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Obesity and FTO: Changing Focus at a Complex Locus

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The fat mass and obesity-associated (*FTO*) gene was placed center stage when common intronic variants within the gene were robustly associated with human obesity. Murine models of perturbed *Fto* expression have shown effects on body weight and composition. However, a clear understanding of the link between *FTO* intronic variants and FTO activity has remained elusive. Two recent reports now indicate that obesity-associated SNPs appear functionally connected not with *FTO* but with two neighboring genes: *IRX3* and *RPGRIP1L*. Here, we review these new findings and consider the implications for future analysis of GWAS hits.

When first cloned in 1999, *Fto* was postulated to have a role in programmed cell death and development. It was identified in the study of the "fused toe" (*Ft*) mouse, a model organism created by insertional mutagenesis that had a 1.6 Mb deletion on chromosome 8 (van der Hoeven et al., 1994). Mice heterozygous for the *Ft* mutation developed fusion of the first to fourth toes of the forelimbs and thymic hyperplasia (Peters et al., 1999). This deletion eliminated six genes (Peters et al., 2002) and three members of the Iroquois gene family (*Irx3*, *Irx5*, and *Irx6*) that form the *IrxB* cluster, as well as three other genes (*Fts*, *Ftm*, and *Fto*), which, at the time, were poorly characterized. *Fts* was initially called "*Ft1*," being the first of the three to be identified; the second proved to be an elusive sequence to characterize technically, so it was labeled the *Fantom* (*Ftm*); the third gene was named "*Fatso*" (*Fto*) because of its large size (Fischer et al., 2008).

Setting a theme that plays throughout the studies of *Fto* biology, subsequent studies of the *Ft* mouse were taken up in trying to determine which of the affected genes in the larger deleted locus were responsible for the component parts of the diverse phenotypes seen in the animal. Furthermore, these initially mysterious genes underwent name changes to reflect evolving knowledge. *Ftm* was found to be a novel basal body protein of cilia involved in Hedgehog (Hh) signaling (Vierkotten et al., 2007). Sequence analysis revealed *Ftm* to be highly homologous to *RPGRIP1* (retinitis pigmentosa GTPase regulator interacting protein 1, a gene encoding a protein with a role in photoreceptor cells of the eye), and it was renamed *Rpgrip1-like* (*Rpgrip1l*). In mice, inactivation of *Rpgrip1l* causes a multiorgan syndromic phenotype with cerebral, renal, and hepatic defects (Vierkotten et al., 2007), while mutations in *RPGRIP1L* are the cause of a group of developmental disorders such as Joubert syndrome type B and Meckel syndrome (Delous et al., 2007).

With the coming of age of technologically advanced genetic association studies, *Fto* underwent a more subtle, but no less significant, name change, with the roots of the labeling acronym reflecting its new association with obesity and metabolism.

A Star Is Born

In the first half of 2007, three independent studies demonstrated a strong association between genetic variance within FTO and human obesity. The gene name morphed into "*fat* mass and *o*besity associated," and the metabolic community had, at last, an example of common genetic variance with robust evidence of association with obesity in the general population.

The Frayling et al. (2007) study initially set out to search for type 2 diabetes mellitus susceptibility genes. In doing so, they identified a common variant in the *FTO* gene that, indeed, predisposed to diabetes but through an effect on body mass index (BMI). This effect on BMI was driven entirely through fat mass and, although not detectable at birth, was fully present by the age of 7 years and persisted through adulthood (Frayling et al., 2007). The major signal for association with BMI was a cluster of SNPs in the first intron of *FTO.* All BMI-associated SNPs were highly correlated with each other, but SNP rs9939609, having the highest genotyping success rate, was studied further. The 16% of adults who were homozygous for the risk allele A weighed close to 3 kg more and had 1.67-fold increased odds of obesity when compared with those not inheriting a risk allele (Frayling et al., 2007).

Complementing and confirming these findings, a contemporaneous report by Dina et al. (2007) took a different approach to discover this association, initially setting out to analyze the distribution of 48 neutral SNPs in a case-control obesity cohort collected from French individuals of European ancestry. While 47 of the 48 showed uniform distribution, the T allele of SNP rs 1121980 (located within the first intron of *FTO*) was strongly associated with severe adult obesity. Building on this observation, Dina et al. demonstrated that several SNPs in the FTO locus were highly associated not only with severe obesity in other adult, European populations but also with severe childhood obesity.

