replicated in cells from 4 individuals total (2 females and 2 males, adults) and mRNA expression is represented as the mean (± SEM) of data points from all four individuals where each data point is the mean of 4-6 technical replicates; paired t-test. All other plots represent means (± SEM) for n=6 per condition; unpaired t-test. * $p<0.05$, ** $p<0.01$, ***$p<0.001$, ****$p<0.0001$

Figure 4.2 *Fto knockdown decreases expression of proximate genes Rbl2 and Rpgrip1l in 3T3-L1 cells but only affects RBL2 in human ASCs.*



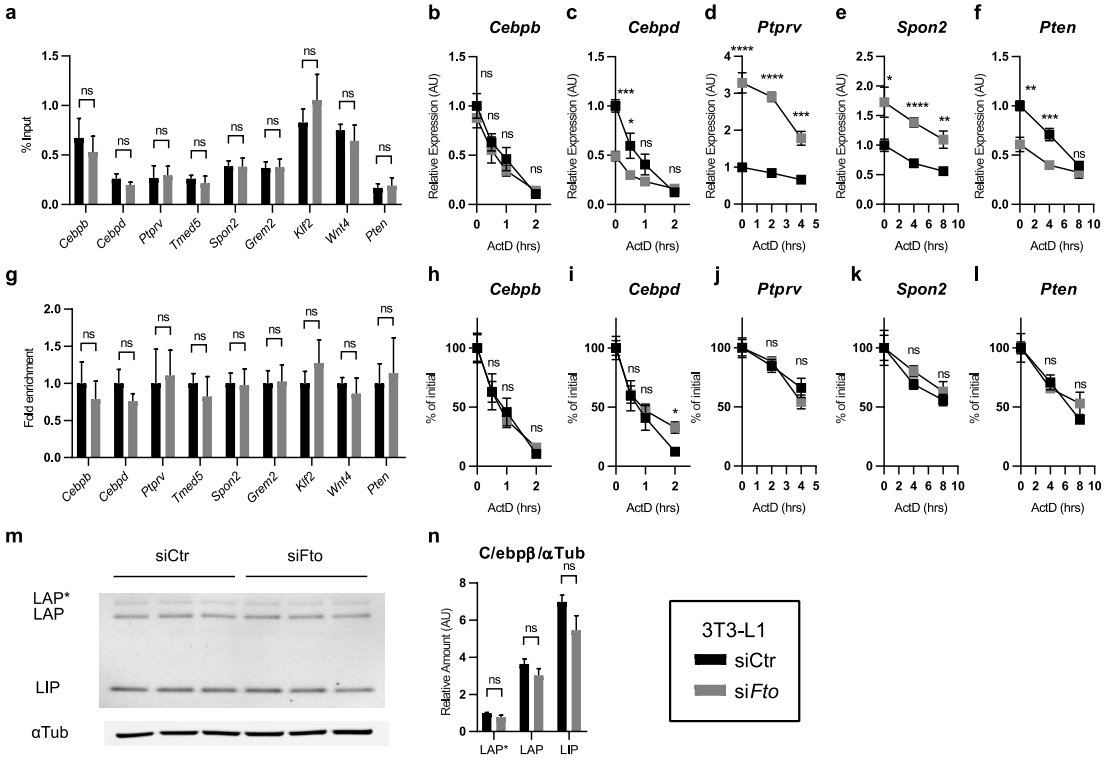
Map of the murine (**A**) and human (**B**) *Fto*/*FTO* gene and vicinal genes created from the GRCm38/mm10 and GRCh38/hg38 assemblies using the UCSC Genome browser: http://genome.ucsc.edu (Kent, Sugnet et al. 2002). Region of linkage disequilibrium surrounding the most significant SNPs shaded gray (Loos and Yeo 2014). qPCR analysis of expression of genes vicinal to *Fto*/*FTO* in 3T3-L1 (**C**; n=6, unpaired t-test) and human ASC (**D**) preadipocytes following *Fto*/*FTO* knockdown. mRNA expression has been normalized to *Rplp0 or RPLP0*. For human ASCs, results have been replicated in cells from 4 individuals total (2 females and 2 males) and mRNA expression is represented as the mean (± SEM) of datapoints from all four individuals where each datapoint is the mean of 4-6 technical replicates; paired t-test. ** $p<0.01$, ***$p<0.001$

Figure 4.3 *Identification of Fto-targeted genes in 3T3-L1 preadipocytes.*



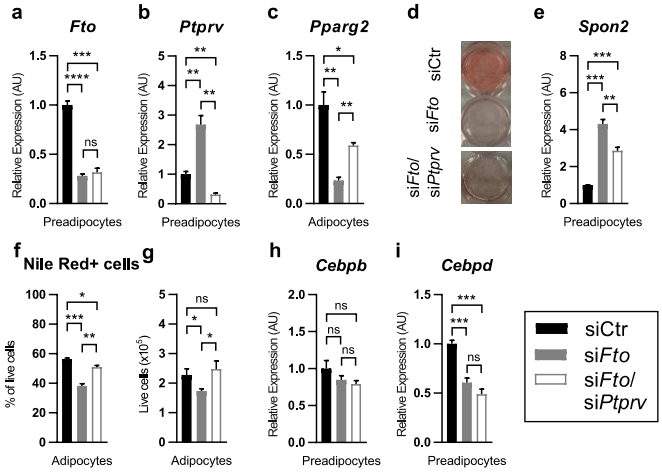
Volcano plot (**A**) of RNAseq analysis of 2-day post-confluent 3T3-L1 preadipocytes following *Fto* knockdown. Differentially expressed genes ($p<0.05$ following Bonferroni correction for multiple testing) are marked in red. Genes with ≥2-fold increase or ≤0.5-fold decrease and unadjusted $p<10^{-5}$ indicated in green. Peak mRNA expression of *Cebpb* (2 hrs; **B**) and *Cebpd* (1 hr; **C**) after adding differentiation media to 2-day post-confluent 3T3-L1 preadipocytes. qPCR confirmation (**D**) of the most significantly up- or downregulated candidate genes in post-confluent 3T3-L1 preadipocytes, following knockdown of *Fto* vs non-targeted controls (n=6; unpaired t-test; representative of 2 independent experiments); listed in order of effect size observed by RNAseq (most downregulated to most upregulated). Expression of homologs of Fto-targeted genes in human ASC preadipocytes (**E**) and mature adipocytes (**F**) following *FTO* knockdown identified by RNAseq in murine 3T3-L1 preadipocytes. Expression of cell cycle arrest genes in human ASC (**G**) preadipocytes and (**H**) mature adipocytes. nd=none detected. Three different primer pairs were used to examine PTPRVP expression in human ASCs. mRNA expression has been normalized to *RPLP0* and is represented as the mean (± SEM) of data points from four individuals (2 females and 2 males, adults) where each data point is the mean of 6 technical replicates; paired t-test. Plots represent mean (± SEM); * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$

Figure 4.4 *Candidate genes regulated by Fto do not exhibit altered m6A patterns, transcript stability or translation in 3T3-L1 preadipocytes.*



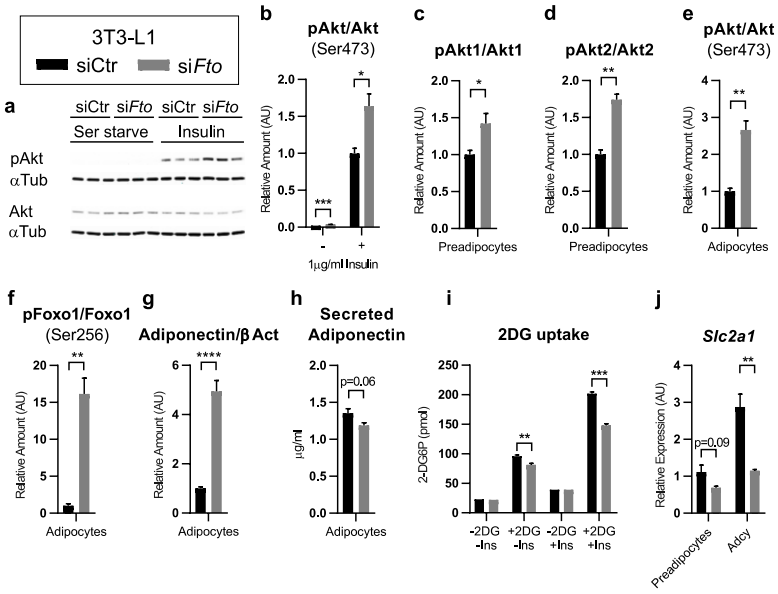
qPCR of Fto-regulated genes in 2-day post-confluent 3T3-L1 preadipocytes after pulldown with anti-m6A antibody following *Fto* knockdown, normalized to transcript levels in input sample (**A**) and calculated as fold-change (**B**; n=3-4 per condition). mRNA expression of *Cebpb* (**B**&**H**), *Cebpd* (**C**&**I**), *Ptprv* (**D**&**J**), *Spon2* (**E**&**K**) and *Pten* (**F**&**L**) after treatment with Actinomycin D (ActD) to inhibit transcription, reported as expression relative to *18s* rRNA levels (**B**-**F**) and calculated as a percentage of initial expression (**H**-**L**). Protein levels of C/ebpβ isoforms, measured by western blot (**M**), quantified in (**N**). All plots represent means (± SEM) for n=4-6 per condition except where noted; unpaired t-test. * p<0.05, ** p<0.01, ***p<0.001, ****p<0.0001

Figure 4.5 *Knockdown of siFto-upregulated Ptprv restores adipogenesis.*



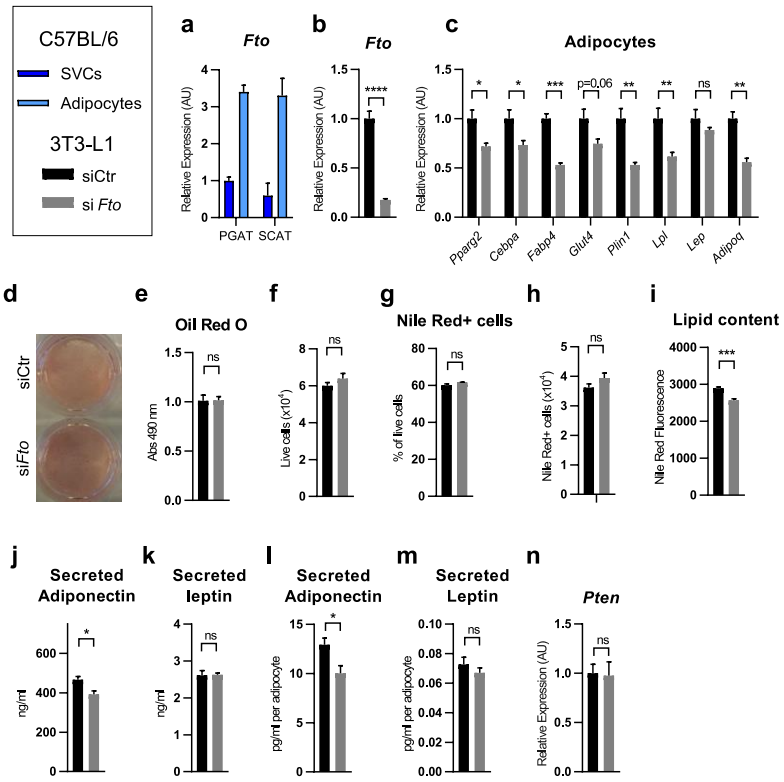
qPCR analysis of *Fto* (**A**), *Ptprv* (**B**), *Spon2* (**E**), *Cebpb* (**H**) and *Cebpd* (**I**) expression in 3T3-L1 preadipocytes and *Pparg2* (**C**) expression in mature adipocytes following single *Fto* knockdown (solid gray bars) or double *Fto*/*Ptprv* expression (open gray bars) prior to confluence and differentiation. Mature adipocytes stained for Oil Red O (**D**) and % of live cells positive for Nile Red staining analyzed by flow cytometry (**F**). Total number of live cells collected by flow cytometry (**G**) after differentiation. All plots represent means (± SEM) for n=3-4 per condition; unpaired t-test. * $p<0.05$, ** $p<0.01$, ***$p<0.001$, ****$p<0.0001$

Figure 4.6 *Fto knockdown increases Akt signaling but glucose uptake is impaired in 3T3-L1 cells.*



Western blot (**A**) and quantification (**B**) of phospho-Akt (Ser473) normalized to total Akt in 3T3-L1 preadipocytes after 4hrs of serum starvation followed by treatment with or without 1µg/ml insulin for 15 minutes. Quantification of western blots for phospho-Akt1/total Akt1 (**C**) and phospho-Akt2/ total Akt2 (**D**) in serum-starved preadipocytes treated with insulin for 15 mins. Quantification of western blots for phospho-Akt/total Akt (**E**) and phospho-Foxo1 (Ser256)/total Foxo1 (**F**) in mature 3T3-L1 adipocytes treated with 1µg/ml insulin for 15 mins. Quantification of western blot for adiponectin (**G**) in mature 3T3-L1 adipocytes and ELISA of adiponectin (**H**) secreted into 1ml of culture media 3 days after the previous media change (cells were cultured in a 12 well plate). 3T3-L1 preadipocytes (2 days post-confluence) were serum starved overnight and then glucose starved for 40 mins prior to stimulation with or without insulin (1µM) for 20 mins. This was followed by addition of 2-deoxyglucose (2-DG) for 20 mins (n=3 for 2-DG treated conditions, n=1 for 2-DG untreated negative controls). Cells were lysed and 2-DG concentrations were assayed (**I**). mRNA expression of *Slc2a1* (**J**) in 3T3-L1 preadipocytes and mature adipocytes. mRNA expression was normalized to *36B4*. pAkt (total, pAkt1 and pAkt2), Akt (total, Akt1 and Akt2), pFoxo1 and Foxo1 protein bands were first normalized to αTub before determining the ratios of phospho/total protein levels. Adiponectin bands were normalized to βAct. n=3-6 per condition except where indicated; unpaired t-test. * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$

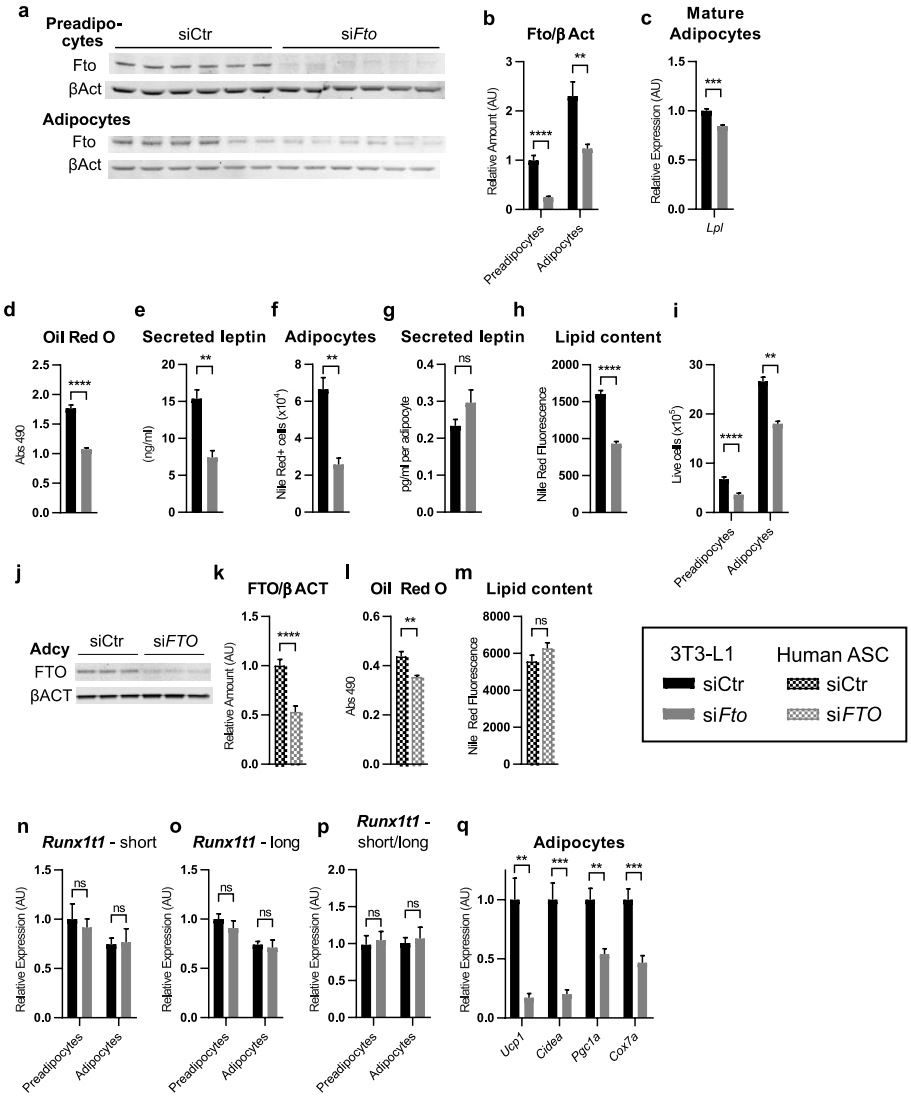
Figure 4.7 *Fto knockdown in mature 3T3-L1 cells decreases adipocyte lipid storage and endocrine function.*



