Cell Metabolism
Article

PDK1 Deficiency in POMC-Expressing Cells Reveals FOXO1-Dependent and -Independent Pathways in Control of Energy Homeostasis and Stress Response

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DOI 10.1016/j.cmet.2008.01.006

SUMMARY

Insulin- and leptin-stimulated phosphatidylinositol-3 kinase (PI3K) activation has been demonstrated to play a critical role in central control of energy homeostasis. To delineate the importance of pathways downstream of PI3K specifically in pro-opiomelanocortin (POMC) cell regulation, we have generated mice with selective inactivation of 3-phosphoinositide-dependent protein kinase 1 (*PDK1*) in POMC-expressing cells (PDK1$^{\Delta POMC}$ mice). PDK1$^{\Delta POMC}$ mice initially display hyperphagia, increased body weight, and impaired glucose metabolism caused by reduced hypothalamic POMC expression. On the other hand, PDK1$^{\Delta POMC}$ mice exhibit progressive, severe hypocortisolism caused by loss of POMC-expressing corticotrophs in the pituitary. Expression of a dominant-negative mutant of FOXO1 specifically in POMC cells is sufficient to ameliorate positive energy balance in PDK1$^{\Delta POMC}$ mice but cannot restore regular pituitary function. These results reveal important but differential roles for PDK1 signaling in hypothalamic and pituitary POMC cells in the control of energy homeostasis and stress response.

INTRODUCTION

Pro-opiomelanocortin (POMC)-expressing neurons are of critical importance for control of food intake, energy expenditure, and glucose metabolism (Cowley et al., 2001; Parton et al., 2007). The prohormone POMC is cleaved into α-melanocyte-stimulating hormone (α-MSH), which, when secreted, binds to the melanocortin 3 and 4 receptors (MC3R and MC4R) on second-order neurons, some of which are located in the paraventricular nucleus of the hypothalamus (Coll et al., 2004). MC4R activation decreases food intake and increases energy expenditure, and MC4R agonists provide a potential avenue for treatment of obesity. Conversely, mutations in the *POMC* or *MC4R* genes cause massive early-onset obesity in humans, further supporting a crucial role for melanocortins in energy homeostasis (Farooqi et al., 2000; Krude et al., 1998). Alternative cleavage of POMC results in β-endorphin production, which also decreases food intake independently of MC4R signaling (Appleyard et al., 2003).

POMC expression, POMC neuron firing, and thus ultimately α-MSH release are under tight control of peripheral hormones such as leptin and insulin, as well as nutrients such as glucose (Könner et al., 2007; Mercer et al., 1996; Parton et al., 2007; Pinto et al., 2004). Leptin signals by activating the signal transducer and activator of transcription 3 (Stat3), which activates POMC expression by recruiting histone acetylases to the *POMC* promoter (Kitamura et al., 2006). Concomitantly, leptin increases the firing rate of POMC neurons by activating nonspecific cation channels, although the exact molecular mechanisms mediating this effect have yet to be fully elucidated (Cowley et al., 2001). Leptin stimulation also leads to phosphatidylinositol-3 kinase (PI3K) activation in POMC neurons, and the acute anorectic effect of intracerebroventricularly applied leptin can be inhibited by PI3K inhibitor pretreatment (Niswender et al., 2001).

Pharmacological inhibition of PI3K in the central nervous system also prevents the acute anorectic effect of centrally administered insulin, although the cell type responsible for this effect has yet to be identified (Niswender et al., 2003). In POMC neurons, insulin strongly activates PI3K (Plum et al., 2006; Xu et al., 2005). Surprisingly, this leads to accumulation of the PI3K product phosphatidylinositol 3,4,5-trisphosphate (PIP_3), subsequent PIP_3-mediated opening of ATP-dependent potassium (K_{ATP}) channels, and thus electrical silencing of POMC neurons (Plum et al., 2006). Moreover, leptin is not able to overcome the hyperpolarization induced by insulin. Accordingly, chronic activation of PI3K by deletion of the PIP_3 phosphatase PTEN leads to diet-sensitive hyperphagia and obesity (Plum et al., 2006). On the other hand, deletion of the *PTEN* gene in all leptin-responsive neurons as characterized by the expression of ObRb results in enhanced sympathetic innervation of white adipose tissue (WAT), leading to transdifferentiation of WAT to brown adipose tissue (Plum et al., 2007).

Besides controlling K_{ATP} channel activation, presumably via generation of PIP_3, which directly binds the K_{ATP} channel, PI3K activation leads to phosphorylation and activation of the downstream kinase AKT, which upon activation translocates to the

nucleus and phosphorylates and inactivates the transcription factor FOXO1. In the absence of PI3K activation, FOXO1 is thought to negatively affect *POMC* transcription by recruitment of histone deacetylases (Kim et al., 2006; Kitamura et al., 2006). Thus, there is an apparent contradiction in that both activation and inhibition of PI3K in POMC neurons result in positive energy balance. Nevertheless, the effect of reduced PI3K signaling in POMC neurons has not been directly addressed.

