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

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Summary

Common polymorphisms in the first intron of *FTO* are associated with a 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, 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 conveying 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 polymorphisms (SNPs) within a ~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 et al., 2007; Scuteri et al., 2007; Meyre 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 et al., 2010). In mice and rats, manipulations of *Fto* gene expression have produced

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changes in body weight, but not in a consistent direction (Stratigopoulos et al., 2008, 2011; Wang et al., 2011; Tung et al., 2010; Church et al., 2010), and humans heterozygous for null alleles of *FTO* show no consistent effects on body weight (Meyre et al., 2010). *RPGRIP1L* (Retinitis Pigmentosa GTPase Regulator-Interacting Protein-1 Like) is located >100bp 5' in the opposite transcriptional orientation of *FTO* (Fig. 1A), and encodes a protein localized at the transition zone of the primary cilium (Williams et al., 2011; Liu et al., 2011). Functional derangements of the primary cilium in Bardet-Biedl and Alström syndromes are associated with obesity (Baker et al., 2009). We have previously demonstrated that fasting reduces hypothalamic expression of *Rpgrip1l* in mice and is restored by exogenous leptin administration (Stratigopoulos 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 et al., 2013) – that increases promoter activity and expression of *RPGRIP1* *Lin vitro*, and the obesity-risk allele of intronic SNP rs8050136 (Scuteri et al., 2007) located in the CUX1 binding site lowers the affinity of the CUX1 p110 isoform for DNA (Stratigopoulos 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* knock down of *Rpgrip1l* reduces Lepr-b localization in the vicinity of the cilium and decreases leptin signaling (Stratigopoulos 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 et al., 2007), but based on the allele-dosage effect of the *FTO* intronic SNPs on human adiposity, and our linking 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. We also describe a series of experiments investigating a possible mechanism for an impact of hypomorphism at *Rpgrip1l* on Lepr-b signaling that could account for these systemic phenotypes.

Results

***Rpgrip1l*^{+/-} mice display increased adiposity due to increased food intake**

Deletion of *Rpgrip1l* in mice, as well as biallelic *RPGRIP1L* mutations in Joubert patients, is linked with severe brain and craniofacial abnormalities (Vierkotten et al., 2007; Arts et al., 2007; Delous et al., 2007). In addition, *Rpgrip1l*-deleted embryos display polydactyly (Vierkotten et al., 2007), which is also characteristic of mutations in cilia-related proteins related to human syndromic obesity (e.g. Zaghoul & 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 (Fig. 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 knock-in allele, in agreement with the embryonic lethality previously reported (Vierkotten et al, 2007). *Rpgrip1l* mRNA and protein levels in the

hypothalamus of heterozygous mice were decreased by ~50%, and *Rpgrip11* mRNA was also decreased by ~50% in subcutaneous fat (Fig. 1C), demonstrating that these mice are systemically heterozygous for *Rpgrip11* (*Rpgrip11*^{+/-}). Conversely, *Fto* mRNA remained unchanged in the hypothalamus and subcutaneous fat of *Rpgrip11*^{+/-} mice, and mRNA levels of nearby genes *Chd9*, *Rbl2*, *Aktip*, *Irx3-5* in the hypothalamus of *Rpgrip11*^{+/-} mice were equivalent to control mice. *Rpgrip11*^{+/-} mice on a C57BL6/J background generated viable *Rpgrip11*^{+/-} mice at expected Mendelian ratios. By 10 weeks of age, male and female *Rpgrip11*^{+/-} mice fed regular chow (9% of calories as fat) *ad libitum* were ~10% heavier than +/+ animals (Fig. 2A, S1A). Thereafter, we concentrated on male *Rpgrip11*^{+/-} mice. At 18 weeks of age, male *Rpgrip11*^{+/-} mice fed regular chow were ~12% heavier due to a ~40% difference in fat mass (Fig. 2B–D). After 1 week of *ad libitum* feeding of a High Fat Diet (HFD; 65% of calories as fat), 19-week old male *Rpgrip11*^{+/-} mice were ~15% heavier (~4 grams) and had ~80% more body fat (~2.5 grams) and 6% more lean mass (~1.5 grams) than *Rpgrip11*^{+/+} littermates (Fig. 2E–G). Energy intake was significantly increased (~23%) in *Rpgrip11*^{+/-} mice consuming the HFD during the 12h light phase compared with *Rpgrip11*^{+/+} littermates (Fig. S1B). No statistically significant differences in energy expenditure or physical activity were detected between *Rpgrip11*^{+/-} and *Rpgrip11*^{+/+} mice when ingesting the high-fat diet (Fig. S1C–D). After 18 weeks of *ad libitum* feeding of regular chow followed by 7 days of the high fat diet, male *Rpgrip11*^{+/-} mice had ~82% more subcutaneous fat, ~65% more perigonadal and perirenal fat, and ~50% more mesenteric fat than +/+ littermates (Fig. 2H). The ~2-fold increase in serum leptin levels of male *Rpgrip11*^{+/-} mice correlated with their increased adiposity (Fig. S1E–F). No notable differences by genotype were observed in blood glucose levels of fed or fasted *Rpgrip11*^{+/-} and *Rpgrip11*^{+/+} mice (Fig. S1G).

Rpgrip11^{+/-} male mice fed regular chow showed increased food intake as early as 4 weeks old (Fig. 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 *Rpgrip11*^{+/-} mice (Fig. 2J, S1H–I). By 5 weeks of age, after being switched to the high-fat diet for 1 week, *Rpgrip11*^{+/-} mice ate more and displayed ~20% lower energy expenditure compared to the control group (Fig. 2K, S1J). At this age, body weight, body composition and physical activity levels were indistinguishable between *Rpgrip11*^{+/-} and control mice (Fig. 2J, S1K). During a 5-day period of high fat feeding, a single intra-peritoneal dose of leptin (4 µg/g) administered at the start of the dark cycle led to acute suppression of feeding for ~2 hours in the control group (Fig. 2K). In contrast, *Rpgrip11*^{+/-} mice started consuming food again at an earlier time point (~1 hour after leptin administration at 19:00). There was no statistically significant difference in cumulative food intake during the acute suppression of food intake by leptin for the two experimental groups from 19:19 through 20:11. On the other hand, *Rpgrip11*^{+/-} mice ate more than littermate controls between 20:37 through 21:29 (slopes: 0.0034 versus 0.0016 grams/min; *p*=0.03) compared with the feeding behavior of the two groups prior to leptin administration. After leptin administration, *Rpgrip11*^{+/-} mice ate ~0.25g (~21%) more HFD than control mice over the ensuing 12h dark cycle period (Fig. 2K). Following leptin administration, *Rpgrip11*^{+/-} and *Rpgrip11*^{+/+} mice consumed ~44% and ~46% less food respectively over the 12h dark cycle period (19:00–07:00) compared with food intake during the light cycle (10:00–19:00) (Fig. 2K). Administration of

saline 3, 5, and 7h before leptin administration did not affect the feeding behavior of either genotype. At the end of the 5-day high-fat feeding period, there were no statistically significant changes in body weight and composition between *Rpgrip11*^{+/-} mice and control group (Fig. 2J).