The last of this triumvirate undertook a genome-wide association study in the genetically isolated population of Sardinia to identify genetic variants associated with obesity-related quantitative traits (Scuteri et al., 2007). Again, a number of common variants in FTO were associated with BMI, hip circumference, and total body weight.

The significant challenges were clear from the beginning. *FTO* was widely expressed throughout the body (Frayling et al., 2007; Dina et al., 2007; Gerken et al., 2007), and, at the time, nothing was known about function or potential pathways. All of these

initial reports made it clear that there was no ready mechanism to explain how the predisposing intronic variants affected function or expression of *FTO*. Indeed, right from the start, the possibility that the underlying mechanism involved neighboring genes or other, as-yet-unidentified, more distant genes was raised.

However, hot on the heels of these initial findings, there followed a report that gave some important clues to FTO biology. Bioinformatics analysis indicated that FTO shared sequence motifs with Fe(II)- and 2-oxoglutarate-dependent oxygenases, with assays using recombinant murine Fto showing that the protein could catalyze Fe(II)- and 2OG-dependent DNA demethylation, the preferred substrate being 3-methylthymine in DNA. Fto was also found to be localized to the nucleus and highly expressed in hypothalamic regions with critical roles for the control of energy balance in a nutritionally dependent manner (Gerken et al., 2007).

Subsequently, genome-wide association studies (GWASs) for obesity-related traits in a myriad of European ancestry populations confirmed that multiple SNPs at the *FTO* locus were associated with BMI (Graff et al., 2013; Bradfield et al., 2012; Lindgren et al., 2009; Meyre et al., 2009; Scherag et al., 2010; Speliotes et al., 2010; Thorleifsson et al., 2009; Wheeler et al., 2013; Willer et al., 2009). Many other reports found the association of *FTO* SNPs with BMI in non-European-derived populations, including most populations of Asian ancestry, as well as Hispanic/Latino populations and Pima Indians (reviewed by Loos and Yeo, 2014).

Studies in populations of African ancestry, in which the *FTO* gene shows significant differences in allele frequency and linkage disequilibrium (LD) patterns, have proven to be insightful. Although several of the previously identified variants appear not to have significant association with BMI in these populations, other intronic variations around the FTO locus (such as rs3751812 and rs9941349) show strong evidence of association (Adeyemo et al., 2010; Hassanein et al., 2010). Furthermore, the most significant value in the report by Peters et al. (2013) came from a SNP (rs56137030) that had not been highlighted in previous studies. A recent meta-analysis by Monda et al. (2013) of data derived from populations of African ancestry also provided support for shared BMI loci across populations.

The association of *FTO* SNPs with obesity-related traits in children and adolescents was also extended (Bradfield et al., 2012; Meyre et al., 2009; Scherag et al., 2010). While SNPs in *FTO* did not influence birth weight (Horikoshi et al., 2013; Jess et al., 2008; Kilpeläinen et al., 2011a), longitudinal studies revealed that the effect on body weight appeared during early childhood, reaching its peak at young adulthood (Graff et al., 2013; Hardy et al., 2010; Sovio et al., 2011).

This initial wave of association studies was soon followed by reports considering whether *FTO* SNPs associated with more specific regulators for energy homeostasis, such as food intake or physical activity.

Data pointing to a link with food intake began to emerge. Obesity-associated *FTO* SNPs were found to be associated with increased energy intake (Cecil et al., 2008; Speakman et al., 2008; Timpson et al., 2008), increased intake of dietary fat (Park et al., 2013; Timpson et al., 2008) or protein (Sonestedt et al., 2009), increased appetite and reduced satiety (Wardle et al., 2008, 2009), and loss of control over eating (Tanofsky-Kraff et al., 2009). This link with food intake was not seen in every study; for example, a report by Stutzmann et al. (2009) failed to find an association of the *FTO* rs 1421085 C allele with eating behavior traits in a large European cohort of children and adults. However, a recent GWAS of macronutrient intake in more than 70,000 individuals identified the BMI-increasing allele of *FTO* SNPs as highly significantly associated with increased protein intake (Tanaka et al., 2013).

