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Forkhead protein FoxO1 mediates Agrp-dependent effects of leptin on food intake

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Leptin controls food intake by regulating the transcription of key neuropeptides in the hypothalamus. The mechanism by which leptin regulates gene expression is unclear, however. Here we show that delivery of adenovirus encoding a constitutively nuclear mutant FoxO1, a transcription factor known to control liver metabolism and pancreatic beta-cell function, to the hypothalamic arcuate nucleus of rodents results in a loss of the ability of leptin to curtail food intake and suppress expression of *Agrp*. Conversely, a transactivation-deficient FoxO1 mutant prevents induction of *Agrp* by fasting. We also find that FoxO1 and the transcription factor Stat3 exert opposing actions on the expression of *Agrp* and *Pomc* through transcriptional squelching. FoxO1 promotes opposite patterns of coactivator-corepressor exchange at the *Pomc* and *Agrp* promoters, resulting in activation of *Agrp* and inhibition of *Pomc*. Thus, FoxO1 represents a shared component of pathways integrating food intake and peripheral metabolism.

Leptin controls energy homeostasis partly through direct actions in the hypothalamic arcuate nucleus[1,2]. The anorectic actions of leptin require activation by phosphatidylinositol 3 kinase (PI3K)[3,4], as well as binding of signal transducer and activator of transcription 3 (Stat3) to Ser1138 of the intracellular domain of the leptin receptor (encoded by *Lepr*)[5]. Activation of Stat3 can be shown in response to leptin[6], and conditional ablation of Stat3 in the brain curtails the effects of leptin[7]. Nonetheless, it is unclear whether Stat3 directly activates transcription of genes encoding neuropeptides in response to leptin. It is also unclear how the PI3K- and Stat3-dependent actions of leptin are integrated in various neuronal subpopulations that show differential sensitivity to leptin[1].

The forkhead box-containing protein of the O subfamily (FoxO)-1 regulates metabolism and cellular differentiation in a PI3K-dependent manner[8]. Here, we sought to determine whether FoxO1 controls metabolism through the regulation of neuropeptide-dependent food intake. We show that FoxO1 is physiologically expressed in key leptin target neurons in the arcuate nucleus, and that it translocates from the cytoplasm to the nucleus in response to fasting. Nuclear translocation is triggered by dephosphorylation, and can be mimicked by a phosphorylation-defective mutant. To investigate the physiologic consequences of FoxO1 translocation, we used stereotactic delivery of constitutively nuclear FoxO1 to this region and showed that it blunts the effects of leptin on food intake and prevents leptin-induced suppression of Agouti-related protein (encoded by *Agrp*) expression. Conversely, a transcriptionally inactive FoxO1 mutant prevents the induction of *Agrp* by fasting, whereas *Foxo1* haploinsufficiency is associated with increased leptin sensitivity. We show that FoxO1 competes with Stat3 for binding to neighboring consensus DNA sequences in the *Agrp* and *Pomc* (encoding pro-opiomelanocortin) promoters to activate exchange of the coactivator Cbp/p300 and corepressors nuclear hormone corepressor (NcoR) and histone deacetylase (Hdac)-1, indicating that transcriptional squelching is a mechanism integrating PI3K and cytokine signaling in the control of food intake.

RESULTS

FoxO1 translocation in Agrp and Pomc neurons

Lepr and insulin receptors (encoded by *Insr*) are expressed in selected areas of the brain[9,10], including a majority of Pomc and Agrp neurons[11]. Immunohistochemistry with a FoxO1-specific antibody showed that FoxO1 is expressed in a majority of cells in the arcuate nucleus (ARH), ventromedial hypothalamus (VMH) and dorsomedial hypothalamus (DMH). In contrast, FoxO1-positive cells account for only a small fraction of cells in the hippocampus (HPC), neocortex (NC) and thalamic paraventricular nucleus (PVN; **Fig. 1a**).

To investigate whether Pomc and Agrp neurons express FoxO1, we used *Pomc*-Cre[12] and *Agrp*-Cre transgenic mice[13] to identify Pomc and Agrp neurons by way of Cre-mediated recombination. We intercrossed these two transgenic lines with *Rosa26*-Gfp transgenic mice. In the resulting progeny, Cre-mediated excision of a *lox*P-STOP cassette leads to expression of Gfp in Pomc and Agrp neurons. As shown previously[12,13], we detected numerous Gfp-expressing neurons in *Pomc*-Cre::*Rosa26*-Gfp and *Agrp*-Cre::*Rosa26*-Gfp mice. Coimmunostaining with antibodies to FoxO1 and Gfp indicated that these neurons express FoxO1 (**Fig. 1b**,**c**). Subcellular localization of FoxO1