POMC is also expressed in two specialized cell types in the pituitary, namely corticotrophs in the anterior lobe and melanotrophs in the intermediate lobe. In melanotrophs, POMC is cleaved into α-MSH, which when released into circulation binds to the MC1R on melanocytes of the skin to control pigmentation (Rees, 2003). In corticotrophs, POMC is cleaved into adrenocorticotrophic hormone (ACTH). Expression and release of ACTH is stimulated by stress stimuli and, upon stimulation of MC2R on the adrenal gland, increases synthesis and release of the steroids corticosterone in rodents and cortisol in humans (Bornstein and Chrousos, 1999; Dallman, 1984; Simpson and Waterman, 1988). Cortisol has many effects; notably, it can induce insulin resistance, stimulate food intake, and increase body weight, whereas a lack of circulating cortisol leads to increased leptin and melanocortin sensitivity, anorexia, weight loss, and impaired stress tolerance (Drazen et al., 2003; Jacobson, 1999). Accordingly, restoration of physiologic corticosterone concentrations in POMC knockout mice, which have no circulating corticosterone due to failure of adrenal gland development, leads to even more pronounced hyperphagia and obesity compared with unrestored POMC knockout mice (Smart et al., 2006).

The role of PI3K in corticotrophs and melanotrophs is poorly understood. In vitro data indicate that inhibition of PI3K induces apoptosis in pituitary tumor cell lines partially via regulation of the proapoptotic *Zac1* gene. Neither chronic PI3K activation nor deletion of the insulin receptor on corticotrophs affects pituitary architecture or stress response in vivo (Könner et al., 2007; Pagotto et al., 1999; Plum et al., 2006; Theodoropoulou et al., 2006).

Although there are multiple isoforms of regulatory and catalytic subunits of PI3K, only one isoform of 3-phosphoinositide-dependent protein kinase 1 (PDK1) has been identified thus far (Alessi et al., 1997; Williams et al., 2000). PDK1 is recruited to the cell membrane by PIP_3 and, among multiple other targets, phosphorylates AKT, thereby activating it. Thus, to address the effect of kinase signaling downstream of PI3K on energy homeostasis and stress response in vivo, we generated mice lacking PDK1 selectively in POMC-expressing cells ($PDK1^{\Delta POMC}$) using Cre/loxP-mediated recombination.

RESULTS

Generation of $PDK1^{\Delta POMC}$ Mice

To achieve this goal, we crossed mice transgenic for Cre driven by the *POMC* promoter (POMC-$Cre^{+/-}$) with mice carrying the floxed *PDK1* allele ($PDK1^{fl\Delta neo/fl\Delta neo}$). Mice heterozygous for both alleles were crossed to $PDK1^{fl\Delta neo/fl\Delta neo}$ mice. We thereby generated mice homozygous for the loxP-flanked *PDK1* allele that also carried the POMC-Cre transgene (genotype POMC-$Cre^{+/-}$:$PDK1^{fl\Delta neo/fl\Delta neo}$), i.e., $PDK1^{\Delta POMC}$ mice. Littermates of $PDK1^{\Delta POMC}$ mice negative for Cre, genotype $PDK1^{fl\Delta neo/fl\Delta neo}$, were used as controls. Additionally, we ascertained that the presence of the POMC-Cre bacterial artificial chromosome had no effect on body weight, as shown previously (see Figure S1A available online) (Balthasar et al., 2004; Lawlor et al., 2002). To visualize POMC cell-specific Cre-mediated recombination, we used two reporter mouse strains that express enhanced GFP or β-galactosidase (lacZ) only after Cre-mediated recombination. Double immunohistochemical analysis of $lacZ^{\Delta POMC}$ mice revealed the presence of immunoreactive PDK1 protein in ~70% of lacZ-positive POMC neurons of $lacZ^{\Delta POMC}$ animals but in only ~8% of lacZ-positive POMC neurons of lacZ:$PDK1^{\Delta POMC}$ mice, indicating that *PDK1* deletion occurred with ~90% efficiency in POMC-expressing cells (Figures 1A and 1B). As expected, since POMC neurons make up only a very small proportion of the hypothalamus, immunoblot analyses of control and $PDK1^{\Delta POMC}$ mice showed no difference in PDK1 protein content in lysates obtained from total brain or peripheral tissues (Figure 1C). Similarly, deletion of the *PDK1* allele was only found in DNA extracts from the arcuate nucleus of the hypothalamus and the pituitary, where POMC is expressed, but not in the hippocampus or peripheral tissues (Figure 1D).

To assess whether PDK1 deficiency affects hypothalamic POMC cell differentiation and/or survival, we quantified the number of hypothalamic GFP-positive POMC cells in control GFP reporter animals ($GFP^{\Delta POMC}$) and GFP reporter animals lacking PDK1 in POMC cells (PDK1:$GFP^{\Delta POMC}$) both in young mice at the age of 4 weeks and in older mice at the age of 12 weeks. This analysis revealed no difference in the total number of POMC neurons between control and $PDK1^{\Delta POMC}$ mice (Figures 1E and 1F).