Other studies have consistently shown that *FTO* SNPs are not associated with physical activity levels (Ahmad et al., 2010; Franks et al., 2008; Speakman et al., 2008; Vimaleswaran et al., 2009). However, low physical activity has been reported to accentuate the effect of *FTO* risk alleles on obesity in French adults (Cauchi et al., 2009), with a large-scale meta-analysis of published and unpublished data of more than 200,000 adults and 20,000 children (Kilpeläinen et al., 2011b) concluding that the association of *FTO* SNPs with the odds of obesity are reduced by close to a third in physically active adults. Phenotypic variability in BMI for a given *FTO* genotype was also reported by Yang et al.'s (2012) report highlighting the potential of environmental modifiers to influence the "genetic burden" of the *FTO* risk alleles.

Model Organisms and *Fto*

The phenotype of mice with a global germline loss of *Fto* was first reported in 2009 (Fischer et al., 2009). *Fto* null mice suffered from a high perinatal mortality and a postnatal period characterized by reduction in both body length and body weight, the latter being a result of reduced fat and lean mass. They were reported to have both an increase in food intake and an increase in metabolic rate. Later analyses of several independent murine models of *Fto* loss also confirmed this pattern of postnatal growth retardation, but one report has shown that, when corrected for alteration in body composition, there was no difference in energy expenditure in mice globally lacking *Fto* (McMurray et al., 2013).

In 2010, Cox and colleagues in Harwell, UK, generated mice globally expressing additional copies of the *Fto* gene (Church et al., 2010). This ubiquitous overexpression of *Fto* increased body and fat mass, with the obese phenotype more marked on a high-fat diet. Food intake was significantly increased, but energy expenditure and physical activity were unaltered in these mice.

Reports of more specific regional and temporal *Fto* perturbation soon emerged. Gao et al. (2010) used a conditional allele to delete *Fto* in the nervous system. Their findings that this resulted in similar phenotypes as that of the whole body deletion pointed to *Fto* having a crucial role in the central nervous system to regulate postnatal growth. Following from the finding that *Fto* expression in the arcuate nucleus of the hypothalamus was nutritionally regulated (Gerken et al., 2007; Stratigopoulos et al., 2011; Poritsanos et al., 2011), we targeted this region with stereotactically delivered adeno-associated virus and demonstrated that overexpression decreased food intake while knockdown of *Fto* increased food intake (Tung et al., 2010).

McMurray et al. (2013) also used a conditional allele to globally delete *Fto* at 6 weeks of age. Development in the weeks after was characterized by a loss of lean mass but an increase in fat mass (McMurray et al., 2013). Intriguingly, regional deletion of *Fto* in mediobasal hypothalamus of adult mice did affect feeding behavior but did not affect body composition, which is

suggestive of *Fto* expressed in nonhypothalamic sites having a role in controlling lean mass.

Thus, although many questions remained unanswered, from these animal data at least, it seemed plausible that *Fto* had a role in the regulation of body composition and energy balance.

Novel Functions, Unexpected Roles

How then does one to try and link these murine and association studies with a common molecular mechanism, particularly one involving the demethylase activity reported by Gerken et al. (2007)? The family of demethylase enzymes had long been known to have a role in the repair of methyl lesions in DNA and RNA (Ougland et al., 2004; Lee et al., 2005) but had little to link them directly to control of metabolism and body composition. Further studies followed that confirmed that FTO catalyzed the demethylation of 3-methylthymine in single-stranded DNA, as well as 3-methyluracil (3meU) (Gerken et al., 2007; Jia et al., 2008) and 6-methyl adenosine (6meA) (Jia et al., 2011) in single-stranded RNA.

The crystal structure of FTO was resolved and showed an N-terminal catalytic domain and a C-terminal domain of unknown function (Han et al., 2010). The specificity for single-stranded nucleic acids appeared to come from an L1 loop, not present in other members of the AlkB family, that acted to hinder double-stranded nucleic acids from entering the catalytic pocket (Han et al., 2010).

The finding that 6meA, the most common modified nucleoside found in messenger RNA (mRNA) (Desrosiers et al., 1974), was demethylated by FTO, with 50-fold greater affinity than 3meU (Jia et al., 2011), gave rise to a hypothesis in which FTO influenced metabolism by posttranscriptional modification of RNA message. This idea was featured in two studies in 2013 (Hess et al., 2013; Karra et al., 2013) that explored how FTO's putative demethylase action might affect complex human phenotypes.