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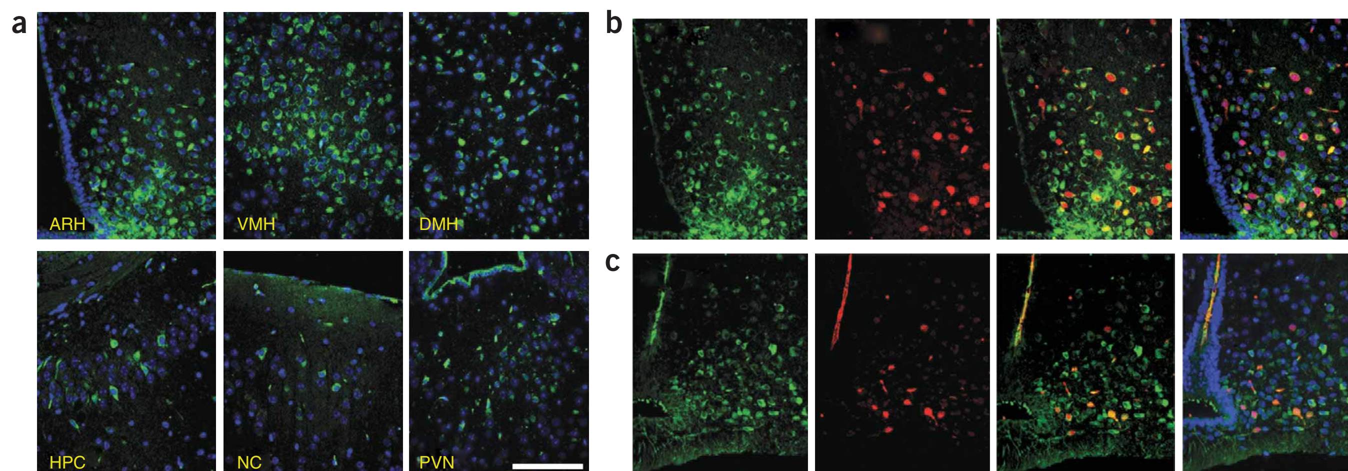

Figure 1 FoxO1 is expressed in Pomc and Agrp neurons. (**a**) Immunohistochemistry with FoxO1-specific antibody (green) and DAPI (blue). ARH, arcuate nucleus; VMH, ventromedial hypothalamus; DMH, dorsomedial hypothalamus; HPC, hippocampus; NC, neocortex; PVN, paraventricular nucleus. Scale bar, 50 μm. (**b**,**c**) Double immunohistochemistry with FoxO1-specific (green) and Gfp-specific (red) antibodies in hypothalamic sections dissected from *Pomc*-Cre::*Rosa26*-Gfp (**b**) or *Agrp*-Cre::*Rosa26*-Gfp mice (**c**). Blue indicates DAPI staining.

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was regulated by fasting-refeeding in Agrp neurons. Of these neurons, 65% showed nuclear localization of FoxO1 after a 6-h fast, as opposed to ~25% of refed mice, indicating that localization of FoxO1 is dependent on physiological hormonal and nutrient cues (**Supplementary Fig. 1** online).

Delivery of constitutively nuclear FoxO1 to the ARH

To investigate whether FoxO1 mediates the action of leptin on transcription of *Agrp* and *Pomc in vivo*, we microinjected adenovirus encoding a constitutively nuclear mutant FoxO1 (FoxO1-ADA) into the ARH of rats and tested the ability of leptin to inhibit food intake and regulate expression of *Agrp* and/or *Pomc*. This mutation prevents nuclear exclusion of FoxO1 by PI3K agonists[14]. We acknowledge that this approach does not allow for targeted expression in specific cell types; nonetheless, it has proved extremely useful in elucidating hypothalamic function[15–20].

To assess the specificity of adenoviral delivery, we isolated brains from mice injected with FoxO1-ADA–encoding adenovirus and performed immunohistochemistry with antibody to hemagglutinin. The FoxO1-ADA construct has an N-terminal tag encoding a viral hemagglutinin epitope. We detected hemagglutinin immunoreactivity in the hypothalamic ARH, but not in NC, HPC, PVN or DMH (**Fig. 2a**). To

