Detailed Functional Characterization of a Waist-Hip Ratio Locus in 7p15.2 Defines an Enhancer Controlling Adipocyte Differentiation

HIGHLIGHTS
An enhancer active during adipogenesis is located in an obesity GWAS locus
The enhancer responded strongly to insulin and isoprenaline
Mutation of the enhancer by CRISPR-Cas9 decreased adipocyte differentiation
Knockout of CHI3L1 decreased adipocyte differentiation

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Detailed Functional Characterization of a Waist-Hip Ratio Locus in 7p15.2 Defines an Enhancer Controlling Adipocyte Differentiation

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SUMMARY
We combined CAGE sequencing in human adipocytes during differentiation with data from genome-wide association studies to identify an enhancer in the SNX10 locus on chromosome 7, presumably involved in body fat distribution. Using reporter assays and CRISPR-Cas9 gene editing in human cell lines, we characterized the role of the enhancer in adipogenesis. The enhancer was active during adipogenesis and responded strongly to insulin and isoprenaline. The allele associated with increased waist-hip ratio in human genetic studies was associated with higher enhancer activity. Mutations of the enhancer resulted in less adipocyte differentiation. RNA sequencing of cells with disrupted enhancer showed reduced expression of established adipocyte markers, such as ADIPOQ and LPL, and identified CHI3L1 on chromosome 1 as a potential gene involved in adipocyte differentiation. In conclusion, we identified and characterized an enhancer in the SNX10 locus and outlined its plausible mechanisms of action and downstream targets.

INTRODUCTION
According to estimations by the World Health Organization, more than 1.9 billion adults were overweight and ~13% of the world’s adult population was obese in 2016. In addition, 41 million children younger than 5 years were overweight or obese. In parallel with this, dramatic increases of downstream consequences of obesity, such as insulin resistance (IR), type 2 diabetes (T2D), and cardiovascular disease are also expected over the next decade, which will have a devastating impact on global health. Once considered as a problem limited to high-income countries, obesity, IR, and associated conditions are now dramatically increasing also in low- and middle-income countries, especially in urban settings. Heart disease and stroke are the leading causes of morbidity and mortality, being the underlying cause of death for ~13 million people in 2010, or one in four deaths worldwide (Lozano et al., 2012). Hence, identifying molecular mechanisms underlying obesity and risk of cardiovascular disease and T2D are important public health priorities.

Genome-wide association studies (GWAS) have been tremendously successful in identifying loci associated with complex traits (Visscher et al., 2017). As a result of large meta-analyses of GWAS led by the GIANT consortium, more than 500 loci have been associated with body mass index (BMI), waist-hip ratio (WHR), and other obesity traits (Visscher et al., 2017). However, the causal variants, genes, or downstream mechanisms have mostly been annotated as expression quantitative trait loci (eQTL) analyses (Graff et al., 2017; Justice et al., 2017; Shungin et al., 2015), and also as NFE2L3, MIR148A, or CBX3 based on the gene nearest to the lead GWAS variant from the study in question. The enhancer is localized in an independent haplotype block tagged by the lead GWAS variant rs3902751 that spans 48 kb from rs10245353 to rs1451385 (Figure 1A).
Figure 1. Genetic Features of the 7p15.2 Region Tagged by GWAS Potentially Involved in Adipogenesis Centered around rs1451385

(A) Physical map of the region showing the position of annotated genes, MIR148A, and genetic polymorphisms (SNPs, annotated with their rs identifiers) associated with obesity and other related phenotypes. The region is delimited by the distal gene NPVF and the proximal gene SNX10. Genome-wide significant SNPs include lead variants associated with WHR (rs10245353, rs39022751, rs1534696, Shungin et al., 2015; rs1055144, rs7798002, rs1451385, Heid et al., 2010) and BMI (rs10261878; Monda et al., 2013). rs1451385 is located in a haplotype block of 48 kb (LD r² > 0.8) that is delimited by the rs10245353 and rs1451385. rs10261878 (associated with BMI) is not included in this haplotype block; instead it is linked to a haplotype block that includes MIR148A. The SNP rs1534696 at SNX10 is located in an independent haplotype. SNPs indicated by # are located at transcriptional peaks as indicated by CAGE. The dotted line represents 17 SNPs with a weak association with BMI (-log p values >3.5; Speliotes et al., 2010).

(B) Transcriptional signals during adipogenesis of mesenchymal stem cells visualized using the ZENBU browser (http://fantom.gsc.riken.jp/zenbu/). The variant rs1451385 is located in a transcriptionally active region of 400 bp delimited by CAGE25894547 and CAGE25894945. The directions of the transcription are indicated with green and purple arrows for forward and reverse directions, respectively. The intensities of the green and purple peaks correspond to the amount of CAGE-RNAs captured during the entire induction of adipogenesis.

(C) CAGE transcriptional signals colored by intensity on the black background and ordered from top to bottom from 1 h after induction of adipogenesis to day 14. The signals are aligned with the corresponding peaks in (B). A transcriptional promoter-like and an enhancer RNA (eRNA) region are predicted by FANTOM5 CAGE data and indicated with white filled and dotted arrows, respectively. The transcriptional like-promoter sequence is annotated as a POL2-binding site by ENCODE chromatin immunoprecipitation sequencing data, and the enhancer region is annotated as an enhancer according to the Regulatory Elements from ORegAnno at the UCSC Genome Browser in various cell lines.

(D) Numerical quantification of the CAGE signals as in (C) showing the three biological replicates for each time point. Maximal transcription is scored in both directions at 12 h post-induction.
In this study, we identified and characterized this active enhancer. Disrupting it causes the loss of differentiation capacity from precursor cells to mature adipocytes. Furthermore, combining CRISPR-Cas9 gene editing with global RNA sequencing (RNA-seq), we identified and characterized potential downstream targets of the enhancer. We aimed to disentangle the molecular mechanisms behind one of the first and strongest GWAS signals associated with obesity-related traits.

RESULTS

In Silico Analyses Highlight a Regulatory Region Tagged by GWAS Potentially Involved in Adipocyte Biology

Using the GWAS catalog (www.ebi.ac.uk/gwas/), we identified all lead variants associated with obesity-related traits (WHR, BMI, lipid levels, glucose-related traits) at the time of study initiation (October 2015). Next, we selected all common single nucleotide polymorphisms (SNPs) in high linkage disequilibrium ($r^2 > 0.8$) with the lead variants and searched for overlaps with annotated enhancers using FANTOM5 CAGE data from adipogenesis (Arner et al., 2015; Ehrlund et al., 2017) favoring (1) enhancers with high gene expression during induction of adipogenesis from mesenchymal stem cells and (2) enhancers showing higher expression levels in adipocytes and preadipocytes than in other cell types. This approach identified 14 putative adipocyte-specific enhancers with supporting evidence from GWAS of obesity-related traits. Of these, nine had weak human genetics support (non-genome-wide significant association and/or reported in a small GWAS without further replication), two were associated with proinsulin and low-density lipoprotein cholesterol, respectively (making them less attractive for studies in adipocytes), and two were upstream of NEGR1 (which is an already well-studied gene). In contrast, the enhancer in 7p15.2 had strong evidence for involvement in adipogenesis from FANTOM5, represented a very strong GWAS signal associated with WHR adjusted for BMI, and had supporting evidence from transcription-binding motifs and chromatin immunoprecipitation sequencing from ENCODE, but little was known about its function.

In this GWAS locus, the FANTOM5 enhancer data highlighted a bidirectional transcription start site active during adipocyte differentiation flanking rs1451385, suggesting that this SNP is located in a functional element of relevance for adipogenesis, as well as fat distribution (Figures 1B–1D). Furthermore, data from ENCODE and the Roadmap Epigenomics Project show that rs1451385 colocalizes with a DNase1 hypersensitive site and with dynamic changes of chromatin acetylation of histone 3 lysine 27 (H3K27ac), suggesting an active regulatory element in this region. The sequence comprising rs1451385 co-immuno-precipitates with the following transcription factors: CTCF (CCCTC-binding factor), USF1 (upstream transcription factor 1), IRF1 (interferon regulatory factor 1), and POLR2A (RNA polymerase II subunit A). USF1 is a transcription factor controlling expression of several genes involved in lipid and glucose homeostasis (Putt et al., 2004) that has been linked to familial combined hyperlipidemia (Pajukanta et al., 2004). USF1 binds to E-box motifs (5'-CACGTG-3'), and it is positioned about 23 nucleotides from a pyrimidine-rich region (Massari and Murre, 2000). The rs1451385 polymorphism is located 2 bp upstream of the E-box motif consensus sequence (5'-ACACGTGA-3'), which is located 23 nucleotides upstream of a 31-nucleotide-long pyrimidine-rich region (23 pyrimidines of a total of 31 nucleotides). Furthermore, analyses of nucleotide-binding sequences using PROMO (Messeguer et al., 2002) show differences in putative binding sites for several transcription factors. Specifically, the T allele of rs1451385, which is in perfect LD with the risk allele (A) at rs3902751 associated with increased WHR (Shungin et al., 2015), confers an extra VDR (vitamin D receptor)-binding site, a FOXP3 site, and a PXR-1:RXR-alpha (a nuclear receptor involved in metabolism sensing)-binding site. Finally, rs1451385 is a borderline significant eQTL ($p = 6.7 \times 10^{-8}$) in subcutaneous adipose tissue in GTEx (www.gtexportal.org/home) for AC003090.1 (ENSG00000223561.2), a long intergenic noncoding RNA (lincRNA) with unknown function, located 100 kb from rs1451385. However, due to its low expression in adipose tissue, it is unlikely that this lincRNA plays an important role in adipogenesis. There were no other eQTLs for subcutaneous or visceral fat in this locus.