Fto and Dopamine

Hess et al. (2013) described a role for *Fto* in controlling the dopaminergic circuitry within the midbrain. The idea to explore a putative *Fto*-dopamine link came about because the phenotype of the *Fto* null mouse closely matched the description of mice lacking dopamine receptor type 2 (D2R) (Sibley, 1999). Their studies found that *Fto* null mice responded differently to control animals after administration of cocaine, a drug that can inhibit the dopamine transporter and, thereby, increase synaptic dopamine levels. In response to cocaine, *Fto*-deficient mice failed to show an increase in locomotor activity; had less c-*fos* activation in the ventral tegmental area, caudate putamen, and nucleus accumbens; and had a blunted increase in extracellular dopamine. Further studies, in which pharmacological agents known to act through the dopaminergic system were administered to a range of genetically engineered animals, confirmed that *Fto* loss impairs D2R-like signaling in a cell-autonomous manner.

An elegant component to this story combined methylated RNA immunoprecipitation with bioinformatics to try and link these novel findings with previous data on the demethylase action of FTO. By identifying transcripts containing m6A in midbrains and striatum from control and *Fto*-deficient mice, Hess et al. (2013) found that the transcripts selectively methylated in *Fto* null mice were disproportionately linked to synaptic transmission and cell signaling. Furthermore, in the absence of Fto, both mRNA and protein of key regulators of dopaminergic neuron activity showed increased methylation.

The identification of FTO-specific adenosine methylation on a subset of mRNA involved in dopamine signaling pathway was the first in vivo demonstration of FTO as a functional m6A demethylase. These observations also provided the first indication that FTO may play a tissue- or even cell-type-specific role by regulating specific subsets of mRNA relevant to the physiological function of the tissue, and this might help explain how a single protein can appear to have such widespread influence over a range of seemingly disparate functions.

Ghrelin and the Brain: Is FTO the Missing Link?

The findings of the Hess et al. (2013) study gave *Fto* a potential role in the reward systems. Following shortly afterward, a study by Karra et al. (2013) pursued a mechanistic link between *FTO* SNPs and gut peptides. They hypothesized that *FTO* variants might not only influence circulating levels of hormones known to have a role in appetitive behavior but might also affect the neuronal response to food cues.

Their studies focused on small numbers of young, normal-weight volunteers divided into two matched groups on the basis of their genotype at the rs9939609 locus, either high risk (AA) or low risk (TT). Compared to TT subjects, after a test meal, the AA group was reported to have a less marked fall in acyl ghrelin and an attenuated suppression in a visual analog hunger score.

Analysis of mRNA from peripheral blood cells after a period of fasting showed that subjects with the AA genotype had, in comparison to the TT group, a 1.5-fold increase in *FTO* expression, a 2.5-fold increase in ghrelin precursor GHRL mRNA abundance, and a reduction in m6A methylation of ghrelin mRNA. fMRI analysis showed between-genotype differences in a number of brain regions recognized to have roles in energy homeostasis and reward related activity.

A further potential link to ghrelin was suggested by data from in vitro studies where *FTO* overexpression increased not only ghrelin and ghrelin O-acyltransferase mRNA but also total and acyl ghrelin concentrations in cell lysates. *FTO* overexpression was also reported to reduce m6A methylation of ghrelin mRNA.

These data led Karra et al. (2013) to the suggestion that the association of *FTO* risk allele with food intake may be mediated through ghrelin, with the risk allele increasing *FTO* expression, which, in turn, reduces m6A ghrelin mRNA demethylation and, thereby, alters ghrelin production. The differential responses on fMRI observed in subjects with SNP were certainly striking because such phenomena have only been demonstrated previously in situations with drastic physiological differences (De Silva et al., 2012), such as comparing lean versus obese individuals or fed versus fasted state, or in the exogenous administration of appetite-modulating hormones (e.g., ghrelin, PYY, GLP-1, and leptin).

While this work is of interest, it is worth noting that leucocytes are not recognized as the natural home for understanding gut hormone biology, and the sizeable changes in mRNA levels in white cells were not reflected in differences in the amount of circulating active acyl-ghrelin seen in the fasted state. Although there are measurable relative changes in white cell levels, the absolute contribution from these cells to total biological active

ghrelin may not be large. It remains to be determined how, if at all, risk genotype affects ghrelin expression and production in the stomach and whether what is happening in a leucocyte bears any relationship to what might be happening within the antrum of the stomach. Additionally, it is worth noting that several studies have failed to reveal any influence of the *FTO* genotype on mRNA level of *FTO*; although mRNA expression of *FTO* in adipose tissue has been shown to be greater in obese individuals, this difference did not extend to *FTO* expression in blood cells. The fact that mice globally lacking *Fto* have unchanged levels of acylated ghrelin in both the fed and fasted states also suggests that *Fto* levels contribute little to circulating levels of ghrelin.

