Deficiency of *Irx5* protects mice from diet-induced obesity and associated metabolic abnormalities

Joe Eun Son¹, Kyoung-Han Kim^{3,4*}, Chi-chung Hui^{1,2,*}

¹Program in Developmental & Stem Cell Biology, The Hospital for Sick Children,
Toronto, ON M5G 0A4, Canada

²Department of Molecular Genetics, University of Toronto, Toronto, ON M5S 1A8,
Canada

³University of Ottawa Heart Institute, Ottawa, ON, Canada

⁴Department of Cellular and Molecular Medicine, University of Ottawa, Ottawa, ON,
Canada

*Corresponding author. E-mail: hankim@uottawa.ca & cchui@sickkids.ca

Abstract

Obesity, a leading cause of several metabolic abnormalities, is mainly due to an imbalance of energy homeostasis. *IRX3* and *IRX5* have been suggested as determinants of obesity in connection with the intronic variants of *FTO*, the strongest genetic risk factor of polygenic obesity in humans. Although the causal effects of *Irx3* on obesity and its related metabolic consequences have been demonstrated *in vivo*, the metabolic function of *Irx5* remains unclear. In this study, using mice homozygous for an *Irx5*-knockout (*Irx5*^{KO}) allele, we show a direct link between *Irx5* expression and regulation of body mass/composition and energy homeostasis. *Irx5*^{KO} mice are leaner and resistant to diet-induced obesity and associated metabolic abnormalities, primarily through the loss of adiposity with an increase in basal metabolic rate with adipose thermogenesis and lower food intake. Furthermore, our long-term feeding analysis found that *Irx3* mutant mouse lines also have less food intake, indicating that lower caloric intake also contributes to their lean phenotype. Together, these results demonstrate that *Irx5* is critical for energy homeostasis and regulation of body mass/composition and suggest that it likely acts in other tissues beyond adipocytes.

INTRODUCTION

Obesity is a global health threat with increased risk for various chronic conditions, including diabetes and cardiovascular diseases^{1,2}. Although lifestyle changes have driven its prevalence to epidemic proportions, genetic factors play an important role in influencing which individuals within a population are more likely to develop obesity in response to a particular environment^{1,3,4}. In the last few decades, tremendous efforts have been made to identify genetic factors involved in obesity. Among genetic variations in the human genome, genome-wide association studies have revealed that single-nucleotide polymorphisms (SNPs) in the first intron of the Fat mass and obesity-associated gene (*FTO*) to be the strongest risk factors in human obesity⁵⁻⁹.

Recent studies using chromatin conformation capture techniques have demonstrated that two homeobox genes, *IRX3* and *IRX5*, in the vicinity of *FTO* directly mediate the effects of obesity-risk variants of *FTO* in multiple tissues, including adipose tissue and brain on body mass and composition regulation in humans¹⁰⁻¹³. Gene editing studies and expression quantitative trait loci (eQTL) analysis further identified that an obesity-associated variant of *FTO* influences causally the expression levels of *IRX3* and *IRX5* in the human brain, human hypothalamic arcuate-like neurons derived from human induced pluripotent stem cells (hiPSCs), and human adipocyte progenitor cells, supporting the notion that these noncoding variants of *FTO* act as long-range regulatory elements of *IRX3* and *IRX5*^{10,12}. Consistent with this, a deletion of ~20 kb element spanning the orthologous obesity-associated interval of *Fto* in mice also results in down-regulation of both *Irx3* and *Irx5* in preadipocytes and developing hypothalamus¹⁰.

Collectively, these studies suggest that obesity-associated variants of *FTO* regulate the expression of *IRX3* and *IRX5*, contributing to metabolic changes.