The Sequence Comprising the GWAS Signal Shows High Enhancer Activity

We used a luciferase reporter assay to test the regulatory function of the region surrounding rs1451385. Based on phylogenetic conservation (Figure 1E), we cloned a 266-bp fragment (from now on called
Enh#385) into the reporter vector pGL4.10 (Promega) with a minimal promoter upstream of the luciferase gene. The two variants (C/T) of rs1451385 were cloned in both directions in relation to the luciferase gene. As a positive control, we chose a human 411-bp enhancer from chromosome 12 that consistently increases luciferase expression 3-fold in a variety of cell lines (Cavalli et al., 2016).

We tested the four variants of Enh#385, the positive and empty controls in several cell lines. In all tested cell lines, we found a strong luciferase activity indicating a functional DNA fragment (Figures 2A–2D). The activity varied depending on the cell line and the Enh#385 variant used. The positive control enhancer increased luciferase activity to about 4-fold on average in all the cell lines tested, whereas Enh#385 increased luciferase activity up to 300-fold when compared with the empty vector. We observed an overall
higher activity of the T allele, which is on the same haplotype as the WHR-increasing variant (Shungin et al., 2015).

The Enhancer Enh#358 Is Potentiated by Insulin and Isoprenaline

Next, we tested the transcriptional response of Enh#385 to insulin and isoprenaline in the reporter assay. Such a response would indicate a role of the enhancer in adipocyte biology and/or IR given that both compounds have profound effects on adipocytes and are involved in IR. Isoprenaline, also called isoproterenol, is a non-selective β-adrenoreceptor agonist that increases intracellular cAMP activity, stimulates lipolysis, and inhibits insulin-stimulated glucose transport, whereas insulin stimulates glucose uptake via Glut4 translocation and activation.

In both human hepatocytes (HepG2) and preadipocytes (SGBS), we observed a strong induction of luciferase activity with both substances (Figures 2E and 2F). The induction was suppressed with simultaneous treatment in HepG2 (Figure 2E). This suppression might reflect the opposite physiological effect of the lipogenic insulin and the lipolytic isoprenaline. At a molecular level, this type of suppression could be explained by competitive binding mechanisms, squelching, or transcriptional interference (Kamei et al., 1996; Levine and Manley, 1989; Manna and Stocco, 2007; Step et al., 2014; Yang-Yen et al., 1990; Zhang and Teng, 2001), resulting in insulin-induced factors competing with isoprenaline-induced factors for binding to Enh#385 (Figure S1). The formation of heterogeneous complexes could block the enhancer activity; a similar model has been proposed for scaffold protein complexes (Ferrell, 2000).

Molecular Dissection of the Enhancer

To define the boundaries and functional components of the enhancer, we used a luciferase assay to assess 20 independent constructs corresponding to different parts of Enh#385 with the T allele in forward direction (Figure 3A). Owing to poor transfection efficiency of SGBS cells, we restricted these experiments to HepG2 cells. To assess potential biotechnological applications of Enh#385, we included the enhancer of human CMV (cytomegalovirus), which is the most powerful enhancer currently used in commercial applications as a comparison. Based on phylogenetic conservation, we defined six sequential blocks (Figure 3A). Luciferase expression showed construct Enh#385-17 as having the highest enhancer activity (Figure 3B). The intensity of this construct was twice that of the original Enh#385 (Enh#385-11) but only 36% of the CMV enhancer. Furthermore, we tested the inducible properties of the most potent constructs (Figure 3C). Enh#385-11 showed the highest response to insulin and isoprenaline, whereas the CMV enhancer was not affected by either substance. In summary, our results indicate the presence of a core enhancer region centered around rs1451385.

CRISPR-Cas9 Mutation of the Enhancer Impairs Lipid Accumulation in Differentiating SGBS Cells

To study the function of the enhancer, we generated two independent mutations in undifferentiated preadipocytes using CRISPR-Cas9 (Figure S2).

After supplementation with adipogenic agents, we compared adipocyte differentiation of wild-type (WT) and mutated cultures after knockout (KO) of Enh#385 (KO-385-V11 and KO-385-V3). The WT cells differentiated to mature adipocytes in a normal fashion as evidenced by accumulation of lipid droplets, whereas the mutated cells showed highly reduced differentiation capacity (Figure 4A). Quantification of lipid droplets showed a 5-fold reduction of droplets in Enh#385 KO cells after 19 days of differentiation when compared with cells edited with a guide targeting a random intergenic region (Ctrl-24) or WT cells (Figure 4B). Additional experiments with independent CRISPR-Cas9 transductions provided similar results when measuring lipid content with oil red O staining (Ramirez-Zacarias et al., 1992) (Figure S3) or staining with the fluorophore BODIPY 493/503 (Majithia et al., 2014; Warnke et al., 2011) (data not shown).

Markers of Adipocyte Differentiation Are Reduced in Cell Cultures with Disrupted Enhancer

The impairment in differentiation was confirmed at the gene expression level by RT-qPCR of three adipocyte markers with divergent functions: the phosphoenolpyruvate carboxykinase 1 kinase (PCK1), involved in glucose metabolism; the lipoprotein lipase (LPL), involved in metabolism of fat; and the secreted adipokine adiponectin (ADIPOQ), involved in the control of fat metabolism and insulin sensitivity. In addition, insulin receptor (INSR) was included in this hypothesis-driven experiment owing to the potent induction by insulin of Enh#385. After 8 days of differentiation measured by RT-qPCR, all three adipocyte markers were strongly
reduced and INSR was slightly reduced in the KO-385-V3 culture (Figure 4C; Figure S4); these results were subsequently confirmed by RNA-seq (next section).

**RNA Sequencing Highlights Several Genes Showing Differential Expression after Mutation of the Enhancer**

To understand the downstream consequences of the editing of Enh#385, we compared gene expression between WT and Enh#385-mutated cells after 8 days of induced differentiation by global RNA-seq. We included a culture transduced with a single-guide RNA targeting an unrelated intergenic region as a comparison to cope with the effect of transduction and the ectopic expression of the Cas9 nuclease. We focused on the most differentially expressed genes across the whole transcriptome to address downstream effects of Enh#385 and to help disentangle the role of this element on adipocyte differentiation (Figure 5A; Table S1). Among the top differentially expressed genes, we selected 20 genes for technical and biological validations (Table 1; Figure 5A). Technical validations were done by RT-qPCR (i.e., using a different technique than RNA-seq). The technical replication was high: differential gene expression assessed by RT-qPCR was confirmed for 19 of 20 genes when using cells mutated with the same CRISPR-Cas 9 vector that was used in cell cultures undergoing RNA-seq (downstream mutant [M(ds)] KO-385-V3). One gene, HYDIN, was impossible to consistently amplify by RT-qPCR, probably owing to its low expression in SGBS cells. Although the two methods correlated very well, we noted that especially for genes with low expression,
the RNA-seq technology was more accurate than RT-qPCR. Two types of biological validations were done: first, using an independent mutation generated with a different CRISPR-Cas9 vector (KO-385-V11) targeting the sequence just upstream of rs1451385 (hence, annotated M(us) in Table 1) and second, using an innocuous lentivirus containing only GFP to assess a potential effect of the transduction protocol on gene expression (annotated as GFP in Table 1). Biological validation was confirmed for 11 of the remaining 19 genes when using the independent CRISPR-Cas9 upstream mutation, but when we evaluated the potential effects of the lentiviral transduction protocol per se on gene expression, we found that HEPH, NDN, and RCAN2 were downregulated in transduced cells (without the Enh#385 mutation), suggesting that the differential expression of these genes was, at least in part, due to the transduction protocol, rather than due to the mutation in Enh#385 (Table 1 and Figure 5B).

Next, to investigate a potential role in adipogenesis of the eight differentially expressed genes passing both technical and biological validation, we examined their expression in undifferentiated preadipocytes (Table 1). We assumed that only genes that were differentially expressed already in preadipocytes could be responsible for the impairment in adipogenesis, whereas a large number of differentially expressed genes represent the consequence of altered adipogenesis of the mutated cultures rather than causal genes. After comparisons of gene expression by RT-qPCR between undifferentiated downstream mutants, upstream mutants, GFP-transduced cells, and WT cells, we found that only chitinase-3-like protein 1 (CHI3L1) on...
chromosome 1 and reelin (RELN) on chromosome 7 were differentially expressed in undifferentiated pre-adipocytes (at least 2-fold difference; Table 1). Several genes with strong prior evidence for involvement in metabolic disease, such as ITIH5, PCK1, MLXIPL, and LPL, we re on ly expressed in mature adipocytes, and hence unlikely to be causal in driving the decrease in adipogenesis, but rather be a result of the difference in cell fate between the Enh#385 KO and WT cells (Table 1; Figure 5B). In subsequent experiments using global gene expression analyses, we were able to extend our search of causal genes and identified altered pathways important for adipogenesis (see below, Table S4 and Figure 7).

Ablation of CHI3L1 Decreases Adipogenesis in Differentiating SGBS Cells

To evaluate the direct role of the two consistently differentially expressed genes on adipogenesis, we used CRISPR-Cas9 to disrupt CHI3L1 and RELN in SGBS cells (Figure S9). After induction of adipogenesis, the CHI3L1-KO cells showed a significant reduction in lipid accumulation, whereas RELN-KO cells did not differ when compared with controls with regard to differentiation capacity (Figure 6). The phenotype similarity obtained by direct mutation of CHI3L1 and reduction of CHI3L1 via mutation of Enh#385 indicates that CHI3L1 and the enhancer are linked by functional interaction circuits. RELN was upregulated in Enh#385 mutant cells; therefore an increase of lipid accumulation in RELN-KO cells could be expected. We did not detect any differences in lipid accumulation between the WT and RELN-KO cells, but we cannot rule out that the overexpression of this gene could reduce the rate of adipocyte differentiation.

