Preserved Energy Balance in Mice Lacking FoxO1 in Neurons of Nkx2.1 Lineage Reveals Functional Heterogeneity of FoxO1 Signaling within the Hypothalamus

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ABSTRACT

Transcription factor FoxO1 regulates energy expenditure, food intake, and hepatic glucose production. These activities have been mapped to specific hypothalamic neuronal populations using cell type-specific knockout experiments in mice. To parse out the integrated output of FoxO1-dependent transcription from different neuronal populations and multiple hypothalamic regions, we used transgenic mice expressing Cre recombinase from the $Nkx2.1$ promoter to ablate loxP-flanked $Foxo1$ alleles from a majority of hypothalamic neurons ($Foxo1^\text{KO}_\text{Nkx2.1}$ mice). This strategy resulted in the expected inhibition of FoxO1 expression, but only produced a transient reduction of body weight as well as decreased body length. The transient decrease of body weight in male mice was accompanied by decreased fat mass. Male $Foxo1^\text{KO}_\text{Nkx2.1}$ mice show similar food intake to wild-type controls, and while female knockouts eat less, they do so in proportion to reduced body size. Energy expenditure is unaffected in $Foxo1^\text{KO}_\text{Nkx2.1}$ mice, though small increases in body temperature are present. Unlike other neuron-specific $Foxo1$ knockouts, $Foxo1^\text{KO}_\text{Nkx2.1}$ mice are not protected from diet-induced obesity. These studies indicate that, unlike the metabolic effects of highly restricted neuronal subsets (POMC, NPY/AgRP, Sf-1), those of neurons derived from the Nkx2.1 lineage occur in either a FoxO1-independent fashion or are compensated for through developmental plasticity.
INTRODUCTION

The physiological relevance of cerebral insulin action remains incompletely understood (1, 2). The insulin receptor is expressed throughout the brain (3), and genetic manipulation of insulin receptors and components of the insulin signaling pathway have resulted in phenotypes of altered body weight (4), fertility (4), and counterregulatory response to hypoglycemia (5, 6). We have previously shown that hypothalamic insulin receptors are implicated in the regulation of hepatic glucose production (HGP) and energy expenditure (EE) (7). Insulin signaling through insulin receptor substrate 2 (Irs2) in the brain (8) leads to activation of phosphatidylinositol triphosphate kinase (Pi3k) (9), where convergence with the leptin signaling pathway occurs. A common effector of both insulin and leptin signaling is forkhead box O1 transcription factor, FoxO1 (10, 11).

Various neuron type-specific manipulations of FoxO1 have resulted in clearly defined roles of FoxO1 in the actions of insulin and leptin. Both FoxO1 and the leptin-responsive transcription factor Stat3 regulate key promoters in orexigenic AgRP and anorexigenic Pomc neurons (12), and delivering constitutively active FoxO1 to the hypothalamus increases food intake and body weight (13, 14). Within AgRP neurons, FoxO1 affects both eating behavior and insulin’s ability to regulate HGP, and indirect evidence implicates the orphan G-protein coupled receptor Gpr17 as a FoxO1 target responsible for its orexigenic effects (15), in addition to neuropeptides AgRP (16) and Npy (13). Within POMC neurons, FoxO1 enhances food intake by decreasing processing of POMC-derived anorexigenic peptides through inhibition of Carboxypeptidase E (Cpe) expression (17). Outside the arcuate nucleus (ARC), FoxO1 decreases energy expenditure and increases transcription of steroidogenic factor 1 (Sf1) in the ventral
medial nucleus of the hypothalamus (VMH) (18). From these data, the overarching function of FoxO1 in the hypothalamus appears to be anabolic.

While these data provide a necessary anatomic-functional map of FoxO1 actions, they don’t address the broader question of the overall role of FoxO1 in vivo. To fill this gap in knowledge, we sought to determine if combined inactivation of FoxO1 in multiple neuronal types, as would be expected to occur in response to feeding, would enhance the anorexigenic effects of FoxO1 removal from individual cell populations. To accomplish this goal, we used mice expressing Cre recombinase throughout a majority of hypothalamic neurons by way of the Nkx2.1 promoter. This transgenic line allows expression of Cre recombinase within multiple cellular subtypes within the hypothalamus (19). Nkx2.1-cre is expressed as early as E10.5 (20, 21), and is expressed in arcuate Npy and POMC neurons (22). We report here that mice with a genetic knockout of FoxO1 in the hypothalamus display mild decreases in body weight early in life that normalize as compensatory mechanisms exert their effects with age.
RESEARCH DESIGN AND METHODS