Fto expression (**A**) in the isolated preadipocyte-containing stromal vascular fraction (SVCs; dark blue) and mature adipocytes (light blue) of perigonadal (PGAT) and subcutaneous (SCAT) adipose tissue of 4-month-old male C57BL/6 mice. mRNA expression of *Fto* (**B**) three days after electroporation of mature 3T3-L1 adipocytes with siRNA targeted against *Fto*. mRNA expression of *Pparg2*, *Cebpa*, *Fabp4*, *Glut4*, *Plin2*, *Lpl*, *Lep* and *Adipoq* (**C**). Representative image (**D**) and quantification (**E**) of Oil Red O lipid staining. Total number of live cells (**F**), percentage of cells stained positive for Nile Red (**G**), number of Nile Red positive cells (**H**) and amount of lipid per Nile Red positive cell (**I**) acquired by flow cytometry. Adiponectin (**J**) and leptin (**K**) secretion into cell culture media, and amount of adiponectin (**L**) and leptin (**M**) secreted, normalized to total number of adipocytes (**N**). mRNA expression of *Pten* after *Fto* knockdown. mRNA expression was normalized to *36B4* in in vitro experiments and to the arithmetic mean of *36B4*, *bAct*, *Gapdh* and *Ppia* in adipose tissue expression analyses. n=4-5 per condition; unpaired t-test. * $p<0.05$, ** $p<0.01$, ***$p<0.001$, ****$p<0.0001$

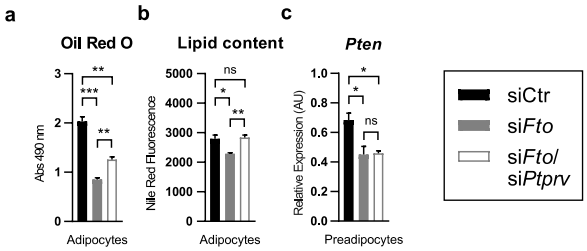
Supplementary Figures

Supplementary Figure 4.1 *Knockdown of Fto/FTO prior to differentiation impairs adipogenesis in 3T3-L1 adipocytes and human adipose stromal cells (ASCs) but does not affect Runx1t1 splicing or induce brown adipose tissue-associated gene expression in 3T3-L1 adipocytes.*



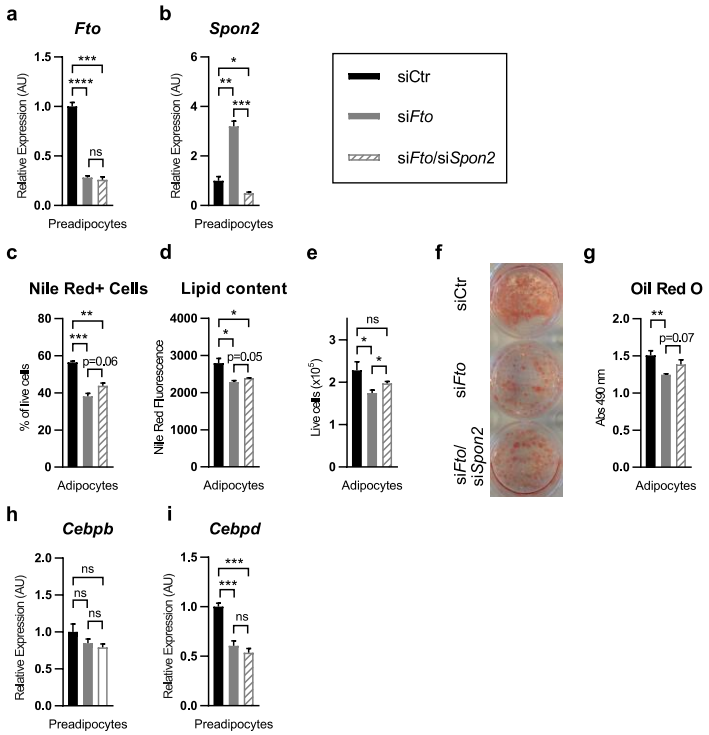
Representative images (**A**) and quantification (**B**) of western blots for Fto and bAct protein in 3T3-L1 preadipocytes and mature adipocytes following *Fto* knockdown prior to confluence. mRNA expression of *Lpl* in mature 3T3-L1 adipocytes (**C**). Quantification (**D**) by absorbance at 490 nm of extracted Oil Red O stain of mature adipocytes. ELISA analysis of total leptin secretion into media (**E**) by mature adipocytes and leptin secretion (**G**) normalized by number of adipocytes (determined by flow cytometry; **F**). Median Nile Red fluorescence intensity (**H**) of Nile Red positive cells and number of live cells (**I**) normalized to counting beads measured by flow cytometry in adipocytes. Representative images (**J**) and quantification (**K**; n=3/condition) of western blot for FTO and βACT protein in human ASCs differentiated into mature adipocytes following *FTO* knockdown. Quantification (**L**) by absorbance at 490 nm of extracted Oil Red O stain and median Nile Red fluorescence intensity (**M**) of Nile Red positive human ASCs. Transcript levels of short (**N**) and long (**O**) *Runx1t1* splice variants as well as the ratio of short/long *Runx1t1* (**P**) in 3T3-L1 preadipocytes and mature adipocytes. mRNA levels of brown adipocyte markers *Ucp1*, *Cidea*, *Pgc1a* and *Cox7a* in 3T3-L1 adipocytes (**Q**). RNA expression is normalized to *Rplp0*. Plots represent means (± SEM) for n=4-6 per condition except where noted; unpaired t-test.

Supplementary Figure 4.2 *Knockdown of Ptprv in siFto-treated 3T3-L1 adipocytes restores adipogenesis.*



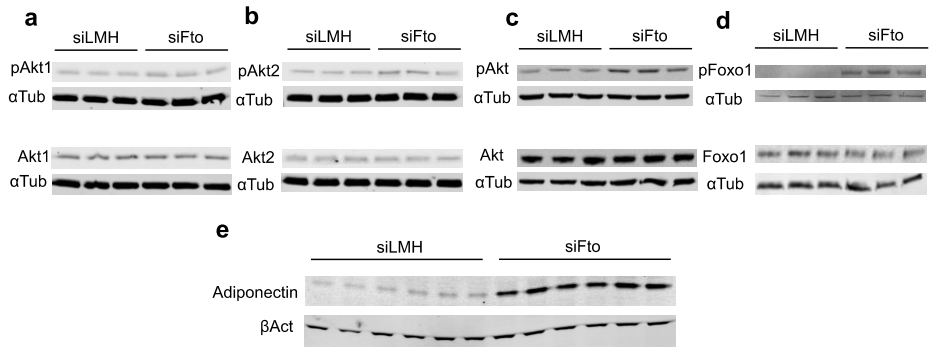
Quantification by absorbance at 490 nm of extracted Oil Red O stain (**A**) in mature 3T3-L1 adipocytes following single *Fto* knockdown (solid gray bars) or double *Fto*/*Ptprv* expression (open gray bars) prior to differentiation. Total number of live cells (**B**) normalized to counting beads measured by flow cytometry after differentiation. *Pten* (**C**) expression in 3T3-L1 preadipocytes. All plots represent means (± SEM) for n=3-4 per condition; unpaired t-test. * p<0.05, ** p<0.01, ***p<0.001, ****p<0.0001

Supplementary Figure 4.3 *Knockdown of Spon2 in siFto-treated 3T3-L1 adipocytes restores adipogenesis.*



qPCR analysis of *Fto* (**A**), *Spon2* (**B**), *Cebpb* (**H**) and *Cebpd* (**I**) expression in 3T3-L1 preadipocytes following single *Fto* knockdown (solid gray bars) or double *Fto*/*Spon2* expression (hatched gray bars) prior to confluence and differentiation. Percentage of mature adipocytes stained for Nile Red (**C**) and median Nile Red fluorescence in the adipocyte population (**D**) analyzed by flow cytometry. Total number of live cells collected by flow cytometry (**E**) after differentiation. Representative image (**F**) and quantification (**G**) of Oil Red O staining of mature adipocytes. All plots represent means (± SEM) for n=3-4 per condition; unpaired t-test. * $p<0.05$, ** $p<0.01$, ***$p<0.001$, ****$p<0.0001$

Supplementary Figure 4.4 *Fto knockdown increases Akt signaling in 3T3-L1 cells.*



Western blots of phospho-Akt1, total Akt1 and αTub (**A**) as well as phospho-Akt2, total Akt2 and αTub (**B**) in 3T3-L1 preadipocytes after 4hrs of serum starvation followed by treatment with or without 1µg/ml insulin for 15 minutes. Western blots for phospho-Akt (Ser473), total Akt and αTub (**C**) and phospho-Foxo1 (Ser256), total Foxo1 and αTub (**D**) in mature 3T3-L1 adipocytes treated with 1µg/ml insulin for 15 mins. Western blot for adiponectin and βAct (**E**) in mature 3T3-L1 adipocytes. n=3-6 per condition.

Supplementary Tables

Supplementary Table 4.1 *qPCR primers Mm*

Mm:	**Forward**	**Reverse**
36B4 (Rplp0)	ACCTCCTTCTTCCAGGCTTTGG	CGAAGGAGAAGGGGGAGATGTT
ActB	CGGGCTGTATTCCCCTCCAT	GGGCCTCGTCACCCACATAG
Gapdh	CTGGAGAAACCTGCCAAGTATGATG	GAGACAACCTGGTCCTCAGTGTAGC
Ppia	CTTCGAGCTGTTTGCAGACAAAGTT	GGAGGAACCCTTATAGCCAAATCCT
Fto	GGCGGCTTTAGTAGCAGCAT	CCAAGTGTCTTCAAGCTCCTC
Cebpb	CAAGCTGAGCGACGAGTACA	AGCTGCTCCACCTTCTTCTG
Cebpd	GACGAGAGCGCCATCGACTT	CCGCTTTGTGGTTGCTGTTG
Pparg2	TTTGAAAGAAGCGGTGAACCA	CGAAGTTGGTGGGCCAGAAT
Cebpa	GCCATGCCGGGAGAACTCTA	GGGCTCTGGAGGTGACTGCT
Fabp4	TTGGTCACCATCCGGTCAGA	TCCACCACCAGCTTGTCACC
Glut4	AGCTGTGCTTGGCTCCCTTC	CCCAGCCACGTTGCATTGTA
Plin1	GTGTACAGGGTGCCAGCAA	CTCTGCAGGCCAACTCATTG
Lep	CGAGGAATCGTTCTGCAAATCC	GCCAGGTTAAGTGCAGCTATCACA
Adipoq	CAGGCCGTGATGGCAGAGAT	GTGGCCCTTCAGCTCCTGTC
Lpl	CCACAGCAGCAAGACCTTCG	TACAGGGCGGCCACAAGTTT
Chd9	CCCCACTGTCTACCAACCAT	TGTCCCAGAACCATCACTCT
Rbl2	TACCGCAGCATGAGCGAGAG	GCGGTCCCTTTGCTCACAGT
Aktip	GAACCCTTTGTGGAGCATGT	TTCGTGGAGGACTGGTTTTC
Rpgrip1l	ATTGCAGCGTGTCAGTTGAG	TGCTGACTCGACAATTGCAT
Irx3	AGCTGCCCATCTTCCCACAG	CGGGTCCCCGAACTGGTACT
Irx5	CCGGCTACAACTCGCACCTC	CCAAGGAACCTGCCATACCG
Crnde	GTCCAGGACGAAGACTTCTGA	CCTCCAAACATGACACCAGC
Adi1	CTATCACCGCTTCACACTGGA	GGCCGGTTGTATGGTGTCC
Lpcat2	GTCCAGCAGACTACGATCAGT	GCAGCAAAATTATTCCAACCAGT
Clca1	GAAGCAACAAGGTGTTCCACCA	GCACGCCCTTGTGACACAGT
Mlec1	AGCCTCGGACTATGGCATGA	TGTACCGCTCTGTCTGATATAGG
Akr1c14	GTGTGGTACTAAACGATGGTCAC	CAAATAAGCGGAGTCAAAATGGC
Abhd13	TTCCCGCCTTTATGTTCCCAT	TGTCTCCAGTGTATCTCACCAA
Pofut1	CGGCCCTATGTGGGCATTC	AAGCCATGAAGTGTGACCCTG
B4galt1	CTGGCTGTATTATTTGCATCCCA	AGCTCGATTGAACATGGTGTC
Tmed5	CAAGGCCAAGAAGACTGGAA	TTGTATGTGGCCACTTTTGC
Rmnd5a	TGCGAAAAATTTCCAGCCATT	CCCTTGTCGCAGGTACACGA
Zfp617	AGGACAGAAATTGGGAGAACAGA	TACACATGGCTTTCACAGGGC

Cd2ap	CTCTCCACAAAATGAGGACGAA	CTTATTGTTCAGGGTTCCACTCC
Cops4	TCTGGCGGGCAAATATCGTC	GATCACGAGGCTGACATTCTC
Txndc16	AGAAGCGTCAAGGTACTGTGG	GAAGGTCTTCCAGGGTGGTAA
Pten	ACACCGCCAAATTTAACTGC	CGTCCCTTTCCAGCTTTACA
Yap1	TGAGATCCCTGATGATGTACCAC	TGTTGTTGTCTGATCGTTGTGAT
Rell1	GGTCGGCCAGATTGTCCAG	TGACAGGGCTTTCAATATCGC
Grem2	GGTAGCTGAAACACGGAAGAA	TCTTGCACCAGTCACTCTTGA
Spon2	ACCGGGCAGACTTCCACAGA	AGTCAGGGCTGGGGACAATG
Ptprv	AGGTAGTCAGTATGTCTTGGAGG	GGCACGCAGTGTTAGGTGA
Grpr155	CTTGTGGGCGTGCTTCTGATA	TGACACCCCAGCTATGAGAATG
Tusc5	CCTTGCCATTGCCTCTTGCT	GGTCCCCCTGCTGCACACTA
Rhob	GTGCCTGCTGATCGTGTTCA	CCGAGAAGCACATAAGGATGAC
Wnt4	GCCGGGCACTCATGAATCTT	CACCCCGTGACACTTGCACT
Klf2	CTCGCACCTAAAGGCGCATC	GTGGCGGGTAAGCTCGTCAG
Slc2a1	TTCTCTGTCGGCCTCTTTGT	GAGAAGCCCATAAGCACAGC
Runx1t1 S	GCGAACTCCAGACAGGGTTACATGG	CCACCATGTCCATGATGCAG
Runx1t1 L	GAACTCCAGACAGAACCAAAGAAAAT	CAGGCCATTTGGCTGGTAGG
Ucp1	CGGGCATTCAGAGGCAAATC	ATGGCTCTGGGCTTGCATTC
Cidea	AGTAGCCGGCGTGGGGTGAT	CCTCCAGCACCAGCGTAACC
Pgc1a	ACCAGCCTCTTTGCCCAGAT	CCGCTAGCAAGTTTGCCTCA
Cox7a	CAGGCTCTGGTCCGGTCTTTT	TCCCCCGCCTTTCAAGTGTA
18S	GTAACCCGTTGAACCCCATT	CCATCCAATCGGTAGTAGCG

Supplementary Table 4.2 *qPCR primers Hs*

Hs:	**Forward**	**Reverse**
36B4 (RPLP0)	GAAGACAGGGCGACCTGGAA	TCTGCTTGGAGCCCACATTG
FTO	GACTGCCGAGGAACGAGAGC	GGGGTCAGATAAGGGAGCCAAG
CEBPB	CAAGAAGACCGTGGACAAGC	AGCTGCTCCACCTTCTTCTG
CEBPD	ATCGACTTCAGCGCCTACAT	CGCCTTGTGATTGCTGTTGA
PPARG	TCATGCTTGTGAAGGATGCAA	TGTGGATCCGACAGTTAAGATCACAT
CEBPA	GCCATGCCGGGAGAACTCTA	TCTGCAGGTGGCTGCTCATC
FABL4	CACAAAATGTGTGATGCTTTTGTAGG	GCCACTTTCCTGGTGGCAAA
GLUT4	GTGCTTGGCTCCCTGCAGTT	CTCCCCAGCCACGTCTCATT
PLIN1	TGCAGAGCGCCAGTAGCTTG	GCGGGGATCTTTTCCTCCAG
LEP	TGACACCAAAACCCTCATCAAGA	GGTGGAGCCCAGGAATGAAG
ADIPOQ	CGGGATTTCACCATGTTGTCC	TCATGACCGGGCAGAGCTAA
CHD9	CAAAGTCAGGCTCGGAGTTG	CCCAGAACCATCGCTCTTCT
RBL2	ACGCTGGAGGGAAATGATCT	TGCCATGTCTTCCCACTTCT
AKTIP	AGGCTGGTGGGTAAAGTGAA	TTCTTTGGTGCAGTTCGTGG
RPGRIP1L	ACTGCAGGAGACTTGCCTGT	CCAGTTCCTCACGACTGACA
IRX3	GGAGCTGCCCATCTTCCC	CATACGGGTAGAAGGCGGG
IRX5	ACCTGGAGAAGAACGACGAG	GCGGCTCCTTAAAATCCGAG
CRNDE	TTTCCGGAGTAGAGCCCTTG	TTTCCAGTGGCATCCTCCTT
PTPRVP (Cousin, Courseaux et al. 2004)	CCTAGAATCCCAGACATTGGCA	GCTGGTTGTTGCTTGGAGGTT
PTPRVP (Doumont, Martoriati et al. 2005)	GTTGATGCCTTACAACCTGTGGCG	AGCTGCTTCACGCGCCTCTGTT
PTPRVP	ACACCTGGATGCAACCTCTC	GGTGTAGCCTGGGATGAAGT
TMED5	GGGAGAACAGGCACAAGAAC	TGATGGATTCCAGGATGTCT
SPON2	CGCTGATGAAGGAGATCGAG	ATGCGCACCACAAACGAG
GREM2	CAGAGAAGGCAGAGGGAGAG	CCAGGAACAAGGACAGGGAA
KLF2	TGCGGCAAGACCTACACCAA	GTCTGAGCGCGCAAACTTCC
WNT4	CCTTCGTGTACGCCATCTCT	TCAGAGCATCCTGACCACTG
PTEN	ACACCGCCAAATTTAATTGC	TAGGGCCTCTTGTGCCTTTA
GADD45A	ACGAGGACGACGACAGAGAT	GCAGGATCCTTCCATTGAGA
MDM2	ATGAAAGCCTGGCTCTGTGT	CCTGATCCAACCAATCACCT
CDKN1A	GACTCTCAGGGTCGAAAACG	GGATTAGGGCTTCCTCTTGG

Chapter 5 Genome-wide meta-analysis uncovers novel loci influencing circulating leptin levels

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Author contributions:

* These authors contributed equally to this work.