Hyperpolarization of POMC Neurons by Insulin Is PDK1 Independent

Next, we performed electrophysiological analyses of GFP-positive neurons from $GFP^{\Delta POMC}$ and GFP:$PDK1^{\Delta POMC}$ reporter mice. Although the resting membrane potential of POMC neurons lacking PDK1 was slightly depolarized, there was no significant difference between control and knockout reporter neurons regarding spontaneous firing rate, membrane resistance, or capacitance, the latter of which is also an indirect measure of neuron size (Figures 2A–2D).

We then tested the response to insulin, which in POMC neurons induces opening of K_{ATP} channels, resulting in hyperpolarization and a consequent reduction of the firing rate (Plum et al., 2006). Insulin stimulation significantly hyperpolarized GFP-positive neurons of $GFP^{\Delta POMC}$ and GFP:$PDK1^{\Delta POMC}$ reporter mice and robustly reduced their firing rate (Figure 2E). As in control POMC neurons, incubation with the K_{ATP} channel blocker tolbutamide restored firing rate in POMC neurons of GFP:$PDK1^{\Delta POMC}$ reporter mice (Figure 2F). Taken together, these findings indicate that insulin-stimulated POMC cell hyperpolarization is PI3K dependent but PDK1 independent, consistent with a model in which PIP_3 directly activates K_{ATP} channels in POMC neurons.

Increased Body Weight and Hyperphagia in Young $PDK1^{\Delta POMC}$ Mice

To investigate whether PI3K activation in POMC neurons plays another PDK1-dependent role in energy homeostasis, we monitored body weight of control and $PDK1^{\Delta POMC}$ mice fed either a normal chow diet (ND) or a high-fat diet (HFD). Shortly after

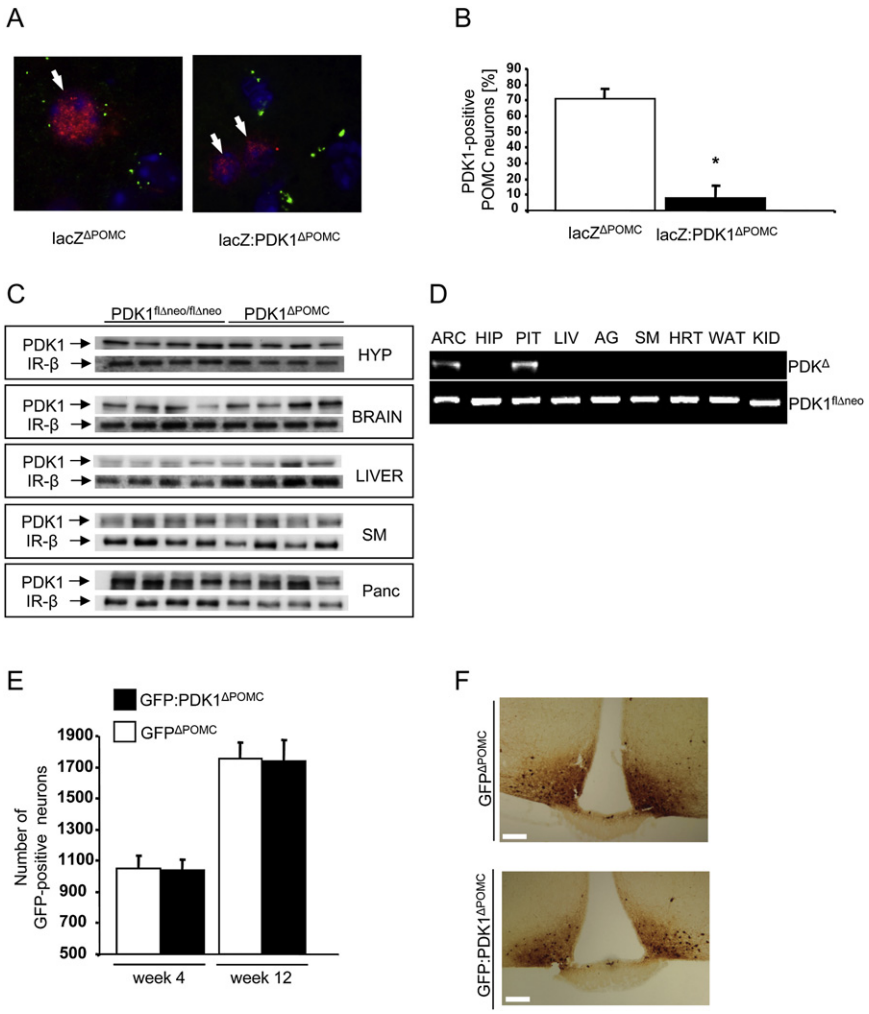

Figure 1. Specificity of *PDK1* Deletion in PDK1$^{\Delta POMC}$ Mice

(A) Detection of PDK1 in POMC neurons by immunohistochemistry. Using lacZ$^{\Delta POMC}$ and lacZ:PDK1$^{\Delta POMC}$ reporter mice, coimmunohistochemistry for β-galactosidase and PDK1 was performed, and the number of POMC cells staining positive for PDK1 was counted for at least 100 neurons from two mice per genotype. Red, lacZ (POMC neurons); green, PDK1; blue, DAPI. Original magnification, ×630.