**Maintenance and care of mouse colony.** Mice were housed in a specific pathogen free (SPF) animal facility, fed Picolab diet 5053 (Purina, Richmond, IN), or D124921 60% High Fat diet (Research Diets, Inc., New Brunswick, NJ) and kept under 12-hr light and dark cycle. Nkx2.1-cre mice were obtained from Jackson Laboratories (Bar Harbor, ME) and backcrossed to C57BL/6J background for 8 generations. Mice with loxP-flanked FoxO1 alleles have been previously described (23). All procedures were approved by the institutional animal care and utilization committee (IACUC) at Columbia University.

**Real Time PCR.** Total RNA was isolated using the PerfectPure RNA tissue kit (5Prime, Gaithersburg, MD) and complementary DNA was made using qScript (Quanta Biosciences, Gaithersburg, MD), both according to manufacturer instructions. Real time PCR (qPCR) was performed using GoTaq qPCR master mix (Promega, Madison, WI) on a C1000 Thermal Cycler with CFX96 Real Time PCR Detection System (BioRad, Hercules, CA). Pooled samples were used to generate serial 1:4 dilutions for standard curves. Primer sequences are available upon request.

**Tissue RTPCR.** Total RNA was isolated using the PerfectPure RNA tissue kit (5Prime, Gaithersburg, MD) and complimentary DNA was made using qScript (Quanta Biosciences, Gaithersburg, MD), both according to manufacturer instructions as described above. Complementary DNA was added to the FoxO1 primers previously described (23) and mixed with KAPA 2x2G Fast Readymix and dye (Kapa Biosystems, Woburn, MA). PCR was performed with the following program: 95°C for 4 minutes (1 cycle), 94°C for 30 seconds, 58°C for 30 seconds, 72°C for 45 seconds (38 cycles), 72°C for 10 minutes (1 cycle), 4°C finish.
Electrophoresis was performed on a 2% agarose gel and ethidium bromide staining was visualized by UV light.

**Metabolic Cages and Body Composition.** Mice were acclimated for 2 days in cages and indirect calorimetry, activity, and food intake was measured during the light and dark cycles using the TSE Labmaster Platform (TSE Systems, Chesterfield, MO) as previously described (15). For fasting-refeeding experiments, food was removed at 17:00 and replaced the next day at 08:00.

**Serum Analytes.** Blood was removed from the tail of mice either overnight fasted, ad libitum fed, or overnight fasted followed by 4-hr refeeding. Blood was collected via heparinized capillary tubes and centrifuged to isolate serum. Serum was analyzed using mouse insulin ELISA (Mercodia, Uppsala, Sweden), leptin ELISA (Millipore, Billerica, MA), active Ghrelin ELISA (Millipore, St. Charles, MO) and kits for cholesterol E (Wako, Richmond, VA), Infinity triglycerides (Thermo Scientific, Middletown, VA), and NEFA (Wako, Richmond, VA) according to manufacturer’s instructions.

**Neuropeptides.** AgRP was measured by RIA using an antibody (kindly provided by Dr. Gregory Barsh, Stanford University School of Medicine, Stanford, CA) directed at the C-terminal end of the molecule. The RIA was performed as previously described using synthetic human AgRP$_{83-132}$ for the standard and iodinated tracer (Phoenix Pharmaceuticals Inc., Burlingame, CA) (24). NPY was measured with RIA kit (Phoenix Pharmaceuticals) and α-MSH and β-endorphin protein were measured as previously described (17, 24).

**Immunohistochemistry and microscopy.** Liver tissue was fixed in formalin overnight, transferred to 70% ethanol, and paraffin embedded. Sections were cut to 5 μ and hematoxylin and eosin (H&E) staining was performed. Brightfield images were taken using a Nikon eclipse
E400 microscope. For brain tissue, mice were perfused with PBS followed by 4% paraformaldehyde (PFA). Brains were removed and fixed in 4% PFA overnight, followed by placement into 30% sucrose in PBS solution for 2 days. Free-floating sections were cut to 20 μm using a cryostat and mounted onto glass slides using Prolong Gold anti-fade reagent (Life Technologies, Grand Island, NY). Fluorescent images were either taken using a Nikon Eclipse 80i microscope with images merged using Adobe Photoshop CS4, or Zeiss LSM710 Confocal microscope with images merged using Zen 2011 software (Carl Zeiss MicroImaging Inc., Thornwood, NY). Antibodies used were for FoxO1 (C29H4, Cell Signaling, Danvers, MA), Nkx2.1/Ttf1 (rabbit ab76013, mouse ab140245, Abcam, Cambridge, MA), and Npy (ab30914, Abcam).