Diminished acute pStat3 response to leptin and reduced number of ciliated cells in the hypothalamus of *Rpgrip11*^{+/-} mice

In response to a single dose (2μg/g) of intraperitoneal leptin, the number of pStat3-positive hypothalamic neurons was ~20% and ~35% lower in *Rpgrip11*^{+/-} compared with *Rpgrip11*^{+/+} mice fed HFD *ad lib* at 5 and 19 weeks of age, respectively (Fig. 3). Moreover, in *Rpgrip11*^{+/-} 19 week old mice, ~20% fewer Lepr-positive cells were positive for the presence of type III A denylyl Cyclase (ACIII) in the axonemal part of the cilium (Bishop et al., 2007) (Fig. 4A). By staining of hypothalamic tissue with an anti-LEPR antibody tested for specificity *in vitro* (Stratigopoulos et al., 2011) and *in vivo* (Fig. S1L), we observed that only ~55% of Lepr-positive cells in 19-week old *Rpgrip11*^{+/-} mice (compared with ~90% in control mice) exhibited Lepr localization to the vicinity of the cilium (Fig. 4B, Movies S1–2), suggesting impaired subcellular trafficking of Lepr. Moreover, in 5-week old wild-type mice fed HFD *ad lib* and administered leptin (2μg/g) intraperitoneally, only ~30% of neurons with diffuse Lepr distribution were pStat3-positive, whereas ~70% of cells with localized Lepr distribution were pStat3-positive (Fig. 4C).

Rpgrip11 interacts with Lepr-b and coordinates leptin signaling

RPGRIP1L encodes a 1,253-amino-acid-long protein that contains several protein-protein interaction domains (Fig. 5A). These comprise five coiled-coil domains (CC1, CC2, CC3, CC4, CC5), which are possibly involved in homopolymer formation (Zhao et al., 2003); a C2 domain (C2 N'terminal-C2C'terminal), which is a Protein Kinase C-like domain that interacts with Nephrocystine 4 and facilitates recruitment of other proteins to the base of the cilium by RPGRIP1L (Arts et al., 2007, Williams et al., 2011); and a domain in the C' terminus homologous to the RPGR (Retinitis Pigmentosa GTPase Regulator)-interacting RID domain of RPGRIP1. RPGR is a component of the ciliary base and physically interacts with RPGRIP1L (Khanna et al., 2009). Because Lepr-b localizes to the vicinity of the cilium upon leptin stimulation, and *Rpgrip11* knock-down diminishes leptin signaling *in vitro* (Stratigopoulos et al., 2011), we hypothesized that RPGRIP1L physically interacts with LEPR-b and there by co-ordinates leptin signaling. We examined three skin-derived primary fibroblast cell lines (UW15, UW04-3 and UW04-4) with compound heterozygous mutations in the RPGRIP1L C2 domain derived from two patients with Joubert Syndrome (Fig. 5A). These mutations severely disrupt – or are predicted to disrupt – nephrocystin-4 binding (Arts et al., 2007; Doherty et al., 2010). Control primary skin fibroblasts from healthy individuals transiently expressing murine *Lepr-b* or stably expressing human *LEPR-b*-display single primary cilia [assessed by staining for acetylated tubulin, or ADP-Ribosylation factor-Like protein 13B (ARL13B)] and dose-dependently phosphorylate STAT3 in response to leptin (Fig. 5B, D). *RPGRIP1L* mutant fibroblasts had ~50% and ~70% fewer ARL13B-positive and ACIII-positive cilia respectively, which were, on average, ~50% longer (Fig. 5B, C). Joubert fibroblasts transiently expressing *Lepr-b* or stably expressing *LEPR-b* failed to phosphorylate STAT3 in a dose-dependent manner in response to leptin (Fig. 5D). *Lepr-b*

localized to the vicinity of the cilium upon leptin stimulation of primary skin fibroblasts from unaffected individuals (Fig. 5E). In contrast, in RPGRIP1L mutant fibroblasts, Lepr-b did not do so; in these cells, Lepr-b localization appeared to be perinuclear. The expression of intact RPGRIP1L in mutant fibroblasts partially restored the number of ACIII-positive cilia, localization of Lepr-b around the base of the cilium, and pSTAT3 levels in response to leptin (Fig. 5C–E). By immunofluorescence, primary fibroblasts from unaffected individuals localized RPGRIP1L in the transition zone of the cilium (Fig. 6A). In contrast, all UW15 Joubert fibroblasts did not incorporate RPGRIP1L in the transition zone. In primary fibroblasts from unaffected individuals, and in hypothalamic neurons of 19-week-old male B6 mice following IP administration of leptin, transiently expressed Lepr-b and endogenous Lepr, respectively, partly co-localized with endogenous RPGRIP1L in the vicinity of the cilium, upon leptin treatment (Fig. 6B, Movie S3). In agreement with a possible physical interaction between RPGRIP1L and LEPR-b implied by immunofluorescence, Lepr-b co-immunoprecipitates with RPGRIP1L at a higher concentration in primary fibroblasts treated with leptin compared with untreated cells (Fig. 6C). In contrast, Lepr-b does not co-immunoprecipitate with intraflagellar transport proteins, Ift46 or IFT20 (Lucker et al., 2010; Follit et al., 2006) in the presence of leptin (Fig. 6C), suggesting that Lepr-b interacts selectively with RPGRIP1L in the transition zone of the cilium. It has been previously reported that TGN46, a marker for the *post* and *Trans*-Golgi Network (TGN), co-localizes with LEPR-bin ARPE-19 cells (Seo et al., 2009). Levels of *Tgn46* mRNA are high in mouse hypothalamus (Allen Brain Atlas; <http://mouse.brain-map.org/gene/show/21892>). In the arcuate hypothalamus of 19-week old male wild type mice administered leptin via a single IP injection, Tgn46 protein co-localized with Lepr in the vicinity of the cilium (Fig. 6D). Co-localization of Lepr and Tgn46 persisted in cells with more diffuse Lepr distribution (Fig. S1M), suggesting that assembly of the TGN near the base of cilium is required for the aggregation of Lepr in the vicinity of the cilium. In the same mice, Lepr subcellular localization at the TGN in arcuate hypothalamic cells was also evidenced by staining with another TGN marker, ARF4 (ADP-Ribosylation Factor 4) (Fig. 6E), a small GTPase that localizes to the TGN and regulates aspects of trafficking to the cilium (Lim et al., 2011).