(B) Quantification of PDK1 expression in POMC neurons from lacZ$^{\Delta POMC}$ and lacZ:PDK1$^{\Delta POMC}$ mice.

(C) Western blot analysis of PDK1 and IR-β subunit expression (loading control) in hypothalamus (HYP), whole brain (BRAIN), liver (LIVER), skeletal muscle (SM), and pancreas (Panc) of PDK1$^{fl\Delta neo/fl\Delta neo}$ and PDK1$^{\Delta POMC}$ mice (n = 4 per group).

(D) Detection of deletion of the *PDK1* allele in PDK1$^{\Delta POMC}$ mice. DNA was extracted from the arcuate nucleus (ARC), hippocampus (HIP), pituitary (PIT), liver (LIV), adrenal gland (AG), skeletal muscle (SM), heart (HRT), white adipose tissue (WAT), and kidney (KID) of a PDK1$^{\Delta POMC}$ mouse. Using a PCR strategy, the deleted allele could be detected only in the arcuate nucleus and the pituitary, but not in the other tissues (upper bands).

(E) POMC cell counts in the arcuate nucleus of GFP reporter mice showed no difference between GFP$^{\Delta POMC}$ and PDK1:GFP$^{\Delta POMC}$ reporter mice at 4 and 12 weeks of age (n = 3 per genotype at each age). Results are expressed as number of neurons staining positive for GFP.

(F) Cre-mediated recombination was visualized by immunohistochemistry for GFP in brains of GFP$^{\Delta POMC}$ and PDK1:GFP$^{\Delta POMC}$ mice. Representative sections for GFP$^{\Delta POMC}$ and PDK1:GFP$^{\Delta POMC}$ mice are shown. Scale bars = 50 μm.

Displayed values are means ± SEM. *p < 0.05.

weaning, male and female PDK1$^{\Delta POMC}$ mice fed either diet presented slightly but significantly increased body weight (Figures 3A and 3B; Figures S1B and S1C). However, body weight of control and PDK1$^{\Delta POMC}$ mice converged over time (Figures 3A and 3B). Consistent with the increased body weight of young PDK1$^{\Delta POMC}$ mice, these animals also exhibited elevated serum leptin and glucose concentrations at 8 weeks of age (Figures 3C and 3D). To address whether the initially increased body weight of PDK1$^{\Delta POMC}$ mice resulted from increased energy intake, we determined food intake in these mice at 8 weeks of age, which revealed significant hyperphagia (Figure 3F).

Analysis of hypothalamic neuropeptide expression in PDK1$^{fl\Delta neo/fl\Delta neo}$ and PDK1$^{\Delta POMC}$ mice revealed a significant reduction in POMC expression in the absence of any alteration in the expression of the orexigenic neuropeptides agouti-related protein (AgRP) and neuropeptide Y (NPY) as well as the thyrotropin-releasing hormone (TRH) (Figure 3G; Figure S2A). Taken together, these data indicate that PDK1$^{\Delta POMC}$ mice initially develop transiently increased body weight as a consequence of hyperphagia caused by reduced hypothalamic POMC expression.

Since the body weight of male PDK1$^{\Delta POMC}$ mice was not distinguishable from that of PDK1$^{fl\Delta neo/fl\Delta neo}$ mice starting at 10 weeks of age, we next determined parameters of energy homeostasis in older mice. Surprisingly, food intake of PDK1$^{\Delta POMC}$ mice was not significantly different from controls at 10 weeks of age (Figure 3F). Strikingly, at 18 weeks of age, PDK1$^{\Delta POMC}$ mice exhibited significantly reduced epigonadal fat-pad mass and lower serum leptin concentration but unchanged body weight (Figures 3D and 3E). Hypothalamic expression of NPY, AgRP, and TRH was again unchanged (Figure S2A) at 18 weeks of age, while hypothalamic POMC expression was still reduced by 80% in PDK1$^{\Delta POMC}$ mice compared to controls (Figure 3G). Thus, progressive reduction of body weight, food intake, serum leptin concentration, and epigonadal fat-pad mass occurred in the presence of constantly reduced hypothalamic POMC expression.