**Body Temperature and Cold Exposure.** Following overnight fast at room temperature, body temperature was measured rectally using Precision Thermometer 4600 (YSI, Yellow Springs, OH). Mice were then placed at 4°C for 4 hours and temperature was remeasured while mice remained under acute cold exposure.

**Liver TG and Cholesterol.** Liver tissue was homogenized and TG and cholesterol were isolated via extraction as previously reported (25). TG was measured with Infinity Triglycerides reagent (Thermo Scientific, Middletown, VA), and cholesterol with cholesterol E kit (Wako, Richmond, VA).

**Statistics.** Experiments were quantitated by two-tailed Student T-test or one-way ANOVA using Prism software (Graphpad Software, La Jolla, CA). Results are presented as means ± SEM and statistical significance is defined as $P<0.05$. 
RESULTS

Generation of mice lacking FoxO1 in Nkx2.1 neurons. To probe the function of FoxO1 in the hypothalamus, we generated a conditional null allele by crossing Foxo1lox/lox with Nkx2.1-cre mice. To assess recombination, we introduced a Rosa26-Tomato reporter allele, and surveyed fluorescence patterns in the CNS. We observed diffuse fluorescence in the hypothalamus (Supplemental Figure 1A) (19), as well as in the hippocampus and cortex (Supplemental Figure 1B), as reported (http://cre.jax.org/Nkx2/Nkx2.html) (19). Nkx2.1-cre was expressed in tanycytes lining the third ventricle, but not in glial cells (Supplemental Figure 1C). Deletion of FoxO1 did not result in compensatory increases in mRNA encoding other isoforms, Foxo3a and 4 (Supplemental Figure 2A). Double immunofluorescence revealed colocalization of Foxo1 and Nkx2.1 protein in many neurons, as well as neurons independently labeled with either Foxo1 or Nkx2.1 (Supplemental Figure 2B). Expression of the Nkx2.1-cre recombinase decreased the levels of Foxo1 in Nkx2.1-labeled cells. Reverse transcription of RNA isolated from peripheral tissues followed by PCR amplification of the resulting complimentary cDNA revealed Nkx2.1-cre expression in hypothalamus and lung, but not in other organs (Supplemental Figure 2D).

Characterization of Foxo1KO<sub>Nkx2.1</sub> mice. Starting as early as at 6 weeks of life, Foxo1KO<sub>Nkx2.1</sub> mice of both sexes weighed less than wild-type (WT) counterparts (Figure 1A and 1B). In males, body weights of Foxo1KO<sub>Nkx2.1</sub> mice caught up with WT mice by 5 months of age. The convergence of body weights in male Foxo1KO<sub>Nkx2.1</sub> mice at 5 months of age could be due to compensatory mechanisms, such as decreasing numbers of POMC neurons, as we have reported in a different model of FoxO1 ablation (26). In males, the transient decrease of body weight is due to a proportional as well as absolute decrease of fat mass, while the proportion of lean mass
is higher as measured at 3 months of age (Figure 1C-D, 1F-G). Fluid mass was not affected in Foxo1KO\textsuperscript{Nkx2.1} mice (Figure 1E, 1H). Female Foxo1KO\textsuperscript{Nkx2.1} mice also showed a decrease of fat mass though not significant, and a larger decrease of lean mass, all of which were proportional to the decreased body weight (Supplemental Figure 3).

We did not observe changes in metabolic parameters. Two-month old Foxo1KO\textsuperscript{Nkx2.1} mice have normal fasting and \textit{ad libitum} fed glucose levels (Table 1). There are no differences in serum insulin, triglyceride (TG), or free fatty acid (FFA) levels between WT and Foxo1KO\textsuperscript{Nkx2.1} mice. Fasted serum levels of these metabolites are also similar in 7-month-old mice (Supplemental Figure 4).