Expression of genes conveying effects on energy homeostasis in hypothalami of *Rpgrip1l*^{+/-} mice

We assessed by RT-PCR the expression levels of selected genes with known actions on ingestive behavior in the hypothalami of *Rpgrip1l*^{+/-} and wild type mice that had been fed chow for 18 weeks followed either by high-fat diet for 7 days, or fed high-fat diet for 6 days and subsequently fasted for 24h. As expected, expression of orexigenic genes Neuropeptide Y (*Npy*), Agouti-Related Protein (*AgRP*) and Pro-Melanin-Concentrating Hormone (*Pmch*) were increased in hypothalami of fasted wild type mice by ~40%, ~50% and ~30%, respectively (Fig. 7A). Importantly, *Npy* expression was ~40% higher in HFD-fed *Rpgrip1l*^{+/-} mice compared to *Rpgrip1l*^{+/+} control mice, consistent with the increased food intake displayed by *Rpgrip1l*^{+/-} mice fed high-fat diet and the proposed impact of RPGRIP1L on leptin signaling. As expected, mRNA levels of anorexigenic pro-opiomelanocortin (*Pomc*) decreased by ~30% in fasted wild type compared with fed wild type mice. In contrast, expression of *Pomc* was not decreased in fasted *Rpgrip1l*^{+/-} mice

and expression of the a-Msh receptor (*Mc4r*) was ~27% lower in fed *Rpgrip11*^{+/-} compared with fed *Rpgrip11*^{+/+} mice, suggesting dysregulation of leptin signaling.

We assessed the expression of feeding-related peptides in the hypothalamus of 5-week old *Rpgrip11*^{+/-} and *Rpgrip11*^{+/+} high fat-fed male mice in response to a single intraperitoneal dose of leptin (4 µg/g) at the start of the dark cycle (Fig. 7B, C). *Rpgrip11*^{+/-} mice ate more than wild type animals ~2 hours after the administration of leptin (Fig. 7B), and *Npy* and *AgRP* expression levels were ~45% and ~35% higher, respectively, in *Rpgrip11*^{+/-} compared with *Rpgrip11*^{+/+} mice (Fig. 7C). *Pomc* expression was also ~70% higher in *Rpgrip11*^{+/-} compared with *Rpgrip11*^{+/+} mice 2 hours after leptin administration. mRNA measurements were taken shortly before *Rpgrip11*^{+/-} mice, unlike *Rpgrip11*^{+/+} mice, exhibited reduced food intake (red asterisk, Fig. 2K). Therefore, the increase in *Pomc* expression in *Rpgrip11*^{+/-} mice may have caused this reduction in food intake.

Discussion

Primary cilia are ubiquitous in the brain, including the hypothalamus, hippocampus and olfactory bulb (Bishop et al., 2007). The melanin-concentrating hormone receptor 1 (*Mchr1*) localizes to neuronal primary cilia. *Mchr1* fails to do so in mice lacking the ciliary proteins, BBS2 or BBS4, implicated in Bardet-Biedl Syndrome, a disorder associated with obesity and anosmia (Berbari et al., 2008). Adult mice with impaired ciliary function due to absence of functional *Kif3a* in *Pomc*-expressing neurons, display increased food intake leading to obesity (Davenport et al., 2007). Knocking down ciliary proteins BBS1 or BBS2 in human retinal pigment epithelial cells disrupts LEPR-b trafficking to the TGN, resulting in retention of LEPR-b molecules in large cytoplasmic vesicles (Seo et al., 2009). In the present study, *Lepr*-b partially co-localized and co-immunoprecipitated with RGPRIPL *in vitro*, and *Lepr*-b co-localized with TGN-specific markers in arcuate hypothalamic cells. Additional evidence that LEPR-b may be part of a cilium-related protein complex includes the observations that LEPR-b co-localizes at the ciliary basal body (Han et al., 2014), co-immunoprecipitates with ciliary basal-body-associated BBS1 *in vitro* (Seo et al., 2009), and our finding that *Lepr*-b co-localizes with ARF4, which participates in mediating trafficking of molecules to the cilium (Lim et al., 2011). A model that includes LEPR-b clustering for optimal JAK/STAT signaling (Zabeau et al., 2004; Peelman et al., 2006) may provide a mechanism for the apparent functional role of cilia in leptin signaling by virtue of the convening of LEPR molecules in close proximity to each other. Our findings in the arcuate nucleus of *Rpgrip11*^{+/-} mice and fibroblasts from Joubert patients with regard to physical size of cilia, clustering of *Lepr*-b, and pStat3 generation in response to exogenous leptin, are consistent with this formulation. Nevertheless, the data presented here suggest that convening of LEPR molecules does not occur in the axonemal part of primary cilia. The mechanism underlying enhanced leptin signaling that involves *Lepr*-b localization in the vicinity of, but not within, the cilium is unclear. The possibility that TGN assembly near the base of the cilium is important for efficient trafficking and clustering of *Lepr*-b to the cell membrane vicinal to the cilium warrants further investigation.

AcIII hypothalamic mRNA levels in *Rpgrip11*^{+/-} and *Rpgrip11*^{+/+} mice are indistinguishable (Fig. S1N). Therefore, the reduction of *AcIII*-positive cilia in the hypothalamus of mice

hypomorphic for *Rpgrip11*, as well as in Joubert fibroblasts that fail to segregate RPGRIP1L at the transition zone, suggests that RPGRIP1L facilitates the transport of ACIII to the cilium. In agreement with this inference, mouse fibroblasts derived from embryos lacking proteins associated with the transition zone - Tectonic 1&2, Cc2d2a or Tmem67 - fail to localize AcIII in their cilia (Garcia-Gonzalo et al., 2011). Adenylyl cyclases catalyze the synthesis of cyclic 3353-AMP (cAMP) from ATP, and elevated cAMP levels in the hypothalamus have been reported to reduce the anorexigenic effect of leptin (Zhao et al., 2002; Fukuda et al., 2011). Moreover, congenital systemic deletion of *AcIII* is associated with increased bodyweight (Wang et al., 2009), suggesting a potential role of AcIII in the increased food intake of *Rpgrip11*^{+/-} mice that may be partially dependent on reduced leptin signaling. However, it is unclear whether some or all of the dose-dependent effect of *Rpgrip11* on energy balance is mediated by AcIII; the phenotypes of mice heterozygous for a null allele of *AcIII* have not been reported. Given the plethora of receptors and signaling molecules that convene in or around the cilium (Mukhopadhyay & Jackson, 2013), it is likely that multiple signaling pathways are affected by *Rpgrip11* hypomorphism. The implication of AcIII in energy homeostasis in this context is certainly consistent with this formulation.

LEPR-b signaling, which is diminished in *Rpgrip11*^{+/-} mice, is important for development of energy homeostasis-related neuronal circuitry in the hypothalamus (Bouret et al., 2004, 2012). It is possible that physiological sensitivity to leptin-mediated by the cilium and RPGRIP1L is important for the development of the feeding neuro circuitry. In support of this hypothesis, induced ablation of ciliary formation in adult mice (week 8) has no apparent effect on responses in feeding behavior to exogenous leptin (Berbari et al., 2013). Nevertheless, transient perturbation of ciliary formation in the mediobasal hypothalamus of adult mice by stereotaxic injection of siRNA specific to ciliary structural components resulted in diminished response to exogenous leptin leading to increased food intake, suggesting that hypothalamic primary cilia modulate leptin sensitivity independent of their role (s) in neurodevelopment (Han et al., 2014).