CHI3L1, also known as YKL-40, is a secreted glycoprotein coupled with stress-induced cellular responses (Ling and Recklies, 2004). Protein levels of CHI3L1 have been associated with several pathogenic processes including schizophrenia, asthma, obesity, and cancer, but the biological function of YKL-40 in specific tissues is largely unknown (Kyrgios et al., 2012; Ober et al., 2008; Zhao et al., 2007). Experimental data have shown that mice deficient in Chi3l1 develop less visceral obesity and have smaller adipocytes; in contrast,
| Gene Symbol | Full Gene Name | Log2FC | p Value* | Adjusted p | Log2FC | p Value* | Log2FC | p Value* | Log2FC | p Value* | Log2FC | p Value* | Log2FC | p Value* | Log2FC | p Value* | Comment                  |
|-------------|----------------|--------|----------|------------|--------|----------|--------|----------|--------|----------|--------|----------|--------|----------|--------|----------|--------------------------|
| GAS7        | Growth arrest-specific 7 | −3.45  | 4.2 × 10−10 | 3.8 × 10−13 | −3.19  | 1.2 × 10−3 | 0.67  | 2.1 × 10−1 |        |          |        |          |        |          |        |          | Not validated              |
| CH3L1       | Chitinase 3-like 1 cartilage glycoprotein-39 | −2.03  | 1.9 × 10−3 | 5.7 × 10−6 | −3.46  | 4.0 × 10−3 | −1.57 | 8.0 × 10−5 | 0.88  | 3.9 × 10−3 | −2.12 | 5.4 × 10−3 | −1.05 | 2.4 × 10−3 | −0.12 | 7.4 × 10−1 | Putative causal gene       |
| HEPH        | Hephaestin     | −2.87  | 2.3 × 10−2 | 1.8 × 10−5 | −5.47  | 4.9 × 10−5 | −2.24 | 1.4 × 10−3 | −2.82 | 1.6 × 10−2 |        |          |        |          |        |          | Downregulated by transduction |
| NDN         | Necdin melanoma antigen MAGE family member | −2.15  | 1.9 × 10−3 | 1.4 × 10−4 | −4.71  | 3.8 × 10−4 | −1.88 | 4.2 × 10−3 | −2.70 | 9.8 × 10−4 |        |          |        |          |        |          | Downregulated by transduction |
| H2AFY2      | H2A histone family member Y2 | −2.62  | 2.0 × 10−1 | 3.4 × 10−3 | −2.70  | 4.5 × 10−3 | 0.92  | 3.6 × 10−2 |        |          |        |          |        |          |        |          | Not validated M(u)          |
| MID2        | Midline 2      | −2.67  | 1.1 × 10−3 | 1.4 × 10−4 | −4.35  | 3.4 × 10−4 | 0.60  | 6.8 × 10−2 |        |          |        |          |        |          |        |          | Not validated M(u)          |
| RCAN2       | Regulator of calcineurin 2 | −2.63  | 1.5 × 10−5 | 1.1 × 10−3 | −4.19  | 2.5 × 10−3 | −3.20 | 3.9 × 10−5 | −3.35 | 3.1 × 10−2 |        |          |        |          |        |          | Downregulated by transduction |
| RELN        | Reelin         | 1.82   | 1.7 × 10−1 | 3.9 × 10−4 | 1.41   | 4.2 × 10−3 | 1.92  | 1.5 × 10−3 | 0.44  | 4.2 × 10−3 | 1.48  | 2.4 × 10−2 | 1.06  | 3.4 × 10−2 | 0.83  | 7.8 × 10−2 | Putative causal gene       |
| SGCD        | Sarcoglycan delta 35 kDa dystrophin-associated glycoprotein | −3.43  | 2.4 × 10−3 | 5.5 × 10−5 | −2.03  | 4.4 × 10−3 | 0.10  | 8.9 × 10−3 |        |          |        |          |        |          |        |          | Not validated M(u)          |
| ROR2        | Receptor tyrosine kinase-like orphan receptor 2 | −2.91  | 2.8 × 10−3 | 3.9 × 10−5 | −1.39  | 5.2 × 10−3 | 0.63  | 5.0 × 10−3 |        |          |        |          |        |          |        |          | Not validated M(u)          |

Table 1. Validation and Filtering of Candidate Genes for Further Studies among 20 Top-Ranked Differentially Expressed Transcripts (Continued on next page)
| Gene Symbol | Full Gene Name | Log2FC | p Value* | Adjusted p | Log2FC | p Value* | Log2FC | p Value* | Log2FC | p Value* | Log2FC | p Value* | Log2FC | p Value* | Log2FC | p Value* | Comment |
|-------------|----------------|--------|----------|------------|--------|----------|--------|----------|--------|----------|--------|----------|--------|----------|--------|----------|---------|
| MYH2        | Myosin heavy chain 2 skeletal muscle adult | -2.77  | 2.8 × 10⁻⁵ | 4.1 × 10⁻⁵ | -1.65  | 3.9 × 10⁻² | 0.47   | 3.7 × 10⁻¹ |        |          |        |          |        |          |        |          | Not validated |
| ITH5        | Inter-alpha-trypsin inhibitor heavy chain family member 5 | -1.32  | 2.9 × 10⁻⁵ | 1.9 × 10⁻¹ | -3.17  | 4.6 × 10⁻⁵ | -5.01  | 1.0 × 10⁻⁵ | -1.56  | 8.1 × 10⁻³ |        |          |        |          |        |          | Adipocyte specific |
| HYDIN       | HYDIN axonemal central pair apparatus protein | -3.22  | 3.2 × 10⁻⁵ | 6.3 × 10⁻² | ND     |          |        |        |        |          |        |          |        |          |        |          |          |
| PCK1        | Phosphoenolpyruvate carboxykinase 1 soluble | -1.90  | 4.1 × 10⁻³ | 3.7 × 10⁻⁴ | -2.03  | 7.7 × 10⁻³ | -2.94  | 2.9 × 10⁻³ | 0.70   | 4.5 × 10⁻¹ |        |          |        |          |        |          | Adipocyte specific |
| MLXIP       | MLX-interacting protein-like | -1.67  | 5.3 × 10⁻³ | 8.6 × 10⁻¹ | -1.51  | 2.7 × 10⁻² | -0.73  | 5.3 × 10⁻² | -0.62  | 2.8 × 10⁻¹ |        |          |        |          |        |          | Adipocyte-specific |
| PLA2G2A     | Phospholipase A2 group 1A platelets synovial fluid | -1.51  | 6.1 × 10⁻³ | 2.9 × 10⁻³ | -1.32  | 2.2 × 10⁻³ | -2.81  | 3.1 × 10⁻⁴ | 1.25   | 8.4 × 10⁻³ | -0.17  | 8.0 × 10⁻¹ | -0.42  | 4.1 × 10⁻¹ |        |          | Adipocyte specific |
| PPAPDC1A    | Phosphatidic acid phosphatase type 2 domain-containing 1A | 1.31   | 7.9 × 10⁻³ | 2.7 × 10⁻¹ | 2.18   | 4.7 × 10⁻⁵ | 1.45   | 3.8 × 10⁻² | 0.55   | 3.0 × 10⁻¹ | -0.05  | 9.2 × 10⁻⁵ | -0.75  | 5.3 × 10⁻² | -0.45  | 1.8 × 10⁻¹ | Preadipocyte specific |
| LPL         | Lipoprotein lipase | -1.40  | 1.3 × 10⁻³ | 4.1 × 10⁻² | -2.04  | 2.5 × 10⁻⁵ | -2.90  | 2.1 × 10⁻¹ | -0.07  | 4.7 × 10⁻¹ |        |          |        |          |        |          | Adipocyte specific |

Table 1. Continued

(Continued on next page)
| Gene Symbol | Full Gene Name | Log2FC | p Value* | Adjusted p | Log2FC | p Value* | Log2FC | p Value* | Log2FC | p Value* | Log2FC | p Value* | Log2FC | p Value* | Comment |
|-------------|----------------|--------|----------|------------|--------|----------|--------|----------|--------|----------|--------|----------|--------|----------|---------|
| DYRK1B      | Dual-specificity tyrosine-Y-phosphorylation regulated kinase 1B | –1.61  | 1.8 x 10^{-1} | 2.7 x 10^{-1} | –2.43  | 8.9 x 10^{-1} | –0.15  | 5.7 x 10^{-1} |        |          |        |          | Not validated M(ds)/WT |
| INSR        | Insulin receptor | –0.83  | 8.1 x 10^{-1} | 2.7 x 10^{-1} | –0.89  | 2.6 x 10^{-1} | 0.56   | 8.4 x 10^{-2} |        |          |        |          | Not validated M(us)/WT |

Table 1. Continued

Genes were selected for confirmatory RT-qPCR analyses based on differential expression (DE) in RNA-seq analyses, excluding genes wherein DE was caused by the transduction procedure and genes with very low gene expression (see Transparent Methods supplemental file for details). The criteria for filtering out false-positives and selecting candidate genes for downstream experiments based on the RT-qPCR validation were three-fold: (1) A technical validation using RNA from the same cells as those used for the global transcriptome analysis requiring significant differentially expressed in the same direction as in RNA-seq for successful validation (columns 6 and 7 [M(ds)/WT]). (2) A biological validation using RNA from an independent mutation (columns 8 and 9 [M(us)/WT]). (3) A control of potential transduction effects in which the candidate genes should not show differential expression comparing non-transduced WT cells and cells transduced with a lentivirus expressing only GFP (columns 10 and 11 [GFP/WT]). Validated genes were further interrogated for possible involvement in adipogenesis by studying their expression in undifferentiated cells. Genes not expressed in preadipocytes or with a >2-fold difference in expression between the differentiated and undifferentiated state were considered adipocyte specific (Figure 5B), and likely a consequence of the phenotype and not causative genes. RT-qPCR data of three independent experiments; p values comparing expression in mutated cells with WT cells (as log2 fold change [FC]) were calculated by Student’s t test with Benjamini-Hochberg correction (columns 7, 9, 11, 13, 15, and 17). * P-values are from two-sample Welch t-tests, unadjusted for multiple testing.
its overexpression induced adiposity (Ahangari et al., 2015). The precise molecular mechanism of \textit{CHI3L1} on adipogenesis has not been determined, but a direct role on the extracellular matrix through type I collagen has been proposed (Iwata et al., 2009; Mariman and Wang, 2010). It is possible that \textit{CHI3L1} is required for the remodeling of the extracellular matrix that precedes the adipocyte differentiation process. In a mouse model of osteomyelitis, partial restoration of osteogenesis has been achieved by suppressing \textit{Chi3l1} (Chen et al., 2017). This suggests that \textit{CHI3L1} might play a key role in cell fate decision (Figure 7).