**Energy expenditure studies.** We postulated that the decreased body weight was at least partly due to increased energy expenditure (EE) in Foxo1KO\textsuperscript{Nkx2.1} mice, as altered FoxO1 expression in Sf-1 neurons in the ventromedial hypothalamus leads to increases in energy expenditure (18). We analyzed Foxo1KO\textsuperscript{Nkx2.1} mice using metabolic cages for indirect calorimetry and food intake measurements during the period (at 4 months of age) in which male Foxo1KO\textsuperscript{Nkx2.1} mice remain lighter (body weight in this cohort 23.52±1.47 vs. 25.29±0.96 grams) and lean (body fat % 7.8±0.5 vs. 9.1±0.2). However, measurements of respiratory exchanges demonstrated similar levels of VCO\textsubscript{2} and VO\textsubscript{2} between WT and Foxo1KO\textsuperscript{Nkx2.1} mice in either the dark or the light phases of the light cycle (Figure 2A-B). 12-hr respiratory exchange rates (RER) and locomotor activity levels were also similar (Figure 2C-D). To study metabolic flexibility in substrate utilization, we fasted mice overnight and then refed them. Neither VCO\textsubscript{2} nor VCO\textsubscript{2} were different between WT and Foxo1KO\textsuperscript{Nkx2.1} mice in this experiment (Figure 2E-F). Both groups of animals dropped their respiratory quotient similarly following fasting, and rebound equally upon refeeding (Figure 2G).
Female Foxo1KO\textsuperscript{Nkx2.1} mice exhibited the same substrate utilization as males, and showed no differences from WT controls (Supplemental Figure 5).

**Food intake studies.** With no discernible differences in EE, we ascertained the role of food intake in the leanness of Foxo1KO\textsuperscript{Nkx2.1} mice. Male mice exhibited normal absolute and body weight-normalized food intake (Figure 3A-B). Female Foxo1KO\textsuperscript{Nkx2.1} mice, on the other hand, exhibited decreased overall food intake, but normal intake proportional to their decreased size (Figure 3C-D). In both males and females, rebound food intake after an overnight fast mirrored *ad libitum* food intake, with no difference in males (Figure 3E-F), but weight-proportional decreases in females (Figure 3G-H).

FoxO1 regulates the transcription of anorexigenic and orexigenic genes, as well as enzymes that process the neuropeptide products of those genes (12, 13, 17). While anorexigenic genes *Agrp* and *Npy* significantly decreased in male Foxo1KO\textsuperscript{Nkx2.1} mice, there were no differences in *Pomc* levels (Figure 4A). Since the decreases in Agrp and Npy were ~50%, we postulated that Nkx2.1-cre may not target all Agrp/Npy neurons, consistent with Nkx2.1-cre-driven GFP-reporter colocalization studies (22). However, previous use of the Nkx2.1-cre revealed near complete targeting of leptin-sensitive neurons, as visualized by reductions in phosphor-Stat3 upon deletion of the leptin receptor (19). To quantify the proportion of Agrp/Npy neurons targeted by the Nkx2.1-cre, we introduced the Npy-GFP reporter into the WT and Foxo1KO\textsuperscript{Nkx2.1} mice. We found virtually no Npy-GFP in the hypothalamus of Foxo1KO\textsuperscript{Nkx2.1} mice (Figure 4B), though Npy-GFP was observed in other parts of the brain such as the cortex (Supplemental Figure 6A). Of note, the Nkx2.1-cre (as visualized by inclusion of the Tomato reporter) did not co-localize with Npy-GFP outside of the hypothalamus. The Tomato reporter
under Nkx2.1-cre served as evidence of cre-driven recombination within the hypothalamus, and Npy-GFP in non-hypothalamic areas provided evidence of proper genetic cross and sufficient GFP fluorescence for detection.

With the dramatic decrease in Npy-GFP expression in Foxo1KO\textsuperscript{Nkx2.1} mice, we hypothesized that, while Agrp/Npy transcripts appeared decreased but still present in the hypothalamus, the protein was reduced or unstable. We measured levels of Agrp and Npy neuropeptides and found significant decreases that correlated with transcript levels (Figure 4C). Levels of Pomc-produced neuropeptides α-Msh and beta-endorphin (β-Ep) were not altered in the Foxo1KO\textsuperscript{Nkx2.1} mice, consistent with normal Pomc transcript levels. The amount of Npy protein present in the arcuate nucleus and PVN revealed persisting levels in Foxo1KO\textsuperscript{Nkx2.1} mice that did not match the difference in the Npy-GFP reporter (Supplemental Figure 6B). We considered that Npy-GFP reporter activity may be compromised by the loss of a cofactor that binds to the Npy promoter fragment affected by insertion of the transgene, thus we looked at alternate signaling pathways that may interact with Foxo1 on the NPY promoter. The ghrelin receptor is located in hypothalamic Agrp/Npy neurons, and elicits a similar orexigenic effect upon stimulation by ghrelin (27). But we did not observe differences in serum levels of active ghrelin after either fasting or refeeding (Figure 4D), nor did we find differences in ghrelin receptor transcript or protein levels (not shown).