For a full list of authors please refer to:

https://www.nature.com/articles/ncomms10494#author-information

T.O.K. and R.J.F.L. conceived and designed the study. T.O.K. and Q.S. performed the meta-analyses. J.F.M.C., A.A.S., C.A.L., Y.Z. and R.L.L. carried out the knockdown studies in mouse adipose tissue explants. T.O.K., J.F.M.C., A.A.S., Q.S., J.K., M.F.F., P.W.F., C.M.L., R.L.L. and R.J.F.L. wrote the manuscript.

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https://www.nature.com/articles/ncomms10494#abstract

Abstract

Leptin is an adipocyte-secreted hormone, the circulating levels of which correlate closely with overall adiposity. Although rare mutations in the leptin (*LEP*) gene are well known to cause leptin deficiency and severe obesity, no common loci regulating circulating leptin levels have been uncovered. Therefore, we performed a genome-wide association study (GWAS) of circulating leptin levels from 32,161 individuals and followed up loci reaching $P<10^{-6}$ in 19,979

additional individuals. We identify five loci robustly associated ($P<5 \times 10^{-8}$) with leptin levels in/near *LEP*, *SLC32A1*, *GCKR*, *CCNL1* and *FTO*. Although the association of the *FTO* obesity locus with leptin levels is abolished by adjustment for BMI, associations of the four other loci are independent of adiposity. The *GCKR* locus was found associated with multiple metabolic traits in previous GWAS and the *CCNL1* locus with birth weight. Knockdown experiments in mouse adipose tissue explants show convincing evidence for *adipogenin*, a regulator of adipocyte differentiation, as the novel causal gene in the *SLC32A1* locus influencing leptin levels. Our findings provide novel insights into the regulation of leptin production by adipose tissue and open new avenues for examining the influence of variation in leptin levels on adiposity and metabolic health.

Introduction

Leptin is an adipocyte-secreted hormone that influences long-term regulation of energy homeostasis by informing the brain about the amount of stored body fat (Ahima, Prabakaran et al. 1996, Montague, Farooqi et al. 1997). Circulating leptin levels correlate closely with measures of adiposity, such as body fat mass and body mass index (BMI) (Shah and Braverman 2012). Yet, at any given level of adiposity, there is substantial variation in circulating leptin levels (Considine, Sinha et al. 1996), of which estimated 30–50% is explained by genetic factors (Narkiewicz, Szczech et al. 1999, Rice, Chagnon et al. 2002, Liu, Butler et al. 2010).

Rare homozygous loss-of-function mutations in the leptin-encoding gene (*LEP*) cause leptin deficiency that leads to hyperphagia and severe obesity, which can be corrected by exogenous leptin administration (Farooqi, Jebb et al. 1999). Leptin-deficient children are born with a normal birth weight but exhibit rapid weight gain in the first few months of life. They show marked abnormalities of T-cell number and function, and have high rates of childhood infection (Farooqi, Matarese et al. 2002). Hypothalamic hypothyroidism is present, characterized by a low free thyroxine and high serum thyroid-stimulating hormone levels (Strobel, Issad et al. 1998). Pubertal development generally does not occur due to hypogonadotropic hypogonadism (Strobel, Issad et al. 1998). Individuals heterozygous for leptin mutations exhibit a partial leptin deficiency with higher body fat than control individuals (Farooqi, Keogh et al. 2001).

Candidate gene studies, typically small in size, have reported associations of two common variants (A19G (rs2167270, minor allele frequency (MAF) 35%) and G2548A (rs7799039, MAF 49%)) in the promoter or 5′-untranslated region of *LEP* with circulating leptin levels in the general population, but these results are inconclusive (Hager, Clement et al. 1998, Le Stunff, Le Bihan et al. 2000, Mammes, Betoulle et al. 2000, Ben Ali, Kallel et al. 2009,

Fourati, Mnif et al. 2013). The same *LEP* variants have been studied for association with obesity, but a meta-analysis of the published results (n_{A19G}=918 and n_{G2548A}=2,174) found no evidence of such association[17]. Candidate gene studies of *LEP* were published before the human genome sequence was extensively characterized and are therefore restricted to the variants known at that time. Furthermore, although *LEP* is an obvious candidate, variants in other genes may also influence circulating leptin levels by regulating leptin production, secretion, clearance or response. Identification of such leptin-regulating genes could provide novel insights into mechanisms that regulate energy homeostasis and neuro-endocrine function (Ahima, Prabakaran et al. 1996, Montague, Farooqi et al. 1997).

In this study, we sought to identify genetic loci associated with circulating leptin levels by a genome-wide meta-analysis. Given the strong correlation between leptin and adiposity, we also examined genome-wide associations with circulating leptin levels adjusted for BMI, to identify loci associated with leptin levels independent of BMI.

Methods

Main analyses

Study design. We conducted a two-stage meta-analysis to identify leptin-associated loci in adults of European ancestry. In stage 1, we performed a meta-analysis of 23 GWAS (*n*=32,161) (**Supplementary Table 5.1**) for BMI-unadjusted and BMI-adjusted circulating levels of leptin. Stage 2 included 13 additional studies (*n*=19,979), which provided either *de novo* or *in silico* data for the lead SNPs of the independent loci reaching $P<1 \times 10^{-6}$ in Stage 1 (**Supplementary Table 5.5**). Secondary meta-analyses were conducted in men (*n*=13,363) and women (*n*=18,698) separately, and with adjustment for body fat percentage (assessed by dual-energy X-ray absorptiometry or bioimpedance analysis) instead of BMI (*n*=18,980). The study-specific descriptive statistics are presented in **Supplementary Table 5.23**.

Stage 1 genome-wide association analyses. Following study-specific quality control measures, the genotype data were imputed using the HapMap Phase II CEU reference panel (**Supplementary Table 5.24**). Directly genotyped and imputed variants were then tested for association with logarithmically transformed leptin (ng ml^{-1}), adjusting for age, age (Montague, Farooqi et al. 1997) and any necessary study-specific covariates (for example, genotype-derived principal components) in a linear regression model. The analyses were performed with and without additional adjustment for BMI. In studies that had assessed body fat percentage with bioimpedance analysis or dual-energy X-ray absorptiometry, additional analyses were performed with adjustment for body fat percentage. The analyses were performed in men and women separately. In studies that included closely related individuals, regression coefficients were also estimated in the context of a variance component model that modeled relatedness in men and women combined, with sex as a covariate.

Before performing meta-analyses on the data from individual studies, SNPs with poor imputation quality scores (r^2-hat <0.3 in MACH, proper-info <0.4 in IMPUTE, INFO <0.8 in PLINK) or with a minor allele count <6 were excluded for each study (**Supplementary Table 5.24**). The genotype data for the leptin-associated lead SNPs was of high quality with a median imputation score of ≥0.94 (**Supplementary Table 5.26**). The fifth percentile for all SNPs was ≥0.80, except for the previously established rs900400 SNP near *CCNL1*.

All individual GWAS were genomic control corrected before meta-analyses. Individual study-specific genomic control values ranged from 0.977 to 1.051. Fixed effects meta-analyses were then conducted using the inverse variance-weighted method implemented in METAL. The genomic control values for the meta-analysed results were 1.050, 1.026 and 1.022 in the BMI-unadjusted meta-analyses of all individuals, men and women, and 1.046, 1.022 and 1.015 in the BMI-adjusted meta-analyses, respectively. Using the LD score regression method (Farooqi, Wangensteen et al. 2007) in the Stage 1 meta-analyses suggests that the observed inflation is not due to population substructure. The regression intercept, which estimates inflation after removing polygenic signals, was 0.994 for BMI-unadjusted and 1.004 for BMI-adjusted meta-analyses of men and women combined.

Selection of SNPs for follow-up. We used a pairwise distance criterion of ±500 kb and r^2<0.1 between SNPs that reached $P<10^{-6}$in the meta-analysis of BMI-adjusted or -unadjusted meta-analysis of leptin levels in Stage 1 in men and women combined or separately, to select loci forward for follow-up in Stage 2. We tested the association of the lead SNPs in up to 19,929 adults of white European ancestry in Stage 2.

Stage 2 follow-up of the loci reaching $P<10^{-6}$ in Stage 1. Association results were obtained from 13 studies that had not been included in the Stage 1 meta-analyses

(**Supplementary Table 5.5**). Samples and SNPs that did not meet the quality control criteria defined by each individual study were excluded. Minimum genotyping quality control criteria were defined as Hardy–Weinberg equilibrium $P>10^{-7}$, call rate >90% and concordance >99% in duplicate samples in each of the follow-up studies.

We tested the association between the SNPs and leptin in each Stage 2 study using approaches similar to those described for the Stage 1 studies. We subsequently performed a meta-analysis of β-coefficients and s.e. from Stage 2 using the inverse variance fixed effects method. The final meta-analysis combined GWAS results from Stage 1 with the Stage 2 results. The conventional *P*-value threshold of $<5 \times 10^{-8}$ in the combined Stage 1 and Stage 2 meta-analysis was used to determine genome-wide significance.

Identifying genes and biological pathways at associated loci

Cross-trait look-ups. To further examine the relationship between the leptin-associated loci and anthropometric and metabolic parameters, we acquired association results for the loci in or near *LEP*, *SLC32A1*, *GCKR*, *CCNL1* and *COBLL1* from nine GWAS meta-analysis consortia: ADIPOGen (BMI-adjusted adiponectin), BCGC (body fat percentage), DIAGRAM (T2D), Early growth genetics (birth weight, early-onset obesity), ICBP (systolic and diastolic blood pressure), GIANT (height, BMI, waist–hip ratio adjusted for BMI), GLGC (circulating levels of high-density lipoprotein cholesterol, low-density lipoprotein cholesterol, triglycerides and total cholesterol), MAGIC (fasting glucose, fasting insulin) and ReproGen (age at menarche) (**Supplementary Table 5.7**).

National Human Genome Research Institute GWAS Catalog look-ups. To identify the associations of the leptin-associated loci in published GWAS, we extracted previously reported

GWAS associations within 500 kb and r^2>0.7 with any of the lead leptin-associated SNPs, from the GWAS Catalog of the National Human Genome Research Institute (www.genome.gov/gwastudies) (**Supplementary Table 5.11**).

Overlap with functional regulatory elements. We used the Uncovering Enrichment Through Simulation method to combine the leptin association data with the Roadmap Epigenomics Project segmentation data (Hayes, Trynka et al. 2015). The pipeline chose 10,000 sets of random SNPs among HapMap2 SNPs with a MAF>0.05 and that matched the original input SNPs based on proximity to a transcription start site and the number of LD partners (r^2>0.8 in individuals of European ancestry in the 1000 Genomes Project). The LD partners were combined with their original lead SNP to create 10,000 sets of matched random SNPs and their respective LD partners. These sets were intersected with the 15-state ChromHMM data from the Roadmap Epigenomics Project and resultant co-localizations were collapsed from total SNPs down to loci, which were then used to calculate an empirical *P*-value when comparing the original SNPs with the random sets. In addition to examining overall enrichment for all leptin-associated loci combined, we examined the variant-specific overlap with regulatory elements for each of the leptin-associated index SNPs and variants in strong LD (r^2>0.8).

Expression quantitative trait loci. We examined the *cis*-associations of the leptin-associated loci with the expression of nearby genes in the lymphocytes, skin, liver, omental fat, subcutaneous fat and brain tissue (**Supplementary Table 5.8**). Conditional analyses were performed by including both the leptin-associated SNP and the most significant *cis*-associated SNP in the association model for a given transcript. To minimize the potential for false positives, we only considered associations that reached study-specific Bonferroni-corrected significance threshold (P<0.05/(total number of transcripts tested)).

Pathway analyses

GRAIL analyses: We used GRAIL to identify genes near the leptin-associated loci having similarities in the published scientific text using PubMed abstracts as of December 2006 (Raychaudhuri, Plenge et al. 2009). The leptin loci were queried against HapMap release 22 for the European panel and we controlled for gene size.

DEPICT analyses: We used DEPICT to identify the most probable causal gene at a given associated locus, reconstituted gene sets enriched for BMI associations, and tissues and cell types in which genes from associated loci are highly expressed (Pers, Karjalainen et al. 2015). We clumped GWAS-based meta-analysis summary statistics using 500 kb flanking regions, LD r^2>0.1 and excluded SNPs with $P \geq 1 \times 10^{-5}$. HapMap Project Phase II CEU genotype data were used to compute LD and genomic coordinates were defined by genome build GRCh37.

Knockdown of genes in mouse adipose tissue explants

Materials. Expression analyses were performed on PGAT and SCAT from 4-month-old male C57BL/6J mice (derived from Jackson, Stock number 000664) fed chow (Purina PicoLab 5058) or high-fat diet (Research Diets, Inc., D12492i, 60% kcal from fat), to increase adiposity and circulating leptin levels. We measured expression of genes located ±100 kb of each lead variant or genes including SNPs with r^2>0.4 with the lead variant. *Tiparp* was included after its identification by eQTL analysis, and *Ucn* and *Mpv17* were included based on their proximity to variants with r^2>0.4 with the lead variant.

For knockdown experiments, 15-week-old male C57BL/6J mice fed high-fat diet *ad libitum* starting at 6 weeks of age were purchased from Jackson Laboratory (Stock Number 380050, Bar Harbor, ME). Animals were maintained at Columbia University animal facility for up to an additional 5 weeks until they reached ~30% fat mass as determined by time-domain NMR (Minispec Analyst AD; Bruker Optics, Silberstreifen, Germany).

Mice were maintained at an ambient temperature of 22 °C–24 °C with a 12-h dark–light cycle (lights on at 0700, h) in a pathogen-free barrier facility. The protocol was approved by the Columbia University Institutional Animal Care and Use Committee.

Electroporation and culture of adipose tissue explants. Non-fasted mice were killed at around 20 weeks of age at ~1000, h. PGAT was dissected and minced into 1- to 2-mm fragments. These fragments were evenly distributed into three replicates per control or knockdown condition. Approximately 7–11 fragments were added per well (for a total amount of ~80 mg tissue) in 12-well culture dishes containing 1 ml M199 with Antibiotic-Antimycotic (Anti-Anti, 5 ×; Invitrogen). Following a 20-min incubation in 5 × Anti-Anti media, tissue fragments were washed twice with 1 ml PBS and then transferred to 4 mm Gene Pulser cuvettes (Bio-Rad) and electroporated in 400 μl PBS with 1 nmol siRNA against *Lep*, *Adig*, *Ift172*, *Mpv17*, *Tiparp* or *Cobll1* (Stealth siRNA, Invitrogen). Non-targeting sequences were used as negative controls (Invitrogen). Electroporation was performed with a Gene Pulser XceII (Bio-Rad) using 50 V, 10^2 wave pulses, with a pulse length of 30 ms and 0.1 ms between pulses (Puri, Chakladar et al. 2007). The tissue fragments were subsequently cultured at 37 °C in 5% CO_2 in 12-well plates for 20 h in basal media consisting of M199 media with 10% fetal bovine serum (Invitrogen) plus 1 × Anti-Anti before stimulation for 12 h with basal media plus 7 nM insulin and 25 nM dexamethasone (both from Sigma), to maintain leptin expression in the explants at

levels comparable to those of *in vivo* tissues (Lee, Wang et al. 2007). Knockdown was considered successful if candidate expression was decreased by ≥30%. The effect of insulin and dexamethasone on expression of candidate genes was determined using the same mincing and culturing strategy without electroporation.

Measuring mRNA levels and leptin and adiponectin secretion. Total RNA was isolated using TRIzol reagent (Invitrogen) and reverse transcribed using Transcriptor First Strand cDNA Synthesis Kit (Roche) using both OligoDT and random hexamer primers. Lightcycler 480 SYBR Green I Master was used for quantitative PCR assays (Roche). Expression of murine homologues of candidate genes in PGAT and SCAT was determined using the $2(-\Delta\Delta C(T))$ method (Livak and Schmittgen 2001). Gene expression in the knockdown experiments was calculated by Lightcycler 480 software (Roche) based on a standard curve. Primers used are listed in **Supplementary Table 5.25**. Culture media was collected from the same samples used for RNA analyses. Following the 12 h insulin/dexamethasone stimulation, secreted leptin and adiponectin were measured using the Perkin-Elmer AlphaLISA kits for mouse leptin and adiponectin (according to the manufacturer's protocol). Not all samples were included for adiponectin measurement due to the discontinuation of the AlphaLISA kit by Perkin-Elmer.

Statistics. Each gene knockdown was tested on tissue from 5 to 13 different mice, as indicated. Control and knockdown samples from each mouse were treated as matched pairs. Each data point represents the mean of three replicates from a single mouse. Differences between control and knockdown conditions were calculated by two-way repeated measures analysis of variance using GraphPad Prism 6. *P*-values <0.05 were considered significant.

Results

Stage 1 genome-wide meta-analysis in 32,161 individuals

We first performed a meta-analysis of the results from genome-wide associations between ~2.5 million genotyped and HapMap-imputed single-nucleotide polymorphisms (SNPs) and circulating leptin levels, including up to 32,161 individuals of European descent from 23 studies (**Supplementary Table 5.1**). After logarithmic transformation that normalized the distribution of leptin levels and adjusting for age and sex, we carried out association analyses within each study and subsequently meta-analysed the study-specific results. To identify loci associated with circulating leptin levels independently of adiposity, we performed a meta-analysis of genome-wide associations in which we additionally adjusted for BMI. We also performed secondary genome-wide meta-analyses in men (n=13,363) and women (n=18,698) separately, as women generally have higher leptin levels than men, primarily due to larger percentage of body fat and greater subcutaneous fat storage (Hellstrom, Wahrenberg et al. 2000).