Secondary Hypocortisolism in PDK1$^{\Delta POMC}$ Mice

Given the paradoxical decline of initially increased body weight and hyperphagia in the presence of constantly repressed *POMC* transcription, we decided to investigate possible mechanisms underlying this phenotype. We noticed that old PDK1$^{\Delta POMC}$ mice performed better than control mice during glucose tolerance tests and showed significantly increased insulin sensitivity during insulin tolerance tests (Figure S1D). Moreover, analysis of glucose-stimulated insulin secretion revealed that although

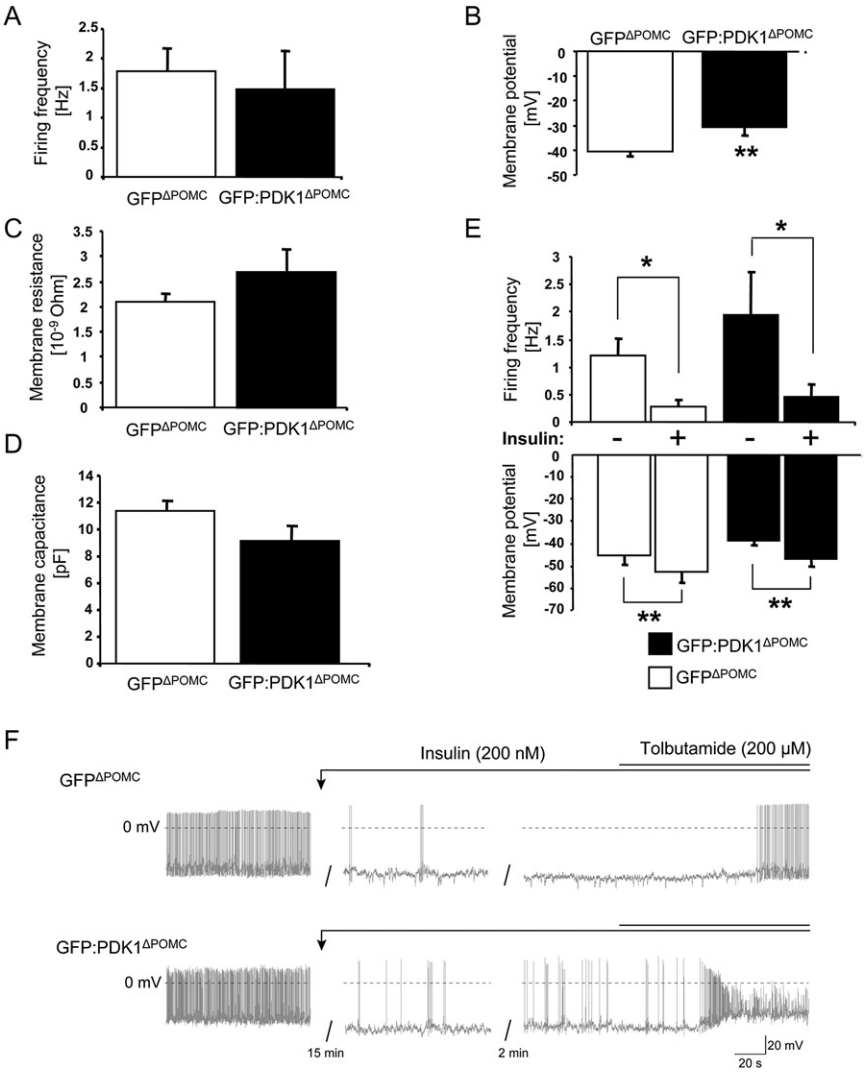

Figure 2. Hyperpolarization of POMC Neurons by Insulin Is PDK1 Independent

(A) Spontaneous firing rate of identified POMC neurons in ARC slices from GFP$^{\Delta POMC}$ (n = 30 neurons) and GFP:PDK1$^{\Delta POMC}$ (n = 12 neurons) mice.

(B) Mean resting membrane potential of identified POMC neurons in ARC slices from GFP$^{\Delta POMC}$ (n = 30 neurons) and GFP:PDK1$^{\Delta POMC}$ (n = 12 neurons) mice.

(C) Mean membrane resistance of identified POMC neurons in ARC slices from GFP$^{\Delta POMC}$ (n = 30 neurons) and GFP:PDK1$^{\Delta POMC}$ (n = 12 neurons) mice.

(D) Mean membrane capacitance of identified POMC neurons in ARC slices from GFP$^{\Delta POMC}$ (n = 25 neurons) and GFP:PDK1$^{\Delta POMC}$ (n = 11 neurons) mice.

(E) Firing frequency and membrane potential of identified POMC neurons in ARC slices from GFP$^{\Delta POMC}$ and GFP:PDK1$^{\Delta POMC}$ mice before and after application of 200 nM insulin (n = 6–7 neurons per group).

(F) Representative recordings of identified POMC neurons in ARC slices from a GFP$^{\Delta POMC}$ and a GFP:PDK1$^{\Delta POMC}$ mouse before and 15 min after 200 nM insulin stimulation, followed by addition of 200 µM tolbutamide.