Loss-of-Function Mutations in Humans

Despite all these emerging data, there remained the recurring uncertainty of what these findings brought to our greater understanding of human disease, the primary driver for undertaking the genetic studies in the first place.

Important data on the potential consequences of FTO loss in humans came from a study of a consanguineous family in which affected individuals presented with a previously unreported autosomal-recessive syndrome characterized by polymalformation and childhood mortality (Boissel et al., 2009). Genome-wide autozygosity screening identified a unique region of shared homozygosity on chromosome 16q12. Further genotype and haplotype analyses reduced the critical region to a 6.5 Mb interval encompassing 28 genes. After sequencing coding regions and splice junctions of all known and putative genes in this linked region, the only mutation found was a homozygous single-nucleotide variation at complementary DNA position 947 within the *FTO* gene. Affected individuals carried a homozygous R316Q mutation in FTO, with loss of this highly conserved arginine residue rendering FTO enzymatically null in two different in vitro assays; the first based on the conversion of the 2-oxoglutarate to succinate and the second based on the ability of FTO to demethylate 3-methylthymine in DNA. The affected individuals homozygous for a catalytically inactive FTO also had, like *Fto* null mice, an early growth retardation phenotype but, unlike the *Fto* null mice, also had developmental abnormalities in the central nervous or cardiovascular systems. These differences are not readily explained, and the molecular mechanisms whereby the mutant FTO leads to the severe phenotype observed these patients remain unknown. However, it may be that the point mutation in *FTO* has deleterious gain-of-function effects and/or dominant-negative interference with other biochemical processes that are not seen with the complete deficiency seen in the *Fto* null mouse.

What of other members of this extended family? $Fto^{+/-}$ mice were considered to be resistant to diet-induced obesity (Fischer et al., 2009), so could heterozygous carriage of this deleterious R316Q mutation actually protect against obesity? This also remains uncertain because, although this report comments that none of the parents of the affected children were obese, detailed phenotypic data on the extended family has not been reported.

Other studies have gone on to investigate whether nonsynonymous variants of *FTO* might be enriched in either lean or obese subjects. Meyre et al. (2010) sequenced entire *FTO* coding regions in both subjects with severe obesity and in individuals with lifelong leanness. Interestingly, nonsynonymous mutations were equally common in both the obese and lean cohorts. Furthermore, heterozygous mutations that severely impaired enzymatic activity of FTO were found in both lean and obese subjects who were otherwise clinically unremarkable.

Other studies in lean and obese cohorts of children brought similar findings. In both African American and Chinese Han populations, variants were identified in *FTO*, but the overall frequencies were similar in case and control, with none conferring risk of obesity (Deliard et al., 2013; Zheng et al., 2013).

Taken in isolation, these human data fall some way short of a compelling case for FTO being the candidate mediator of the obesity association. Murine data indicate that an increase in *Fto* expression could potentially lead to obesity, but, to date, only deleterious point mutations have been described in obese patients. However, it may be that there are as-yet-uncharacterized patients in other cohorts with genetically deleterious mutations leading to complete loss of FTO. For example, such an individual may, if one were to phenocopy the mouse model of Fto deficiency, present initially as a "failure to thrive" and thereafter be noted to be of small stature.

An Interesting Neighborhood

As outlined earlier, the initial *FTO* intronic associations, followed by identification of association SNPs in intron 3 (Tönjes et al., 2010) and intron 8 (Adeyemo et al., 2010), set in train a plethora of studies based around *Fto* biology. However, other groups took a different path, with the Leibel group, in particular, choosing to focus on *Rpgrip1l* as being the potential mechanistic link (Stratigopoulos et al., 2008, 2011). They initially took an in silico bioinformatics approach to focus on two intronic SNPs (rs17817449 and rs8050136) and predicted that they were located in a putative binding site for the transcription factor Cutl-like 1 (CUTL1), also known as cut-like homeobox1 (CUX1) (Stratigopoulos et al., 2008). CUTL1 acts as a transcriptional repressor by displacing activators (Skalnik et al., 1991) and/or by recruitment of histone deacetylase 1 (Mailly et al., 1996). Initial in vitro data were promising. In chromatin immunoprecipitation of DNA from human fibroblasts using a CUTL1-specific antibody, a 90-base-pair fragment that included rs8050136 was precipitated. Furthermore, small interfering RNA-mediated reduction of Cutl1 by 70% resulted in *FTO* expression decreasing by 90% and *RPGRIP1L* by 65%.