Two loci, near the *LEP* and *SLC32A1* genes, reached genome-wide significance ($P<5 \times 10^{-8}$) in the BMI-adjusted meta-analysis of men and women combined (**Table 5.1**). To confirm these associations and to identify additional leptin-associated loci, we took forward all independent (pairwise distance >500 kb and $r^2<0.1$) SNPs reaching$P<10^{-6}$ with leptin levels with or without adjustment for BMI in meta-analyses of all individuals combined, men only or women only, for follow-up in stage 2 (**Supplementary Tables 5.2**–**4**).

Stage 2 follow-up in 19,979 individuals identifies five loci

We examined the associations of the loci taken forward from stage 1 in up to 19,979 additional individuals of European descent from 13 studies (**Supplementary Table 5.5**). All studies performed the same association analyses as described in Stage 1; that is, with and without adjustment for BMI and in men and women combined, as well as separately. Finally, after performing a joint meta-analysis of the stage 1 and stage 2 results, five independent SNPs reached genome-wide significance ($P<5 \times 10^{-8}$) in the combined meta-analyses of men and women (**Table 5.1**). In the BMI-adjusted meta-analysis, we confirmed genome-wide significant associations for the loci near *LEP* and *SLC32A1*, and identified an additional locus in *GCKR*. In the BMI-unadjusted meta-analysis, we identified two additional loci near *CCNL1* and in *FTO*. A locus in *COBLL1*, previously identified for association with BMI-adjusted waist–hip ratio (WHR_{adjBMI}) (Shungin, Winkler et al. 2015), blood triglycerides (Global Lipids Genetics, Willer et al. 2013) and risk of T2D (Morris, Voight et al. 2012), reached $P=1 \times 10^{-6}$ with BMI-unadjusted leptin and $P=2 \times 10^{-6}$ with BMI-adjusted leptin levels, with the leptin-increasing allele being associated with lower WHR_{adjBMI}, triglycerides and risk of T2D.

The estimated effects of five of the six loci (in/near *LEP*, *SLC32A1*, *GCKR*, *CCNL1* or *COBLL1*) on leptin levels did not markedly differ in magnitude between the BMI-unadjusted and BMI-adjusted models, suggesting that these associations are not mediated by adiposity *per se* (**Figure 5.1**). In contrast, the association between the *FTO* locus and leptin levels was completely abolished after adjusting for BMI, indicating that the association with leptin is entirely mediated by the well-established association between *FTO* and BMI (Locke, Kahali et al. 2015) (**Figure 5.1**).

BMI is the most commonly used index of adiposity, but it is not a direct measure of adiposity and it does not distinguish between lean and fat body mass. To assess whether adjustment for a more direct measure of adiposity could enhance our ability to identify adiposity-independent loci, we performed secondary analyses in 13 studies that had data on both BMI and body fat percentage assessed by dual-energy X-ray absorptiometry or bioimpedance analysis (*n*=18,980 or 59% of stage 1 sample). The analysis showed no marked differences in the effect sizes between the BMI and body fat percentage-adjusted results for the leptin-associated *LEP*, *SLC32A1*, *CCNL1*, *GCKR*, *COBLL1* and *FTO* loci (**Supplementary Table 5.6**), suggesting that adjustment for BMI as compared with a more direct measure of adiposity did not compromise our ability to identify adiposity-independent leptin-associated loci.

Effects on other traits and potential functional roles

We took forward the genome-wide significant leptin loci near *LEP*, near *SLC32A1*, in *GCKR* and near *CCNL1*, to examine their associations with obesity-related and metabolic traits and to more directly assess their putative roles in the control of circulating leptin. We also took forward the locus near *COBLL1*, given its robust association with WHR_{adjBMI} (Shungin, Winkler et al. 2015), even though it just missed the genome-wide significance threshold for association with BMI-adjusted and BMI-unadjusted leptin levels (**Table 5.1**). As the *FTO*-leptin association was completely accounted for by *FTO*'s association with BMI (**Figure 5.1**), extensively described in the literature (Loos and Yeo 2014), we did not include this locus in our follow-up analyses.

To examine the associations of the identified loci with obesity-related and metabolic traits, we performed look-ups in the data from relevant genetic consortia (**Supplementary Table 5.7**). To study the associations of the leptin-associated loci with the expression of nearby genes,

we performed *cis*-expression quantitative trait locus (eQTL) analyses in several human tissues, including the subcutaneous (*n*=776) and omental fat (*n*=742), liver (*n*=567), lymphocytes (*n*=778), brain (*n*=193) and skin (*n*=667) (**Supplementary Table 5.8**). We also examined the regulatory functions of these loci by studying their enrichment with functional genomic elements in data from the Roadmap Epigenomics Project (Roadmap Epigenomics, Kundaje et al. 2015). Finally, to identify the causal genes in the leptin-associated loci, we performed *ex vivo* knockdown studies of adipocyte-expressed genes using small interfering RNA (siRNA) in explanted mouse adipose tissue.

Common variation near *LEP* regulates leptin levels

The rs10487505 variant (MAF 49%) is located 21 kb from *LEP* (**Figure 5.2A**) and is in modest linkage disequilibrium (LD) (r^2=0.4,D'=0.8) with the A19G (rs2167270, MAF 35%) variant that has been extensively studied in candidate gene studies but whose associations with increased levels of leptin and obesity have been inconclusive (Hager, Clement et al. 1998, Fourati, Mnif et al. 2013). The leptin-increasing allele of the rs10487505 variant has been nominally associated with weight regain after bariatric surgery in a candidate gene-based analysis of 1,443 patients (Sarzynski, Jacobson et al. 2011). Look-ups in consortium data showed a nominally significant association for the leptin-decreasing allele of rs10487505 with higher BMI in the GIANT Consortium (*P*=0.03, *N*=221,677), as well as with increased risk of early-onset obesity (*P*=0.04, *N*=13,848) and higher birth weight (*P*=0.02, *N*=26,836) in the EGG Consortium (**Supplementary Table 5.7**). Although *LEP* is an obvious candidate gene to account for the association with circulating leptin levels, the rs10487505 variant was not associated with *LEP* messenger RNA expression in the omental or subcutaneous adipose tissue (SCAT), liver, lymphocytes, brain or skin (**Supplementary Tables 5.8**&**5.9**).

A variant in strong LD with rs10487505 (rs6979832, r^2=0.98) overlapped with predicted enhancer elements in all three adipose cell lines of the Roadmap Epigenomics Project (Roadmap Epigenomics, Kundaje et al. 2015). Further, a previous study identified a 465-bp adipocyte-specific enhancer region 4.5 kb upstream from the *LEP* transcription start site by using luciferase assays and chromatin state mapping (Wrann, Eguchi et al. 2012). This region harbours rs10249476 that is in modest LD with rs10487505 (r^2=0.4,D'=0.8) and reached the second most significant association with BMI-adjusted leptin levels in stage 1 meta-analysis (P=3 × 10^{-10};n=30,810) (**Figure 5.2A**).

Collectively, although the locus near *LEP* overlaps with predicted enhancer elements, the lack of association with *LEP* transcript expression in the fasting state suggests that other mechanisms may be involved in mediating the association of this locus with leptin levels, such as an effect on *LEP* expression in the fed state (Zhang, Matheny et al. 2002) or an effect on leptin protein secretion.

To validate our knockdown strategy for subsequent analyses of candidate genes in loci other than the locus near *LEP*, we used siRNA against *Lep* in mouse adipose tissue explants. Electroporation of the perigonadal adipose tissue (PGAT) explants with siRNA against *Lep* resulted in a 92% decrease in *Lep* mRNA (P<1 × 10^{-4}) and a 92% decrease in secreted leptin (P=4 × 10^{-4}) (**Figure 5.4A**&**B**, **Supplementary Figure 5.1C**&**D** and **Supplementary Table 5.10**). In addition, to determine whether electroporation with siRNA altered other secretory function(s) of the perigonadal explants, we measured secretion of adiponectin and found no changes associated with *Lep* knockdown (**Figure 5.4D** and **Supplementary Figure 5.1E**).

ADIG may regulate leptin expression

The intergenic rs6071166 variant, ~20 kb from the *SLC32A1* gene (**Figure 5.2C**), reached genome-wide significance for association with BMI-adjusted leptin levels and has not been previously identified for association with any other traits. In look-ups of genome-wide association study (GWAS) consortium data, we did not find significant association with other obesity-related or metabolic traits (**Supplementary Table 5.7**). The rs6071166 variant was not associated with the mRNA expression of nearby genes in the adipose tissue, liver, lymphocytes, brain or skin (**Supplementary Tables 5.8** and **5.9**).

To identify the potential causal gene in this locus using the mouse PGAT explant model described above, we first measured the expression levels of murine homologues of genes surrounding the lead variant associated with circulating leptin levels. We tested PGAT and SCAT of 4-month-old C57BL/6J mice fed chow or high-fat diet (**Figure 5.3C**&**D**). In addition, we analyzed candidate gene expression in other tissues (liver and hypothalamus) that we predicted could play a role in circulating leptin levels via effects on leptin clearance or response (**Supplementary Figure 5.7**). Genes were considered strong candidates if they were highly expressed in adipose tissue and/or if they were regulated by high-fat diet feeding in a manner similar to *Lep*. This analysis identified *adipogenin* (*Adig*) as a candidate gene in the *SLC32A1* locus; *Adig* is highly expressed in the adipose tissue, in contrast to other nearby genes. To test whether *Adig* affected *Lep* expression, we performed *ex vivo* knockdown studies using siRNA against *Adig* in mouse PGAT explants. We found that knockdown of *Adig* decreased *Lep* expression by 26% ($P=4 \times 10^{-4}$) and leptin secretion by 23% ($P=0.003$), consistent with a causal role for *ADIG* in control of circulating leptin levels (**Figure 5.4A**&**B** and **Supplementary Figure 5.2C**&**D**).

ADIG is located ~116 kb from the rs6071166 variant and encodes a cytoplasmic adipocyte protein adipogenin, that is, similar to leptin, highly and specifically expressed in the adipose tissue (Hong, Hishikawa et al. 2005, Kim, Tillison et al. 2005, Ren, Eskandari et al. 2016) and upregulated by treatment with insulin and glucose (Ren, Eskandari et al. 2016). *Adig* expression is also strongly upregulated in murine 3T3-L1 preadipocytes during *in vitro* differentiation into adipocytes (Hong, Hishikawa et al. 2005, Kim, Tillison et al. 2005). Two studies have investigated the effect of *Adig* knockdown on the differentiation of 3T3-L1 cells and expression of *Pparγ2*, a master regulator of adipocyte differentiation, but with conflicting results; whereas the first study found *Adig* knockdown to block adipocyte differentiation and decrease *Pparγ2* expression (Hong, Hishikawa et al. 2005), a later study found no similar changes (Ren, Eskandari et al. 2016). When we measured *Pparγ2* expression following *Adig* knockdown in PGAT explants containing mature adipocytes, we did not see a change as compared with controls (**Supplementary Figure 5.2F**).

Common variation in *GCKR* regulates leptin levels

Variants ($r^2 \geq 0.9$ with our lead SNP rs780093) of the leptin-associated locus in *GCKR* have previously shown genome-wide significant associations with more than 25 metabolic traits; the leptin-increasing allele has been associated with increased fasting glucose and fasting insulin but decreased 2-h glucose and higher high-density lipoprotein cholesterol, and lower total cholesterol, low-density lipoprotein cholesterol, triglycerides, C-reactive protein and circulating uric acid levels, among others (**Supplementary Table 5.11**). The *GCKR* gene encodes a regulatory protein in the liver that inhibits the activity of glucokinase, the enzyme responsible for regulating the uptake, metabolism and storage of circulating glucose (de la Iglesia, Mukhtar et al. 2000). A putative causal variant in this gene is the common nonsynonymous Pro446Leu variant

(rs1260326), for which rs780093 acts as a good proxy (r^2=0.9). Carriers of the glucose-lowering Leu allele have a reduced ability to sequester and inhibit glucokinase and a blunted response to fructose 6-phosphate, both of which favour the generation of free and active cytoplasmic glucokinase (Rees, Wincovitch et al. 2012).

The mechanisms that might link changes in *GCKR* function to leptin levels are not known. As insulin increases leptin secretion from adipocytes (Kolaczynski, Nyce et al. 1996) and the *GCKR* locus is strongly associated with circulating levels of insulin, the association of the *GCKR* locus with leptin levels could be the consequence of the GCKR locus' effect on insulin levels. The leptin-increasing allele of the rs780093 variant was significantly associated with higher levels of fasting insulin in studies included in our stage 2 meta-analyses (P=2 × 10^{-5}, N=8,953). To test whether insulin mediated the association of rs780093 with circulating leptin levels, we analysed the association of rs780093 with leptin, while adjusting for fasting insulin levels. Although the effect size was somewhat attenuated, the association of rs780093 with BMI-adjusted leptin levels remained significant after adjustment for fasting insulin (β=0.047, P=2 × 10^{-4} versusβ=0.034, P=0.004 before and after the adjustment, respectively), suggesting that the association of the *GCKR* locus with leptin is at least in part independent of effects on insulin levels.

Although *GCKR*'s function renders it a potential candidate among the genes in this region, *cis*-eQTL analyses showed association of the leptin-increasing allele of rs780093 with increased expression of the nearby *IFT172* in the liver (P=7 × 10^{-30}), omental fat (P=6 × 10^{-64}) and subcutaneous fat (P=3 × 10^{-52}) (**Supplementary Table 5.9**). The rs780093 variant is, however, only in moderate LD (r^2=0.4) with the peak SNP influencing *IFT172* expression in the region and the peak SNP remained significantly associated with *IFT172*expression after

adjustment for rs780093, whereas the association of rs780093 was abolished after adjustment for the peak SNP (**Supplementary Table 5.9**).

Because of the observations in human tissues, we examined *Ift172* in the mouse explant model. *Ift172* was not highly expressed in mouse PGAT or SCAT and levels were not upregulated by high-fat diet (**Figure 5.3E**&**F**). *Ift172* was, however, upregulated in the liver under high-fat diet feeding (**Supplementary Figure 5.7E**). Knockdown of *Ift172* in PGAT explants decreased *Lep* mRNA expression by 22% (P=0.02), but did not decrease leptin protein secretion (P=0.6) (**Figure 5.4A**&**B** and **Supplementary Figure 5.3C**&**D**). *IFT172* is known to play a major role in assembly and maintenance of primary cilia that act as critical signaling hubs for cellular pathways during development (Halbritter, Bizet et al. 2013). Knockout of *Ift* genes in central neurons causes obesity in mice (Davenport, Watts et al. 2007) and obesity is a clinical feature in two human ciliopathic syndromes, the Alström and Bardet–Biedl syndromes (Zaghloul and Katsanis 2009, Girard and Petrovsky 2011). In the hypothalamus, alterations in the function of the primary cilium lead to impaired leptin signaling (Stratigopoulos, LeDuc et al. 2011). Therefore, we cannot exclude a role for *IFT172* in the regulation of circulating leptin levels.

Another nearby gene, *MpV17* mitochondrial inner membrane protein, is a potential candidate in the region based on its expression in mice fed chow or high-fat diet; *Mpv17* expression was increased by high-fat diet, in a manner similar to *Lep* (**Figure 5.3C**&**D**). However, knockdown of *Mpv17* did not change *Lep* mRNA expression (P=0.2) or leptin secretion (P=0.2) by PGAT explants (**Figure 5.4A**&**B** and **Supplementary Figure 5.4C**&**D**), suggesting that the involvement of *MPV17* in leptin regulation is unlikely.

Locus near *CCNL1* regulates leptin levels and birth weight

The leptin-decreasing allele of rs900400, located 67 kb upstream from *CCNL1* (**Figure 5.2D**), was previously reported for its association with lower birth weight (Horikoshi, Yaghootkar et al. 2013). This cross-phenotype association could indicate a mechanism that is shared between birth weight and leptin levels in adulthood. Fetal adipose tissue is capable of producing leptin (Atanassova and Popova 2000) and fetal leptin levels are correlated with fetal fat mass (Clapp and Kiess 1998, Jaquet, Leger et al. 1998). Placenta provides an additional source of leptin for the fetus, however, and it has been suggested that leptin could mediate fetal growth (Hassink, de Lancey et al. 1997, Tamura, Goldenberg et al. 1998). Assuming that leptin levels track from birth through adulthood, increased leptin levels could drive the association of the *CCNL1* locus with birth weight. Other studies suggest that leptin production is decreased in cultured adipocytes from men born with a low birth weight (Schultz, Broholm et al. 2014). Therefore, the association of the *CCNL1* locus with leptin levels in adulthood could be mediated by its association with birth weight.

Although *CCNL1* is the nearest gene to rs900400, our *cis*-eQTL analyses identified rs900400 as the variant most significantly associated with the expression of another nearby gene, *TIPARP* (**Supplementary Table 5.9**). The *TIPARP* gene encodes a poly (ADP-ribose) polymerase involved in DNA repair. The leptin-increasing allele of rs900400 was associated with lower *TIPARP* expression in omental fat (3×10^{-30}) and subcutaneous fat ($P=7 \times 10^{-58}$) (**Supplementary Table 5.9**). *Tiparp* was also implicated as a causal gene by our expression analysis of mouse adipose tissue and its expression was increased in SCAT and liver in mice fed with high-fat diet (**Figure 5.3H** and **Supplementary Figure 5.7G**). Knockdown of *Tiparp*in mouse PGAT explants did not, however, significantly alter the expression of *Lep* mRNA (P=0.7)

or leptin secretion (P=0.8) (**Figure 5.4A**&**B** and **Supplementary Figure 5.5D&E**). Although we attempted to use SCAT for explant knockdown studies, high intra-depot variability compromised this approach. Interestingly, stimulation of the explants with insulin and dexamethasone increased explant expression of *Tiparp* by 50% (P=0.003) over incubation in basal media alone, in a manner similar to *Lep* expression (**Figure 5.4C** and **Supplementary Figure 5.5A**). Collectively, although *TIPARP* remains a putative causal gene within the locus near *CCNL1*, further evidence is required to confirm its role in the regulation of circulating leptin levels.