Studies using genetic mouse models have demonstrated that *Irx3* and *Irx5* are both involved in metabolic regulation. *Irx3*-knockout mice are lean and display an anti-obesity phenotype with elevated energy expenditure due to adipose browning (i.e., enhanced adipose thermogenesis) as well as a metabolically healthy phenotype compared to wild-type controls^{10,13}, unveiling that *Irx3* plays critical regulatory functions in energy homeostasis. Our recent study with a mouse line harboring *cis*-heterozygous mutant allele of *Irx3* and *Irx5* (*Irx3/5* double heterozygous; *Irx3/5*^{dHet}) further revealed that the cumulative dosage of *Irx3* and *Irx5* is critical for controlling energy homeostasis, particularly feeding regulation and leptin response¹⁴. These data also illustrate the overlapping function of *Irx3* and *Irx5* in energy homeostasis and body weight regulation. A previous study using an *Irx5*-knockout mouse line generated in our lab reported that *Irx5*^{KO} mice exhibit lower body weight with elevated adipose mitochondrial respiration¹⁵. However, the detailed metabolic function of *Irx5*, particularly on body mass/composition, energy homeostasis, as well as metabolic function including glucose homeostasis are not fully understood. Moreover, as *Irx3* and *Irx5* play overlapping roles in feeding regulation, as shown with a reduced food intake phenotype in the *Irx3/5*^{dHet} mice and mice lacking *Irx3* in an *Ins2-Cre*⁺ radial glia-like neural stem cell population¹⁴, whether *Irx5* alone is implicated in food intake regulation remains elusive.

In this study, we examined the metabolic phenotype of *Irx5*^{KO} mice in the presence or absence of HFD challenge, demonstrating that deficiency of *Irx5* protects mice from obesity as well as associated metabolic abnormalities. Furthermore, we

examined the long-term dietary intake of *Irx5*^{KO} mice together with *Irx3*^{KO} mice and *Ins2-Cre;EnR-Irx3* mice (with the hypothalamic expression of a dominant-negative form of *Irx3*) as previous studies reported that these mice exhibit weight loss without changes in food intake^{13,15}. Assessments of energy expenditure and long-term dietary intake revealed that anti-obesity effects in *Irx5*^{KO} mice are primarily attributed to an increase in basal metabolic rate with browning of adipose tissue and a decrease in food intake. We validated that both *Irx3*^{KO} and *Ins2-Cre;Rosa26*^{EnR-Irx3} mice also exhibit a dramatic reduction of total food intake. Our study unveils a critical function of *Irx5* on energy homeostasis, and demonstrates a direct link between *Irx5* expression and regulation of body mass/composition and metabolic pathology.

MATERIALS and METHODS

Animals

All animal experimental protocols approved by the Animal Care Committee of The Toronto Centre for Phenogenomics conformed to the standards of the Canadian Council on Animal Care. *Irx3*^{KO} (*Irx3*^{tauLacZ}) mice, *Irx5*^{KO} (*Irx5*^{eGFP}) mice and *Rosa26*^{EnR-Irx3} were described previously^{20,26}. *Ins2-Cre* (stock # 003573) mice were obtained from Jackson Laboratory. Mice were back-crossed for 10 generations on an outbred CD1 background. The colony was housed in a specific pathogen-free (SPF) facility in ventilated cages with controlled environment settings (21-22 °C, 30%-60% humidity for

standard housing), 12-h light/dark cycles, and free access to water. Mice were randomly assigned for the experimental groups at the time of weaning. Mice were fed either ND (Harlan #2918; 17% fat) or 45% HFD (#D12451, Research Diets).

Metabolic phenotyping experiments

Body weight was measured every week from 3 weeks of age. Body composition was analyzed using an EchoMRI device (Echo Medical Systems). Food intake of individually-housed mice was determined as the difference in weight between Food-In and Food-Out, accounting for food spillage onto the cage floor on subsequent weeks, measured weekly from 3 weeks of age. Energy expenditure was evaluated at 10-12 weeks of age by indirect calorimetry using Oxymax Comprehensive Lab Animal Monitoring System (CLAMS, Columbus Instruments) over periods of 24 h. In brief, oxygen consumption (VO_2), an indicator of energy expenditure, was normalized by the lean mass of individual animals. Mouse plasma insulin and leptin levels were determined by using Rat/ Mouse Insulin ELISA Kit (Millipore, EZRMI-13K for insulin and EZML-82K for leptin) according to the manufacturer's instructions. Plasma triglyceride concentration was determined using the Serum Triglyceride Determination kit (Sigma, TR0100) with triglyceride reagent (Sigma, T2449).