Adult male mice heterozygous for null mutations of *Lepr-b* (*Lep*^{db}) or leptin (*Lep*^{ob}) have ~50% and ~30% increased body fat, respectively, compared to wild type mice (Chung et al., 1998). Likewise, individuals heterozygous for hypomorphic alleles of *LEP* have increased body fat (Farooqi et al., 2001). Reduced LEPR-b (or other weight-homeostatic) signaling as a consequence of alterations in the function of the primary cilium, might account for some or all of the increase in the fat mass of *Rpgrip11*^{+/-} mice compared with their control littermates.

We have previously shown that the protective C allele of rs8050136, one of the *FTO* intronic SNPs associated with increased BMI (Scuteri 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 et al., 2011). As in the case of mouse embryos lacking functional *Rpgrip11*, loss of *Fto* in zebra fish results in multiple developmental defects that are due in part to ciliary structural defects accompanied by dysregulated β -catenin-dependent Wnt signaling (Vierkotten et al., 2007; Huang et al., 2011; Osborn et al., 2014). Similarly, CUX1 p110 controls the expression of genes that participate in the β -

catenin-dependent Wnt pathway (Cadieux et al., 2009). Moreover, CUX1 P110 is a positive regulator of leptin signaling, whereas *FTO* knock-down results in diminished leptin signaling *in vitro* (Stratigopoulos 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 events, 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 looping (Smemo 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 et al., 2014). In humans, the implicated *FTO* allele conveys its impact on adiposity, proportional to allele dose, primarily by effects on energy intake (Cecil et al., 2008; Haupt et al., 2009; Speakman et al., 2008; Wardle et al., 2009; Den et al., 2009; Wardle et al., 2008; Rutters et al., 2010; Tanofsky-Kraff et al., 2009). *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 et al., 2014). It is certainly possible, of course, that intronic *FTO* has effects on the expression of several genes (Jowett et al., 2010). Our data support a quantitatively important role for *RPGRIP1L* in conveying the orexigenic effects of intronic sequence variants in *FTO*.

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.knockoutmouse.org>), and maintained on a C57BL/6J background. Targeting was confirmed by the International Knockout Mouse Consortium. Compound heterozygous *Lepr-b^{cre}/+*, *Gt (ROSA) 26 Sor^{TM2Sho}/+* mice were a gift by Dr. Martin Myers (University of Michigan) (Leshan et al., 2006).

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 high fat (65% of calories as fat; Cat. No

D12492; Open Source Diets™, USA) for one week. An additional 5 *Rpgrip1l^{+/-}* and 7 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 high fat diet for one week. After 2 days of *ad libitum* ingestion of the high fat diet, mice were fasted overnight for 10 hours in order to assure that both groups were identical with regard to metabolic and feeding status. Food was re-introduced and serial intraperitoneal saline injections were performed in all mice to accustom them to handling. 9 hours after re feeding, 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 12h light/12h dark cycle (lights were turned off at 7 PM), 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-Calorimetry System (TSE Systems, USA). Calorimetry was performed on the 18-week old *Rpgrip1l^{+/-}* (n=10) and control (*Rpgrip1l^{+/+}*; n=6) experimental groups for ~72h while fed regular chow *ad libitum*, and then for another 3 days after the groups were switched to the high fat diet *ad libitum*. Calorimetry was also performed on the 3-week old *Rpgrip1l^{+/-}* (n=6) and control (*Rpgrip1l^{+/+}*; n=6) groups for one week while being fed regular chow *ad libitum*, and then for another week after the groups were switched to the high fat diet *ad libitum*. 40h after they were switched to the high fat diet, mice were fasted overnight for 10 hours, administered saline intraperitoneally at 2, 4 and 6 hours after a 10h overnight fast, followed by intraperitoneal leptin administration (4µg/g) at the start of the dark cycle. During this period, food intake and energy expenditure were automatically recorded every 26 minutes by the calorimeter. Mice were allowed to acclimate during the first ~24h. Energy intake was calculated by multiplying cumulative food intake for a 24-hour period by the metabolizable energy present in the high fat (5.24 Kcal/g) diet 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 grams in mass (Halldorsdottir et al., 2009).

Tissue culture

Human primary skin fibroblasts from healthy individuals [Protocol: IRB-AAAK6905(Y1Moo)] were derived and maintained in AmnioMAX™ - II Complete Medium (Invitrogen, Carlsbad, CA) in a humidified atmosphere at 37°C and 5% CO₂. Primary skin fibroblasts from Joubert patients UW15, UW04-3 and UW04-4 were maintained in AmnioMAX™ - II Complete Medium. For the phosphorylated Stat3 assay or immunofluorescence studies, confluent primary skin fibroblasts cells were made quiescent

48h after transfection by incubation in DMEM with 0.5% BSA overnight. Cells were then treated with leptin for 30 minutes or 3 hours prior to protein extraction. Expression vectors were introduced in cultured cells via transfection (using Lipofectamine®, Invitrogen) or transduction (via Lentiviral packaging, Invitrogen).

Immunohistochemistry and cellular Immunofluorescence

Cultured-cell slides were fixed with ice-cold methanol:acetone (1:1). Primary antibodies against LEPR (1–4 µg/ml for immunohistochemistry, 0.5–1 µg/ml for immunofluorescence; Mouse, Human; Cat # AF497, R&D SYSTEMS, Minneapolis, MN), Acetylated tubulin (1:500; Mouse; Cat # T6793-.2ML, Sigma, St. Louis, MO), and RPGRIP1L (1:400; Human and mouse; provided by Daniel Doherty, University of Washington) were used in conjunction with Alexa Fluor® 488, 555 and 647 secondary antibodies (Invitrogen, Carlsbad, CA). ProLong® Gold antifade reagent with either DAPI (Invitrogen) or DRAQ5 (Axxora, San Diego, CA) were used for mounting and nuclear staining. Brain perfusion, cryo sectioning and immunohistochemical analysis of the murine hypothalamus were performed as described elsewhere (Stratigopoulos et al., 2011). Mice were treated with an intraperitoneal injection of leptin (2µg/g of body weight) 30 minutes prior to sacrifice and brain perfusion. For pSTAT3 staining, brain sections were treated with 1% NaOH/1% H₂O₂ solution, 0.3% Glycine and 0.03% SDS prior to antibody treatment. Anti-pSTAT3 (Tyr-705), anti-Arf4 and anti-TGN46 antibodies antibody was purchased from Cell Signaling Technology (1: 200; Cat no. 9131, Danvers, MA), Protein Tech (1:100; Cat no 11673-1-AP, Chicago, IL) and Lifespan Biosciences (1:500; Cat no LS-C124646, Seattle, WA) respectively. Tomato-positive arcuate cryosections from *Lepr-b^{cre/+} Gt(ROSA)26Sor^{TM2Sho/+}* compound heterozygous mice were visualized using a confocal microscope, treated with 50% MeOH for 10 min followed by overnight incubation with PBS at 64°C, and subsequent overnight incubation with anti-LEPR antibody.