Displayed values are means ± SEM. *p < 0.05; **p < 0.01; n.s., not significant.

insulin secretion was lower in these mice compared to PDK1$^{fl\Delta neo/fl\Delta neo}$ mice, their blood glucose concentration was significantly lower than in control groups, further corroborating the finding that PDK1$^{\Delta POMC}$ mice exhibit dramatically increased peripheral insulin sensitivity and subsequent compensatory reduction of insulin secretion (Figures S1E and S1F). Taken together, the relative body weight loss and dramatically increased insulin sensitivity in older mice resemble key clinical features of severe hypocortisolism (Jacobson, 1999).

Strikingly, analysis of plasma corticosterone concentrations revealed a significant reduction in PDK1$^{\Delta POMC}$ mice compared to control mice at as early as 3 weeks of age, and importantly, corticosterone concentrations further decreased over time in PDK1$^{\Delta POMC}$ mice (Figure 4B). Moreover, analysis of stress-induced corticosterone release revealed that PDK1$^{\Delta POMC}$ mice exhibited a dramatic impairment in stress-induced corticosterone production (Figure 4A; Figure S1G). Similarly, injection of an ACTH analog in PDK1$^{\Delta POMC}$ mice could not increase plasma corticosterone to the level of control mice, consistent with adrenal insufficiency (Figure 4C). Taken together, these data indicate that PDK1$^{\Delta POMC}$ mice exhibit critically reduced circulating corticosterone with further progressive loss into adulthood.

Hypothalamic corticotrophin-releasing hormone (CRH) is partially responsible for POMC expression and ACTH release from the pituitary after stress stimuli, but not under basal conditions, as CRH knockout mice show no change in basal pituitary POMC expression (Muglia et al., 2000). There was no difference in hypothalamic CRH expression between PDK1$^{fl\Delta neo/fl\Delta neo}$ and PDK1$^{\Delta POMC}$ mice at any age or on any diet, indicating that hypothalamic circuits are not responsible for the loss of circulating corticosterone in PDK1$^{\Delta POMC}$ mice (Figure S2A).

As changes in the function of corticotrophs of the pituitary were likely responsible for hypocortisolism in PDK1$^{\Delta POMC}$ mice, we next examined the pattern of GFP expression in the pituitary of GFP$^{\Delta POMC}$ mice and GFP:PDK1$^{\Delta POMC}$ mice. This analysis revealed a greater than 90% reduction in GFP-positive corticotroph numbers in GFP:PDK1$^{\Delta POMC}$ mice compared to GFP$^{\Delta POMC}$ mice (Figures 4D and 4E). Consistent with the dramatic reduction in corticotroph number, real-time PCR analysis revealed an ~80% reduction of pituitary POMC mRNA expression in PDK1$^{\Delta POMC}$ mice compared to PDK1$^{fl\Delta neo/fl\Delta neo}$ mice (Figures 4F and 4G). We also noted a dramatic decrease in melanotroph numbers in the intermediate lobe of the pituitary, but, in accordance with the notion that even POMC knockout mice have a relatively faint change in pigmentation and fur color, we did not notice any obvious change in the fur color or skin pigmentation of PDK1$^{\Delta POMC}$ mice (data not shown). Taken together,