COBLL1 or *GRB14* may regulate leptin levels

The intronic rs6738627 variant in *COBLL1* (**Figure 5.2E**) did not reach genome-wide significance for the association with leptin levels (**Figure 5.1** and **Table 5.1**). However, as previous GWAS have shown robust associations of the leptin-increasing allele with a lower WHR_{adjBMI}[19], we chose to take it forward for follow-up analyses, to examine the role of leptin levels in the previous associations.

Look-ups in data from genetic consortia showed a strong association of the leptin-increasing allele of rs6738627 with higher body fat percentage ($P=2 \times 10^{-8}$, n=76,338; **Supplementary Table 5.7**). As reported previously, the rs6738627 variant was also strongly associated with decreased WHR_{adjBMI} ($P=2 \times 10^{-8}$, n=174,672; **Supplementary Table 5.7**), suggestive of a preferential gluteal rather than abdominal fat storage, which may contribute to the association of rs6738627 with increased leptin levels (Van Harmelen, Reynisdottir et al. 1998).

In PGAT and SCAT expression analyses in mice, we found an upregulation of *Cobll1* in high-fat diet-fed mice in both depots (**Figure 5.3I**&**J**). Although knockdown of *Cobll1* in the

perigonadal explants did not influence *Lep* mRNA expression (P=0.2), it did decrease leptin protein secretion by 16% ($P=3 \times 10^{-4}$, **Figure 5.4A**&**B**), suggesting a potential causal role for *Cobll1*. In addition, stimulation of explants with insulin and dexamethasone increased explant expression of *Cobll1* by 78% (P=0.004) over incubation in basal media alone (**Figure 5.4C** and **Supplementary Figure 5.6A**). *COBLL1* is known to be involved in neural tube formation (Carroll, Gerrelli et al. 2003), but its possible functions in adipose tissue are unknown.

In human eQTL analyses, the leptin-increasing allele of the *COBLL1* locus showed an association with lower expression of *GRB14* in omental fat ($P=5 \times 10^{-12}$) and subcutaneous fat ($P=3 \times 10^{-5}$) (**Supplementary Table 5.9**). We did not, however, find high expression of *Grb14* in PGAT or SCAT explants from mice and the levels were not regulated by high-fat diet feeding (**Figure 5.3I**&**J**). The protein product of *GRB14* is the growth factor receptor-bound protein 14 that binds directly to the insulin receptor and inhibits insulin signaling (Depetris, Hu et al. 2005). The adipose tissue expression of *GRB14* may play a role in regulating insulin sensitivity (Cariou, Capitaine et al. 2004). Grb14-deficient mice exhibit improved glucose tolerance, lower circulating insulin levels and increased incorporation of glucose into glycogen in the liver and skeletal muscle (Cooney, Lyons et al. 2004). Both *COBLL1* and *GRB14* are thus possible candidates to account for the association of the *COBLL1* locus with leptin levels.

Enrichment with pathways and regulatory elements

We used the Data-driven Expression Prioritized Integration for Complex Traits (DEPICT) software (Pers, Karjalainen et al. 2015) to identify enrichment of gene sets and pathways across loci reaching $P<1 \times 10^{-5}$ for association with leptin levels. However, none of our findings reached the false discovery rate threshold of 5% (**Supplementary Tables 5.12–5.17**). Next, we used the Gene Relationships Across Implicated traits (GRAIL) tool

(Raychaudhuri, Plenge et al. 2009) to identify genes near the leptin-associated loci having similarities in the text describing them within published article abstracts. However, no statistically significant results were found in these analyses either (**Supplementary Tables 5.18** and **5.19**). Finally, we used the Uncovering Enrichment Through Simulation method (Hayes, Trynka et al. 2015) to test for the overall enrichment of leptin-associated loci reaching $P<10^{-5}$ with ChromHMM annotations for adipose and brain tissues available from the Roadmap Epigenomics Project (Roadmap Epigenomics, Kundaje et al. 2015). However, we did not find significant enrichment of our leptin-associated loci in any chromatin states once corrected for multiple testing (**Supplementary Table 5.20**). The lack of significant findings may be due to the small number of loci identified and the limited knowledge available on leptin-regulating pathways in adipose tissue.

Established adiposity loci and leptin

Circulating leptin levels correlate closely with BMI and other measures of adiposity[3]. The most recent GWAS meta-analysis for BMI, including nearly 340,000 individuals, identified 97 loci that reached genome-wide significance (Locke, Kahali et al. 2015). Of the 97 BMI-increasing loci, 89 showed a directionally concordant association with increased BMI-unadjusted leptin levels ($P_{binomal}=2 \times 10^{-18}$), of which 25 reached nominal significance (**Supplementary Table 5.21**). Previous GWAS of extreme and early-onset obesity have identified 12 genome-wide significant loci (Meyre, Delplanque et al. 2009, Scherag, Dina et al. 2010, Berndt, Gustafsson et al. 2013, Wheeler, Huang et al. 2013). Of these, ten showed a directionally consistent association with increased BMI-unadjusted leptin levels ($P_{binomal}=0.04$), of which five reached nominal significance (**Supplementary Table 5.21**).

We also examined leptin associations for 49 loci identified in GWAS for WHR_{adjBMI}, a measure of body fat distribution independent of overall adiposity (Shungin, Winkler et al. 2015). Of the 49 WHR_{adjBMI}-increasing loci, only 24 showed a directionally concordant association with increased BMI-adjusted leptin levels (**Supplementary Table 5.22**). As the distribution of body fat differs between men and women, we also examined the leptin associations for the 49 WHR_{adjBMI} loci in men and women separately. There was no enrichment of leptin associations in either of the sexes, with 27 loci showing a directionally concordant association with increased leptin levels in men and 20 loci in women (**Supplementary Table 5.22**).

Discussion

In a meta-analysis of genetic association data in up to 52,126 individuals, we identified 5 common loci associated with circulating leptin levels. In addition, a locus near *COBLL1*, previously identified for association with a lower WHR_{adjBMI} (Shungin, Winkler et al. 2015), reached $P=1 \times 10^{-6}$ for association with increased leptin levels. Even though leptin correlates strongly with adiposity, we did not identify loci previously associated with BMI, other than *FTO*, despite having a sample size similar to early GWAS meta-analyses of BMI that identified multiple loci (Willer, Speliotes et al. 2009). On the contrary, five of the six loci we identified were associated with leptin independently of BMI or body fat percentage. Our findings indicate that genetic mechanisms not influencing adiposity may have an important role in the regulation of circulating leptin levels.

Our strongest adiposity-independent leptin signal was near *LEP*, but we also identified leptin-associated variants in four other genomic loci, providing evidence that mechanisms other

than those that involve *LEP per se* may regulate leptin production and release from adipose tissue. In one of these loci, near *SLC32A1*, our knockdown studies indicated a role for *adipogenin*, a gene involved in the regulation of adipocyte differentiation (Hong, Hishikawa et al. 2005, Kim, Tillison et al. 2005). Although adipogenin was identified as a potent regulator of adipogenesis a decade ago (Hong, Hishikawa et al. 2005, Kim, Tillison et al. 2005), our results provide the first evidence linking this function to leptin regulation. We anticipate that our findings will motivate and inform eventual testing of *Adig* by transgenic manipulation in mice.

No clear effect on leptin production was seen following knockdown of candidate genes in the *GCKR* and *CCNL1* loci, which may indicate that the gene implicated by position plays no role in the phenotype, or that the effect was undetectable in our experimental conditions. Alternatively, the association with leptin levels may be explained by effects of non-coding elements on other genes outside the implicated genetic interval, or by inter-species differences. Furthermore, although adipose tissue is the most direct contributor to circulating leptin levels, the effect of the causal gene may be conveyed by another tissue; leptin production and secretion are influenced by insulin, catecholamines and other hormones, as well as paracrine effects of local inflammatory cells on adipocytes (Fried, Ricci et al. 2000).

Although the locus near *SLC32A1* had not been identified previously for association with other traits, the leptin-associated loci in/near *GCKR*, *CCNL1* and *COBLL1* have been associated with multiple obesity-related and metabolic traits (Willer, Speliotes et al. 2009, Saxena, Hivert et al. 2010, Manning, Hivert et al. 2012, Horikoshi, Yaghootkar et al. 2013, Shungin, Winkler et al. 2015). These cross-phenotype associations may either reflect pleiotropy, where a gene product influences multiple traits, and/or mediation effects, where one phenotype is causally related to a second phenotype. For example, the association of the pleiotropic *GCKR* locus with leptin levels

may be partly mediated through *GCKR*'s role in the regulation of glucose homeostasis and insulin levels (Saxena, Hivert et al. 2010, Manning, Hivert et al. 2012), which may influence leptin production and secretion in adipose tissue (Kolaczynski, Nyce et al. 1996). The *COBLL1* locus is strongly associated with decreased WHR_{adjBMI}, indicative of a preferential accumulation of gluteal subcutaneous fat, which may contribute to the observed association with circulating leptin levels (Van Harmelen, Reynisdottir et al. 1998). The identification of the birth weight locus, *CCNL1*, as a leptin-regulating locus may provide an intriguing link between leptin regulation and fetal growth, albeit such a link remains to be more firmly established (Tamura, Goldenberg et al. 1998).

Unraveling the polygenic basis of leptin production could provide opportunities for targeted leptin supplementation in obese individuals. Although leptin therapy is an efficient weight-loss treatment for obese individuals with congenital leptin deficiency, the beneficial effects of leptin supplementation do not translate to all obese patients (Heymsfield, Greenberg et al. 1999). Sensitivity to changes in circulating concentration of leptin may be enhanced at very low values (Farooqi, Keogh et al. 2001) where a relatively small increase in leptin production may be sensed by the homeostatic feedback system that controls energy balance. As a substantial minority of individuals with common forms of obesity, not associated with leptin mutations, have relatively low levels of circulating leptin (Ravussin, Pratley et al. 1997), augmenting leptin levels in this subgroup could be therapeutically worthwhile. Identification of leptin-regulating loci may provide new tools for identifying obese individuals with susceptibility to low leptin levels and who may benefit of leptin treatment.

In 2010, Sun *et al.* (Sun, Cornelis et al. 2010) identified two common non-synonymous SNPs in the leptin receptor (*LEPR*) gene associated with leptin receptor levels. Leptin receptor

plays an essential role in mediating the physiological effects of leptin. Although some studies have described abnormally high circulating leptin levels in carriers of rare *LEPR* mutations (Clement, Vaisse et al. 1998), others have not (Farooqi, Wangensteen et al. 2007). We did not find association between *LEPR* variants and circulating leptin levels, suggesting that common variants in *LEPR* are not important regulators of circulating leptin levels.

Our meta-analyses were limited by the number of available studies with leptin data, imputation by HapMap reference panel for autosomal chromosomes and the fact that we examined additive effects only. In addition, we corrected for adiposity by adjusting for BMI, which is a heterogeneous measure of adiposity as it does not account for individual differences in body fat and lean mass. Future discovery efforts in extended sample sizes based on genome-wide imputation of 1000 Genomes reference panels, which include X and Y chromosomes and which also test for recessive and dominant inheritance, will allow for the discovery of more and lower-frequency variants, and for refining association signatures of already established leptin-associated loci.

In summary, we identified six genetic loci associated with circulating leptin levels, of which five showed associations independently of adiposity. Our findings represent a step forward in the understanding of biological mechanisms regulating leptin production in adipose tissue and open new avenues for examining the influence of variation in leptin levels on adiposity and metabolic health.

Tables

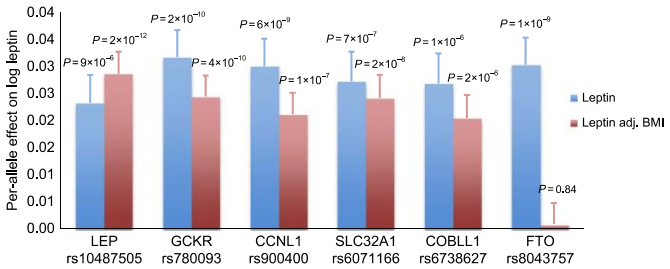
Table 5.1 *Meta-analysis results in men and women combined for the genome-wide significant leptin-associated loci and for the locus in COBLL1.*

SNP	Nearest gene	Chr:Position	Effect/ Other allele	EAF	Leptin unadjusted for BMI								Leptin adjusted for BMI							
					Stage 1		Stage 2		Stage 1+2				Stage 1		Stage 2		Stage 1+2			
					P	N	P	N	Beta	SE	P	N	P	N	P	N	Beta	SE	P	N
rs10487505	*LEP*	7:127647399	G/C	0.50	4.0E-06	29470	2.0E-01	17110	0.023	0.005	9.0E-06	46580	2.7E-11	29255	5.2E-03	16781	0.029	0.004	2.0E-12	46036
rs780093	*GCKR*	2:27596107	C/T	0.61	1.8E-07	32147	3.6E-04	19979	0.032	0.005	2.3E-10	52126	6.3E-07	31802	1.4E-04	19648	0.024	0.004	3.8E-10	51450
rs900400	*CCNL1*	3:158281469	T/C	0.60	7.3E-07	32128	1.7E-03	19685	0.030	0.005	5.6E-09	51813	9.8E-07	31785	1.7E-02	19354	0.021	0.004	1.2E-07	51139
rs6071166	*SLC32A1*	20:36766426	C/A	0.37	2.0E-07	29471	1.6E-01	17007	0.027	0.006	6.6E-07	46478	2.9E-08	29256	3.9E-02	16678	0.024	0.004	1.8E-08	45934
rs6738627	*COBLL1*	2:165252696	A/G	0.37	8.3E-07	25573	8.6E-02	19573	0.027	0.006	1.4E-06	45146	4.6E-06	25229	4.5E-02	19242	0.020	0.004	1.9E-06	44471
rs8043757	*FTO*	16:52370951	T/A	0.40	8.8E-08	32120	2.7E-03	19919	0.030	0.005	1.1E-09	52039	6.1E-01	31776	7.5E-01	19588	0.001	0.004	8.4E-01	51364

Leptin (µg/ml) was logarithmically transformed. The study-specific analyses were performed with linear regression models while accounting for sex, age and age ^{2}. Meta-analyses of the study-specific results were performed using fixed effects meta-analysis. Beta refers to the change in logarithmically transformed leptin per each copy of the effect allele. EAF, effect allele frequency