**Foxo1KO\textsuperscript{Nkx2.1} mice are not protected from diet-induced obesity.** Given the transient decrease of body weight on chow diet in Foxo1KO\textsuperscript{Nkx2.1} mice, we used a diet containing 60% fat (high-fat diet, HFD) to determine if hypothalamic loss of FoxO1 protects from diet-induced obesity. Male Foxo1KO\textsuperscript{Nkx2.1} mice weighed less than WT at the start of the experiment, but the difference in
body weight normalized on HFD (Figure 5A). HFD also normalized body composition
differences between WT and Foxo1KO^{Nkx2.1} mice (Supplemental Table 1), abolishing differences
in fat mass and lean mass content as seen in younger mice. Foxo1KO^{Nkx2.1} mice were slightly
shorter than WT mice at 5 months of age, and the difference reached statistical significance by
10 months (Figure 5H-I). HFD increased body length of mice of both genotypes to the same
extent.

Foxo1KO^{Nkx2.1} mice exhibited a slight but not significant decrease in fasting glucose on
regular chow and HFD (Figure 5B). We saw no differences in fasting serum insulin levels
(Figure 5C). While HFD produced a large increase in fasting leptin levels compared to regular
diet, it resulted in no differences between Foxo1KO^{Nkx2.1} and WT mice (Figure 5D). Serum
cholesterol levels followed a similar pattern, being raised by HFD, but not differently so in the
two genotypes (Figure 5E). In contrast, we saw a slight elevation of serum triglycerides in HFD-
fed Foxo1KO^{Nkx2.1} mice compared to WT (Figure 5F). Serum free fatty acids were slightly
decreased on HFD, but the differences were not statistically significant (Figure 5G).

Neurons located in the DMH project to brown adipose tissue and are implicated in the
acute thermogenic response to cold exposure (28). Impaired leptin signaling in Nkx2.1-
expressing neurons can prevent this response in younger mice (29). We measured body
temperature of WT and Foxo1KO^{Nkx2.1} mice at room temperature and found a trend toward
increased body temperature in Foxo1KO^{Nkx2.1} mice (Figure 6A). These increases may represent
thermogenesis that would not be detected by indirect calorimetry and can account for the
decreased fat content of Foxo1KO^{Nkx2.1} mice. However, when we placed mice at 4°C for 4 hr to
test acute thermogenesis, we failed to see a difference between genotypes (Figure 6A), indicating
that the acute sympathetic response to leptin signaling is intact in Foxo1KO^{Nkx2.1} mice. Brown adipose tissue morphology does not appear to be distinct in Foxo1KO^{Nkx2.1} mice (Figure 6B).

Finally, in the light of elevated plasma TG levels on HFD, we examined hepatic lipid content in WT and Foxo1KO^{Nkx2.1} mice. However, total lipid content was similarly increased by HFD (Figure 6C), and there were no differences in either hepatic triglycerides (Figure 6D) or total cholesterol in 10-month-old mice on regular chow (Figure 6E). Glucose tolerance was normal in Foxo1KO^{Nkx2.1} mice on regular chow (Supplemental Figure 7).
DISCUSSION

We generated Foxo1KO\textsuperscript{Nkx2.1} mice with ablation of FoxO1 in hypothalamic Nkx2.1 neurons. These mice are leaner and smaller than WT mice at a young age, but the lean phenotype normalizes with age. There are modest differences in food intake in female knockouts, while energy expenditure is similar between the two groups. Given that FoxO1 is a downstream effector of both insulin and leptin signaling, we expected that Foxo1KO\textsuperscript{Nkx2.1} mice would be a model of constitutively active, or at least sensitized, insulin and leptin signaling in hypothalamic neurons. In rats, decreasing hypothalamic insulin receptors results in increased food intake, obesity, and anxiety-like behavior (30, 31). A localized knockdown of insulin receptors or insulin signaling in the VMH does not affect body weight, but increases glucagon secretion and results in insulin resistance (32, 33). While hypothalamic insulin receptor signaling overall inhibits hepatic glucose production (34), genetic manipulations of selected hypothalamic neurons reveal opposing actions of insulin in AgRP neurons, where insulin signaling decreases HGP (35), vs. POMC neurons, where insulin appears to increase HGP (7). In addition to HGP, insulin action in POMC neurons increases POMC neuron number in a FoxO1-dependent manner (26).