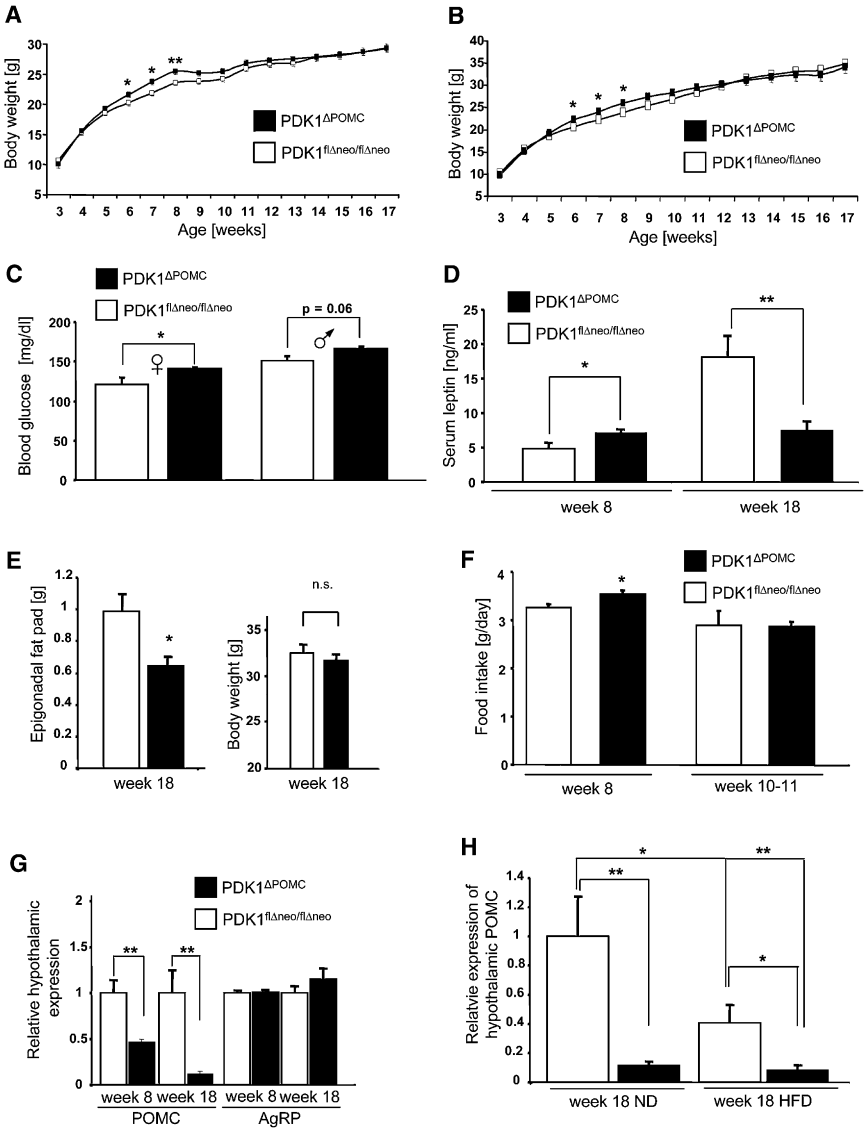

Figure 3. Increased Body Weight, Hyperphagia, and Reduced Hypothalamic POMC Expression in PDK1$^{\Delta POMC}$ Mice

(A) Average body weight of male PDK1$^{fl\Delta neo/fl\Delta neo}$ (□) and PDK1$^{\Delta POMC}$ (■) mice on normal diet (ND) (n = 16–20).

(B) Average body weight of male PDK1$^{fl\Delta neo/fl\Delta neo}$ (□) and PDK1$^{\Delta POMC}$ (■) mice on high-fat diet (HFD) (n = 12–24).

(C) Blood glucose levels in 8-week-old female PDK1$^{fl\Delta neo/fl\Delta neo}$ (left white bar, n = 17), female PDK1$^{\Delta POMC}$ (left black bar, n = 17), male PDK1$^{fl\Delta neo/fl\Delta neo}$ (right white bar, n = 21) and male PDK1$^{\Delta POMC}$ (right black bar, n = 16) mice.

(D) Serum leptin concentrations of male PDK1$^{fl\Delta neo/fl\Delta neo}$ (white bars, n = 17–20) and PDK1$^{\Delta POMC}$ (black bars, n = 10–13) mice at 8 and 18 weeks of age.

(E) Epigonadal fat-pad weight and body weight in male PDK1$^{fl\Delta neo/fl\Delta neo}$ (white bars, n = 21) and PDK1$^{\Delta POMC}$ (black bars, n = 13) mice on ND at 18 weeks of age.

(F) Daily food intake in male PDK1$^{fl\Delta neo/fl\Delta neo}$ (white bars, n = 13, 5) and PDK1$^{\Delta POMC}$ (black bars, n = 7, 4) mice on ND at 8 weeks and 10–11 weeks of age.

(G) POMC and AgRP expression in male PDK1$^{fl\Delta neo/fl\Delta neo}$ (white bars, n = 5–11) and PDK1$^{\Delta POMC}$ (black bars, n = 5–11) mice on ND at 8 and 18 weeks of age as measured by real-time PCR.

(H) Hypothalamic POMC expression in male PDK1$^{fl\Delta neo/fl\Delta neo}$ (white bars, n = 4–5) and PDK1$^{\Delta POMC}$ (black bars, n = 4–5) mice on ND or HFD at 18 weeks of age as measured by real-time PCR.

Displayed values are means ± SEM. *p < 0.05; **p < 0.01.

these findings demonstrate that PDK1 is essential for the survival of corticotrophs and that POMC cell-restricted PDK1 deficiency results in secondary hypocortisolism. Thus, our study reveals that PDK1 plays critical but divergent roles in POMC-expressing cell types: while PDK1 deficiency in hypothalamic POMC neurons affects *POMC* transcription and not cell survival, it primarily controls cell survival of corticotrophs and melanotrophs in the pituitary.

Corticosterone Replacement Prolongs Hyperphagia and Increased Body Weight in PDK1$^{\Delta POMC}$ Mice

To directly address whether progressive hypocortisolism contributes to the normalization of hyperphagia and increased body weight of PDK1$^{\Delta POMC}$ mice, we next aimed to restore circulating corticosterone concentrations in PDK1$^{\Delta POMC}$ mice. To this end, we implanted osmotic minipumps filled with corticosterone in 8-week old mice and monitored corticosterone concentrations, body weight, and food intake. One week after surgery, circulating corticosterone levels were similar between PDK1$^{fl\Delta neo/fl\Delta neo}$ mice and PDK1$^{\Delta POMC}$ mice (Figure 5A). Two weeks after surgery, food intake and body weight were significantly increased in PDK1$^{\Delta POMC}$ mice (Figures 5B and 5C), at a time where there is no significant difference between unrestored PDK1$^{\Delta POMC}$ mice and control mice (Figures 3A and 3F). Moreover, epigonadal fat-pad mass was significantly increased in PDK1$^{\Delta POMC}$ mice 3 weeks after surgery (Figure 5D). Taken together, these data demonstrate that corticosterone restoration aggravates positive energy balance in PDK1$^{\Delta POMC}$ mice, indicating that in unrestored PDK1$^{\Delta POMC}$ mice, the effect of reduced hypothalamic POMC expression is ameliorated by progressive loss of circulating corticosterone.