Glucose and insulin tolerance tests.

For glucose and insulin tolerance tests (GTT and ITT), mice were subjected to intraperitoneal injection of glucose (1 mg/g of body weight) or insulin (0.65 mU/g of body weight, Humulin®) after fasting 16 hours for GTT and 6 hours for ITT with water *ad libitum*. Blood glucose was measured from the tail at 0, 15, 30, 60, and 120 mins post-glucose, using a glucometer (Contour NEXT, Bayer HealthCare).

Gene expression analyses by quantitative PCR

Total RNA was extracted from tissue using RNeasy Mini (Qiagen) or Purelink RNA Micro Kit (Thermofisher), and complementary DNA was synthesized using M-MLV reverse transcriptase (Thermofisher) with oligo(dT). Gene expression assay was conducted using SYBR Green methods on Viia7 (Applied Biosystems), and relative cycle threshold (CT) values were normalized by *Actb* (β -actin). The primers used for qPCR were described in our previous study¹⁴.

Histology

Tissues were fixed in 4% paraformaldehyde (PFA) and embedded in paraffin. Sections of 5 μ m were stained with hematoxylin and eosin (H&E). Images were captured using a Nikon eclipse microscope and analyzed using ImageJ (National Institutes of Health (NIH))

Statistical analysis

Statistical analyses were performed using PRISM 6.0 software (GraphPad). Data are expressed as the means + SEM values. Statistical significance of differences among groups was determined by Student's t-test or One-way analysis of variance (ANOVA) with post-hoc analysis. When ANOVA indicated statistical significance, Tukey's honestly significant difference (HSD) test was used to determine the significantly different means. A probability value of $P < 0.05$ was used as the criterion for statistical significance.

RESULTS

***lrx5* knockout mice are leaner and resistant to high-fat diet induced obesity**

To determine the metabolic function of *lrx5*, we employed a mouse model homozygous for a null allele of *lrx5* (*lrx5*-knockout mice, *lrx5*^{KO}), and performed a diet-induced obesity experiment with high-fat diet feeding in adult mice. We observed a 30-35% lower body weight in *lrx5*^{KO} mice compared to control littermates (wild-type) under normal diet condition (Fig. 1a). This difference becomes more pronounced if animals were subjected to a high-fat diet (HFD), with *lrx5*^{KO} mice showing no significant body weight gain by HFD, contrasting to a ~80% increase in control mice (Fig. 1b). In further analyses, we focused mainly on *lrx5*^{KO} mice fed HFD because they showed a marked difference in weight gain than those fed a normal diet (ND).

In parallel with the body weight data, the percentage of fat mass in *lrx5*^{KO} mice was significantly reduced without obvious change of the lean mass ratio (Fig. 2a), suggesting a selective reduction in fat mass. Consistently, *lrx5*^{KO} mice exhibit a marked

reduction in adiposity with smaller adipose depots (Fig. 2b) as well as decreased adipocyte size (Fig. 2c-d) compared to wild-type mice. These results were corroborated by significantly lower levels of plasma triglyceride (Fig. 2e) as well as leptin (Fig. 2f), which positively correlates with adiposity^{16,17}, in *Irx5*^{KO} mice compared to wild-type mice. These data clearly indicate that mice that are deficient for *Irx5* are lean and display a profound anti-obesity phenotype.