\textbf{Enh#385 Regulates Genes Involved in Adipocyte Biology and Nearby Genes}

We specifically examined the expression of genes linked to adipocyte biology in our RNA-seq data from differentiated cells. We confirmed the reduction in expression of the markers previously measured by

![Figure 6. Adipocyte Differentiation Capacity of Wild-Type and Mutant SGBS Cells Estimated as Accumulation of Neutral Lipids](image)
RT-qPCR and observed reduction of several additional adipocyte markers. For example, LEP, PPARGC1A, PLIN1, CD36, and SLC2A4 (GLUT4) were reduced in mutant differentiated cultures. We noted that expression of some key proteins involved in fat synthesis (FASN), transport of lipoproteins (APOE), and the receptors for ADIPOQ (ADIPOR1 and ADIPOR2) were unaffected by the mutations of the enhancer (Table S2).

Next, we explored whether there were any putative cis-regulated genes among the differentially expressed genes. We scanned the chromosomal region surrounding rs1451385 (±5Mb) for differentially expressed genes. All genes annotated in the UCSC Browser (https://genome.ucsc.edu/), including protein-coding genes and non-coding RNA genes within this interval, were identified and expression levels were compared between mutant and WT cells. This region included a microRNA (miRNA) (MIR148A) and a few genes that are good candidates for involvement in metabolic disease based on prior literature, such as IGF2BP3 (insulin-like growth factor 2 mRNA-binding protein 3) at chr7:23,349,828 (7p15.3) and CREB5 (cAMP responsive element-binding protein 5) at chr7:28,865,511 (7p14.1). The only transcript with a significant differential expression in this large region was NFE2L3 (2.3-fold reduction, p = 0.009). However, as NFE2L3 is expressed at very low levels in SGBS cells, and because we did not find any differential expression in similar experiments using undifferentiated SGBS cells (see below), we did not consider this association further. These results suggest that the enhancer does not act in cis on protein-coding genes, but it should be noted that our global RNA sequence method did not allow quantification of short RNAs, such as miRNAs.

**Causative Genes Regulated by Enh#385 Uncovered by Differential Gene Expression in Undifferentiated Cells**

To extend the list of potential downstream causal genes regulated by Enh#385, we performed another transcriptomics analysis by RNA-seq, this time in undifferentiated SGBS preadipocytes. We compared the two independent mutants of Enh#385 (KO-385-V3 and KO-385-V11) with WT cells and a GFP transduction control (Figure S5; Table S3). In these analyses, we confirmed the downregulation of CHI3L1 (fold change,
and other downstream genes involved in adipogenesis could be located on the same chromosome. We hypothesized that the mediator of the link from Enh#385 to the initiation of the adipogenesis program could be a downstream effector of Enh#385 having a role in adipocyte differentiation, but the mechanistic circuits between the Enh#385 and WNT10B enhancer mutants, thus blocking adipogenesis. The increase of these two important anti-adipogenic regulators, RELN and NDN, was also upregulated in GFP-transduced cells, which may indicate that this differential expression was, at least partly, driven by the transduction procedure. In addition, NDN (Necdin; fold change, 0.42; \( p_{\text{adj}} \), 0.018), associated with the Prader-Willi syndrome in humans, characterized by severe obesity, showed significant DE in undifferentiated preadipocytes, indicating a role as effector transcript downstream of Enh#385. This gene was discarded in the first qPCR validation analysis due to concerns regarding unspecific differential expression induced by the transduction protocol. Comparison of differentiated and undifferentiated WT cells revealed several adipocyte-specific genes (Figure 5B). These genes show higher (or even exclusive) expression in differentiated adipocytes; hence, it is likely that their differential expression in the Enh#385 mutants is a consequence of the lack of adipogenesis, rather than being involved in the causative process.

The most differentially expressed gene was H19, a maternally imprinted lincRNA that interacts with insulin-like growth factor 2 (IGF2), and has been suggested to be a tumor suppressor (Bartolomei et al., 1991; Bell and Felsenfeld, 2000). Defects in the H19/IGF2 imprinting have been associated with Beckwith-Wiedemann syndrome (DeBaun et al., 2002), Silver-Russell syndrome (Bartholdi et al., 2009; Bliik et al., 2006), and Wilms tumor 2 (Steeman et al., 1994). Other top differentially expressed genes included several plausible candidates for involvement in adiposity and/or IR, such as ADH1B, IL8, ACE, IL1B, and IL6 (Figure 5S; Table S3).

Next, we focused on genes linked to adipocyte biology and adipogenesis based on prior literature (Table S4). We found that PPARG was reduced (0.7-fold) in mutant cells, whereas WNT10B, encoding a molecular switch that inhibits adipogenesis, was significantly increased (2.5-fold) (Christodoulides et al., 2006; Ross et al., 2000). It has been shown that WNT10B inhibits PPARG and promotes the expression of the osteoblastogenic transcription factor RUNX2 (Bennett et al., 2005). Consistent with a potential pivotal role of WNT10B in the observed phenotype, we found an increase (1.3-fold) of RUNX2 in the mutated cells. The anti-adipogenic transcription factor KLF2 has also been suggested as contributor in this pathway (Banerjee et al., 2003). Again, consistent with this hypothesis, KLF2 was significantly upregulated (1.4-fold) in our enhancer mutants, thus blocking adipogenesis. The increase of these two important anti-adipogenic regulators, WNT10B and KLF2, acting upstream of PPARG suggests that Enh#385 might function very early in the initiation of the adipogenesis program.

**DISCUSSION**

Our study offers a detailed characterization of a GWAS locus (previously annotated as SNX10) associated with WHR and other obesity-related traits in a series of functional experiments. Our main conclusions are several-fold. First, we identified an enhancer active during adipogenesis that responds strongly to external metabolic stimuli, such as insulin and isoprenaline. The known WHR-raising allele (A at rs3902751) (Shungin et al., 2015) is on the same haplotype as the allele being associated with higher enhancer activity (T at rs1451385), suggesting that higher activity of this enhancer is associated with higher WHR. Second, mutation of the enhancer using CRISPR-Cas9 in preadipocytes dramatically impairs adipocyte differentiation. RNA-seq followed by replication using RT-qPCR in mature adipocytes demonstrated that several adipocyte genes were downregulated in cells with disrupted Enh#385 and highlighted CHI3L1 as a potential downstream target, also downregulated. Third, disruption of CHI3L1 caused a decrease in adipogenesis.

Independently of the allele tested, the enhancement of luciferase expression was higher with Enh#385 cloned in forward direction (Figure 2), suggesting that the cloned DNA fragments may have a significant promoter-like activity. Consistent with this, the signal from the CAGE-sequencing experiment was generally stronger in the forward direction throughout adipogenesis (Figure 1D). However, given the long physical distance to the nearest gene, the regulatory element is most likely to be an enhancer with promoter-like activity. This complex nature of the regulatory element has been observed in a previous study, wherein the sequence of Enh#385 was included in a DNA fragment (976 bp) functionally classified as weak repressor element (Kwaks et al., 2003).

CHI3L1 could be a downstream effector of Enh#385 having a role in adipocyte differentiation, but the mechanistic circuits between the Enh#385 and CHI3L1 function are unclear, especially as they are not located on the same chromosome. We hypothesized that the mediator of the link from Enh#385 to CHI3L1 and other downstream genes involved in adipogenesis could be MIR148A, a non-protein-coding
gene encoding an miRNA localized 95 kb from the enhancer (Figure 1). miRNAs are known to be master regulators of groups of proteins (Stefani and Slack, 2008), and indeed, several miRNAs have been reported to regulate adipogenesis and lipid metabolism (Ahn et al., 2013; Esau et al., 2004; Yang et al., 2011). Indeed, our analysis of enhancer-promoter interactions by HiChIP in a human coronary artery smooth muscle cell line shows connections between the Enh#385 and MIR148A (Figure S6).

Searching for MIR148A targets using TargetScan (http://www.targetscan.org), we found two conserved sequences at the 3’ UTR of WNT10B and two sequences of KLF2 that acts as binding targets for miR-148a (Figure S7). Consistent with this, we observed a significant increase of WNT10B in Enh#385 mutants (Table S4). There is also support in prior literature for involvement of MIR148A and WNT10B in adipogenesis. In mice, miR-148a is upregulated during adipogenesis and downregulated in mature adipocytes of obese animals (Xie et al., 2009). In a recent report using differentiating human adipose-derived mesenchymal stem cells, it was shown that MIR148A induces adipogenesis by suppressing its target gene, WNT10B, an endogenous inhibitor of adipogenesis (Shi et al., 2015). It is tempting to speculate that the increase of WNT10B and KLF2, two anti-adipogenic factors, in our mutants is the result of the reduction of MIR148A (Table S4). This scenario is consistent with the observed increase of RUNX2 and decrease of PPARG in our DE data and with the lack of adipogenesis in the Enh#385 mutant cells. A schematic illustration of this proposed model is shown in Figure 7.