Anti-ARL13B (1:500; Cat no 17711-1-AP, Proteintech, Chicago, IL), anti-Acetylated Tubulin (1:1,000; Cat no T7451, Sigma), and anti-γ Tubulin (1:200; Cat no sc-7396, Santa Cruz) were utilized for the measurement of cilia number and length determination. Fibroblasts were grown for 2 days to ~85–95% confluency, serum-starved for ~48 hours in DMEM, and subsequently fixed in 4% PFA for 15 min followed by 100% methanol for 10 minutes at –20°C.

Isolation of total RNA and nuclear protein extracts

Total RNA was extracted and DNase-treated using the RN easy 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 13g 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 high fat diet for 7 days (4 *Rpgrip11*^{+/-} and 4 *Rpgrip11*^{+/+} males) or fed the high fat diet for 6 days and fasted for 24h (4 *Rpgrip11*^{+/-} and 4 *Rpgrip11*^{+/+} 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 *Rpgrip11* mRNA levels were measured by quantitative PCR as described elsewhere (Stratigopoulos et al., 2011). Similarly, quantitative PCR was performed to validate mRNA levels of *Npy*(F-GACAATCCGGGCGAGGACGC, R-CACCACATGGAAGGGTCTTCAAG), *Pmch*(F-GATCCGTAGCCTTCCCAGCTGAG, R-CCAACATGGTCGGTAGACTCTTCCC), *Pomc*(F-GCCTGGAGAGCAGCCAGTGCCAG, R-GACGTA CTTCGGGGGTTTTTCAGTC), *AgRP* (F-GGGCGTGGCTCCACTGAAGGGCAT, R-CTGCAGTTGTCTTCTTGAGGCCAT), *Mc4r* (F-CACCATGGCATGTATACTTCCCTCC, R-GATCACTAGAATGTTCTCCAACA), *Lepr-b* (F-AACCCCAAGAATTGTTCTCTGG, R-GGAGACCATAGCTGCTGGGACC), *Cdh9* (F-CATGGCTCAAACCTCAGCTGCAA, R-GCAGCTTAGTGTATGTCCCAG), *Rbl2* (F-GGCCTGGAGCAGCTACCGCAG, R-GATACATAGTTTCTTCAGCGGT), *Aktip* (F-GCAGCGCAGTCAACAAACGGC, R-CAGAGCGGTAAGATGGCTGTAC), *Irx3* (F-CAATGTGCTTTCATCAGTGTACG, R-GGATGCTGGACGCCAGGGCTGT), *Irx5* (F-GCACGGATGAGCTCGGCCGCTC, R-GGGTGATATCCCAAGGAACCTG), *Irx6* (F-CTCAGTATGAGTTCAAGGATGCTG, R-CTCCCTTGTGGCATTCTTCCTG) and *Adcy3* (F-CCAAGCACGTGGCTGACGAG, R-CAGAGGACAGCTGGGTAAAGCC) in the hypothalamus of *Rpgrip11*^{+/-} and *Rpgrip11*^{+/+} littermates. mRNA levels were normalized as described elsewhere (Stratigopoulos et al., 2011).

Antibodies

Anti-RPGRIP1L (1:200; Human & Mouse; provided by Daniel Doherty) was used for determining *Rpgrip11* protein levels in *Rpgrip11*^{+/-} mice by Western blotting. Phospho-Stat3 (Tyr705) antibody purchased from Cell Signaling Technology (1:1000; Cat# 9131, Danvers, MA) was also utilized for western blotting. 3-TUBULIN (1:2000; Cat# 05-661; Millipore, Bedford MA) and STAT3 (1:1000; Cat# 9132) were used as internal controls. In murine, tissue, *Lepr* and *AcIII* were stained with antibodies as described elsewhere (Stratigopoulos et al., 2011).

Plasmids

The full-length murine *Rpgrip11c* DNA (fused to *GFP*) under the *CMV* promoter was purchased from Origene (Cat# MG218118; Rockville, MD). The overexpression *Myc*-tagged *Ifi46* and *IFT20c* DNA were also purchased from Origene (Cat # MR204208, RC201337). The mouse leptin receptor isoform b (*Lepr-b*) cDNA (Stratigopoulos et al., 2011) with 3 stop codons at the 3' end (introduced by PCR) was cloned downstream of the

CMV promoter using the pcDNA3.1 Directional TOPO Expression Kit (Invitrogen). The human LEPR-b cDNA was amplified from a plasmid purchased by Sino Biological Inc. (NM_002303.3; Beijing, China) using primers 5'-GCGCTAGCATGATTTGTCAAAAATTCTGTGTGG, 5'ATGGATCCCACAGTTAGGTCACACATCT, and cloned in CD520A-RFP at the *NheI*-*Bam*HI restriction sites after digestion of the insert with *NheI*, *Bam*HI and Mung Bean nucleases (NEB). CD520A-RFP was constructed by cloning RFP - amplified from pDsRed-Monomer-N1 (Clontech) using primers 5'-AATCGGATCCACCGGTCGCCACCAT and 5'-AATTCAGCGCCGCTCTCTGGGAGCCGGAGTG - at the *Bam*HI-*NotI* restriction sites of CD520A (System Biosciences, Mountain View, CAL).

Co-Immunoprecipitation Assay

Primary skin fibroblasts - isolated from a healthy individual - at 80% confluency in a T75 flask were transfected with 23g of specified overexpression construct using Lipofectamine 2000™ reagent (Invitrogen). After 48 h, cells were starved in DMEM with 0.5% BSA for 6h before treatment with leptin (1µg/ml). After 30 minutes or 1 hour of incubation with leptin, cells were lysed (1M Tris-HCl pH=8.0, 5M NaCl, 0.5M EDTA, 10% NP40) in the presence of protease/phosphatase inhibitor (Cat # 1861280; Pierce) and protein was immunoprecipitated by incubation with 5% w/v Protein G Sepharose beads (Cat # 17-0618-01, GE Healthcare Life Sciences, Piscataway, NJ) and anti-GFP (1/200; Mouse; Cat# 11814460001, Roche), anti-Lepr (1/200; Mouse; Cat # AF497, R&D SYSTEMS) or anti-myc (1/200; Mouse; Cat # R951-25, Invitrogen) antibodies. Bound material was detected by immunoblotting with anti-LEPR or anti-GFP antibodies (Sigma).

Statistical analysis

Data are expressed as means +/- standard error. Statistical analysis was performed using Student's T-test (StatView 5.0, SAS Institute Inc.). Levels of statistical significance were set at 2-tailed $p_{\alpha} < 0.05$. All error bars represent Standard Error of the Mean (SEM).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- Mice heterozygous for a null *Rpgrip1l* allele are fatter than wild type controls.
- Leptin signaling is diminished in the hypothalami of *Rpgrip1l*^{+/-} mice.
- The localization of the leptin receptor is perturbed in *Rpgrip1l*^{+/-} mice.
- RRGRIPL may account for part or all of the association of the *FTO* locus with BMI.