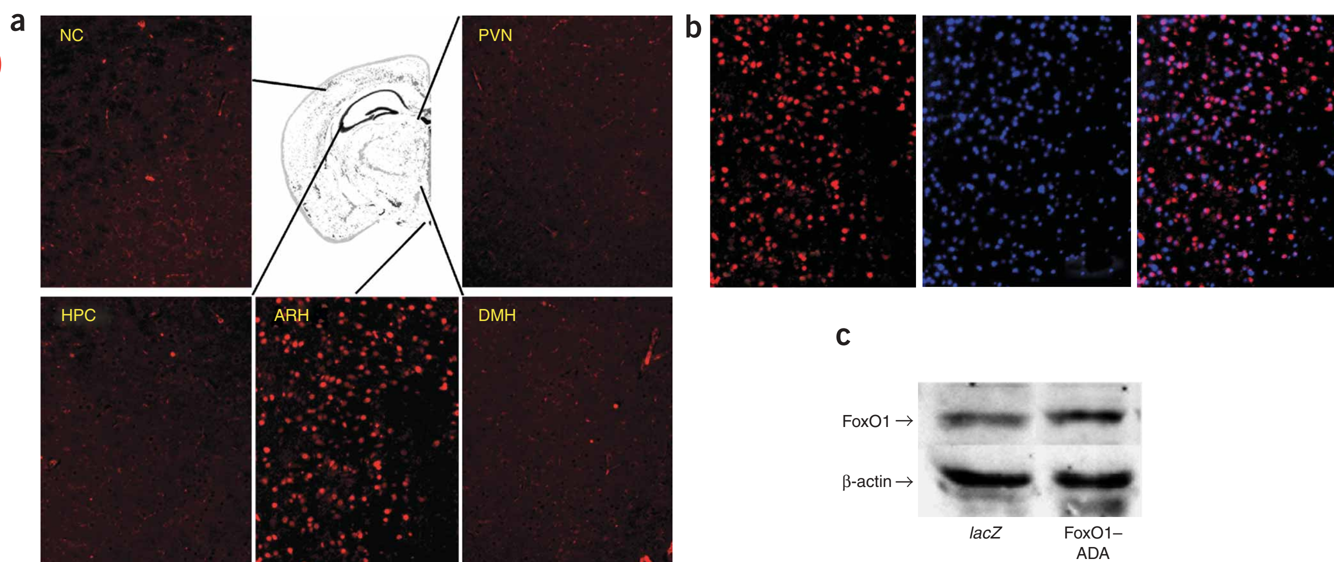

Figure 2 Localization of FoxO1-ADA after stereotactic delivery. (**a**) Immunohistochemistry with antibody to hemagglutinin in brain sections from mice injected with hemagglutinin–FoxO1-ADA–encoding adenovirus (indicated by red). ARH, arcuate nucleus; VMH, ventromedial hypothalamus; DMH, dorsomedial hypothalamus; HPC, hippocampus; NC, neocortex; PVN, paraventricular nucleus. (**b**) Coimmunostaining with antibody to hemagglutinin (red) and DAPI (blue) to determine the efficiency of adenoviral transduction. (**c**) Western blot analysis of FoxO1 expression in rats transduced with *lac*Z-encoding adenovirus or FoxO1-ADA–encoding adenovirus.

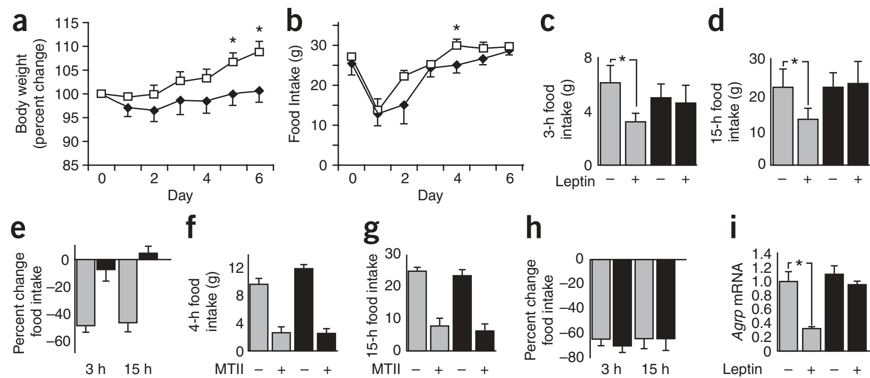

Figure 3 Effects of FoxO1-ADA on food intake and neuropeptide expression. Mean ± s.e.m. percent change in body weight (**a**) and food intake (**b**) in rats receiving *lacZ*-encoding adenovirus (diamonds) or FoxO1-ADA–encoding adenovirus (squares). $n = 7$ for each group. (**c**,**d**) Mean ± s.e.m. total food consumption in response to intracerebroventricular administration of leptin in rats injected with *lac*Z-encoding adenovirus (gray bars) or FoxO1-ADA–encoding adenovirus (black bars). $n = 9$ for each group. (**e**) Data from **c** and **d** plotted as percent inhibition of total food intake. (**f**,**g**) Mean ± s.e.m. food intake in response to intracerebroventricular administration of MT-II in rats injected with *lac*Z-encoding adenovirus (gray bars) or FoxO1-ADA–encoding adenovirus (black bars). $n = 9$ for each group. (**h**) Data from **f** and **g** are plotted as percent inhibition of total food intake. (**i**) Expression of *Agrp* mRNA in rats injected with *lac*Z-encoding adenovirus (gray bars) or FoxO1-ADA–encoding adenovirus (black bars) and then treated with leptin (+) or saline (–).

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measure the efficiency of adenoviral transduction, we coimmunostained with antibodies to hemagglutinin and DAPI. We detected hemagglutinin immunoreactivity in >80% of ARH nuclei (**Fig. 2b**). Expression studies indicated that we achieved an approximately twofold increase in total FoxO1 levels (**Fig. 2c**).