Having raised the possibility that both genes were regulated by CUTL1/CUX1 via a single regulatory site in the first intron of *FTO*, Leibel's group looked, in more detail, at the role of this transcription factor on the different isoforms (the larger P200 and the smaller p100 isoform, generated from p200 by enzymatic cathepsin L activity) (Stratigopoulos et al., 2011).

A series of detailed biochemical and promoter analysis experiments led to a proposed mechanism whereby, together, FTO, RPGRIP1L, and CUX1 facilitate the leptin response within the hypothalamic arcuate nucleus and thereby affect food intake. The model proposed that reduced circulating leptin, as seen in negative energy balance, results in the reduced enzymatic processing of CUX1 P200 and decreased CUX1 P110 protein levels. In turn, this change in transcription factor isoform ratio decreases *RPGRIP1L* and *FTO* expression, reducing leptin signaling through an alteration in leptin receptor recruitment at the cilium

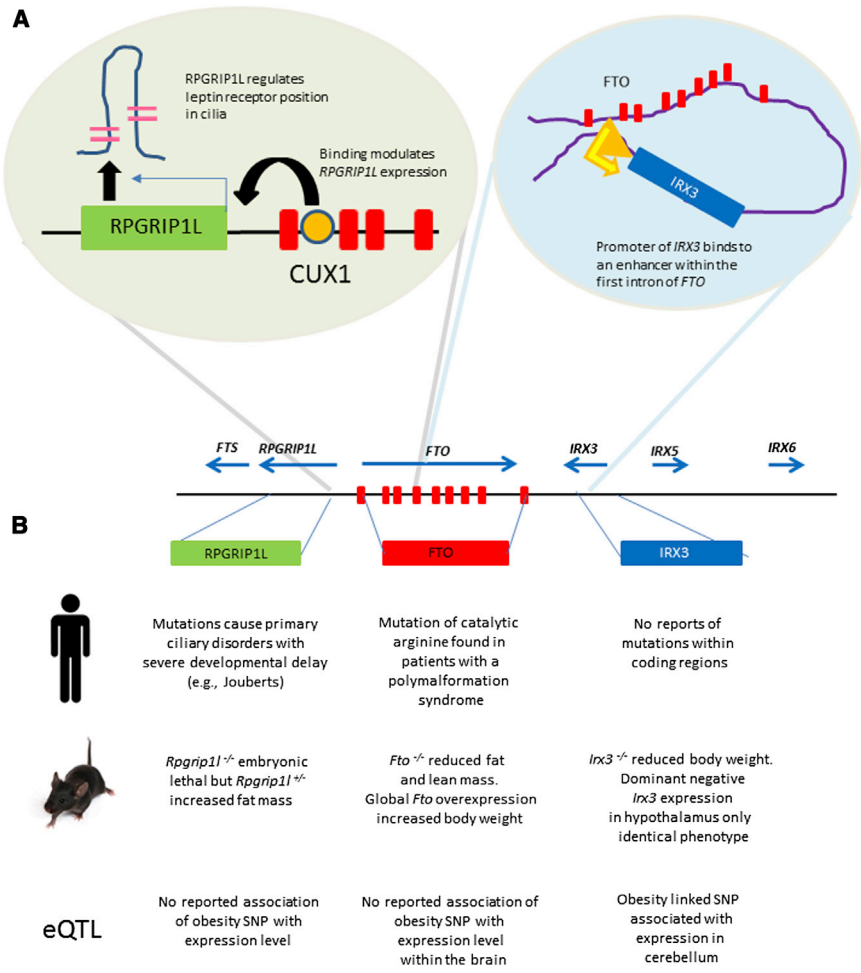

Figure 1. Increasing Functional Complexity around the FTO Locus

(A) Emerging data indicate that the links between intronic variance within FTO and body composition are mediated through functional interactions with neighboring genes. The first intron of FTO contains a binding site for the transcription factor CUX1 which, through regulation of RPGRIP1L expression, modulates leptin receptor localization within neurons. This intron also contains an enhancer sequence which directly binds to the promoter of IRX3.