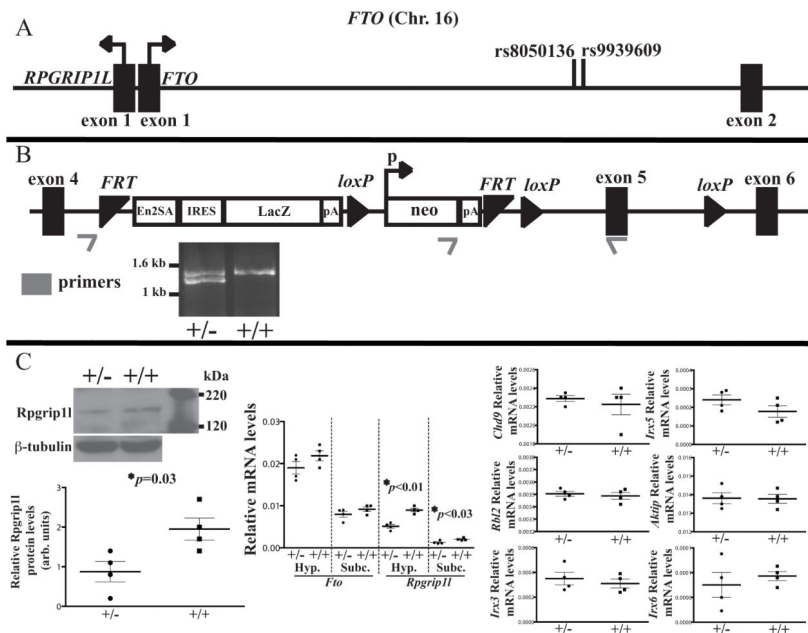


Fig. 1. Targeted disruption of the *Rpgrip11* locus

(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 *Rpgrip11* 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 *Rpgrip11* exon 4 resulting in premature transcriptional termination of the *Rpgrip11* mRNA. Figure not drawn to scale. (C) *Fto* and *Rpgrip11* transcript levels, and Rpgrip11 protein levels assessed by RT-PCR and western blotting respectively in the hypothalamus of *Rpgrip11*^{+/-} (+/-) and *Rpgrip11*^{+/+} (+/+) mice. *Fto* and *Rpgrip11* 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 *Rpgrip11*^{+/-} and *Rpgrip11*^{+/+} mice. Error bars represent SEM.

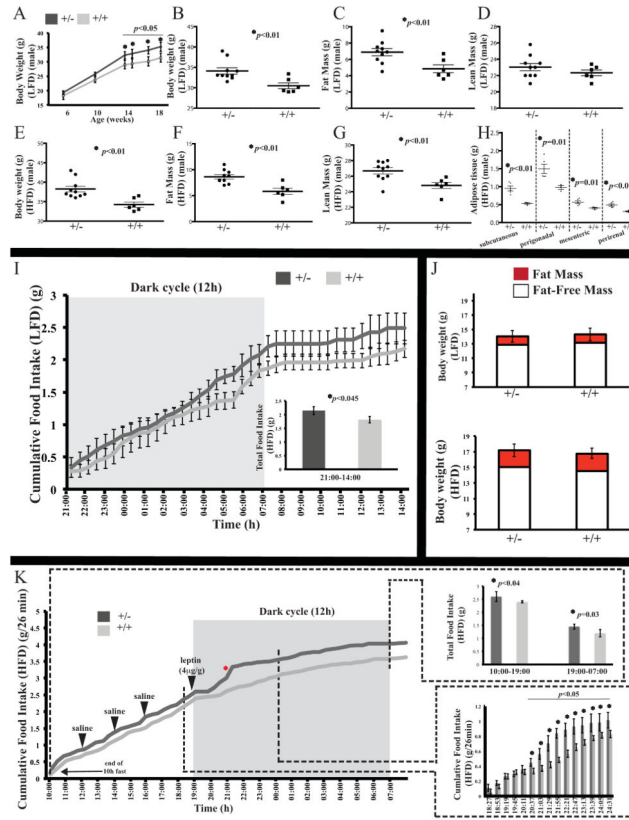


Fig. 2. Positive energy balance in mice heterozygous for a null allele of *Rpgrip11*
 (A) Total body weight increased in male *Rpgrip11*^{+/-} versus *Rpgrip11*^{+/+} mice fed regular chow (LFD) over an 18-week period. Total body weight (B, E), fat mass (C, F), and lean mass (D, G) increased in 18-week old male *Rpgrip11*^{+/-} versus *Rpgrip11*^{+/+} mice fed LFD and a high fat diet (HFD) respectively. (H) Increase in weight of various adipose tissue depots of *Rpgrip11*^{+/-} compared with *Rpgrip11*^{+/+} mice after being fed regular chow for 18 weeks followed by the HFD for 7 days. Food intake (I, K) and body composition (J) of male *Rpgrip11*^{+/-} and *Rpgrip11*^{+/+} mice fed LFD up to 4 weeks of age and subsequently fed HFD for 1 week. (K) While receiving the HFD, male *Rpgrip11*^{+/-} and *Rpgrip11*^{+/+} mice were administered leptin intraperitoneally at the start of the dark cycle resulting in a shorter delay in feeding for the *Rpgrip11*^{+/-} group compared with control mice, that was followed by greater food intake in the *Rpgrip11*^{+/-} mice compared with the control group. After leptin administration, food intake was ~44% and ~46% lower in *Rpgrip11*^{+/-} and *Rpgrip11*^{+/+} mice respectively during the dark cycle (19:00–07:00) compared to the light cycle (10:00–19:00). Red asterisk marks the time point when mRNA measurements were taken (Fig. 7B, C). Error bars represent SEM.