Deletion of *Irx5* protects mice from metabolic abnormalities

Obesity is highly associated with metabolic dysfunctions, including glucose intolerance, insulin resistance and hepatosteatosis¹. As *Irx5*^{KO} mice were lean and resistant to HFD-induced obesity, we examined glucose homeostasis by performing glucose tolerance tests (GTT) and insulin tolerance tests (ITT) at different time points in the presence or absence of HFD. In 8-week-old mice, GTT was indistinguishable between wild-type and *Irx5*^{KO} mice (Fig. 3a). Although ageing and HFD led to glucose intolerance and insulin resistance in wild-type mice, *Irx5*^{KO} mice showed none of these metabolic phenotypes; *Irx5*^{KO} mice exhibited significantly improved glucose homeostasis with smaller glucose excursion in GTT (Fig. 3b-c) and increased insulin sensitivity in ITT (Fig. 3d) with lower plasma insulin levels (Fig. 3e) compared to wild-type controls. Furthermore, *Irx5*^{KO} mice exhibited lower liver weight (Fig. 3f) as well as less lipid accumulation in the liver (Fig. 3g), suggesting that deletion of *Irx5* protects mice from HFD-induced hepatic steatosis. Together, these findings demonstrate that loss of *Irx5* protects mice from obesity-associated metabolic abnormalities.

Deletion of *Irx5* results in increased energy expenditure with adipose thermogenesis

It is well established that maintaining energy homeostasis, a balance between energy expenditure and energy intake, is crucial for regulating body weight/composition¹⁸, and obesity is mainly due to excessive energy intake compared to energy expenditure¹. To elucidate a main driver underlying the lean and anti-obesity phenotype of the *Irx5*^{KO} mice, we next measured the energy expenditure of *Irx5*^{KO} mice. Indirect calorimetric analysis revealed that O₂ consumption in *Irx5*^{KO} mice was increased compared to wild-type control regardless of diet, indicating higher energy expenditure in *Irx5*^{KO} mice (Fig. 4a). Consistently, *Irx5*^{KO} mice showed upregulation of brown adipocyte marker genes in PWAT, including *Ucp1*, *Pgc1α* as well as increased expression of *Adrb3* encoding the β3-adrenergic receptor, suggestive of elevated sympathetic activation (Fig. 4b). *Irx5*^{KO} BAT also exhibited significantly increased expression of *Ucp1* as well as *Pgc1α* compared to those from wild-type mice, indicating activation of BAT by loss of *Irx5* (Fig. 4c). Similar to the phenotypes seen in *Irx3*^{KO} mice¹³ as well as *Irx3/5*^{dHet} mice¹⁴, our data suggest that ‘beiging’ of WAT by higher sympathetic tone and activation of BAT may lead to increased energy expenditure in *Irx5*^{KO} mice and partially account for their leanness and protection from diet-induced obesity.

Deletion of *Irx5* suppresses food intake, and *Irx3* mutant mouse lines also exhibit a similar reduction of food intake

As our recent study has demonstrated that the gene dosage of *Irx3* and *Irx5* is critical for controlling food intake¹⁴, we speculated that potential differences in food intake might contribute to the lean and anti-obesity phenotype of *Irx5*^{KO} mice. Thus, we measured their food intake every week from weaning (*i.e.*, 3-week-old). Contrary to the previous report of no significant difference in energy intake of *Irx5*^{KO} mice¹⁵, we found that *Irx5*^{KO} mice consumed less food (~30%) than wild-type mice (Fig. 5a). These results indicate that, in addition to a higher metabolic rate, reduction of food intake contributed to the lean and anti-obesity phenotype of *Irx5*^{KO} mice. Together, our data suggest that *Irx5* is critical for the regulation of both food intake and energy expenditure.

This finding prompted us to assess possible differences in the food intake of *Irx3* mutant mouse models, *Irx3*^{KO} mice and *Ins2-Cre;EnR-Irx3* mice, as it has been demonstrated that they exhibited weight loss without significant changes in food intake¹³. Long-term dietary intake assessments indeed showed that food intake was suppressed in both *Irx3*^{KO} mice and *Ins2-Cre;EnR-Irx3* mice, compared to their littermate control (Fig. 5b, c), suggesting that their lean phenotypes could also be attributed to lower energy intake.