(B) Summary of data on FTO, RPGRIP1L, and IRX3 from human genetic and murine model studies. Data on IRX3 are notable in that eQTL mapping demonstrates an association of obesity-linked SNPS with IRX3 expression.

and thereby increasing the drive to eat. The proposed model goes on to suggest that, because P110 has reduced affinity for the rs8050136 obesity-risk A allele but higher affinity for the protective C allele, individuals with the C allele would have higher *FTO* and *RPGRIP1L* expression and be relatively more leptin sensitive (Figure 1A).

Building on their previous findings, this group has more recently published a detailed metabolic phenotype of mice lacking one copy of *Rpgrip1l* (Stratigopoulos et al., 2014). As noted previously, homozygous mutations in *Rpgrip1l* led to significant developmental anomalies (Vierkotten et al., 2007), but *Rpgrip1l*$^{+/-}$ mice had a small but statistically significant 10% increase in body weight demonstrable by 10 weeks old (Figure 1B). Analysis of older animals showed this to be driven by an increase in fat but not lean mass. By 19 weeks old, male mice had an increase in both fat and lean mass, with absolute food intake corrected for different body compositions showing *Rpgrip1l*$^{+/-}$ to be hyperphagic.

In Rpgrip1l$^{+/-}$ mice, an acute leptin dose was reported to show a diminished Stat 3 response within the hypothalamus, and an antibody-based analysis showed a reduction of leptin receptor in the vicinity of the cilia.

This group went on to look at fibroblasts from healthy human controls and from a patient with Jouberts syndrome to further determine how RPGRIP1L interacts with the leptin receptor. When leptin receptors were transfected into control fibroblasts, they correctly localized to the cilium with leptin stimulation, and there was a dose-dependent increase in Stats 3 phosphorylation. In contrast, there was both perturbed localization and diminished signaling in *RPGRIP1L*-deficient fibroblasts.

They concluded that alteration in leptin receptor signaling, brought about as a direct consequence of alteration in the function of primary cilium, accounted for the increase in fat mass of *Rpgrip1l*$^{+/-}$ mice. These model organism studies are presented as further supportive evidence that individuals with obesity risk A allele at rs8050136 have reductions in both *RPGRIP1L* and *FTO* expression due to reduction on p110 at cognate CUX1 binding, causing diminished leptin signaling, increased food intake, and adiposity.

Disorders of ciliary biology have long been linked to "syndromic obesity," and these data from the Leibel group now raise the intriguing possibility of primary cilia having a role in common forms of obesity (Stratigopoulos et al., 2008, 2011). However, there remains still that elusive, direct connection between obesity-associated variants and expression of the gene in question: in particular, whether in humans the number of copies of the risk allele associates with changes of expression of *RPGRIP1L* and *FTO*, not only in the direction predicted by the model but also in physiologically relevant tissue.

IRX3: Another Interesting Neighbor

Irx3 was one of the initial genes identified within the *Ft* region but initially did not attract as much attention as its neighbors did. A report by Ragvin et al. (2010) reignited interest in its relationship with FTO. This group focused on the fact that the *FTO* LD block appeared to contain numerous highly conserved noncoding elements (HCNEs) and used computational analysis to determine if these potential regulatory elements might affect target genes elsewhere.

Their initial analysis suggested that the target gene of the HCNE within *FTO* was *IRX3* and, using sequences from the obesity-associated *FTO* LD block in a zebrafish-based, green fluorescent protein reporter assay, found that expression patterns matched those of *IRX3*. As *IRX3* is highly expressed in pancreas, they went on to suggest that at least some of the effect of the *FTO* locus on susceptibility to type 2 diabetes may be through changes in insulin secretion.

Smemo et al. (2014) built on these observations in a comprehensive study bringing together evidence from murine, human, and in vitro studies to make a compelling case for the involvement of IRX3 in the association between human obesity and *FTO*.

They initially investigated *cis*-regulatory interactions between *FTO* and *IRX3*, using chromatin conformation capture in both embryonic (mice and zebrafish) and brain tissue from adult mice. They found that the promoter of *Irx3* strongly interacted with the obesity-associated interval within *FTO* (Figure 1A).