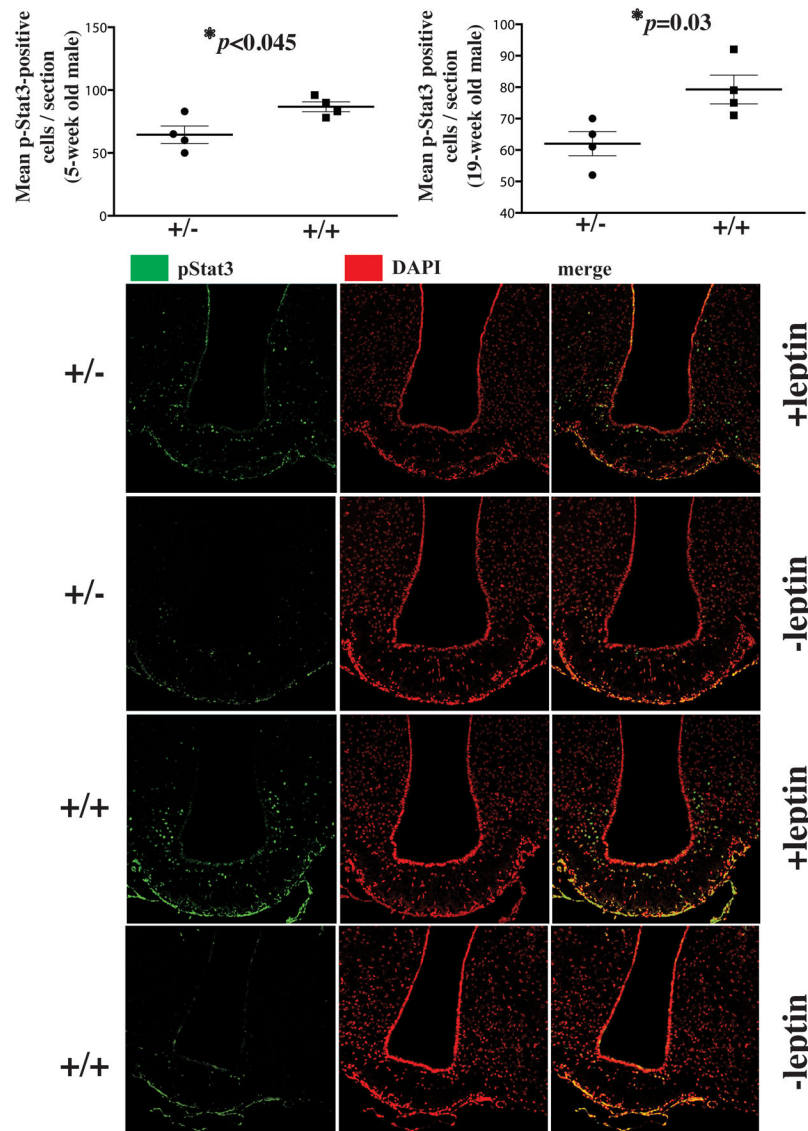


Fig. 3. Reduced leptin signaling in *Rpgrip11*^{+/-} mice

pStat3-positive cells in the hypothalamus of *Rpgrip11*^{+/-} and *Rpgrip11*^{+/+} 5-week and 19-week old male mice fed regular chow to whom leptin (2 μ g/g of body weight) was administered 30 min prior to sacrifice. pStat3-positive cells were counted in 10 μ m-thick cryosections encompassing the whole arcuate hypothalamus. Nuclear stain is shown in red to improve contrast with pStat3-specific green stain. Error bars represent SEM.

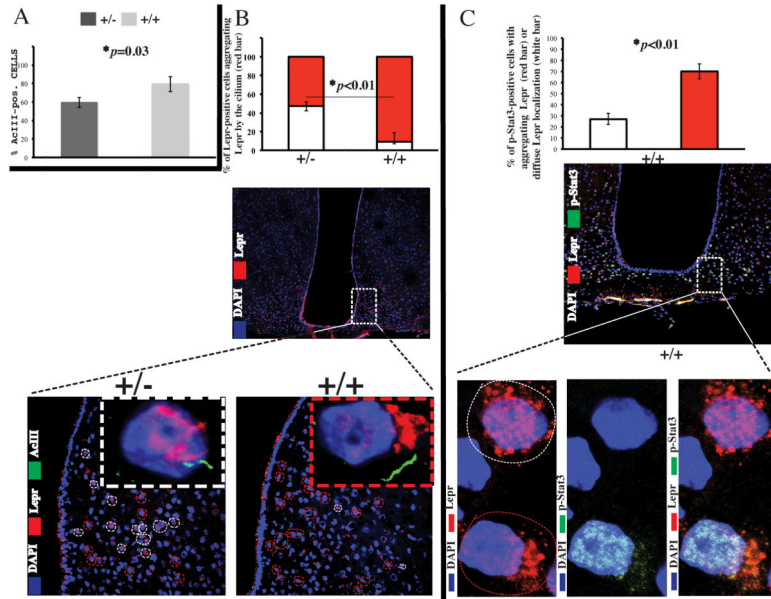


Fig. 4. Reduced AcIII-positive cilia and diminished aggregation of Lepr in the vicinity of cilia in hypothalamic cells of *Rpgrip11*^{+/-} mice

(A) Percentage of AcIII-positive positive cells in Z-stacks of 10µm cryosections spanning the arcuate hypothalamus of 19-week old male *Rpgrip11*^{+/-} and *Rpgrip11*^{+/+} mice fed regular chow. (B) Comparison of frequency of Lepr in the vicinity of cilia in arcuate hypothalamic cells of *Rpgrip11*^{+/+} (red dashed-line circles) versus *Rpgrip11*^{+/-} (white dashed-line circles). See also Movies S1–2. (C) Higher percentage of pSTAT3-positive arcuate neurons with polarized (red dashed-line circle) versus scattered (white dashed-line circle) localization of Lepr in wild type mice administered leptin intraperitoneally. Each bar represents N of 4. Error bars represent SEM.

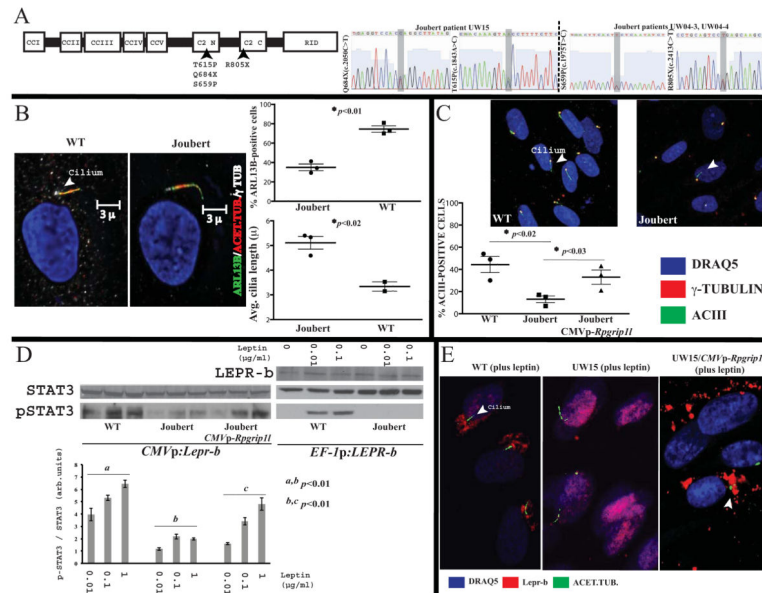


Fig. 5. Reduced ciliogenesis, perturbed *Lepr-b* localization and diminished leptin signaling in human fibroblasts with hypomorphic mutations of *RPGRIP1L*