DISCUSSION

In this report, mouse genetic studies illustrate that *Irx5* plays a critical role in energy homeostasis and deficiency of *Irx5* dramatically modulates both energy

expenditure and food intake resulting in an anti-obesity phenotype. Animals lacking *Irx5* exhibit profound protective effects against HFD-induced obesity and associated metabolic abnormalities, including glucose intolerance, insulin resistance and hepatosteatosis (Fig. 6). However, it is important to note that *Irx5*^{KO} mice are somewhat runted at weaning (*i.e.*, 3-week-old), probably due to developmental defects since *Irx5* is essential for the development and function of many organs, including the heart and bone¹⁹⁻²². Nevertheless, it is important to highlight the robust resistance phenotype against diet-induced obesity and associated metabolic abnormalities conferred by deficiency of *Irx5*.

Our results established that loss of *Irx5* results in increased energy expenditure with elevated adipose thermogenesis in WAT and BAT. In a previous study, a gene network centered on amyloid precursor protein (*App*) has been suggested as an *Irx5*-regulated pathway in the control of mitochondrial respiration in adipocytes¹⁵. Intriguingly, in a recent report, Yao *et al.* has demonstrated that myeloid-specific deletion of *Irx3* can protect mice against diet-induced obesity and metabolic diseases via elevated adaptive thermogenesis and suggested that *Irx3* activities in adipose tissue macrophage (ATM) promote metabolic inflammation to accelerate the development of obesity²³. Since *Irx3* and *Irx5* play cooperative functions with overlapping expression patterns in many developmental processes as well as energy homeostasis and body weight regulation^{14,21,24-27}, it will be of interest to examine whether *Irx3* and *Irx5* also share the regulatory mechanism in both adipocyte and macrophage.

In this study, we showed that *Irx5*^{KO} mice as well as *Irx3*^{KO} and *Ins2-Cre;EnR-Irx3* mice exhibit reduced food intake, which is inconsistent with the observations in previous

reports demonstrating no significant difference in energy intake. We argue that comparing cumulative values measured over long-time intervals (this report), rather than relying solely on daily or short-term measures (previous reports), should provide more reliable insight into the feeding behavior of mutant mice²⁸. Notably, our recent study using *lrx3*^{5^{dHet}} mice and conditional deletion of *lrx3* in the radial-glia like neural stem cell lineage in postnatal mouse hypothalamus revealed that *lrx3* and *lrx5* play a regulatory role in hypothalamic postnatal neurogenesis and leptin response, contributing to feeding control¹⁴. It is possible that sole deletion of *lrx3* or *lrx5* also share this mechanism in the regulation of food intake. Interestingly, another line of *lrx3* knockout mice (*lrx3*^{Δ/Δ}) was found to display a reduced preference for sucrose¹⁰, also suggesting critical involvement of *lrx3* as well as *lrx5* on the regulation of energy intake. These results collectively unveil a critical role of *lrx3* and *lrx5* in food intake regulation, independent of their known adipose being functions. This is meaningful as obesity-risk alleles of *FTO* are often associated with increased food intake^{5,8,29}.

In closing, our diet-induced obesity study here establishes a role for *lrx5* in the control of body mass/composition and associated metabolic abnormalities. Our analysis provides evidence that lower food intake also contributes to a lean phenotype of other *lrx3* mutant mouse lines, which exhibit a lean phenotype with increased metabolic rate. Together, these results suggest that through the control of energy expenditure and food intake, *lrx3* as well as *lrx5* play important regulatory roles in energy homeostasis. Further investigation will be needed to decipher the molecular actions of *lrx3* and *lrx5* in diverse cell types that are involved in the regulation of energy homeostasis.