FoxO1 inhibits leptin's effect on food intake

We investigated the effect of FoxO1-ADA on leptin-dependent feeding behavior in rats. At the end of the experiment, we stained serial hypothalamic sections to monitor adenoviral delivery. The data confirm that expression of hemagglutinin-FoxO1-ADA occurs selectively in the rat ARH (**Supplementary Fig. 2** online). After adenoviral delivery, weight gain was modestly enhanced in rats receiving FoxO1-ADA (**Fig. 3a**). As shown previously[19,21], food intake in rats that received the adenovirus decreased by ~50% the first day after injection, but tended to be higher in rats given FoxO1-ADA compared with rats given an adenovirus encoding *lacZ* (**Fig. 3b**). Five days after adenoviral delivery, we measured the ability of leptin to reduce food intake. Intracerebroventricular administration of leptin reduced food intake by 50% in rats treated with control (*lacZ*-encoding) adenovirus (**Fig. 3c–e**), but not in rats treated with FoxO1-ADA (**Fig. 3c–e**). We obtained similar results when we administered leptin intraperitoneally (**Supplementary Fig. 3** online). In contrast, the anorexigenic response to the melanocortin receptor 3 and 4 agonist MT-II[22] was unchanged, showing that FoxO1-ADA specifically affects Lepr signaling (**Fig. 3f–h**). To determine whether the inhibition of food intake was associated with changes in expression of *Agrp* or *Pomc*, we quantified *Agrp* and *Pomc* mRNA by real-time reverse transcription (RT)-PCR. Leptin decreased *Agrp* mRNA levels by 63% in *lacZ*-injected rats (**Fig. 3i**), whereas it had no effect in FoxO1-ADA–injected rats (**Fig. 3i**). *Pomc* mRNA levels were unchanged by leptin or FoxO1-ADA (**Supplementary Fig. 4** online).

We explored the contribution of FoxO1 to leptin signaling in two loss-of-function approaches. Injection of a dominant-negative FoxO1 mutant lacking the transactivation domain (Δ256)[23] prevented induction of *Agrp* expression caused by fasting (**Fig. 4a**). In *Foxo1* haploinsufficient mice ($Foxo1^{+/-}$)[24,25] (**Supplementary Fig. 5** online), intracerebroventricular administration of leptin nearly doubled the inhibition of food intake observed in wild-type littermate controls (**Fig. 4b**,**c**). Moreover, $Foxo1^{+/-}$ mice showed a complete absence of rebound feeding in response to an overnight fast, unlike their wild-type littermates (**Fig. 4d**). These data indicate that loss of FoxO1 function is associated with increased sensitivity to leptin.

Mechanisms of *Agrp* and *Pomc* regulation by FoxO1

We next investigated the mechanism by which FoxO1 regulates expression of *Agrp*. The *Agrp* promoter contains two forkhead DNA-binding sites adjacent to two Stat3 sites (**Fig. 5a**). *Pomc* also contains a FoxO1 binding site adjacent to a Stat3 site that becomes activated in response to leukemia inhibitory factor (LIF)[26] (**Fig. 5a**). Gel-shift assays using nuclear extracts from rat pituitary-derived AtT20 cells indicated that Stat3 and FoxO1 bind to these sites. Addition of oligonucleotide probes spanning the Stat3 and FoxO1 binding sites of the *Agrp* or *Pomc* promoters produced gel-retarded bands (**Fig. 5b**), one of which was supershifted by the addition of antibody to FoxO1 (**Fig. 5b**). Insulin treatment of intact cells before nuclear extraction prevented FoxO1 binding, consistent with the observation that insulin promotes FoxO1 nuclear exclusion. Nuclear extracts from LIF-treated AtT20 cells yielded a different gel-retardation pattern (**Fig. 5c**), and the antibody to Stat3 caused a supershift (**Fig. 5c**). Because LIF promotes nuclear localization of Stat3, these results indicate that Stat3 binds to these *Agrp* and *Pomc* promoter sequences.

Next, we measured *Agrp* and *Pomc* promoter activities in reporter gene assays. FoxO1 increased *Agrp* reporter activity by 3.2-fold and insulin inhibited this effect. Conversely, Stat3 had no effect on basal *Agrp* reporter activity, but caused a 60% inhibition in the presence of LIF. Coexpression of FoxO1 and Stat3 inhibited FoxO1-induced *Agrp* promoter activity, suggesting that Stat3 competes with FoxO1 for binding to *Agrp* (**Fig. 6a**). The opposite pattern was observed in *Pomc*

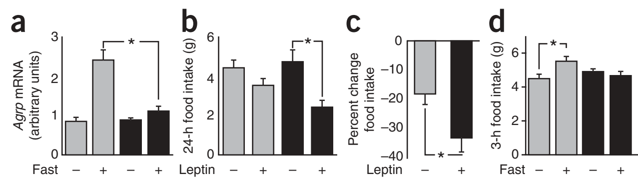

Figure 4 Effects of loss of FoxO1 function on food intake and neuropeptide expression. (**a**) Real-time RT-PCR of *Agrp* expression in rats injected with *lac*Z-encoding adenovirus (gray bars) or FoxO1-Δ256 (black bars) and then fasted (+) or not (–) for 12 h. (**b**) Mean ± s.e.m. food intake in response to intracerebroventricular administration of leptin (+) or saline (–) in wild-type mice (gray bars) and $Foxo1^{+/-}$ littermates (black bars). $n = 6$ for each genotype. (**c**) Data from **b** plotted as percent inhibition of total food intake. (**d**) Mean ± s.e.m. food intake in $Foxo1^{+/-}$ mice after 12-h fast (rebound refeeding). $n = 10$ for each genotype. $^{*}P > 0.05$ by one-factor ANOVA.