(A) *RPGRIP1L* domain organization. Biallelic mutations, Q684X/T615P, and S659P/R805X, in the C2 domain of *RPGRIP1L* disrupt - or predicted to disrupt - nephrocystin-4 binding in Joubert patients UW15 and UW04-3/UW04-4 respectively (Arts et al., 2007; Doherty et al., 2010). (B) Number of cilia positive for ARL13B [membrane-associated protein of the ARL family of small GTPases that regulates ciliary transport (Lim et al., 2011)], acetylated tubulin and γ tubulin is reduced, where axoneme length is increased, in cultured primary skin fibroblasts from Joubert patients compared with primary fibroblasts from healthy individuals. Each data point represents a unique Joubert cell line. (C) Number of AcIII-positive ciliated cells is reduced in cultured primary skin fibroblasts from Joubert patients, and number is partially restored in Joubert fibroblasts transiently expressing *Rpgrip1l*. Each data point represents a Joubert cell line. (D) Western blot showing reduced pSTAT3 levels in whole protein extracts from unaffected compared with UW15 fibroblasts transiently expressing murine *Lepr-b* (*CMVp:Lepr-b*) or stably expressing human *LEPR-b* (*EF-Ip:LEPR-b*), and partially restored pSTAT3 levels in UW15 primary fibroblasts transiently co-expressing *Lepr-b* and *Rpgrip1l*. Results were replicated in Joubert fibroblasts from patients UW04-3 and UW04-4 (data not shown). (E) Immunofluorescence of primary skin fibroblasts from unaffected individuals or UW15 fibroblasts transiently expressing *Lepr-b* or *Lepr-b* and *Rpgrip1l*. *Lepr-b* fails to localize in the vicinity of the cilium in the presence of leptin UW15 fibroblasts transiently expressing *Lepr-b*. *Lepr-b* localization in the presence of leptin is partly restored in UW15 fibroblasts transiently expressing *Lepr-b* and *Rpgrip1l*. Results were replicated in Joubert fibroblasts from patients UW04-3 and UW04-4 (data not shown). Error bars represent SEM.

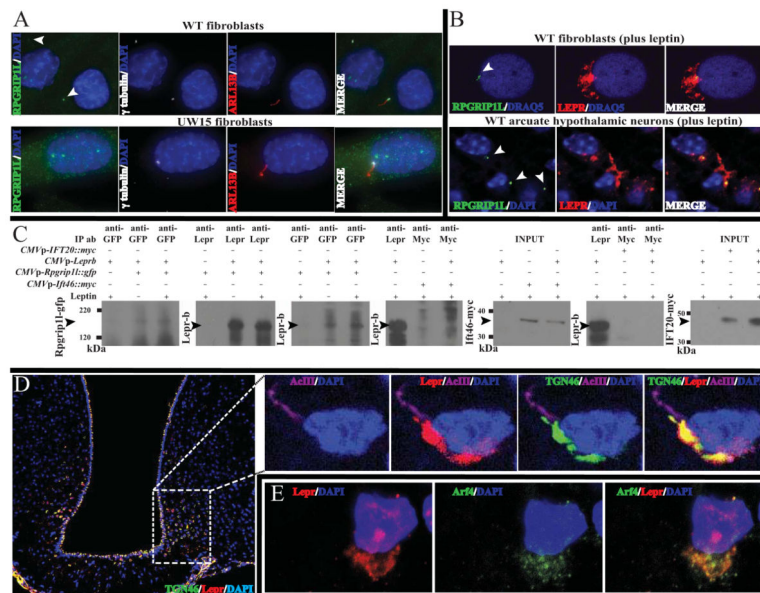


Fig. 6. Co-localization of RPGRIP1L, Lepr, Tgn46 and Arf4

(A) Localization of endogenous RPGRIP1L in ciliated wild type primary fibroblasts. Increased gain of the green channel confirmed the absence of RPGRIP1L at the transition zone of fibroblasts from patient UW15 segregating for biallelic mutations in *RPGRIP1L*. Arrows point at the transition zone. (B) RPGRIP1L co-localizes with Lepr-b transiently expressed in primary fibroblasts from an unaffected subject, as well as endogenous Lepr in arcuate neurons. See also Movie S3. (C) Increased levels of co-immunoprecipitated Lepr-b with GFP-tagged Rpgr11 in the presence of leptin compared with proteins extracts from untreated primary fibroblasts from unaffected human subjects. Lepr-b did not co-immunoprecipitate with myc-tagged ciliary protein Ift46 or IFT20 in the presence or absence of leptin. (D) Subcellular co-localization by immunohistochemistry of Lepr and Tgn46 (See also Fig. S1M) or (E) Lepr and Arf4 in the arcuate hypothalamus of mice stimulated with leptin.

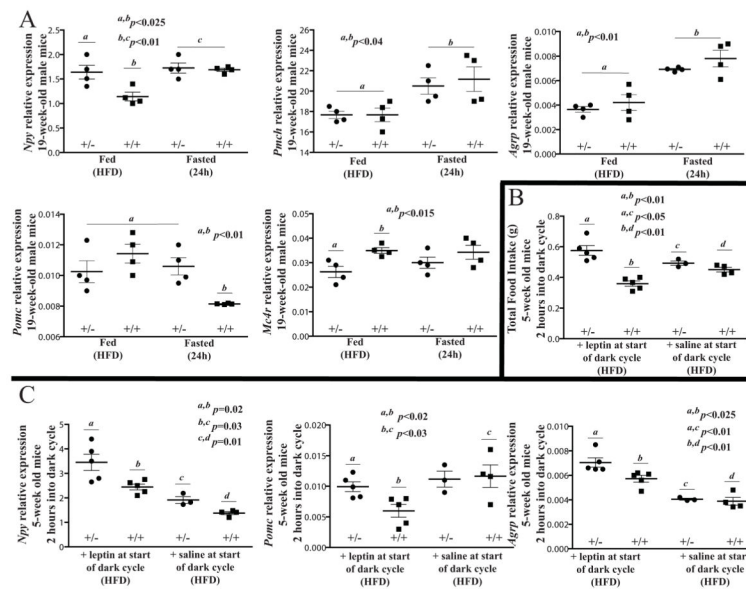


Fig. 7. Alterations in *Npy*, *AgRP*, *Pomc* and *Mc4r* expression in *Rpgrip11*^{+/-} mice
 (A) mRNA levels assessed by RT-PCR using as template total hypothalamic RNA extracted from 4 *Rpgrip11*^{+/-} and 4 *Rpgrip11*^{+/+} mice fed regular chow *ad lib* for 18 weeks followed by the high fat diet (HFD) for 6 days before a 24-hour fast; or 4 *Rpgrip11*^{+/-} and 4 *Rpgrip11*^{+/+} littermates fed regular chow *ad lib* for 18 weeks followed by HFD for a week. Food intake (B) and transcript levels (C) in 8 *Rpgrip11*^{+/-} and 9 *Rpgrip11*^{+/+} mice fed HFD at 4-weeks of age *ad lib* for one week. 5 *Rpgrip11*^{+/-} and 5 *Rpgrip11*^{+/+} mice were administered leptin intraperitoneally at the start of the dark cycle, whereas 3 *Rpgrip11*^{+/-} and 4 *Rpgrip11*^{+/+} mice were administered saline at the same time point. All mice were sacrificed 2 hours after being injected with leptin or saline (red asterisk; Fig. 2K). *Npy*, *AgRP* and *Pomc* mRNA levels were higher in leptin-treated *Rpgrip11*^{+/-} compared with leptin-treated *Rpgrip11*^{+/+} mice. Each data set is annotated with a letter to facilitate comparison. Error bars represent SEM.