Results from an in vivo mouse reporter assay that tested human DNA fragments from the *FTO* obesity-associated region, plus data from studies using a human bacterial artificial chromosome (BAC) spanning the *FTO* locus that included its promoter and the obesity-associated region, clearly showed that *IRX3* relied on long-range regulatory input from *FTO*.

Building on this, a crucial data set missing from previous studies looked at gene expression levels in the cerebellum, a site of high expression of both *FTO* and *IRX3*. This expression quantitative trait loci (eQTL) mapping in human brain samples demonstrated that obesity-linked SNPs such as rs9930506 were associated with *IRX3* expression, but not with expression of *FTO,* thereby directly linking these variants to *IRX3* regulation (Figure 1B).

Returning to mouse models, Smemo et al. (2014) reported that *Irx3* null mice had a significant reduction in body weight, with less fat and lean mass compared to wild-type controls. Transcriptional analysis of tissue indicated that there may be increased sympathetic tone to white adipose tissue, as well as increased activation of brown adipose, leading to increased energy expenditure that contributes to the phenotype seen. Using *Cre-lox* technology to express a dominant-negative Irx3 only within the hypothalamus, this group determined that disrupting Irx3 function in this region alone recapitulated the metabolic phenotype of *Irx3*-deficient mice, thereby supporting the notion that hypothalamic Irx3 is critical in the regulation of body composition.

While this appears to be a compelling set of data, there are still some areas of uncertainty. The cerebellum is not an area of brain normally recognized to be involved in the control of energy expenditure and eQTL analysis using hypothalamic tissue seems to be an important next step. Furthermore, it would be very interesting to determine if overexpression of *Irx3*, either globally or in a more tissue-specific manner, causes obesity in a model organism. Nevertheless, it would appear that IRX3 has a strong candidacy to be a major mediator of the effects of the common intronic SNPs on human adiposity.

Discussion

The technological and computational advances that underpin genetic association studies have brought forth a glut of new information relevant to a whole host of different human phenotypes, complex traits, and disease conditions. They have much potential to reveal previously unanticipated insights, but as powerful as the data generated are, there remains the need to move from an association map into systems, models, and platforms that can be used as tools to get to grips with the biological architecture of the problem under scrutiny.

So, 7 years on from the initial cluster of reports, how far have we come with the poster boy of metabolic GWASs?

The data set from animal studies make a decent case that FTO has a role in the control of energy homeostasis and body composition. However, there is still much to learn. Whereas initial studies understandably focused on regions of the brain that are well characterized to have a role in energy balance, there remains uncertainty not only as to which brain regions are most critical but also the role Fto may play in peripheral tissues such as skeletal muscle and fat.

Taken in isolation, the data on *FTO* from human studies fall some way short from making a compelling case for FTO as a candidate mediator of the obesity association. As described earlier, the most deleterious mutation has been identified in a single extended family with a multisystem phenotype that caused significant morbidity and early mortality. To date, there appear to be no reports of deleterious encoding *IRX3* mutations being found in populations of lean or obese; these would be a welcome addition to the field.

Can we learn wider lessons on how to tackle the ever-growing list of "hits" from metabolically relevant association studies? Although murine models have proven invaluable in the study of monogenic disorders of energy balance and appetitive behavior, it is naive to assume that murine models alone can give us all the answers. A comprehensive perspective that clearly lays out many of the issues surrounding the determination of pathogenicity (or otherwise) of genetic variance can be found in a recent article by MacArthur et al. (2014), where the primacy of robust genetic support for causation is rightly emphasized. Furthermore, the recent paper by Smemo et al. (2014), highlighting the role of IRX3, is an exemplar of how a multilayered combination of techniques and evidence need to be marshaled together. The eQTL data presented are key pieces of evidence, and in the future, when determining if noncoding SNPs are exerting a functional impact through the alteration of gene expression, a stronger emphasis on eQTL mapping seems a sensible step forward. Material contained with the GtEX data set, for example, where a larger number of human tissues have been used for eQTL mapping (http://www.gtexportal.org/home/), is likely to be a valuable resource.

Many of the strongest signals coming from GWASs spotlight genetic loci located some distance from coding exons. Sticking a pin in the nearest named coding sequence and studying the consequence of loss of that gene is one very simple way to move forward. However, if we are to learn anything from the iterative process that the FTO story has been to date, then we learn that it is only by embracing all the technologies at our disposal will we be able to move from GWAS signals to biological understanding.

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