Experimentally, we were able to measure an increase of expression of MIR148A between 0 and 8 days of differentiation, but we could not detect significant differences in expression of MIR148A between WT and Enh#385 KO cells before or 8 days after induction of differentiation. It is possible that a role of MIR148A in regulating adipogenesis is transient, and can only be recorded in a short time window during the differentiation process (and we analyzed differential expression at just two time points). Supporting this interpretation, CAGE-sequencing data (from the FANTOM consortium) measuring the activity of the MIR148A promoter during MSC adipocyte differentiation show only a consistent increase in activity at 4 and 14 days, but not at the rest of the 17 time points, indicating a fluctuating and transient regulation of MIR148A (Figure S8). The inconsistent results in detection of MIR148A in mutant cells could be also due to the negative regulatory feedback loop between MIR148A and one of its targets, the methylase DNMT1 (Figure 7). The methylase downregulates the expression of MIR148A by hypermethylation of its promoter (Hong et al., 2018; Long et al., 2014).

The consistent downregulation of the imprinted gene NDN (Necdin) leads us to hypothesize that imprinting by methylation of NDN could be mediated by the DNMT1-DNMT1-MIR148A circuit. NDN is a particularly interesting gene for adipocyte biology as it is one of the candidate genes in the chromosomal region 15q11-15q13, which results in Prader-Willi syndrome when the paternal copy is deleted. In addition to mild to moderate intellectual impairment and behavioral problems, obesity and T2D are the most common symptoms of Prader-Willi syndrome, owing to insatiable appetite and chronic overeating (MacDonald and Weyrick, 1997). NDN is a predicted target of MIR148A, and its expression is elevated in adipocytes of obese individuals (Bracconi et al., 2010). DNMT1 maintains methylation pattern during adipocyte differentiation, and its silencing accelerated adipogenesis (Londono Gentile et al., 2013). It has been proposed that DNMT1 and MIR148A are regulated by a negative feedback loop that could explain the discrepancy in the published reports (Xu et al., 2013). In fact, the promoter of MIR148A is in proximity of CpG islands, and it is silenced by hypermethylation (Hanou et al., 2010). Our differential expression analysis shows that DNMT1 expression is only slightly increased in mutant cultures; however, it has been reported that the targeting of this gene by MIR148A occurs exclusively at the protein level (Pan et al., 2010), which makes the differences in mRNA a less ideal indicator of enzyme methylation activity. Imprinted genes (identified using the Genomic Imprinting website: http://www.geneimprint.com) were generally not affected by the mutations of Enh#385. However, in addition to NDN, the adipogenesis-linked and imprinted transcript H19 was the most differentially expressed gene in preadipocytes, showing a consistent downregulation in mutated cells (Han et al., 2018; Huang et al., 2016). It is plausible that only loci involved in adipogenesis are accessible to DNMT1, so it should not be expected that all imprinted genes and genes regulated by CpG methylation are downregulated in Enh#385 mutants. The increased activity of this enzyme may lead to the repression of active adipogenic loci controlled by methylation. Our expression analysis shows that the imprinted gene NDN and genes regulated by methylation such as DLK2 (Delta like non-canonical Notch ligand 2) and EBF2 (Early B-cell factor 2), both promoting adipogenesis (Jimenez et al., 2007; Nueda et al., 2007), are consistently downregulated in cells with disrupted Enh#385 (Tables S2 and S4). Thus, despite being limited to a few genes, this methylation link reinforces our model in which MIR148A is the most likely target of the enhancer Enh#385 (Figure 7).
GWAS have been remarkably successful in discovering loci associated with complex traits, but to unlock the transformative potential of these findings, there is a strong need for detailed studies of the molecular mechanisms underlying these signals. We have performed such a study that improves the understanding of adipocyte biology and fat distribution. Although the exact molecular events that link genetic variation in Enh#385 (previously annotated as the SNX10 locus) with the impairment of adipocyte differentiation still need to be characterized in detail, one potential explanation for link of the enhancer with adipogenesis could be cis-acting regulation through the nearby MIR148A. We hypothesize that allelic variants that reduce the activity of Enh#385 could lead to the downregulation of MIR148A. This reduction of MIR148A may produce an increase of the cell fate determinant WNT10B and the subsequent inhibition of adipogenesis. These initial and complex alterations of gene activity could lead to the reduction of CHI3L1 expression, which, as we demonstrate in this work, is essential for the adipocyte maturation. Further studies are needed to establish these potential mechanisms linking Enh#385 to adipocyte differentiation.

Limitations of the Study
The model we propose in which the enhancer Enh#385 acts on MIR148A is based on indirect observations and is not directly proved in this study. Hence, it should be viewed as hypothesis generating, and we do not discard the possibility that the enhancer also acts on other genes or genetic elements.

METHODS
All methods can be found in the accompanying Transparent Methods supplemental file.

SUPPLEMENTAL INFORMATION
Supplemental Information can be found online at https://doi.org/10.1016/j.isci.2019.09.006.

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AUTHOR CONTRIBUTIONS
C.C-L., E.A., and E.I. conceived and designed the study. C.C-L., A.P., and S.H. designed, performed, and analyzed the experiments. C.C-L., M.P., and E.I. analyzed gene expression data. M.W. and C.W. contributed with cell lines and reagents. T.Q. contributed with the chromatin interactome data. C.C-L. and E.I. wrote the manuscript with input from the other authors. All authors approved of the final version of the manuscript.

DECLARATION OF INTERESTS
The authors declare no competing interests.

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Supplemental Information

Detailed Functional Characterization

of a Waist-Hip Ratio Locus in 7p15.2 Defines

an Enhancer Controlling Adipocyte Differentiation

Casimiro Castillejo-Lopez, Milos Pjanic, Anna Chiara Pirona, Susanne Hetty, Martin Wabitsch, Claes Wadelius, Thomas Quertermous, Erik Arner, and Erik Ingelsson
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Detailed functional characterization of a waist-hip ratio locus in 7p15.2 defines an enhancer controlling adipocyte differentiation

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Figure S1. Potential model of inhibition by competitive binding. Related to Figures 2 and 3. Both the stimulation with insulin and isoprenaline (in blue and pink, respectively) potentiated the transcriptional activity of the enhancer. A potential model explaining our findings is that each stimulation generates a specific set of co-activators, and that simultaneous stimulation (in green) results in competitive inhibition of co-activators that annulate the potentiation of the transcription.
Figure S2. Estimation of editing efficiency by allelic isolation and TIDE. Related to Figures 4 and 5, and Table 1.

(A-B) Sequences alignments representing isolated alleles of SGBS cell cultures treated with CRISPR-Cas9 editing vectors; and (C-D) graphs showing the estimation of In-Dels in mutated cultures by Tracking of Indels by Decomposition (TIDE). (A) Sequences from KO-385-V11 cultures targeted upstream the rs1451385 polymorphism (T/C). Each row corresponds to an isolated allele. The guide RNA is delineated at the top in the wildtype sequence and the PAM sequence is indicated in blue. The cutting site of the Cas9 nuclease is three nucleotides upstream of the PAM (note that this construct is targeting the lagging strand) as predicted. The efficiency of mutation in this culture was estimated as 100% with the most abundant allele carrying an A insertion. (B) Isolated alleles from KO-385-V3 cultures targeted downstream of the polymorphism. The PAM sequence is indicated in pink letters in the reference sequence. The cutting site of the Cas9 nuclease is precisely three nucleotides upstream of the PAM sequence. Only one of the recovered alleles was wild type. The alignments were done using the SnapGene software. (C) TIDE analysis of the KO-385-V3 culture sequenced with the forward genomic primer; and (D) sequenced with the reverse genomic primer (Supplementary Table 5). The x-axis indicates the type of mutation (insertion or deletion), and the number of nucleotides inserted or deleted. The efficiency of both mutations (KO-385-V3 and KO-385-V11[not shown]) reached more that 95% according to both evaluations. The most abundant alleles were a four-nucleotide deletion and a one-nucleotide insertion, respectively.
Figure S3. Quantification of lipids by Oil Red O staining. Related to Figure 4. Oil Red O, a lipid-specific dye, was used to determine lipid accumulation in differentiating cells cultures. (Ramirez-Zacarias et al., 1992) Cells were fixed for 1 hour with 10% formalin solution, washed with water, and stained for 2 hours with Oil Red O, followed by exhaustive rinsing with water. The dye was extracted with isopropyl alcohol and its absorbance was read at 510 nm. (A) Absorbance quantification at 5, 10 and 22 days after induced differentiation. Each point represents the mean values of three independent cultures. (B) Representative photographs of wild type, GFP transduced and mutant cultures at day 12. Scale bar, 100 micrometers.
Figure S4. Relative RNA expression by RT-qPCR in differentiated SGBS cells. Related to Figure 4C and Table 1. The expression of the selected genes was done comparing three mutated cultures (K0-385-v11) propagated and differentiated in parallel with four WT cultures and three mutated (KO-385-v3) against four independent WT cultures. Each dot represents the mean of three technical replicates of the same culture. The normalization was done with the housekeeping gene TBP.
Figure S5. Differential gene expression in preadipocytes. Related to Figure 5B. Volcano plot comparing gene expression in preadipocytes between Enh#385 mutated (KO-385-V3 and KO-385-V11) and control (WT and GFP-transduced) cell cultures. Gene with significant differential expression (DE; \(P_{\text{adj}}<0.05\) and abs(log\(_2\) fold change)>1) are in green, while those with abs(log\(_2\) fold change)>1 are in orange. The top 10 differentially expressed genes are in blue, and four highlighted genes (\textit{CHI3L1}, \textit{RELN}, \textit{NDN} and \textit{H19}) are in red.
Figure S6. Circos plot of the chromatin interactome in the cis region around Enh#385. Related to Figure 7. The plot was generated using the Bioconductor function *FitHiC* with HiChIP data obtained from human coronary artery smooth muscle cells. The positions of the Enh#385 defined by the SNP rs1451385 and the *MIR148A* are marked. Chromatin looping tracks are in purple, and open chromatin are shown as bars. Raw and processed data are available at the Gene Expression Omnibus (GEO) under accession GSE101498. The data can be visualized in the WashU Epigenome Browser (https://epigenomewidget.org) with the following session ID: 98051079.
Figure S7. Predicted targets of miR148a that may be involved in initiation of adipogenesis. Related to Figure 7. Conserved sequences at the 3’ UTR regions of WNT10B (A and B) and KLF2 (C and D) that were predicted as targets of MIR148A using Targetscan (http://www.targetscan.org). The predicted targets were found by searching for the presence of conserved 8mer, 7mer, and 6mer sites at the 3’UTR of the genes matching the seed region of MIR148A. Matches to the human WNT10B and KLF2 3’ UTR are shadowed white. The targets in the 3’ UTR of WNT10B are broadly conserved among vertebrates, while the conservation is restricted to primates in the case of KLF2.
Figure S8. Activity of the MIR148A promoter during mesenchymal stem cell adipocyte differentiation. Related to Figure 7. Transcriptional signal of MIR148A during adipogenesis of mesenchymal stem cells visualized using the ZENBU browser (http://fantom.gsc.riken.jp/zenbu/). Signals are ordered from top to bottom from one hour after induction of adipogenesis to day 14.
Figure S9. Sequencing chromatograms of wild type cell cultures and cultures mutated with CRISPR-Cas9. Related to Figure 6. (A) Sequencing chromatograms of the amplified genomic DNA of the target region of CHI3L1: Sequence of wild type cell culture (above) and of CRISPR-Cas9 treated cells (below). The more frequent mutated allele contains a 199 bp insertion at the predicted cutting site of the nuclease. The PAM sequence corresponding to the guide G58 is colored in blue. The central part of the chromatogram is not shown due to space restrictions. (B) Sequences of the RELN targeted region. The more frequent mutated allele contains a 60 bp insertion at the expected site of the PAM sequence corresponding to the guide G64.
### Table S2. Differential expression of genes known to be involved in adipocyte biology after Enh#385 KO in differentiated adipocytes. Related to Figure 7.