The cell type-specific nature of insulin signaling among arcuate nucleus neurons may explain the phenotype of Foxo1KO\textsuperscript{Nkx2.1} mice, where glucose levels and hepatic fat content appear to be normal.

Using targeted inactivation of FoxO1 in AgRP and POMC neurons, we have found FoxO1 to be important in the regulation of body weight and food intake (15, 17). While the Nkx2.1-Cre mouse used in these studies does target these types of neurons, the overlap is not complete, and is expected to leave FoxO1 intact in ~15% of adult POMC neurons and up to 45% of NPY neurons (22), an expectation that is consistent with our finding of ~24% residual Foxo1
mRNA within the hypothalamus (Supplemental Figure 2A). Those neurons unaffiliated with the Nkx2.1 lineage may compensate for the decreased FoxO1 in other neurons and result in a mild phenotype. In addition, these data raise the possibility that the phenotype of FoxO1 knockouts driven by AgRP- and Pomc-cre is not in fact due to the arcuate nucleus, but to other sub-populations of such neurons in the PVN or brainstem (36). Alternatively, the activation of Nkx2.1-cre may selectively reduce a pool of FoxO1 that is regulated by acetylation, not phosphorylation. Mice expressing constitutively acetylated FoxO1 (KR) exhibit an increased body weight and fat mass (37), therefore the opposite body composition profile of the Foxo1KO_{Nkx2.1} mice may be due in part to deletion of the pool of FoxO1 that undergoes acetylation within neurons of Nkx2.1 lineage.

The compensation in the overall bioenergetics profile of Foxo1KO_{Nkx2.1} mice does not appear to affect body length. Interestingly, Kim et al. found downregulation of a cluster of Igf1-related genes when FoxO1 is deleted from the VMH (18), raising the possibility that Foxo1KO_{Nkx2.1} mice have decreased IGF-1 signaling, resulting in decreased length. We also hypothesized that another signaling pathway located within Agrp/Npy neurons, that of ghrelin through the growth hormone secretagogue receptor (Ghsr1a), might be altered in the Foxo1KO_{Nkx2.1} mice and affect body length (27). However, we did not find differences in ghrelin receptor transcript or protein level in Foxo1KO_{Nkx2.1} mice (not shown), and no compensatory change in activated ghrelin levels in the serum were present.

The differences in male vs. female fat composition may be due to differences in innervation of adipose tissue depots. Sexual dimorphism exists in the innervations of abdominal and subcutaneous fat depots from the brain, including leptin– and insulin receptor–expressing neurons (38). These connections may also contribute to the sexual dimorphism seen in body
weight and food intake in mice lacking neuronal insulin receptors (4) or mice lacking Foxo1 in POMC neurons (17). Such dimorphism may reveal a need for gender-specific or individualized treatment when targeting the brain for weight reduction.

Decreased AgRP and Npy are not sufficient alone to manifest body weight dysregulation when altered at an early age, but later deletion of Agrp/Npy in adults has powerful effects on food intake (39). This phenomenon suggests developmental compensation or redundant mechanisms existing within the neonatal brain that ensure a behavioral desire to eat and thrive. Even removal of both AgRP and NPY can be performed with little body weight or food intake phenotype results, though loss of both genes results in disruption of ghrelin signaling (40, 41). As we could not identify changes in ghrelin receptor expression within the hypothalamus or circulating active ghrelin, the decrease in Agrp and Npy observed in the Foxo1KO\textsuperscript{Nkx2.1} mice is not sufficient to elicit this disruption. In future studies, it will be of interest to explore this possibility by inducing a FoxO1 knockout in adult animals by way of inducible cre-mediated recombination.
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There are no conflicts of interest related to the work described in this paper.

Authors’ contributions

GH designed, executed, analyzed experiments, and wrote the manuscript. KM executed experiments. SLW designed and reviewed experiments. DA designed and reviewed experiments, wrote the manuscript.