271

272

273

FIGURE LEGENDS

Figure 1. Lean and anti-obesity phenotype of *lrx5* knockout mice. **a**, Body weight changes of wild-type (WT) and *lrx5*-knockout (*lrx5*^{KO}) mice fed a normal diet (ND) or high-fat diet (HFD) mice. **b**, weight gain in ND or HFD. Data are represented as means ± SEM values. **P* < 0.05; ***P* < 0.01 (ND, WT vs. *lrx5*^{KO}), #*P* < 0.05; ###*P* < 0.01 (HFD, WT vs *lrx5*^{KO}), ns: not significant. One-way analysis of variance (ANOVA) with Tukey's multiple-comparisons tests

Figure 2. Lower adiposity in *lrx5*^{KO} mice. **a**, Fat and lean mass ratio as a percentage of body weight at 16 weeks of age. **b**, Tissue weight of perigonadal white adipose tissue (PWAT), inguinal WAT (IWAT), brown adipose tissue (BAT). **c**, H&E-stained sections of perigonadal white adipose tissue (PWAT), inguinal WAT (IWAT) and brown WAT (BAT) from HFD fed mice. **d**, Frequency distribution of adipocyte diameter in PWAT from HFD fed mice. **e**, Plasma triglyceride level at fasting and postprandial condition from HFD fed mice. **f**, Plasma leptin level at fasting conditions from HFD fed mice. Data are represented as means ± SEM values. **P* < 0.05; ***P* < 0.01 (ND, WT vs. *lrx5*^{KO}), #*P* < 0.05; ###*P* < 0.01 (HFD, WT vs *lrx5*^{KO}), ns: not significant. One-way ANOVA with Tukey's multiple-comparisons tests or two-tailed unpaired *t*-test.

Figure 3. Improved metabolic abnormalities in *Irx5*^{KO} mice.

a-c, Glucose tolerance tests (GTT). The inset bar-graph shows area under curve (AUC). **d**, Insulin tolerance test (ITT). The inset bar-graph shows AUC. **e**, Plasma insulin level at fasting conditions from HFD fed mice. **f**, Liver weight. **g**, H&E-stained sections of liver from HFD fed mice. Data are represented as means $\pm$ SEM values. * $P < 0.05$; ** $P < 0.01$ (ND, WT vs. *Irx5*^{KO}), # $P < 0.05$; ## $P < 0.01$ (HFD, WT vs *Irx5*^{KO}), ns: not significant. One-way ANOVA with Tukey's multiple-comparisons tests or two-tailed unpaired *t*-test.

Figure 4. Increased energy expenditure with adipose thermogenesis in *Irx5*^{KO}

mice. a, O₂ consumption normalized by lean mass. **b-c**, RNA expression analysis of adipose beiging markers in PWAT and BAT from HFD fed mice. $P < 0.05$; ** $P < 0.01$ (ND, WT vs. *Irx5*^{KO}), # $P < 0.05$; ## $P < 0.01$ (HFD, WT vs *Irx5*^{KO}). One-way ANOVA with Tukey's multiple-comparisons tests or two-tailed unpaired *t*-test.

Figure 5. Suppressed energy intake in *Irx5*^{KO} mice as well as *Irx3*^{KO} mice and *Ins2-Cre;EnR-Irx3* mice.

Food intake measurement of **(a)** *Irx5*^{KO}, **(b)** *Irx3*^{KO} and **(c)** *Ins2-Cre;EnR-Irx3* mice. The inset bar graph shows accumulated food intake. Data are represented as means $\pm$ SEM values. * $P < 0.05$; ** $P < 0.01$. Two-tailed unpaired *t*-test.

Figure 6. Schematic model depicting that *Irx5* deficiency protects mice from

obesity and metabolic abnormality. *Irx5*^{KO} mice are lean and exhibit a profound anti-

obesity and metabolically healthy phenotype, primarily through increased basal metabolic rate with activated thermogenesis in adipose tissue and lower food intake.

Acknowledgments The research was supported by the Canadian Institutes of Health Research (MOP-136821) and the Canada Research Chairs program to C.C.H. J.E.S. was supported by a post-doctoral fellowship award from Diabetes Canada. J.E.S. was awarded a Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education (2018R1A6A3A03012237). K.H.K. was supported by the Canadian Institutes of Health Research (PJT-173281) and the Heart and Stroke Foundation of Canada, National New Investigator Award.

Author Contributions J.E.S., K.H.K., and C.C.H. conceived and designed the study. J.E.S. and K.H.K. performed the experiments. J.E.S., K.H.K., and C.C.H. wrote the manuscript.