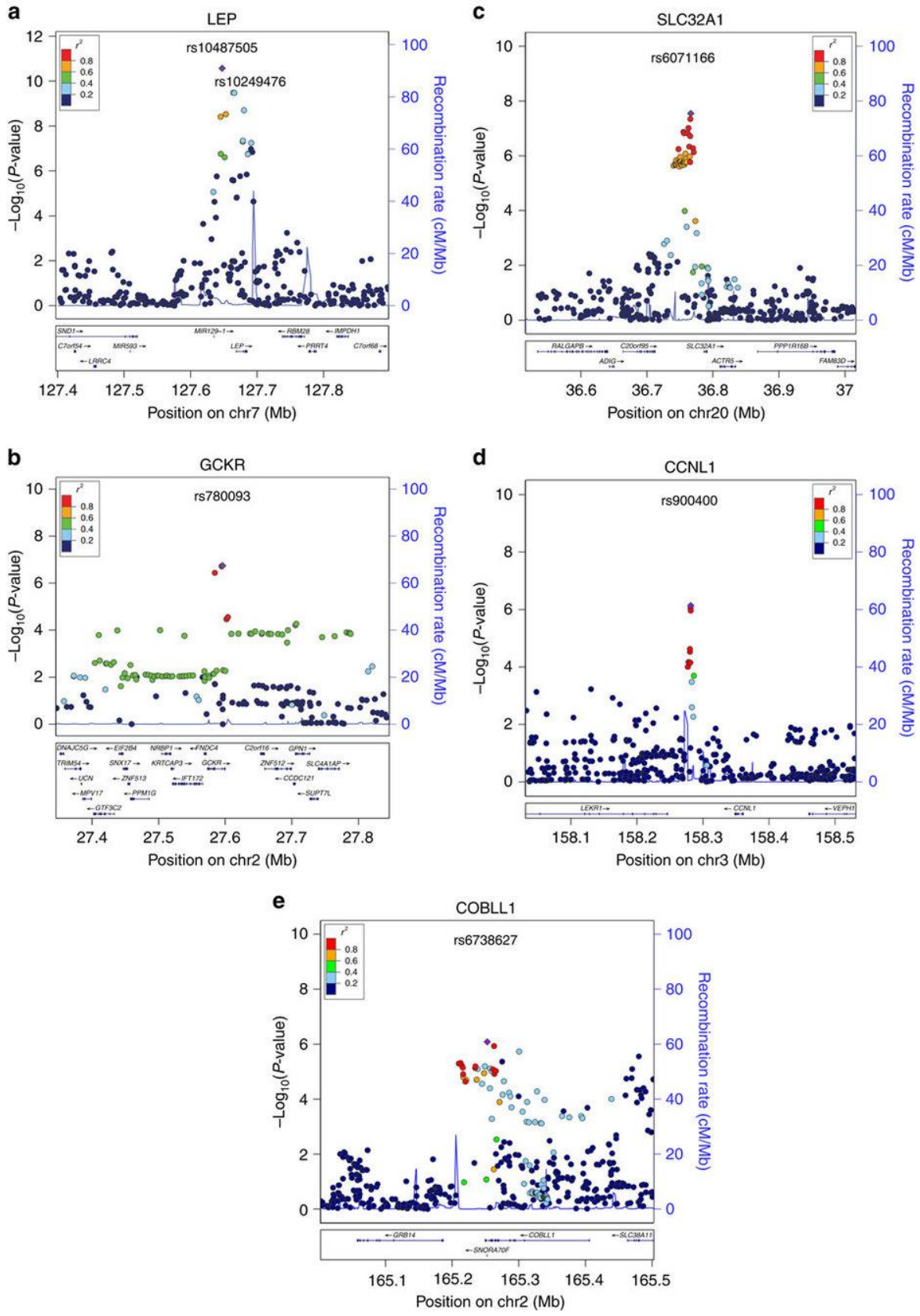
Figures

Figure 5.1 *Meta-analysis results for the leptin-associated loci.*



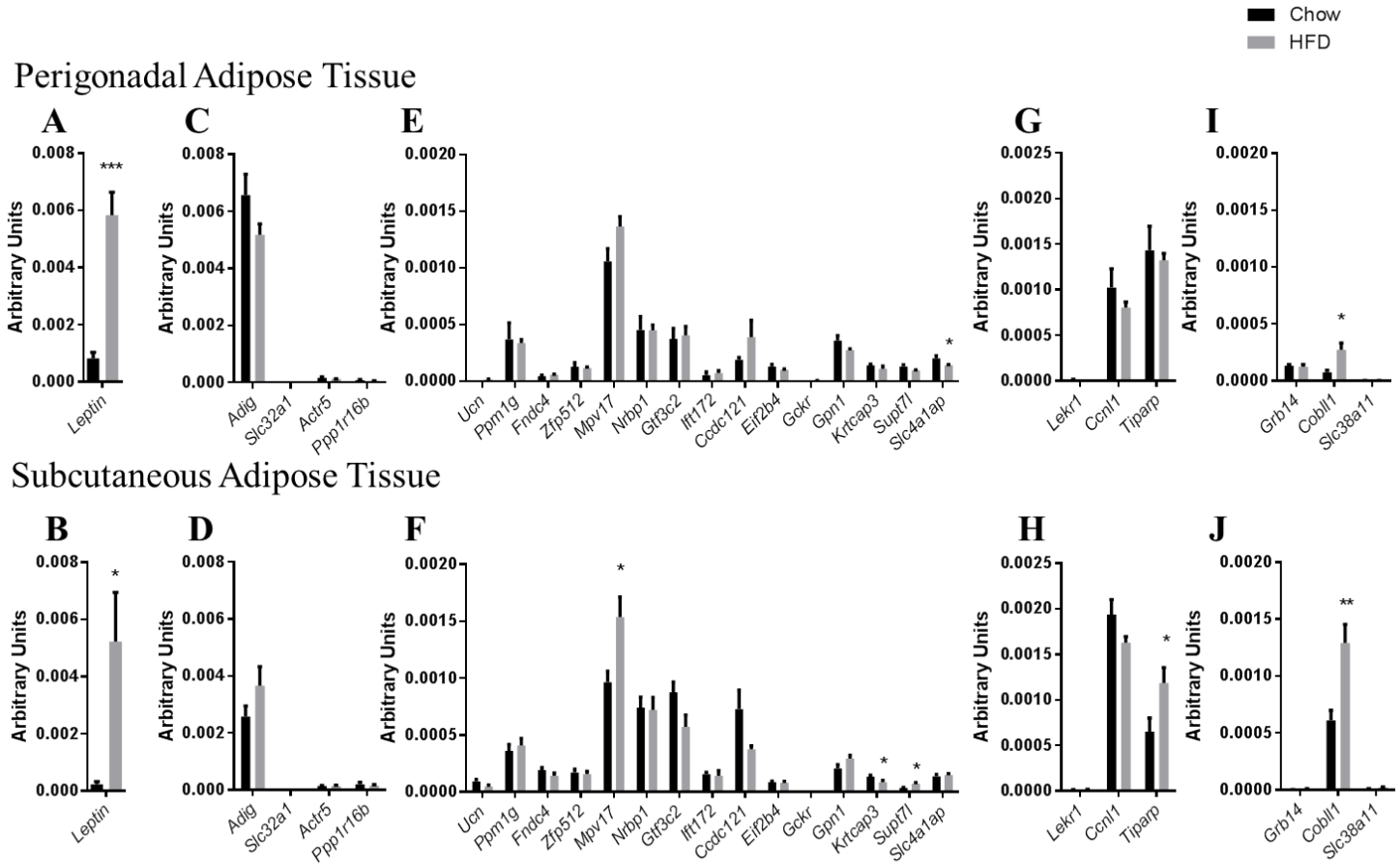
The bars show the additive effect of the loci in or near LEP, GCKR, CCNL1, SLC32A1, COBLL1 and FTO on BMI-unadjusted and BMI-adjusted leptin levels in the meta-analysis of Stage 1 and Stage 2 combined. The error bars indicate s.e.

Figure 5.2 *Regional plots for the loci associated with circulating leptin concentrations.*



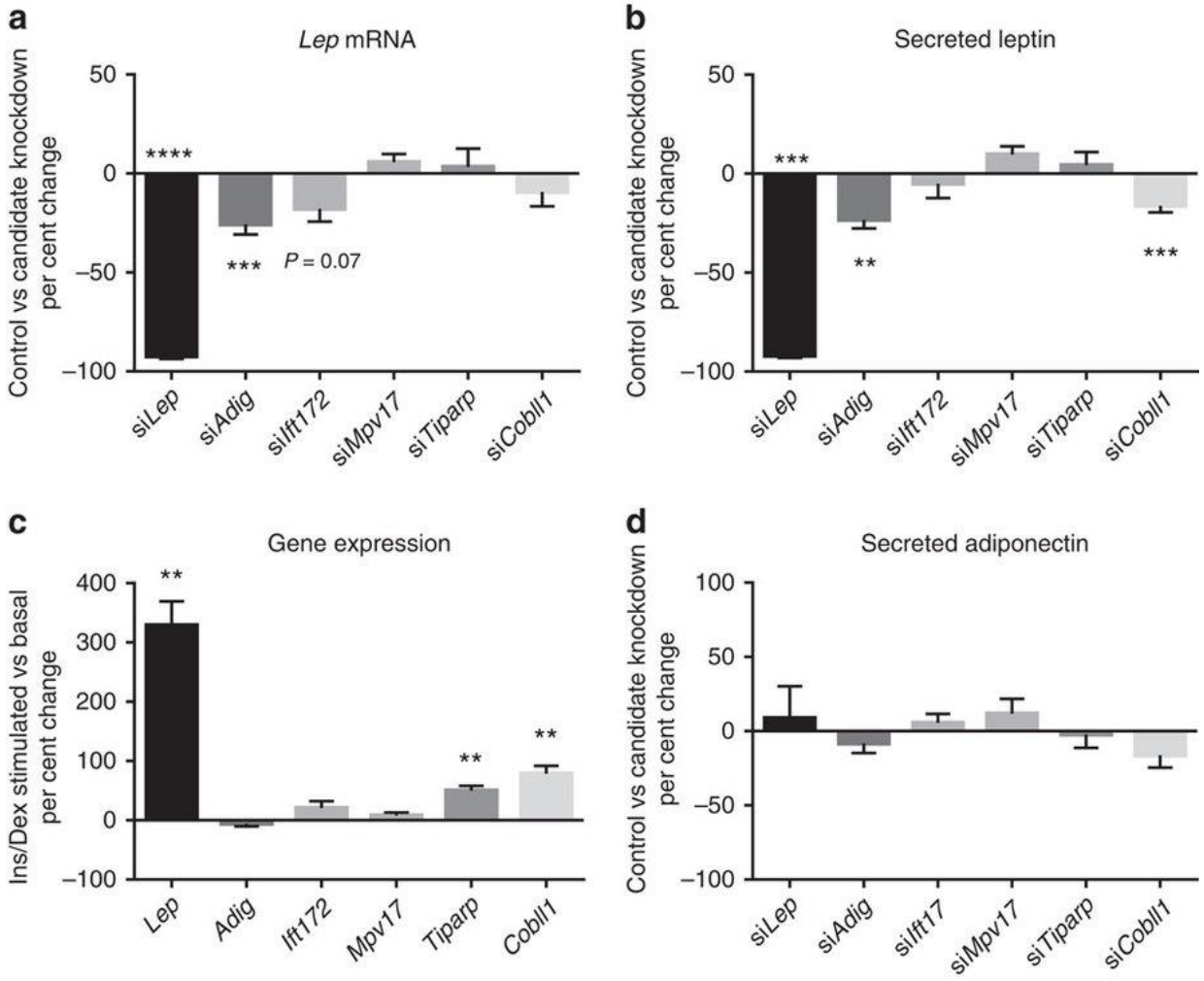
Regional plots for the loci in or near *LEP* (**A**), *GCKR* (**B**), *SLC32A1* (**C**) and *CCNL1* (**D**), which reached genome-wide significance in the combined meta-analysis of Stage 1 and Stage 2 for BMI-unadjusted or BMI-adjusted leptin levels. The *COBLL1* locus (**E**) that reached $P=1 \times 10^{-6}$ with BMI-unadjusted and $P=2 \times 10^{-6}$ with BMI-adjusted leptin levels is also shown. For the locus near *LEP* (**A**), the rs10249476 SNP, located in a previously identified adipocyte-specific enhancer region (Wrann, Eguchi et al. 2012) is indicated.

Figure 5.3 *Expression of murine homologues of candidate genes.*



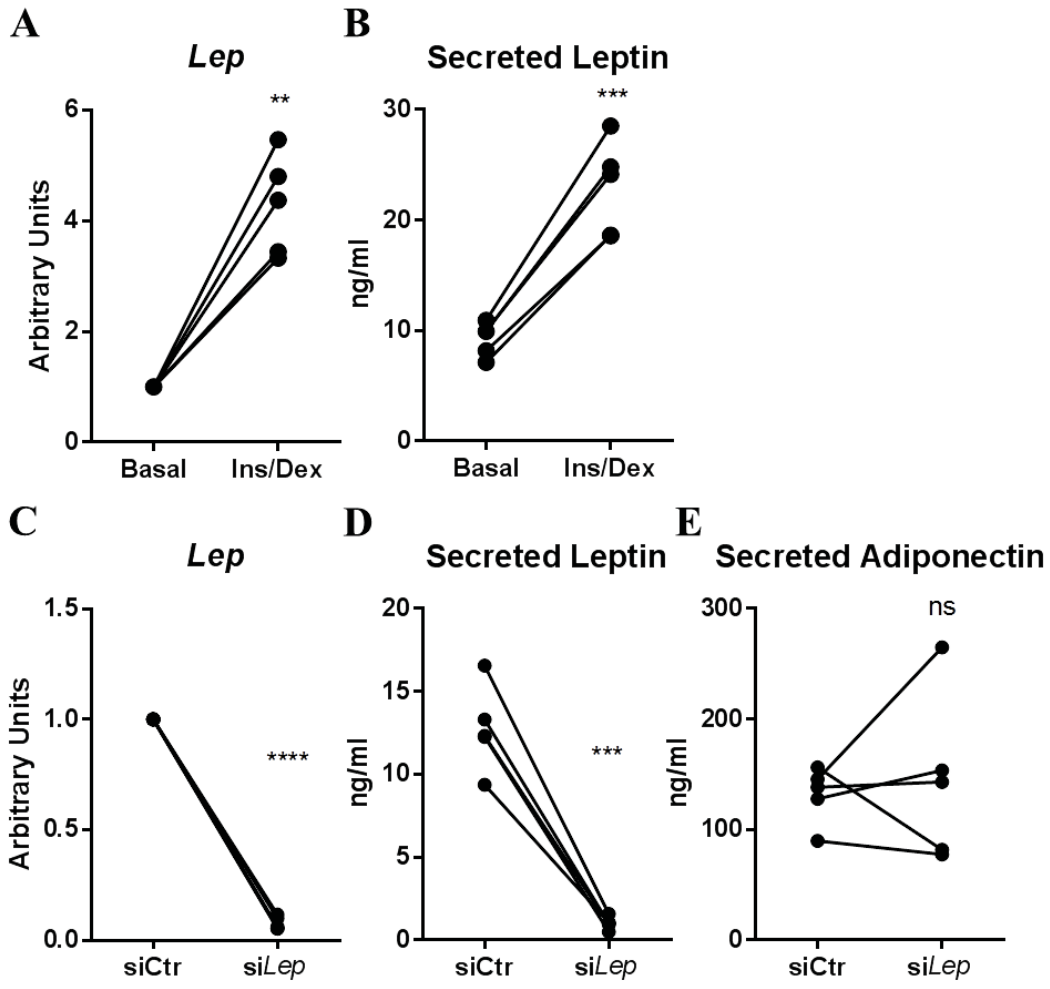
Expression of murine homologues of genes located within *Lep* (**A**&**B**), *Slc32a1* (**C**&**D**), *Gckr* (**E**&**F**), *Ccnl1* (**G**&**H**) and *Cobll1* loci (**I**&**J**) in PGAT and SCAT from 4-month-old mice fed chow (black bars) or high-fat diet (HFD; grey bars). Quantitative PCR (qPCR) transcripts were normalized using *ActB, Rplp0, Gapdh* and *Ppia* as housekeeping genes. *N*=5 mice per group. T-test. **P*<0.05, ***P*<0.01 and ****P*<0.001.

Figure 5.4 *Candidate gene knockdown studies*



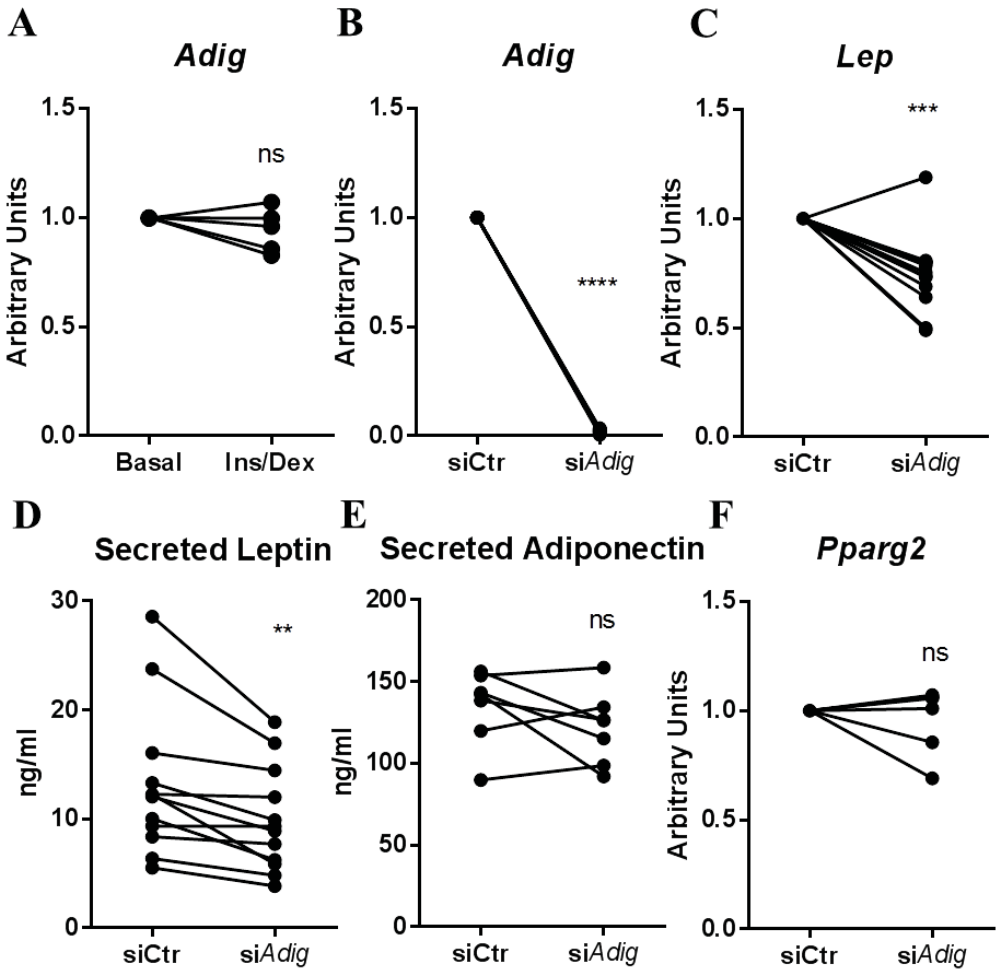
Changes in *Lep* mRNA expression (**A**) and secretion into media (**B**) following candidate gene knockdown in PGAT explants following stimulation with insulin and dexamethasone for 12 h. Gene expression induced by stimulation with insulin and dexamethasone (**C**) N=5–13 mice per group (3 replicates/condition/mouse). Secreted adiponectin was measured as a control for non-leptin secretory function (**D**) N=5 mice per group. Two-way repeated measures analysis of variance (ANOVA). *P<0.05, **P<0.01, ***P<0.001 and ****P<0.0001.