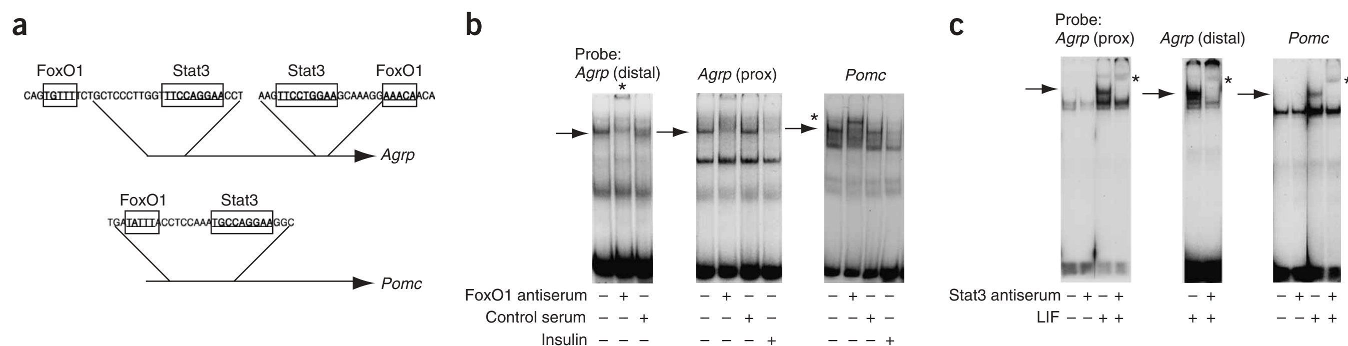

Figure 5 FoxO1 and Stat3 bind to adjacent sequences in the *Agrp* and *Pomc* promoters. (**a**) Map of FoxO1 and Stat3 binding sites in *Agrp* and *Pomc* promoters. (**b**,**c**) We incubated nuclear extracts from AtT20 cells with radiolabeled oligonucleotides spanning the FoxO1 and Stat3 binding sites in the presence or absence of antibodies to FoxO1, Stat3 or nonimmune serum ($n = 3$). Arrows, gel-shifted bands; asterisks, antibody-induced supershifts.

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reporter gene assays. FoxO1 decreased *Pomc* promoter activity by 50% and insulin prevented the effect of FoxO1, whereas Stat3 increased *Pomc* activity by 2.9-fold in the presence of LIF. Coexpression of FoxO1 and Stat3 inhibited Stat3-induced activity of *Pomc* promoter (**Fig. 6b**).

The opposing actions of FoxO1 and Stat3 on *Agrp* and *Pomc* suggest that the two proteins act on the expression of these genes through squelching, that is, by competing for binding to adjacent sites in the two promoters. When we performed gel-shift assays in the presence of varying amounts of the two purified proteins, we detected binding of both to each promoter (**Supplementary Fig. 6** online). To test this hypothesis in intact cells, we performed chromatin immunoprecipitation (ChIP) assays on primary cells isolated from mouse hypothalami. We amplified DNA corresponding to *Agrp* and *Pomc* promoters in chromatin immunoprecipitated by an antibody to FoxO1, but not by control serum (**Fig. 6c**), indicating that FoxO1 is recruited onto the *Agrp* and *Pomc* promoters *in vivo*. Leptin treatment of intact primary hypothalamic cells inhibited FoxO1 binding to *Agrp* and *Pomc* promoters by >90% (**Supplementary Fig. 7** online). Next, we tested whether the effect of leptin was the result of Stat3 nuclear translocation rather than FoxO1 nuclear exclusion. To this end, we overexpressed constitutively active Stat3 (Stat3-CA) in cultured hypothalamic cells. Stat3-CA inhibited FoxO1 binding to *Agrp* and *Pomc* promoters by >90% (**Fig. 6c** and **Supplementary Fig. 7**). These results indicate that FoxO1 is recruited to the *Agrp* and *Pomc* promoters in intact chromatin, and that leptin inhibits FoxO1 binding through Stat3-mediated competition. Conversely, Stat3 was recruited to the *Agrp* and *Pomc* promoters in a leptin-dependent manner (**Fig. 6d**), and its binding was inhibited >90% by overexpression of FoxO1-ADA (**Fig. 6d** and **Supplementary Fig. 7**).