Guarantor Statement

Dr. Domenico Accili is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.
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### Table 1. Metabolic parameters

|                          | N  | WT       | Foxo1KO<sup>Nkx2.1</sup> |
|--------------------------|----|----------|----------------------------|
| Fasting glucose (mg/dL)  | 16 | 90 ± 5   | 94 ± 4                     |
| Fed glucose (mg/dL)      | 16 | 157 ± 6  | 163 ± 6                    |
| Fasting insulin (ng/mL)  | 5  | 0.48 ± 0.15 | 0.40 ± 0.13               |
| TG (mg/dL)               | 6  | 36 ± 4   | 42 ± 4                     |
| FFA (mEq/L)              | 5  | 0.78 ± 0.06 | 0.89 ± 0.06              |

Data show means ± SEM in fasted or refed animals (N=X for each genotype).
FIGURE LEGENDS

Figure 1. Growth curves

(A-B) Body weights of 4- to 20-week-old male (A) and female (B) mice (n >10 for each genotype). (C) Total fat mass, (D) lean mass, and (E) fluid mass in 3-month-old male mice as measured by MRI (n ≥18). (F) Fat mass, (G) lean mass, and (H) fluid mass shown as percentage of total body weight. All results represent means ± SEM. *= P<0.05.

Figure 2. Energy balance in 4-month-old male mice

(A-D) Ad libitum-fed animals. (A) VCO$_2$ and (B) VO$_2$ during a representative 12-hr dark/light cycle. (C) Respiratory exchange ratio (RER) and (D) locomotor activity during a representative 12-hr dark/light cycle. (E-G) Fasting/refeeding experiments. (E) VCO$_2$, (F) VO$_2$, (G) RER before and after fasting and following refeeding in 26 minute increments. Data show means ± SEM.

Figure 3. Assessment of food intake

(A) Total food intake of 4-month-old male mice (n≥7) during the 12-hr dark/light cycle and over 24 hr. (B) Food intake normalized by body weight during 12-hr dark/light cycle and over 24 hr. (C) Total food intake of 4-month-old female mice during the 12-hr dark/light cycle and over 24 hr. (D) Food intake normalized by female body weight during 12-hr dark/light cycle and over 24 hr. (E) Total food intake in males over 24 hr following overnight fast. (F) Food intake in males after overnight fast normalized by body weight. (G) Total food intake in females over 24 hr following overnight fast. (H) Food intake in females after overnight fast normalized by body weight. Data show means ± SEM. *= P<0.05.
Figure 4. Hypothalamic neuropeptides

(A) qPCR measurement of hypothalamic neuropeptide mRNA in overnight-fasted, 5-month-old male WT and Foxo1KO<sup>Nkx2.1</sup> mice (n≥7). (B) NPY-GFP expression in arcuate nucleus of overnight fasted mice. Representative image shown. (C) Protein levels of alpha-MSH, beta-endorphin protein, AGRP, and NPY in hypothalamus of overnight fasted, 5-month-old male WT and Foxo1KO<sup>Nkx2.1</sup> mice (n≥7). (D) Serum levels of active ghrelin following overnight fast or overnight fast followed by 4 hours refeeding. (n≥5). Results are presented as means ± SEM. *= P<0.05.

Figure 5. Metabolic effects of high-fat diet

(A) Body weight, (B) Whole blood glucose, (C) insulin, (C) leptin, (E) cholesterol, (F) triglycerides, and (G) free fatty acids measured in serum of overnight-fasted male mice following 10 week HFD (n=5-8). (H) Ano-nasal body length of males following HFD. (I) Ano-nasal body length of 10-month-old male mice on regular chow diet (n=5-8). Results are presented as means ± SEM. *= P<0.05.

Figure 6. Brown Adipose Tissue and Liver analysis

(A) Rectal body temperature measured at room temperature and following 4-hr of cold exposure at 4°C (n=8). Results are shown means ± SEM. (B) H&E staining of brown adipose tissue (BAT) at room temperature. We show a representative image. (C) H&E staining of liver from WT and Foxo1KO<sup>Nkx2.1</sup> mice following regular chow or HFD. We show a representative image. (D)
Triglycerides and (E) total cholesterol of 7-month-old male mice following overnight fast.

Results are shown as means ± SEM.
Figure 4

A

Fold Induction / Hprt (AU)

Pomc  Agp  Npy

WT  KO

B

WT  KO

C

Peptide (fmol/mg protein)

[Graph showing peptide levels for MSH, BNP, AGRP, and NPY for WT and KO groups, with significant differences indicated by asterisks.]