DECLARATION OF INTERESTS

The authors have no conflicts of interest to disclose.

References

- 1 Gonzalez-Muniesa, P. *et al.* Obesity. *Nat Rev Dis Primers* **3**, 17034, doi:10.1038/nrdp.2017.34 (2017).
- 2 Williams, E. P., Mesidor, M., Winters, K., Dubbert, P. M. & Wyatt, S. B. Overweight and Obesity: Prevalence, Consequences, and Causes of a Growing Public Health Problem. *Curr Obes Rep* **4**, 363-370, doi:10.1007/s13679-015-0169-4 (2015).
- 3 van der Klaauw, A. A. & Farooqi, I. S. The hunger genes: pathways to obesity. *Cell* **161**, 119-132, doi:10.1016/j.cell.2015.03.008 (2015).
- 4 Elks, C. E. *et al.* Variability in the heritability of body mass index: a systematic review and meta-regression. *Front Endocrinol (Lausanne)* **3**, 29, doi:10.3389/fendo.2012.00029 (2012).
- 5 Cecil, J. E., Tavendale, R., Watt, P., Hetherington, M. M. & Palmer, C. N. An obesity-associated FTO gene variant and increased energy intake in children. *N Engl J Med* **359**, 2558-2566, doi:10.1056/NEJMoa0803839 (2008).
- 6 Frayling, T. M. *et al.* A common variant in the FTO gene is associated with body mass index and predisposes to childhood and adult obesity. *Science* **316**, 889-894, doi:10.1126/science.1141634 (2007).
- 7 Dina, C. *et al.* Variation in FTO contributes to childhood obesity and severe adult obesity. *Nat Genet* **39**, 724-726, doi:10.1038/ng2048 (2007).
- 8 Speakman, J. R., Rance, K. A. & Johnstone, A. M. Polymorphisms of the FTO gene are associated with variation in energy intake, but not energy expenditure. *Obesity (Silver Spring)* **16**, 1961-1965, doi:10.1038/oby.2008.318 (2008).
- 9 Tanofsky-Kraff, M. *et al.* The FTO gene rs9939609 obesity-risk allele and loss of control over eating. *Am J Clin Nutr* **90**, 1483-1488, doi:10.3945/ajcn.2009.28439 (2009).
- 10 Sobreira, D. R. *et al.* Extensive pleiotropism and allelic heterogeneity mediate metabolic effects of IRX3 and IRX5. *Science* **372**, 1085-1091, doi:10.1126/science.abf1008 (2021).
- 11 Laber, S. *et al.* Linking the FTO obesity rs1421085 variant circuitry to cellular, metabolic, and organismal phenotypes in vivo. *Sci Adv* **7**, doi:10.1126/sciadv.abg0108 (2021).
- 12 Claussnitzer, M. *et al.* FTO Obesity Variant Circuitry and Adipocyte Browning in Humans. *N Engl J Med* **373**, 895-907, doi:10.1056/NEJMoa1502214 (2015).
- 13 Smemo, S. *et al.* Obesity-associated variants within FTO form long-range functional connections with IRX3. *Nature* **507**, 371-375, doi:10.1038/nature13138 (2014).
- 14 Son, J. E. *et al.* Irx3 and Irx5 in Ins2-Cre(+) cells regulate hypothalamic postnatal neurogenesis and leptin response. *Nat Metab* **3**, 701-713, doi:10.1038/s42255-021-00382-y (2021).
- 15 Bjune, J. I. *et al.* IRX5 regulates adipocyte amyloid precursor protein and mitochondrial respiration in obesity. *Int J Obes (Lond)*, doi:10.1038/s41366-018-0275-y (2018).
- 16 Ostlund, R. E., Jr., Yang, J. W., Klein, S. & Gingerich, R. Relation between plasma leptin concentration and body fat, gender, diet, age, and metabolic covariates. *J Clin Endocrinol Metab* **81**, 3909-3913, doi:10.1210/jcem.81.11.8923837 (1996).
- 17 Howard, B. V., Ruotolo, G. & Robbins, D. C. Obesity and dyslipidemia. *Endocrinol Metab Clin North Am* **32**, 855-867, doi:10.1016/s0889-8529(03)00073-2 (2003).