Genes were selected from published literature and divided in two categories “Adipocyte marker genes” and “Adipogenic genes” based on their function as a constituent part of the adipocyte; or as a gene necessary for the process of differentiation to mature adipocyte, respectively. The first category was subdivided in Adipokine Related genes (AR), genes involved in Glucose Metabolism (GM); Immune-related Genes (IG) and genes involved in Lipid Metabolism (LM). The second category was subdivided in genes involved in Signal Transduction (ST), Transcription Factors (TF) and Long-non-coding RNAs (LR). The relative expression represents the number of transcripts divided by the total number of counts; and provides an estimate of the abundance of the transcript. Fold change (Fold C) represents the difference in relative expression between the Enh#385 KO and WT cultures.

| Gene Symbol | Gene Name | Function | WT Relative Expression | Mutant Relative Expression | Fold Change | Class |
|-------------|-----------|----------|------------------------|---------------------------|-------------|-------|
| LIP | lipin | Adipokine key player in the regulation of energy balance and body weight | 3.6 | 0.9 | 3.9 | AR |
| ADIPOQ | adiponectin | Adiponectin C10 and collagen domain containing | 45.0 | 15.7 | 2.8 | AR |
| ADIPO2 | adiponectin receptor | Adiponectin receptor | 34.1 | 27.1 | 1.2 | AR |
| ADIPORE | adiponectin receptor | Adiponectin receptor | 21.3 | 18.2 | 1.2 | AR |
| SC2A4 | solute carrier family 2 facilitated glucose transporter member 4 | Solute carrier family 2 facilitated glucose transporter member 4 | 0.2 | 0.0 | 0.6 | LM |
| PDK1 | phosphoinositide-3-kinase catalytic subunit, alpha | Regulates carbohydrate biosynthesis | 26.5 | 3.4 | 8.0 | LM |
| PDKA | pyruvate dehydrogenase phosphatase 1 | Pyruvate dehydrogenase phosphatase 1 | 69.6 | 59.3 | 1.2 | LM |
| PDKC | pyruvate dehydrogenase phosphatase 1 | Pyruvate dehydrogenase phosphatase 1 | 199.4 | 212.8 | 1.1 | LM |
| SCD | stearoyl CoA desaturase 1 | Stearoyl CoA desaturase 1 | 14.1 | 14.4 | 1.0 | LM |
| TIGR | triglyceride lipase | Triglyceride lipase activity | 60.6 | 22.9 | 2.7 | LM |
| P2U2 | perilipin 2 | Perilipin 2 | 30.4 | 13.3 | 2.3 | GM |
| CD36 | cluster of differentiation 36 | CD36 | 67.9 | 40.2 | 1.7 | LM |
| FABP4 | fatty acid binding protein 4 | Fatty acid binding protein 4 | 258.8 | 182.1 | 1.4 | LM |
| SEC27A | solute carrier family 27 fatty acid transporter member 2 | Solute carrier family 27 fatty acid transporter member 2 | 2.7 | 2.0 | 1.4 | LM |
| MPZ | myelin protein zero | Myelin protein zero | 19.0 | 15.5 | 1.2 | LM |
| DGAT2 | diacylglycerol O-acyltransferase 2 | Diacylglycerol O-acyltransferase 2 | 23.8 | 20.6 | 1.1 | LM |
| DGAT2A | solute carrier family 27 fatty acid transporter member 2 | Solute carrier family 27 fatty acid transporter member 2 | 19.8 | 18.3 | 1.1 | LM |
| LPL | lipoprotein lipase | Lipoprotein lipase | 41.0 | 37.2 | 1.1 | LM |
| FABP5 | fatty acid binding protein 5 | Fatty acid binding protein 5 | 161.0 | 159.8 | 1.0 | LM |
| FAT1 | fatty acid synthase | Fatty acid synthase | 230.8 | 205.1 | 1.1 | LM |
| SEC27A1 | solute carrier family 27 fatty acid transporter member 1 | Solute carrier family 27 fatty acid transporter member 1 | 40.4 | 40.4 | 1.0 | LM |
| APOE | apolipoprotein E | Apolipoprotein E | 2887.4 | 2899.2 | 1.0 | LM |
| DIO2 | diacylglycerol lipase type II | Diacylglycerol lipase type II | 0.2 | 0.1 | 1.1 | LM |
| AGAT2 | 1-acetylglutamate kinase | Agmatine decarboxylase | 18.7 | 20.2 | 1.1 | LM |
| CRTLA | cluster of differentiation 11a | Cluster of differentiation 11a | 36.0 | 45.3 | 1.3 | LM |
| DGAT1 | diacylglycerol O-acyltransferase 1 | Diacylglycerol O-acyltransferase 1 | 5.1 | 6.3 | 1.2 | LM |
| P2U2 | perilipin 2 | Perilipin 2 | 584.6 | 784.5 | 1.3 | LM |

**Adipokine related genes**

| Gene Symbol | Gene Name | Function | WT Relative Expression | Mutant Relative Expression | Fold Change | Class |
|-------------|-----------|----------|------------------------|---------------------------|-------------|-------|
| DX3 | Delta-like 3 homologue | Delta-like 3 homologue | 0.6 | 0.3 | 0.5 | GM |
| STAM | signal transducer and activator of transcription 7 acute phase response factor | Signal transducer and activator of transcription 7 acute phase response factor | 119.9 | 107.1 | 1.1 | GM |
| SRRT | signal transducer and activator of transcription 7 acute phase response factor | Signal transducer and activator of transcription 7 acute phase response factor | 12.8 | 14.0 | 1.1 | GM |
| SPPC | secreted protein acidic and rich in cysteine | Secreted protein acidic and rich in cysteine | 6296.2 | 5486.5 | 1.1 | GM |
| WNT10B | wingless-type MMTV integration site family member 10b | Wingless-type MMTV integration site family member 10b | 0.2 | 0.1 | 1.1 | GM |
| EBF2 | erythroid b-cell factor 2 | Erythroid b-cell factor 2 | 24.0 | 26.7 | 1.1 | GM |
| PPAR | peroxisome proliferator-activated receptor gamma | Peroxisome proliferator-activated receptor gamma | 41.8 | 43.9 | 1.1 | GM |
| SREBF1 | sterol regulatory element binding transcription factor 1 | Sterol regulatory element binding transcription factor 1 | 11.0 | 9.6 | 1.1 | GM |
| END1 | endogenously expressed | Erythroid b-cell factor 2 | 21.6 | 22.7 | 1.1 | GM |
| GATA2 | GATA binding protein 2 | GATA binding protein 2 | 15.9 | 13.9 | 1.1 | GM |
| CEBPB | CCAAT enhancer binding protein CEBP b | CCAAT enhancer binding protein CEBP b | 1.8 | 2.8 | 1.5 | GM |
| CEBP | CCAAT enhancer binding protein CEBP delta | CCAAT enhancer binding protein CEBP delta | 3.8 | 4.6 | 1.3 | GM |
| RUNX2 | runt-related transcription factor 2 | Runt-related transcription factor 2 | 10.0 | 7.2 | 1.4 | GM |
| JUN | Jun | Jun | 23.8 | 35.1 | 1.4 | GM |
| ZIC1 | zinc finger family member 1 | Zinc finger family member 1 | 0.2 | 0.1 | 1.1 | GM |
| CEBPA | CCAAT enhancer binding protein CEBP alpha | CCAAT enhancer binding protein CEBP alpha | 6.0 | 4.9 | 1.2 | GM |
| PPARG | peroxisome proliferator-activated receptor gamma coactivator 1 alpha | Peroxisome proliferator-activated receptor gamma coactivator 1 alpha | 0.4 | 0.3 | 1.1 | GM |
| PROM16 | promyelocytic leukemia | Promyelocytic leukemia | 0.4 | 0.3 | 1.1 | GM |
| H19 | H19 | H19 | 14530.4 | 12119.1 | 1.2 | GM |
Table S4. Differential expression of genes known to be involved in adipocyte biology after Enh#385 KO in differentiated adipocytes. Related to Figure 7. Genes were selected from published literature and divided in two categories “Adipocyte marker genes” and “Adipogenic genes” based on their function as a constituent part of the adipocyte; or as a gene necessary for the process of differentiation to mature adipocyte, respectively. The first category was subdivided in Adipokine Related genes (AR), genes involved in Glucose Metabolism (GM); Immune-related Genes (IG) and genes involved in Lipid Metabolism (LM). The second category was subdivided in genes involved in Signal Transduction (ST), Transcription Factors (TF) and Long-non-coding RNAs (LR). The relative expression represents the number of transcripts divided by the total number of counts; and provides an estimate of the abundance of the transcript. Fold change (Fold C) represents the difference in relative expression between the Enh#385 KO and WT cultures.
### Primers used for genome amplification of the CRISPR-Cas9 targets and TIDE sequencing