Supplementary Figure 5.1 *Effects of Lep knockdown on leptin transcription and secretion in perigonadal adipose tissue explants from mice fed with high fat diet.*



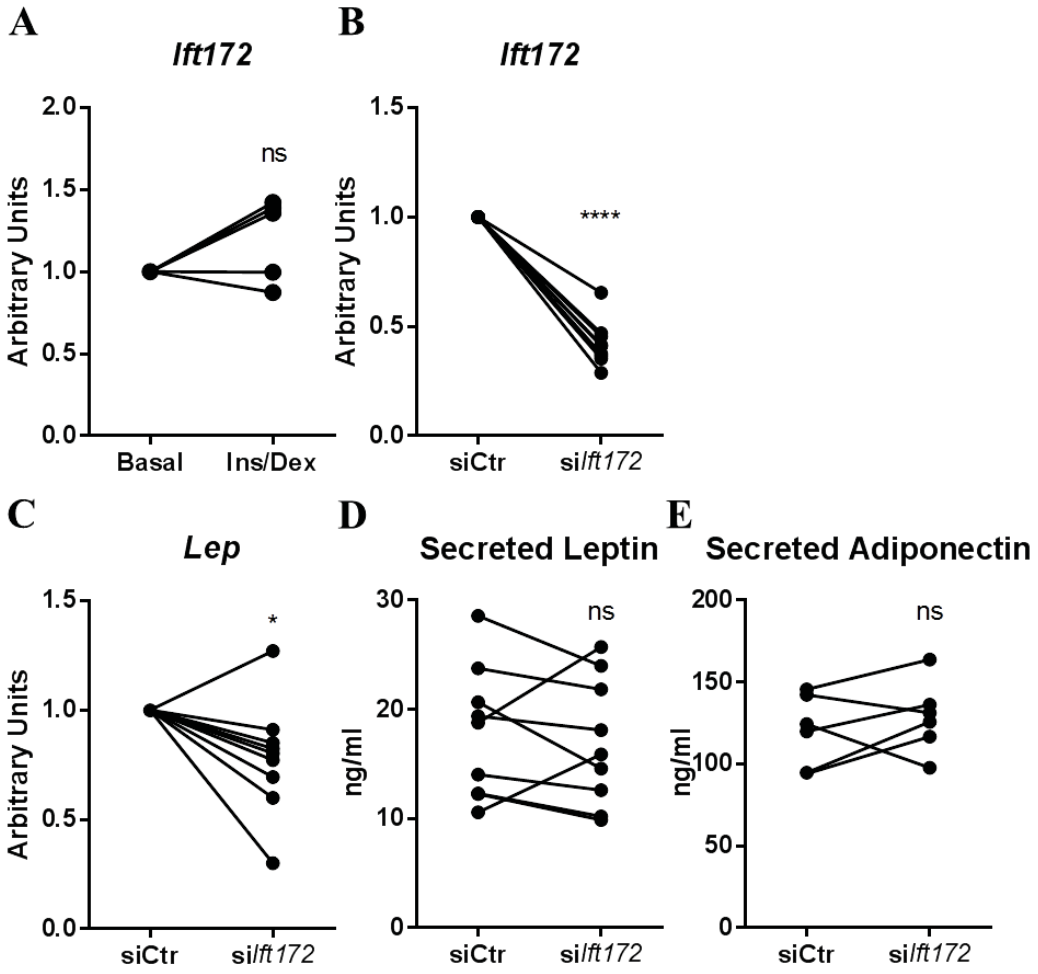
Lep transcription (**A**) and secretion (**B**) by explants in the basal or Ins/Dex stimulated state. N=5 mice per group. *Lep* knockdown (**C**) decreased LEP secretion (**D**) following stimulation with insulin and dexamethasone for 12 hours. Adiponectin secretion (**E**) remained unchanged. N=5 mice per group. Each point represents the average of 3 samples. 2 way repeated measures ANOVA. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

Supplementary Figure 5.2 *Effects of Adig knockdown on leptin transcription and secretion in perigonadal adipose tissue explants from mice fed with high fat diet.*



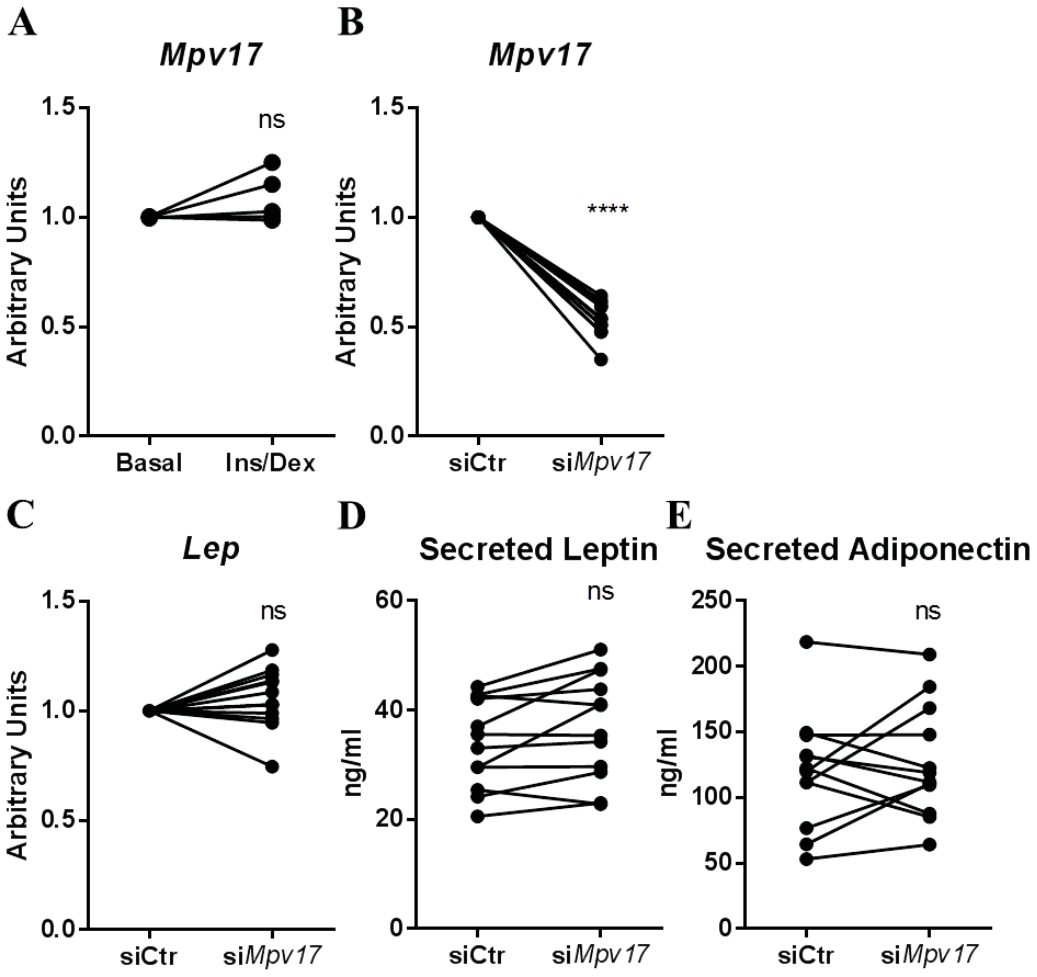
Adig expression (**A**) by explants in the basal or Ins/Dex stimulated state. N=5 mice per group. *Adig* knockdown (**B**) decreased both *Lep* expression (**C**) and leptin secretion (**D**) following stimulation with insulin and dexamethasone for 12 hours. N=12 mice per group. Adiponectin secretion (**E**) remained unchanged. N=7 mice per group. *Pparg2* expression (**F**) was unchanged as well. N=6 mice per group. Each point represents the average of 3 samples. 2 way repeated measures ANOVA. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

Supplementary Figure 5.3 *Effects of Ift172 knockdown on leptin transcription and secretion in perigonadal adipose tissue explants from mice fed with high fat diet.*



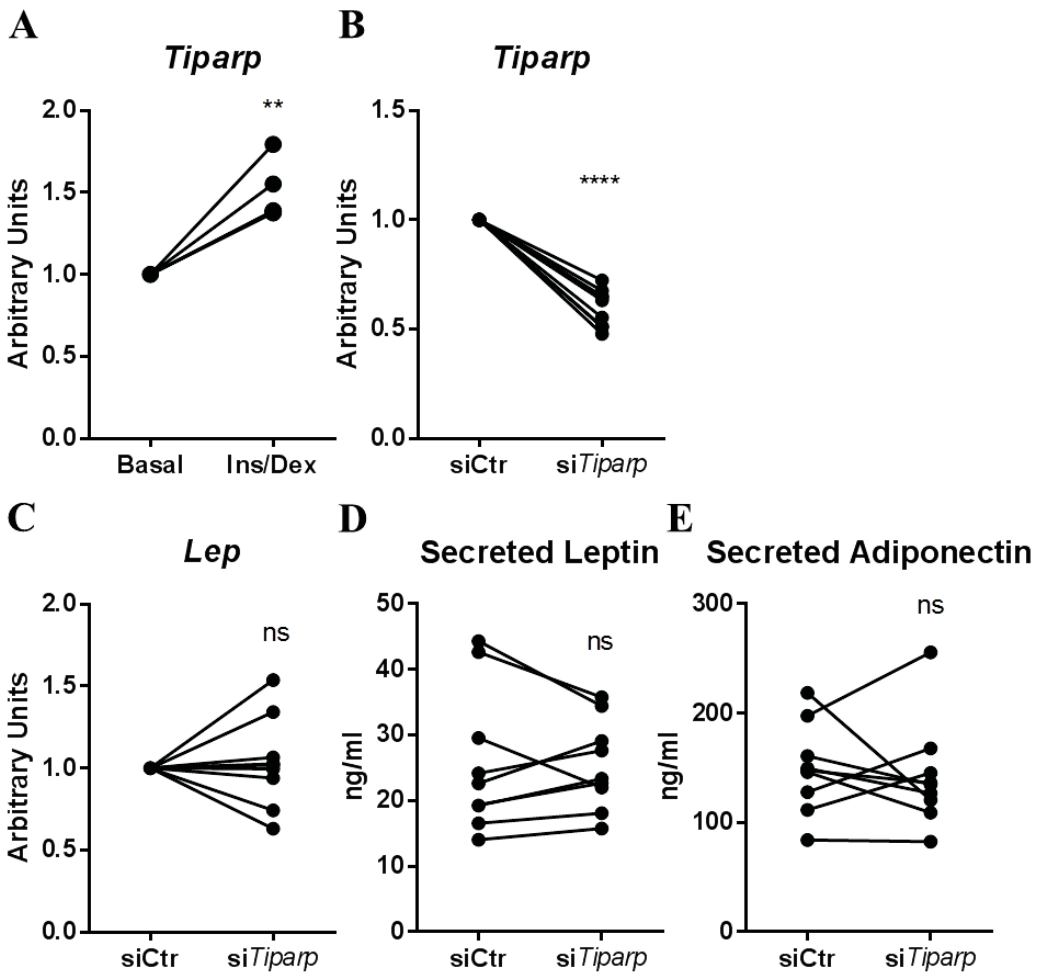
Ift172 expression (**A**) by explants in the basal or Ins/Dex stimulated state. N=5 mice per group. *Ift172* knockdown (**B**) decreased *Lep* expression (**C**) but did not change leptin secretion (**D**) following stimulation with insulin and dexamethasone for 12 hours. N=9 mice per group. Adiponectin secretion (**E**) remained unchanged. N=6 mice per group. Each point represents the average of 3 samples. 2 way repeated measures ANOVA. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

Supplementary Figure 5.4 *Effects of Mpv17 knockdown on leptin transcription and secretion in perigonadal adipose tissue explants from mice fed with high fat diet.*



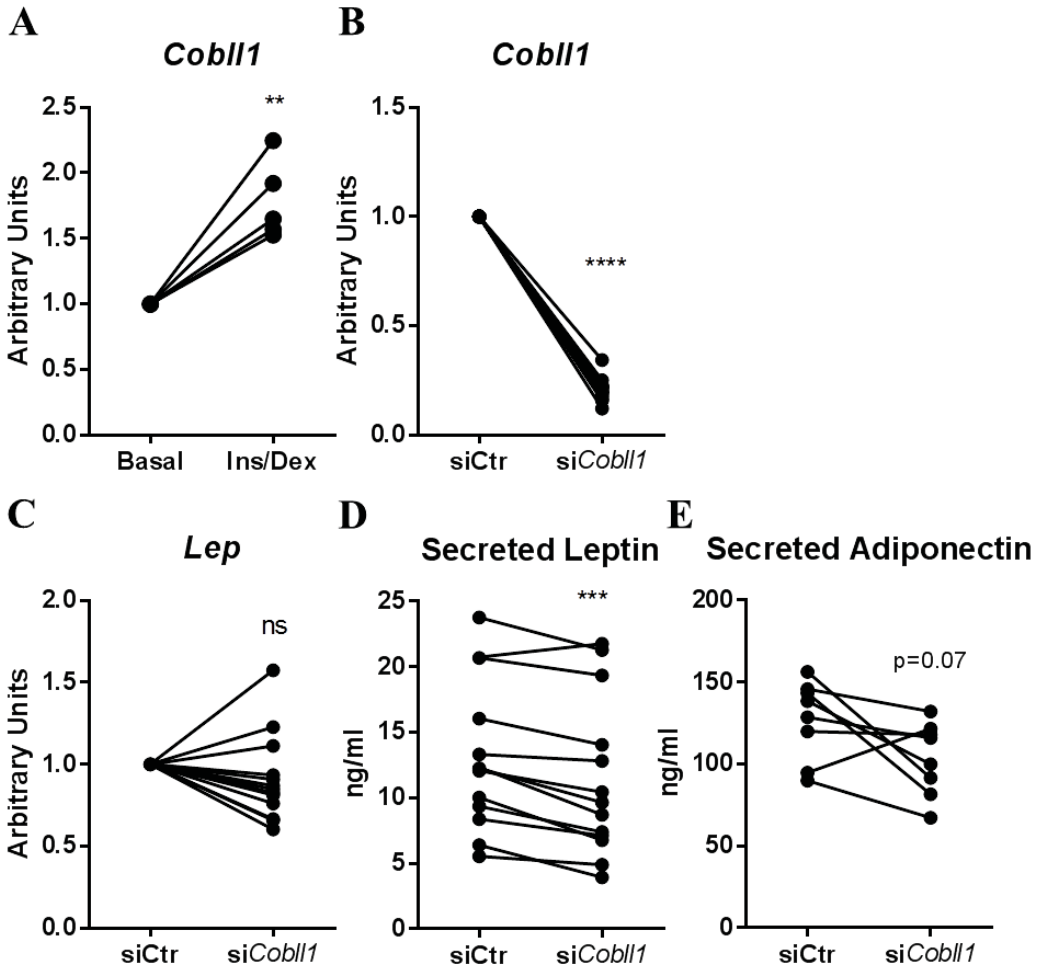
Mpv17 expression (**A**) by explants in the basal or Ins/Dex stimulated state. N=5 mice per group. *Mpv17* knockdown (**B**) did not change *Lep* expression (**C**) or secretion (**D**) following stimulation with insulin and dexamethasone for 12 hours. Adiponectin secretion (**E**) remained unchanged. N=12 mice per group. Each point represents the average of 3 samples. 2 way repeated measures ANOVA. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

Supplementary Figure 5.5 *Effects of Tiparp knockdown on leptin transcription and secretion in perigonadal adipose tissue explants from mice fed with high fat diet.*



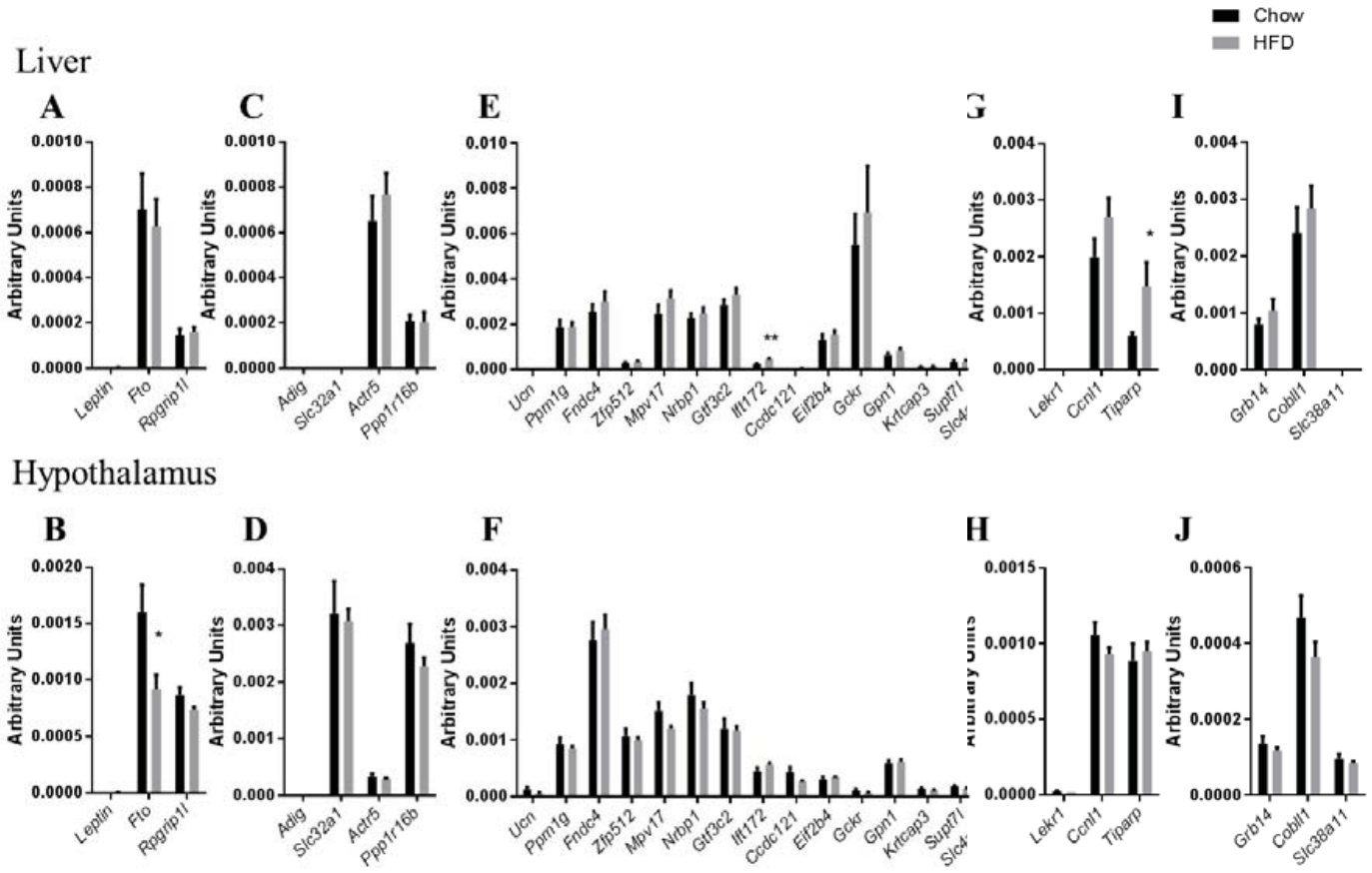
Tiparp expression (**A**) by explants in the basal or Ins/Dex stimulated state. N=5 mice per group. *Tiparp* knockdown (**B**) did not change *Lep* expression (**C**) or secretion (**D**) following stimulation with insulin and dexamethasone for 12 hours. Adiponectin secretion (**E**) remained unchanged. N=9 mice per group. Each point represents the average of 3 samples. 2 way repeated measures ANOVA. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

Supplementary Figure 5.6 *Effects of Cobll1 knockdown on leptin transcription and secretion in perigonadal adipose tissue explants from mice fed with high fat diet.*



Cobll1 expression (**A**) by explants in the basal or Ins/Dex stimulated state. N=5 mice per group. *Cobll1* knockdown (**B**) did not change *Lep* expression (**C**) but decreased Lep secretion (**D**) following stimulation with insulin and dexamethasone for 12 hours. N=13 mice per group. Adiponectin secretion (**E**) remained unchanged. N=8 mice per group. Each point represents the average of 3 samples. 2 way repeated measures ANOVA. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001

Supplementary Figure 5.7 *Expression of murine homologs of candidate genes.*



Expression of murine homologs of genes located within *LEP* (**A**&**B**), *SLC32A1* (**C**&**D**), *GCKR* (**E**&**F**), *CCNL1* (**G**&**H**), and *COBLL1* loci (**I**&**J**) in liver and hypothalamus from 4-month-old mice fed chow (black bars) or high fat diet (HFD; gray bars). qPCR transcripts were normalized using *ActB, 36B4, Gapdh* and *Ppia* as housekeeping genes. N=5 mice per group. T-test. *p<0.05, **p<0.01.