Regulation of coactivator-corepressor exchange by FoxO1

Next, we sought to determine the mechanism by which FoxO1 exerts opposing actions on *Agrp* and *Pomc* promoters. To this end, we studied coactivator-corepressor exchange in cells overexpressing FoxO1-ADA or *Foxo1*-specific siRNA (**Fig. 6e**). The latter effectively suppressed expression of FoxO1 (**Fig. 6e**). Under basal conditions and after expression of FoxO1-ADA, we detected the nuclear coactivator Cbp/p300 bound to the *Agrp* promoter (**Fig. 6e**). In contrast, inhibition of FoxO1 expression resulted in ablation of Cbp/p300 binding and recruitment of the nuclear corepressor NcoR and the histone deacetylase Hdac1 (**Fig. 6e**). These data indicate that FoxO1 binding favors an open chromatin structure at the *Agrp* promoter, whereas FoxO1 inhibition results in histone deacetylation and binding of corepressors, leading to repression of *Agrp*. We observed the opposite pattern at the *Pomc* promoter. Here, overexpression of FoxO1-ADA was associated with Hdac1 or NcoR binding and decreased Cbp/p300 recruitment (**Fig. 6e**). FoxO1 inhibition by siRNA prevented binding

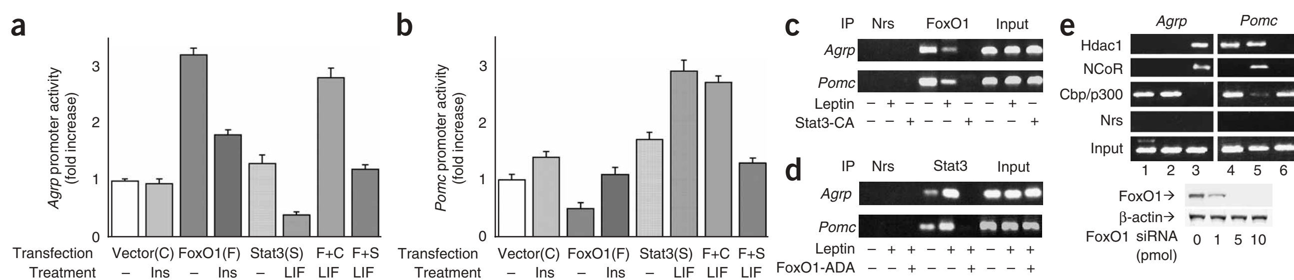

Figure 6 FoxO1 and Stat3 effect opposing actions on *Agrp* and *Pomc* promoters. (**a**) Mean ± s.e.m. *Agrp*-driven luciferase activity in AtT20 cells cotransfected with FoxO1 or Stat3 ($n = 6$). (**b**) Mean ± s.e.m. *Pomc*-driven luciferase activity in AtT20 cells cotransfected with FoxO1 or Stat3 ($n = 6$). (**c**,**d**) *Agrp* and *Pomc* ChIP assays in primary cell cultures from mouse hypothalamus using antibodies to FoxO1, Stat3 or nonimmune serum. In some experiments, we transduced cells with Stat3-CA or FoxO1-ADA adenoviruses. We amplified DNA using primers spanning the FoxO1 and Stat3 binding sites in the *Agrp* or *Pomc* promoters. Input represents DNA extracted from chromatin before immunoprecipitation. (**e**) Upper panel, ChIP assays in primary mouse hypothalamic cells transduced with control *lacZ*-encoding adenovirus (lanes 1 and 4), FoxO1-ADA (lanes 2 and 5) or *Foxo1* mRNA (5 pmol; lanes 3 and 6). Antibodies are indicated on the left. Nrs, nonimmune rabbit serum. Input, DNA extracted from chromatin before immunoprecipitation. Lower panel, efficiency of FoxO1 inhibition by siRNA. Western blot with antibodies to FoxO1 or β-actin in cells transfected with increasing amounts of *Foxo1*-specific siRNA ($n = 3$).

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of Hdac1 or NcoR and increased binding of Cbp/p300 (**Fig. 6e**). This is consistent with the observation that FoxO1 acts as a repressor of *Pomc* transcription.

DISCUSSION

The molecular mechanism by which leptin controls food intake is unclear. PI3K mediates insulin and leptin signaling in the hypothalamus[3,27]. But neither the effectors of PI3K signaling on neuropeptide expression nor the mechanism by which leptin promotes PI3K activity are known[28]. Indeed, measurements of tris-phosphorylated phosphatidyl inositol in response to insulin and leptin indicate that the actions of insulin and leptin are similar in Pomc neurons, but opposite in Agrp neurons[27]. We propose that leptin signaling through Jak2-Stat3 inhibits *Agrp* expression by squelching FoxO1-dependent transcription of *Agrp*. We propose that the inhibition of FoxO1 function by leptin is independent of PI3K, but our data should not be construed to suggest that other aspects of leptin signaling, such as regulation of neuronal activity and synaptic connectivity, are also independent of PI3K. It is known that insulin and leptin have similar biologic effects. But they seem to signal primarily through different pathways, the PI3K pathway for insulin and the Jak-Stat pathway for leptin. Thus far, the prevailing explanation for the biochemical mechanism of the shared biologic actions of insulin and leptin was that the two hormones could signal across each other's pathway, albeit less potently than through their own. Our data indicate another mechanism by which the two pathways interact, namely by transcriptional squelching of their distal effectors, FoxO1 (for insulin) and Stat3 (leptin). The data provide also a general mechanism by which hormone–growth factor signaling interacts with cytokine signaling. Although direct adenoviral delivery has a number of limitations, including the induction of tissue inflammation and the inability to target specific cell types or accurately control protein expression levels, the demonstration of Foxo1 translocation in response to fasting and the use of haploinsufficient mice to confirm the main conclusions of the adenoviral studies provide robust physiological correlates to support our conclusions.