| Target  | Forward primer       | Reverse primer               |
|---------|----------------------|------------------------------|
| Enh#385 | TTGAGTTGAACATTATGTAGCGTTT | TGAACAGTAAACATTACGAAAAATTTAG |
| RELN    | CCCCTCCATGTCGACACA   | GGAGATGAGGATTTAAAACAACCCA   |
| CHI3L1  | CCAAGGCACCTAGCATATCA | CTTCTAGCCCACCACATTTCC       |

### Primers used for RT-qPCR validation

| Gene symbol | Forward primer       | Reverse primer               |
|-------------|----------------------|------------------------------|
| GAS7        | ACGTTCCCTCACCCAGACA  | TTCAGCTGTTCCTGGAAGCAGTAG     |
| CHI3L1      | CAACCCTGAAGACTCTTGTGTGTGT | GCAAGTGGCTCCACCCATCAAA    |
| HEPH        | TGATGGAGCCACTCGAGTCTA | CCCCTATCCGGTTCTGTC         |
| NDN         | CTGCGGAAGCTCTCATCCT  | CCAGGACCTCCATGTATTGAGCATCT |
| LPL         | GTGATTCTAATTTGACGACTGACT | GCCAGGCTCTCTTCTTCTTCT    |
| H2AFY2      | AGAACAGCTGAGAGACCATCAA | TGGGCTGAGATGCTTTGAG       |
| MID2        | CACGGTTTCTCATGCTGCAA  | ACCCTCTAGCACGTTCCTTCTCT    |
| RCAN2       | GTGCGGATTCTGCAGGTCTT  | TCGGCTGAGATTTAGGATTG       |
| RELN        | CCCAAATGCTGCTGGAAGTGTG | ATGCCACCTGCTGCTGGAAG      |
| ITIH5       | CCGAGGCAATCTGACACTGT  | GAACCTCAGATTCCTCTGAGAAAA   |
| SGCD        | GCCGAAGCTGCTGCAATATGGA | AGACCTTCTGCTGCTCCTCT       |
| MYH2        | GCCGGACTCTGATAAGGACCAA | ACACCAAGACTTCTTTCTTCTCAAG |
| ROR2        | GATGGGTTCTGCCAGCCTTA  | CGACAGGGCTGAGACAGT         |
| PCK1        | GGAAGAATAAGGAGTGAGCTCAGA | GCCCTCAAAGATAATGCCTCCTCA   |
| MLXIPL      | GGGTTACCCATACAACACCAAGC | TGGACACCTAATCTCTCTCAAG     |
| PLA2G2A     | CGGAGGTTCTGCAACGACTCTA | ACTGAGGGCTGCTCCTTCTTCT    |
| PPAPDC1A    | GCATGATTGCGACTACAAGCAT | GCTGGAGCTGCACTAAA           |
| HYDIN       | TGCTGGATTATGGAAGATTTTCCT | GATGAGTCTCTTGAGGACTGAA     |
| DYRK1B      | ATCAAGACCTCAAACAGCCACG | GCCCAAGATGTAGTCATGG         |
| INSR        | CTGAGAGGCTACCTGCTGCTT | AGTGGCGGACATGCACAAACTTT    |
| TBP         | GCCAGAGGTTTCTGAGTTTG  | CGAGCTGCATTAGGCTCATTG       |
| ADIPOQ      | CCTGGTGAAGGATGAGAAAGA | CATAGGCCACCTTCTCAGGTT       |

Table S5. Primers sequences for genome amplification of the CRISPR-Cas9 targets and for RT-qPCR. Related to Figures 4, 5 and 6, and Table 1.
**Transparent Methods**

**Cell cultures and differentiation**

The human preadipocyte SGBS cell line was cultured as previously described (Fischer-Posovszky et al., 2008) in OF-serum containing medium composed of DMEM/F12 (1:1) medium (Life Technologies) supplemented with 10% FCS, 17 μM biotin, 33 μM pantothenic acid and 1% penicillin/streptomycin/amphotericin B (Gibco). Cells were maintained at 37°C and 5% CO₂.

To promote adipose differentiation, cells were seeded into a 24-well plate at a density of 2.5x10⁵ cells per well and cultured in Quick-Diff medium consisting of serum-free medium supplemented with transferrin (0.01 mg/ml), insulin (20 nM), cortisol (100 nM), triiodothyronine (0.2 nM), dexamethasone (25 nM), isobutyl-1-methylxanthine (IBMX) (250 μM) and the PPARγ-agonist rosiglitazone (2 μM) (BioNordika, Sweden). After 4 days, the medium was removed and replaced by 3FC medium composed of serum-free medium supplemented with transferrin (0.01 mg/ml), insulin (20 nM), cortisol (100 nM) and triiodothyronine (0.2 nM). The medium was changed every 4 days, and the differentiation process was documented by microscopy every 4 days.

The HEK293T (ATTC Cat# CRL-3216, RRID: CVCL_0063), HEla (CLS Cat# 300194/p772_HeLa, RRID: CVCL_0030) and HepG2 (CLS Cat# 300198/p2277_Hep-G2, RRID: CVCL_0027) cells were cultured in RPMI 1640 medium supplemented with 10% FBS, 2 mM L-glutamine and 1% penicillin/streptomycin/amphotericin B (Gibco).

**Evaluation of enhancer activity**

*Cloning of reporter constructs.* A 266 bp genomic fragment containing the polymorphism rs1451385 was amplified with the primers (5´caggtaccGTTGAACATTATGTAGC 3´) and (5´ggagatcTTGAACATTATGTAGC 3´) and cloned into the KpnI and BglII sites of the pGL4.10 firefly luciferase reporter (Promega). The minimal promoter of pGL4.26 (Promega) was inserted upstream of the luciferase gene. The 266 bp fragment was cloned in inverse position by amplification with the primers (5´cgggtaCCTGAGGAGTAGTATGC 3´) and (5´tcagatctCCTGAGGAGTAGTATGC 3´) and inserted into the same KpnI and BglIII sites. In order to test both alleles, the cloned allele (T) was mutated to (C) by conventional PCR mutagenesis using the primers (5´CAATATCCACCACCACTGAGTGCCTAATTGAGGCGGTAC 3´) and (5´CGACCTCACGATACCTGAGTGGATATTGCACAGA 3´). The vectors used in our typical reporter assay were the following: forward direction-allele T, forward direction-allele C, reverse
direction-allele T, reverse direction-allele C, empty vector containing only the minimal promoter, and a positive control vector. As a positive control, we chose a human 411-bp enhancer from chromosome 12 that consistently increases luciferase expression about three-fold in a variety of cell lines (Cavalli et al., 2016). The positive enhancer control was amplified with the primers 5’-GCTGTGGGGGAAGAGGTATTC-3´ and 5’-GAGAGACTCCCCCACCTTCA-3’.

Quantification of enhancer activity. Enhancer activity was examined by luciferase reporter gene assays in the following human cell lines: HepG2 (hepatocytes), HeLa (cervical cancer cells), HEK293 (embryonic kidney) and SGBS (preadipocytes). Co-transfections were done in 48-well plate at 80% confluence using Lipofectamine 3000 (Life Technologies) with 150 ng of reporter construct and 10 ng Renilla luciferase reporter vector to normalize the transfection efficiency. Cells were lysed 24 h after transfection, and ratios between Firefly luciferase and Renilla luciferase activity were measured with a dual luciferase assay (Promega) using a Varioskan Lux Microplate Reader (Thermo). For reporter experiments where inducible reagents were used (insulin and/or isoprenaline), the measurements were done 48 hours after transfection, corresponding to 24 hours of inducible treatment.

Replicates. Each mean value is the result of measuring 12-14 biological replicates, using three different cell cultures and two different plasmid preparations.

Genome editing
We performed CRISPR-Cas9 genome editing in the human preadipocyte SGBS cell line to alter the sequence adjacent to rs1451385. The guide RNAs (gRNA) were designed using the online tools: http://www.broadinstitute.org/rnai/public/analysis-tools/sgrna-design and http://crispr.mit.edu/. The DNA oligos were purchased from IDT (Integrated DNA Technologies), annealed and cloned into the BsmBI site of the lentCRISPRv2 lentiviral vector as previously described. (Shalem et al., 2014) Correct insertion was verified by Sanger sequencing. To rule out off-target effects, we induced mutations of the enhancer with two separate guide RNAs. We also searched for potential off-target sites in the human genome for both guides. (Bae et al., 2014) For guide G385-11, there were two possible off-targets with two mismatches; while for guide G385-3T/C, we found seven possible off-targets with two mismatches. No sites with precise match or with one mismatch were observed. We further searched the regions of these nine potential off-target sites (localized in intergenic regions) for known genes involved in adipocyte biology, but we did not find any adipocyte-related genes close to
these potential off-target sites. Using our differential expression data, we have also checked for alteration of gene expression in the proximity of these potential off-target sites, but we were unable to detect any such effects.