- 18 Spiegelman, B. M. & Flier, J. S. Obesity and the regulation of energy balance. *Cell* **104**, 531-543, doi:10.1016/s0092-8674(01)00240-9 (2001).
- 19 Tan, Z. *et al.* IRX3 and IRX5 Inhibit Adipogenic Differentiation of Hypertrophic Chondrocytes and Promote Osteogenesis. *J Bone Miner Res* **35**, 2444-2457, doi:10.1002/jbmr.4132 (2020).
- 20 Costantini, D. L. *et al.* The homeodomain transcription factor *Irx5* establishes the mouse cardiac ventricular repolarization gradient. *Cell* **123**, 347-358, doi:10.1016/j.cell.2005.08.004 (2005).
- 21 Gaborit, N. *et al.* Cooperative and antagonistic roles for *Irx3* and *Irx5* in cardiac morphogenesis and postnatal physiology. *Development* **139**, 4007-4019, doi:10.1242/dev.081703 (2012).
- 22 Cain, C. J. *et al.* Loss of Iroquois homeobox transcription factors 3 and 5 in osteoblasts disrupts cranial mineralization. *Bone Rep* **5**, 86-95, doi:10.1016/j.bonr.2016.02.005 (2016).
- 23 Yao, J. *et al.* Macrophage IRX3 promotes diet-induced obesity and metabolic inflammation. *Nat Immunol*, doi:10.1038/s41590-021-01023-y (2021).
- 24 Cohen, D. R., Cheng, C. W., Cheng, S. H. & Hui, C. C. Expression of two novel mouse Iroquois homeobox genes during neurogenesis. *Mech Dev* **91**, 317-321, doi:10.1016/s0925-4773(99)00263-4 (2000).
- 25 Kim, K. H., Rosen, A., Bruneau, B. G., Hui, C. C. & Backx, P. H. Iroquois homeodomain transcription factors in heart development and function. *Circ Res* **110**, 1513-1524, doi:10.1161/CIRCRESAHA.112.265041 (2012).
- 26 Li, D. *et al.* Formation of proximal and anterior limb skeleton requires early function of *Irx3* and *Irx5* and is negatively regulated by *Shh* signaling. *Dev Cell* **29**, 233-240, doi:10.1016/j.devcel.2014.03.001 (2014).
- 27 Fu, A. *et al.* Dynamic expression patterns of *Irx3* and *Irx5* during germline nest breakdown and primordial follicle formation promote follicle survival in mouse ovaries. *PLoS Genet* **14**, e1007488, doi:10.1371/journal.pgen.1007488 (2018).
- 28 Ellacott, K. L., Morton, G. J., Woods, S. C., Tso, P. & Schwartz, M. W. Assessment of feeding behavior in laboratory mice. *Cell Metab* **12**, 10-17, doi:10.1016/j.cmet.2010.06.001 (2010).
- 29 Speakman, J. R. The 'Fat Mass and Obesity Related' (FTO) gene: Mechanisms of Impact on Obesity and Energy Balance. *Curr Obes Rep* **4**, 73-91, doi:10.1007/s13679-015-0143-1 (2015).

Figure 1. Lean and anti-obesity phenotype of *lrx5* knockout mice

Figure 2. Lower adiposity in *Irx5*^{KO} mice

Figure 3. Improved metabolic abnormalities in *Irx5*^{KO} mice

Figure 4. Increased energy expenditure with adipose thermogenesis in *Irx5*^{KO} mice

Figure 5. Suppressed energy intake in *lrx5*^{KO} mice as well as *lrx3*^{KO} mice and *Ins2-Cre;EnR-lrx3* mice

Figure 6. Schematic model depicting that *Irx5* deficiency protects mice from obesity and associated metabolic abnormalities