The ability of FoxO1 to effect opposite changes on Agrp and Pomc transcription seems to result from its role in promoting coactivator-corepressor exchange. Thus, stimulation of *Agrp* expression correlated with recruitment of Cbp/p300 and inhibition of nuclear corepressor NcoR and Hdac1 binding, whereas at the *Pomc* promoter we detected an opposite pattern. An alternative, and not mutually exclusive, mechanism for the FoxO1-Stat3 interaction proposed here is that the two proteins are involved in direct interactions[29]. But we did not detect coimmunoprecipitation of FoxO1 with Stat3 in AtT20 cells during our studies.

The lack of effects on *Pomc* expression in our *in vivo* experiments is likely to reflect experimental and methodological differences between our approach and previous reports on *Pomc* regulation by leptin. We studied short-term changes occurring after a physiological fast, whereas changes in expression of *Pomc* mRNA in rodents require prolonged starvation (2 d), which corresponds to >3 d of fasting in humans, or prolonged (5–7 d) leptin treatment in $Lep^{-/-}$ (*ob*/*ob*) mice[30–32]. Nonetheless, the fact that the inhibition of food intake by leptin was prevented by FoxO1-ADA provides a strong indication that this pathway is physiologically relevant. The data are consistent with the phenotype of *Pomc*-Cre and *Rip*-Cre deletion of Irs2 (ref. 33). Because *Agrp* mRNA was altered in opposite ways in response to gain- and loss-of-function FoxO1 mutants, our data support a model in which Agrp is the primary mediator of the acute effects of leptin on food intake. Our data also dovetail with knockout experiments showing that ablation of Agrp neurons in adult mice inhibits feeding behavior[34,35]. Our data are also further consistent with the observation in *Lepr* knock-in mice that Stat3 signaling is crucial for regulation of *Agrp* by leptin *in vivo* (and *Pomc in vitro*). In contrast, *Lepr* knock-in mice show no effects on regulation of neuropeptide Y (encoded by *Npy*) regulation[5]. Notably, there is no FoxO1 site in the *Npy* promoter and FoxO1-ADA injection did not affect expression of *Npy* (data not shown).

FoxO1 is considered a mediator of stress resistance in insulin-producing pancreatic beta cells[36]. If the FoxO1-mediated stress response is biochemically akin to that observed here for neuropeptide-dependent feeding, then this suggests that mechanisms to preserve body weight are part of an ancestral stress-resistance pathway. This may explain why mammals are arguably better predisposed to preserve body weight than to lose it[37]. It will be interesting to determine whether abnormalities of FoxO1 function affect the development of human obesity.

METHODS

Vectors and siRNA. We have previously described expression vector encoding Myc-tagged FoxO1[24] and adenoviral vector encoding hemagglutinin epitope–tagged FoxO1-ADA (with the mutations T24A, S253D and T316A), FoxO1-Δ256 (ref. 23) and control *lacZ*. C.W. Schindler (Columbia University) provided the Phen-Leu-Asp-Gly (FLAG) epitope-tagged Stat3 expression vector. We constructed Stat3-CA (with the mutations A662C and N664C) using the Quick Change kit (Stratagene). The FoxO1-specific siRNA sequence is 5′-ACGGAGGATTGAACCAGTATA-3′. We transfected siRNA using Lipofectamine-plus reagent (Invitrogen).

Immunofluorescence. We fixed mouse brain tissue overnight in 4% paraformaldehyde, embedded the samples in paraffin and cut 5-μm thick sections. We immunostained samples using a mixture of rabbit polyclonal antibodies to FoxO1, containing antibody 9462 (Cell Signaling), H-128 (Santa Cruz Biotechnologies) and 3587 (ref. 38), each used at a dilution of 1:100. We detected Pomc- and Agrp-expressing neurons using a *ROSA26*-Gfp reporter mouse (B6;129-Gt(ROSA)26Sor^{tm2Sho}, The Jackson Laboratories) intercrossed with *Pomc*-Cre[12] or *Agrp*-Cre[13] mice, followed by immunostaining with a Gfp-specific antibody (1:100; Santa Cruz Biotechnologies). We used FITC-conjugated rabbit-specific and CY3-conjugated mouse-specific IgG to detect FoxO1 and Gfp, respectively.