The gRNA sequences were: G385-11, atattcacagctgctgcaAGG for construct V11-385; G385-3T/C, gtagcctcgtgtcagTGG for construct V3-385; aatggccatctccccctTGG for RELN and gtagcctgtgaaagtCGG for CHI3L1 (PAM sequences in capitals). Lentiviruses expressing Cas9 and the gRNAs were generated in HEK293T cells by co-transfection of the packaging plasmids psPAX.2 and psMD2.G (Addgene). Supernatants containing lentiviruses were harvested 24 and 48 h post-transfection. Confluent SGBS cells were subsequently exposed to lentiviruses (with polybrene; 8 μg/ml) and selected with puromycin (1 μg/ml) for 72 h.

**Assessment of editing efficacy**

Mutation efficacy was estimated by two methods: first, cloning and sequencing of isolated alleles; and second, by analysis of the sequence chromatograms of the amplified target region using Tracking of Indels by DEcomposition (TIDE). The first method gives information about the frequency and the type of mutations, while the second method gives information of indel mutations but neither the nature of the indel or possible substitutions are counted. We verified that both methods correlated well in our CRISPR-Cas9 transductions (Supplementary Figure 2). We chose TIDE as the standard method for our mutation assessments of independent cell cultures.

For isolation of mutated alleles, cells resistant to puromycin were propagated and an aliquot corresponding to 1×10⁶ cells was withdrawn for genomic DNA preparation. PCR amplifications of the target sequence were carried out with 20 ng genomic DNA in 50 μl reaction using Platinum Taq polymerase (Thermo Fisher) and the primers (5´ TTCCATGCGTTGGAAGTCCATT-3´ and 5´ CGGCCTTGAGGAGTAGTATGC-3´). PCR conditions were 3 min at 94°C (1x), followed by (2x) touchdown cycles of 30 s at 94°C, 20 s at 59°C, 58°C, 57°C and 30 s at 72°C and ending with (27x) of 30 s at 94°C, 20 s at 56°C and 30 s at 72°C, and a final extension at 72°C for 5 min. The PCR products were purified using the PCR DNA and Gel Band purification kit (GE Healthcare) and cloned using the TOPO TA Cloning kit in the pCR™ 2.1 vector (ThermoFisher). Individual bacteria clones were Sanger sequenced using universal primers present in the vector. Sequences were aligned to a wild type clone and the frequency of editing was estimated. Independent transductions were assessed for efficiency of genome editing using Tracking of Indels by DEcomposition (TIDE) as described previously (Brinkman et al., 2014) (Supplementary Figure 2). PCR products were purified using the SequalPrep™
Normalization Plate (Invitrogen). An aliquot of 30 ng DNA was Sanger sequenced in both directions using the amplification primers (Supplementary Figure 2). For primers sequences, see Supplementary Table 5. The chromatograms were analyzed using the TIDE tool, available at https://tide.nki.nl.

Image quantification of lipid accumulation

We observed some limitations using the classical Oil Red O staining method for quantification of lipid content in our SGBS cell cultures. During the rinsing step, small oil droplets detached from some cells and floated to the surface where they were discarded during subsequent washing steps. This loss was more pronounced when cultures were well-differentiated. To cope with this problem, we decided to measure the oil droplets by cell imaging and normalize by the number of cell nuclei. Lipid content was estimated using phase contrast images and/or images stained with Bodipy 493/503 (4, 4-difluoro-1, 3, 5, 7, 8- pentamethyl-4-bora-3a, 4a-diaza-s-indacene) and captured with the Phase or GFP channel, respectively. To quantify the number of cells, the nuclei were stained with Hoechst 33342 (Thermo Fisher). Before imaging, the culture medium was replaced by PBS containing a solution of Bodipy®493/503 (2 µM) and Hoechst® 33342 (1.6 µM). The EVOS FL Auto-fluorescent microscope was used to image the cells on the phase contrast channel and with the EVOS® DAPI Light Cube to capture the nuclei and GFP Light Cube to capture stained lipids. Cell counting was performed on the DAPI channel. Cultures were differentiated in triplicates and at least 25% of each culture well was automatically recorded and the pictures analyzed at batch mode using ImageJ 1.50i (ImageJ, RRID: SCR_003070).

The following macros for ImageJ were used for quantification of lipids stained with Bodipy 493/503 and recorded with the GFP channel; or without staining and recorded using phase contrast. The number of cells was determined with Hoechst staining. The photographs were taken automatically using 10X magnification in an EVOS FL Auto2 fluorescence microscope.

*Bodipy 10x:*

run("8-bit");
run("Auto Local Threshold...", "method=Bernsen radius=15 parameter_1=0 parameter_2=0 white"); run("Measure");
Phase contrast:
run("8-bit");
run("Auto Local Threshold...", "method=Sauvola radius=15 parameter_1=0 parameter_2=0 white");
setOption("BlackBackground", false);
run("Dilate");
run("Analyze Particles...", "show=Ellipses");
run("Fill Holes");
run("Measure");

Hoechst staining:
run("Smooth");
setOption("BlackBackground", false);
run("8-bit");
run("Auto Local Threshold...", "method=Bernsen radius=15 parameter_1=0 parameter_2=0 white");
run("Analyze Particles...", "size=20-700 circularity=0.50-1.00 show=Outlines display clear summarize");

RNA sequencing
Total RNA was isolated with RNeasy Mini kit (Qiagen). Three independent RNA samples were analyzed from each type of differentiated SGBS cells: Wild type cells (WT), cells transduced with lentivirus expressing a gRNA targeting an unrelated intergenic region and cells transduced with lentivirus expressing Cas9 and a sgRNA targeting the sequence downstream of the polymorphism rs1451385 (V3-385). To assess the effect of insulin, we treated all our fully differentiated cell cultures with or without 100 nM insulin for 24h. This resulted in a total of 18 RNA samples of differentiated cells being used in the first experiment. Additionally, to study differential gene expression in undifferentiated preadipocytes, we used duplicate samples of wild type cells, cells transduced with lentivirus expressing GFP (GFP), cells mutated downstream of rs1451385 (V3-385) and cells mutated upstream of rs1451385 (V11-385), i.e. 8 samples in total. The total RNAs were subjected to amplification using The Ion AmpliSeq Transcriptome Human Gene Expression Mini Kit (Thermo Fisher) and run in an IonProton instrument (Thermo Fisher) at the Uppsala Genome Center. The average number of reads per sample was > 1.2x10^7. RNA-Seq reads were quality-controlled and analyzed using Torrent Suite Software (Thermo Fisher) with standard settings. Differential expression of genes between KO and control conditions was assessed using the DESeq2 R package from
Validation using RT-qPCR

RNA was extracted using RNeasy and cDNA synthesized using the SuperScript IV Reverse Transcriptase (Fisher Scientific) kit according to the manufacturer’s instructions. Real-time PCR was performed using the MX 3005 Strata quantitative PCR (Stratagene) according to the supplier’s manual (Stratagene). PCR amplification was detected using the SYBR™ Green master mix (Fisher Scientific). Three biological replicates were used and converted to cDNA. Each cDNA sample was run in triplicate and raw data were normalized to the housekeeping gene TBP4. Primers sequences are described in Supplementary Table 5.

We selected genes for confirmatory RT-qPCR analyses using the following criteria:

1) Differential expression in mature adipocytes: Genes whose expression was significantly altered after differentiation to adipocytes comparing three mutated cultures (M(ds)) against three wildtype (WT) cultures analyzed with RNA-seq (Table 1, column 4[adjP]) were further investigated. In order to increase statistical power and discard false positives due to transduction effects, we used the ranking of DE genes obtained from the comparison of six mutated cultures (M (ds)) with twelve control cultures (six wildtype cultures and six cultures with mutants cells from a CRISPR-Cas9 mutation of a regulatory element on chromosome 1 with very low effect on global gene expression). The significance of this comparison was estimated by Student’s t-test with unpaired values and the Welch correction for unequal variance (Table 1, column 5 [p-val*]).

2) Discarding genes whose differential expression was potentially caused by the transduction procedure, i.e. genes differentially expressed only in the transduced cultures (e.g. EMX2, EMX2OS).

3) Discarding genes with an overall very low gene expression (e.g. MXRA5).

4) Adding a few wildcard genes from the top 50 DE genes from RNA-seq with high biological potential (e.g. ITIH5, MLXIPL, PPAPDC1A, Dyrk1B, INSR).

The criteria for filtering out false positives and selecting candidate genes for downstream experiments based on the RT-qPCR validation were three-fold: (1) A technical validation using RNA from the same cells as those used for the global transcriptome analysis requiring significant DE in the same direction as in RNA-seq for successful validation (Table 1, columns 6-7 [M(ds)/WT]). (2) A biological
validation using RNA from an independent mutation (Table 1, columns 8-9 [M(up)/WT]). (3) A control of potential transduction effects in which the candidate genes should not show differential expression comparing non-transduced WT cells and cells transduced with a lentivirus expressing only GFP (Table 1, columns 10-11 [GFP/WT]). Finally, validated genes were further interrogated for possible involvement in adipogenesis by studying their expression in undifferentiated cells. Genes not expressed in preadipocytes or if there was a >2-fold difference in expression between the differentiated and undifferentiated state were considered adipocyte-specific (Figure 5B), and likely a consequence of the phenotype and not causative genes. RT-qPCR data of three independent experiments, p-values (p-val) comparing expression in mutated cells with WT cells were calculated by Student’s t-test with Benjamini-Hochberg correction (Table 1, columns 7, 9, 11, 13, 15 and 17).

**Supplemental References**

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