D

Active Ghrelin (pg/mL)

[Graph showing active ghrelin levels for fasted and refed conditions for WT and KO groups, with significant differences indicated by asterisks.]
### Supplemental Table 1. Body composition on HFD

|                  | WT-RD    | WT-HF    | KO-RD    | KO-HF    |
|------------------|----------|----------|----------|----------|
| Fat Mass (g)     | 2.1 ± 0.1| 7.5 ± 1.9| 2.2 ± 0.3| 6.3 ± 1.2|
| Fat Mass % (g/BWT)| 7.4 ± 0.4| 21.3 ± 4.7| 7.9 ± 0.9| 19.1 ± 3.2|
| Lean Mass (g)    | 21.9 ± 0.6| 21.7 ± 0.5| 20.4 ± 0.4| 21.0 ± 0.5|
| Lean Mass % (g/BWT)| 76.9 ± 0.4| 65.9 ± 3.0| 75.9 ± 0.6| 66.7 ± 2.0|
| Fluid Mass (g)   | 2.2 ± 0.1| 2.4 ± 0.1| 2.1 ± 0.1| 2.4 ± 0.1|
| Fluid Mass % (g/BWT)| 7.7 ± 0.3| 7.5 ± 0.6| 7.8 ± 0.2| 7.8 ± 0.4|

Data show means ± SEM ad libitum fed animals.
SUPPLEMENTAL FIGURE LEGENDS

Supplemental Figure 1. Nkx2.1-Cre expression

(A) ROSA26/Tomato reporter expression in hypothalamus activated by Nkx2.1-cre. (B) ROSA26/Tomato reporter expression in hippocampus and cortex activated by Nkx2.1-cre. (C) ROSA26/Tomato reporter expression (red) in arcuate activated by Nkx2.1-cre with immunofluorescence for astrocyte marker GFAP (in green). No colocalization is present.

Supplemental Figure 2. Foxo1 deletion by Nkx2.1-cre

(A) Real time PCR from hypothalamic tissue after overnight fast. (n=5WT, 12KO). Results shown as fold induction compared to WT and normalized to Hprt. All results represent means ± SEM. *= P<0.05. (B) Co-immunofluorescence for Foxo1 (green) and Nkx2.1 (red) in arcuate nucleus of wildtype (left) and Foxo1KO_Nkx2.1 mice after overnight fast. (C) Ethidium bromide staining showing null band and non-recombined loxP bands in WT and KO mouse tissues. Total RNA was extracted from tissue followed by reverse transcription and 40 cycles of PCR followed by electrophoresis on agarose gel. Null bands only appear in hypothalamus and lung tissue, as expected previous references using Nkx2.1-cre mouse.

Supplemental Figure 3. Female body composition

(A) Total fat mass, (B) lean mass, and (C) fluid mass in 3-month-old female mice as measured by MRI. (D) Fat mass, (E) lean mass, and (F) fluid mass shown as percentage of total body weight. All results represent means ± SEM. *= P<0.05.
**Supplemental Figure 4. Male metabolic parameters at 7 months of age**

(A) Insulin, (B) leptin, (C) cholesterol, (D) triglycerides, and (E) free fatty acids measured in serum of male mice after overnight fasting or overnight fasting followed by 4 hours refeeding. All results represent means ± SEM. *= P<0.05.

**Supplemental Figure 5. Energy balance in 4-month-old female mice**

(A-C) Ad libitum-fed animals. (A) VCO$_2$ and (B) VO$_2$, and (C) Respiratory exchange ratio (RER) during a representative 12-hr dark/light cycle. (D-F) Fasting/refeeding experiments. (D) VCO$_2$, (E) VO$_2$, (F) RER before and after fasting and following refeeding in 26 minute increments. Data show means ± SEM.

**Supplemental Figure 6. Npy reporter and protein in cortex and hypothalamus**

(A) NPY-GFP (green) and Nkx2.1-cre driven ROSA26/Tomato reporter (red) in cortex of Foxo1KO$^{Nkx2.1}$ mouse. No co-localization of NPY-GFP and Tomato is present. (B) Immunofluorescence for NPY protein in arcuate nucleus (left) and PVN (right) in WT and Foxo1KO$^{Nkx2.1}$ mice. Representative image is shown.

**Supplemental Figure 7. Glucose tolerance test**

Glucose tolerance test in WT and Foxo1KO$^{Nkx2.1}$ mice. Mice were fasted overnight then injected IP with glucose bolus. Data show means ± SEM.
Supplemental Figure 5

A

B

C

D

E

F

254x338mm (72 x 72 DPI)
Supplemental Figure 6

A

NPY-GFP
Nkx2.1

B

WT

WT

KO

KO

254x338mm (72 x 72 DPI)
Supplemental Figure 7

![Graph showing glucose levels over time for WT and KO groups](image-url)