Cell culture and isolation of primary hypothalamic cultures. We cultured AtT20 cells (American Type Culture Collection) in Ham F12K medium supplemented with 15% horse serum and 2.5% FCS. We microdissected mouse hypothalami, dissociated cells by pipetting and incubated them in L15 medium containing 0.05% trypsin, EDTA for 1 h at room temperature (25 °C). After filtration through a 0.4-mm mesh and centrifugation, we resuspended cells in L15 complete medium. In some experiments, we treated cells with 10 mM leptin for 12 h.

Gel-shift assays. We performed electromobility gel-shift assays as described[39], using the oligonucleotide probes 5′-TAGTGATATTTACCTCCAAATGCCAGGAAGGCAG-3′ (–381 to –346 of *Pomc*) and 5′-GGGAACAGTGTTTTCTGCTCCCTTGGTTTCCAGGAACCTTAGGTA-3′ (–363 to –319 of *Agrp*) and 5′-TTGACAAAGTTCCTGGAAGCAAAGGAAACAACATGTCCTAGGG-3′ (–130 to –86 of *Agrp*). The FoxO1 (TG/ATTT) and Stat3 (TTC/AT/CG/TGGAA) binding sites are underlined. In some experiments, we treated cells with 100 nM insulin or 1 nM LIF for 20 min. For antibody-induced supershift, we incubated nuclear extracts with FoxO1-, Stat3-specific

antibodies or nonimmune serum for 1 h before adding radiolabeled probe to the reaction mixture.

ChIP assays. We performed ChIP assays in isolated mouse hypothalamic cells using the following primers: 5′-TCTTGAC AGCCTCTGTTGTCTCCC-3′ and 5′-CTGTGCGAGGCAGGCCTAG TTCTG-3′ (–497 to –474 and –260 to –283 of *Pomc*, respectively); 5′-CCTGAAAGCTTTGTCCTCTGAAGC-3′ and 5′-GCAGAACCTAG GGATGGGTCATGC-3′ (–401 to –378 and –8 to +16 of *Agrp*, respectively). For coactivator and corepressor ChIP, we used a different set of primers: 5′-ACTCCAAAAGGTAGCCTGCCTTGG-3′ and 5′-GATGTTCAGTGGCCTCTCTTAGTC-3′ (–532 to –509 and +40 to +63 of *Pomc*, respectively). We used antibodies to FoxO1 (H-128), Stat3, Cbp, NcoR (Santa Cruz Biotechnologies), Hdac1 (Cell Signaling) and p300 (Upstate Biotechnologies)[40].

Luciferase assays. We transfected AtT20 cells with *Pomc* (–646 to +65)- or *Agrp* (–401 to +16)-driven luciferase along with control, FoxO1-ADA or Stat3 expression vectors. We cotransfected plasmid pRSV-*lacZ* as a control of transfection efficiency. We treated cells with 100 mM insulin or 1 mM leptin for 12 h.

mRNA isolation and real-time PCR. We obtained brain micropunches of ARH from individual rats as previously described[21]. We isolated total RNA using Trizol (Invitrogen) and synthesized single-strand cDNA using Superscript (Invitrogen). We performed real-time PCR with amplification primers corresponding to rat *Agrp* and *Pomc* sequences as described[40]. Primer sequences are available upon request.

Rat brain stereotactic microinjection. We studied Sprague-Daley rats (Charles River Laboratories)[13]. We targeted the bilateral arcuate nucleus using a double-guide cannula system (Plastics One). We performed adenovirus microinjection 4 d after insertion of cannula to allow for postoperative recovery. We used a microinjection system (Stoelting Corporation) to administer 1 μl adenovirus (1.8–8.8 $\times$ 10^{10} plaque-forming units/ml) at a rate of 100 nl/min for 5 min. We then removed the microinjector and chronically implanted a stainless-steel guide into the third ventricle for leptin injection. We used 1 μg (for mice) or 3 μg (for rats) of recombinant mouse leptin.

Food intake studies. After placing a double-guide cannula for adenovirus injection, we implanted a 22-G single-guide cannula into the third ventricle (intracerebroventricular) of rats. Five days after injection of FoxO1-ADA or *lacZ*-encoding adenoviruses, we measured 3-h and 15-h food intake from 18:00 to 21:00 and 18:00 to 09:00, respectively. At 17:00 on the following day, we injected 3 μg leptin or 1.5 μg MT-II into the third ventricle, followed by measurement of food intake after 3 and 15 h. The Institutional Animal Care and Use Committees of Columbia University and Albert Einstein College of Medicine approved all procedures.

Statistical analysis. For the assessment of food intake and mRNA levels in rats, we measured at least seven rats for each group and carried out descriptive statistics and analysis of variance (ANOVA) followed by Fisher test using the Statsview software.

Note: Supplementary information is available on the Nature Medicine website.

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COMPETING INTERESTS STATEMENT
The authors declare that they have no competing financial interests.

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