Chapter 6 Concluding Remarks

While the association between intron 1 alleles of the *FTO* locus and adiposity is statistically extremely strong, ten years of research into the molecular genetics of this locus have not revealed a clear explanation for this association. A number of genes in the vicinity of the intronic alleles have been implicated in a variety of mechanisms that may – possibly concordantly – contribute to the adiposity/obesity associations (**Fig. 4.1.1**).

Genes distant from the *FTO* obesity-associated locus may convey effects on adiposity

RBL2, a gene 270 kb from the *FTO* intronic adiposity signals has been implicated on the basis of eQTL studies showing increased *RBL2* expression in lymphocytes of individuals segregating for the obesity-risk allele of rs8050136 in the first intron of *FTO* (Jowett, Curran et al. 2010). We observed a decrease in *RBL2* expression in both murine 3T3-L1 and human preadipocyte-containing adipose tissue-derived stromal cells following *FTO* knockdown (**Chapter 4**, **Fig. 4.4.2**). RBL2 might play a role in the cardiac and craniofacial malformation syndrome seen in humans with *FTO* mutations, and in the runting and postnatal lethality in $Fto^{-/-}$ mice.

Other genes located further from the *FTO* intronic adiposity signals have also been implicated by eQTL studies. Expression of *IRX3* (approximately 500 kb away) is elevated in cerebellum of individuals segregating for the *FTO* risk allele at rs9930506. Expression of *IRX3* and *IRX5* (approximately 1.2 Mb away) were also elevated in cultured preadipocytes from individuals with this same risk allele (Smemo, Tena et al. 2014, Claussnitzer, Dankel et al.

2015). This regulation has been attributed to long-range genetic interactions between the *FTO* locus and the *IRX3* promoter identified using circular chromosome conformation capture followed by high throughput sequencing (4C-seq) (Smemo, Tena et al. 2014). Additionally, the risk allele of rs1421085 in the first intron of *FTO* disrupts binding of a transcriptional repressor, ARID5B. Knockdown of ARID5B in preadipocytes isolated from humans segregating for the protective rs1421085 allele increases *IRX3* and *IRX5* expression, further supporting the assertion that the *FTO* locus participates in long-range interactions with its neighboring genes (Claussnitzer, Dankel et al. 2015). *Irx3*$^{-/-}$ mice exhibit reduced lean and fat mass, due to elevated energy expenditure. This increase in energy expenditure is attributed to "browning" of the adipose tissue which promotes thermogenesis by the uncoupling of oxidative phosphorylation (Smemo, Tena et al. 2014, Claussnitzer, Dankel et al. 2015). However, the increased adiposity of individuals segregating for the susceptibility allele of rs9939609 in the *FTO* locus is due primarily to increased food intake (Cecil, Tavendale et al. 2008, Speakman, Rance et al. 2008, Wardle, Carnell et al. 2008, Tanofsky-Kraff, Han et al. 2009, Herman and Rosen 2015). *Irx3* is reportedly regulated by both *Rpgrip1l* (Vierkotten, Dildrop et al. 2007) and *Fto* (Ronkainen, Huusko et al. 2015), further complicating the molecular physiology of transcriptional control attributed to the *FTO* locus.

FTO and *RPGRIP1L* may convey effects of the *FTO* obesity-associated locus

The location of the obesity-associated SNPs in *FTO* suggests that they might regulate FTO itself, a purported RNA m6A demethylase and/or *RPGRIP1L*, a gene vicinal to *FTO* that encodes a critical component of the primary cilium. eQTL studies have not demonstrated an association between *FTO* or *RPGRIP1L* expression and *FTO* risk allele genotypes in any human

tissues (Kloting, Schleinitz et al. 2008, Wahlen, Sjolin et al. 2008, Grunnet, Nilsson et al. 2009, Zabena, Gonzalez-Sanchez et al. 2009, Jowett, Curran et al. 2010, Lappalainen, Kolehmainen et al. 2010, Smemo, Tena et al. 2014, Claussnitzer, Dankel et al. 2015). However, human eQTL studies are limited by the developmental stage and cellular heterogeneity of tissues being studied (Pastinen and Hudson 2004), a caveat highlighted by our data regarding the developmental timing of FTO and RPGRIP1l in adipose tissue development and function. Variants rs8050136 and rs1421085 in the first intron of *FTO* have been identified by our laboratory to regulate expression of *FTO* and *RPGRIP1L* by differential binding of the transcriptional regulator, CUX1 (Stratigopoulos, Padilla et al. 2008, Stratigopoulos, LeDuc et al. 2011, Stratigopoulos, Burnett et al. 2016). CUX1 p200 repressor binding results in reduced expression of *RPGRIP1L* and *FTO* in fibroblasts and induced pluripotent stem cells of individuals segregating for *FTO* risk alleles (Stratigopoulos, LeDuc et al. 2011, Stratigopoulos, Burnett et al. 2016).

Work from our laboratory has demonstrated that a primary site of Fto and Rpgrip1l function is in the hypothalamus, where these genes are metabolically regulated: decreased upon fasting and restored by leptin administration due to regulation of the proteolytic activity of Cathepsin L which performs Cux1 p200 processing (Stratigopoulos, LeDuc et al. 2011). These data confirm a role for these molecules in the regulation of food intake and energy expenditure governed by hypothalamic circuitry. Studies in rats support these inferences: in adult male Wistar rats, overexpression of *Fto* by injection of an AAV-vector into the ARC and PVH decreased food intake whereas *Fto* knockdown in the ARC increased food intake (Tung, Ayuso et al. 2010).

FTO and *RPGRIP1L* exhibit opposing effects in adipose tissue

We propose that the *FTO* locus also contributes to adipose tissue development and function. And that these actions – mediated by RPGRIP1L and FTO – influence the cellular anlagen of developing adipose tissue (RPGRIP1L) and the expansiveness of the adipose tissue depot under circumstances of positive energy balance (FTO).

We show that both RPGRIP1L (**Chapters 2**&**3**) and FTO (**Chapter 4**) have cell-autonomous effects on adipogenesis and adipocyte physiology. Knockdown of these genes induces reciprocal effects on adipogenesis. *Rpgrip1l* knockdown increases adipogenesis (**Chapter 3**), whereas *Fto* knockdown inhibits adipogenesis (**Chapter 4**). When *Fto* is knocked down in murine 3T3-L1 cells, *Rpgrip1l* expression is decreased. When expression of both genes is downregulated in this case, the effect of *Fto* knockdown predominates and these cells exhibit decreased lipid filling and adipocyte effector gene expression, such as *Pparg2*, *Cebpa*, *Glut4*, *Fabp4*, *Lpl* and *Atgl*.

RPGRIP1L may act to limit adipose tissue expansion

Rpgrip1l is located in the transition zone of the cilium, where it directs traffic to and from the ciliary compartment. Primary cilia, while present in preadipocytes, are lost during differentiation. Hence, we propose that Rpgrip1l functions primarily in early stages of adipogenesis. This inference is consistent with our knockdown studies, in which *Rpgrip1l* knockdown prior to differentiation enhanced adipogenesis, whereas knockdown in mature cells had no obvious effect (**Chapter 3**).

In **Chapter 2** we describe elevated adiposity in a mouse congenitally systemically hypomorphic for *Rpgrip1l* due to hyperphagia (Stratigopoulos, Martin Carli et al. 2014). The loss of hypothalamic activity of *Rpgrip1l*, which normally inhibits hyperphagia by facilitating leptin signaling, is the likely mechanism for this hyperphagia. However, we propose that the permissive effects of hypomorphism for *Rpgrip1l* on expansion of adipocyte precursors during development, facilitates the adipose tissue depot accommodation to storage of excess ingested calories (**Chapter 3**)**.** We knocked out *Rpgrip1l* in mature adipocytes of mice using the Adiponectin-Cre driver to excise a loxP-flanked exon 5 of *Rpgrip1l* and did not observe any difference in body weight between Adipoq-Cre;*Rpgrip1l*$^{fl/fl}$ mice and their *Rpgrip1l*$^{+/+}$ littermates, when fed either chow or high fat diet. We interpret this result as further evidence that in adipose tissue Rpgrip1l may function primarily during developmental initiation of adipogenesis, at which point primary cilia are sensing environmental signals which determine cellular developmental fates. Critical to further testing of this hypothesis is the development of sufficiently specific preadipocyte Cre.

FTO may convey effects of the *FTO* obesity-associated locus on type 2 diabetes

In 3T3-L1 cells and human ASCs, we detected upregulation of *Fto* expression throughout adipogenesis (**Chapter 4**). Additionally, *Fto* expression was elevated in isolated mature adipocytes compared to the preadipocyte-containing SVC fractions of perigonadal and subcutaneous depots of C57BL/6 mice.

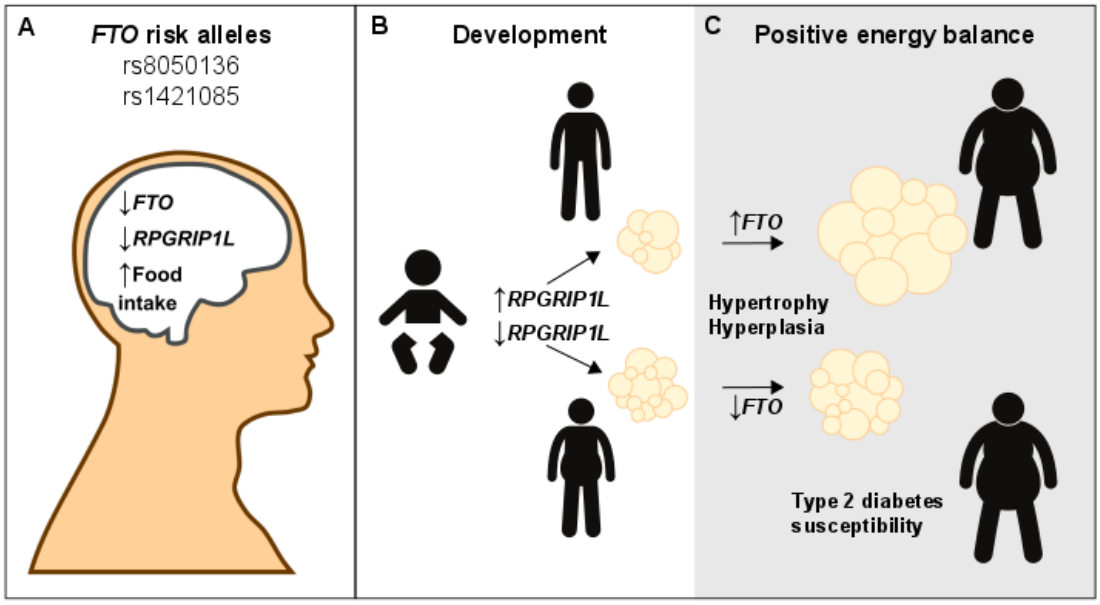
We propose that Fto functions mainly in mature adipocytes to maintain their critical lipid handling and possibly endocrine functions. We observed anti-adipogenic effects when *Fto* was knocked down before adipogenesis in 3T3-L1 cells and human ASCs. We also observed decreased lipid content in 3T3-L1 cells when *Fto* was knocked down after adipogenesis. *Fto*$^{-/-}$

mice are not lipodystrophic early in life, indicating that the molecule is not critical for early/embryonic development of adipose tissue. Embryonic development of adipose tissue is regulated by mechanisms distinct from those which govern adipogenesis in adult mice (Jeffery, Church et al. 2015, Wang, Tao et al. 2015). The late lipodystrophy seen in *Fto*$^{-/-}$ mice (loss of adiposity, accompanied by elevated blood concentrations of glucose, triglycerides and cholesterol) is indicative of a cell autonomous derangement of adipocyte function (Church, Lee et al. 2009, Fischer, Koch et al. 2009, Ronkainen, Huusko et al. 2015). Similar metabolic defects are seen in *Pparg* knockout mice, which exhibit impaired adipocyte lipid storage and utilization (Wang, Mullican et al. 2013). The decrease in *Pparg2* expression following *Fto* knockdown is consistent with this hypothesis, as Pparg2 expression is critically required for adipocytes to maintain their lipid handling and endocrine functions. Our results, demonstrating altered regulation of C/ebpβ- and C/ebpδ-targets following *Fto* knockdown, are consistent with a report implicating Fto as a transcriptional coactivator of C/ebp response elements acting via reactivation of methyl-inhibited transcription in conjunction with C/ebpβ (Wu, Saunders et al. 2010). Contrary to reports indicating that the main function of Fto is m6A RNA demethylation (Zhao, Yang et al. 2014), we did not find evidence of altered RNA m6A patterns or changes in the stability or translation of Fto-regulated targets *Cebpb*, *Cebpd*, *Ptprv*, *Spon2* or *Pten*

These distinctive and discrete effects of RPGRIP1L and FTO on adipocyte development and function are illustrated in **Fig. 4.6.1**. In individuals segregating for the rs8050136 and rs1421085 *FTO* obesity risk alleles, binding of the CUX1 p200 isoform decreases *RPGRIP1L* and *FTO* expression. In the hypothalamus, one consequence of this transcriptional regulation is increased food intake. We propose that RPGRIP1L and FTO play complementary roles in

adipose tissue, contributing, respectively, to developmental adipogenesis, and adipose tissue expansion in response to positive energy balance.

Figure 6.1 *RPGRIP1L and FTO modify the adiposity and metabolic effects conveyed by FTO risk alleles.*



A primary effect of *FTO* obesity risk alleles at rs8050136 and rs1421085 is to decrease *FTO* and *RPGRIP1L* expression in response to CUX1 p200 binding. Decreased expression of *FTO* and *RPGRIP1L* increases food intake and adiposity (**A**). Peripherally mediating/modifying this central effect on energy balance are decreases of *RPGRIP1L* and *FTO* expression in preadipocytes and mature adipocytes, respectively. During development of adipose tissue depots, decreased *RPGRIP1L* expression in preadipocytes of individuals segregating for *FTO* risk alleles (lower section) causes adipose depots to expand preadipocyte pools that are accommodative of increased fat storage (**B**). As adipocytes mature they express increased levels of *FTO*. However, in older individuals segregating for the rs8050136 and rs1421085 *FTO* obesity risk alleles that also suppress *FTO* expression, this lack of *FTO* upregulation, which may be further exacerbated in individuals maintaining positive energy balance (**C**), impairs the lipid handling and endocrine functions of adipocytes which can lead to the eventual loss of adipose tissue, ectopic lipid

storage and the ensuing metabolic sequelae. This formulation is consistent with the association of the *FTO* locus with T2D beyond the risk attributable to increased adiposity. There may also be anatomic regional differences in the FTO-mediated effects on adipocyte replacement/expansion, further contributing to the effects of FTO genotypes on insulin sensitivity and diabetes risk.

The *FTO* obesity-associated locus does not mediate food intake by altering leptin production by adipocytes

Adipose tissue development and function are critical determinants of metabolic health. In addition to the developmental effects on adiposity mediated by RPGRIP1L, and the effects conveyed on adipocyte function and survival related to FTO, the *FTO* locus could impact systemic energy homeostasis by modifying production of humoral signals which are integrated centrally to regulate energy balance. In **Chapter 5**, we explored molecular modifiers of adipocyte production of leptin identified by GWAS (Kilpeläinen, Carli et al. 2016) as reductions in leptin production by adipocytes could increase obesity risk (Zhang, Proenca et al. 1994). Knockdown of *Adig* and *Cobll1*, homologs of genes near the *SLC32A1* and *COBLL1* leptin-associated loci, respectively, both decreased the amount of leptin produced by mouse adipose tissue explants cultured *in vivo*. The *FTO* locus was associated with elevated circulating leptin concentrations but this association was abrogated when corrected for BMI, indicating that this locus does not contribute to adiposity by dysregulating leptin production. Our *in vitro* findings are consistent in this regard, as knockdown of *Rpgrip1l* and *Fto* in 3T3-L1 cells did not affect leptin production per adipocyte.

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