Restoration of Energy Homeostasis in PDK1$^{\Delta POMC}$ Mice by FOXO1 Inhibition In Vivo

Next, we aimed to define the pathways that act downstream of PDK1 to regulate hypothalamic POMC expression and/or corticotroph survival in the pituitary. Many kinases and transcription factors act downstream of PDK1, notably AKT, mTOR, S6K1, SGK1, and PKC isoforms, and thus could contribute to the observed phenotype (Mora et al., 2004). Yet one of the major mediators of PI3K signaling is the transcription factor FOXO1. FOXO1

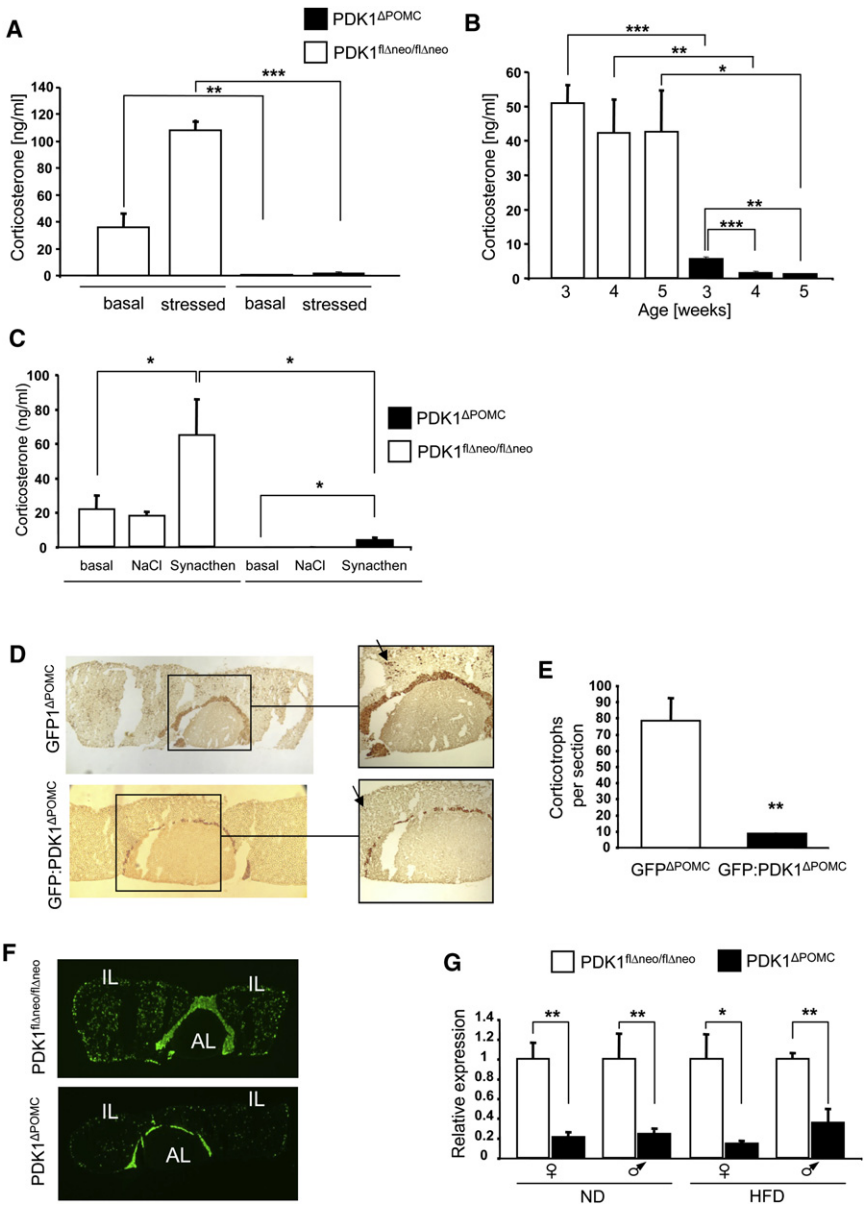

Figure 4. Reduced Plasma Corticosterone and Adrenal ACTH Insensitivity in PDK1$^{\Delta POMC}$ Mice

(A) Plasma corticosterone levels of male PDK1$^{fl\Delta neo/fl\Delta neo}$ (white bars, n = 6) and PDK1$^{\Delta POMC}$ (black bars, n = 5) mice on ND before and after a stress test at 12 weeks of age.

(B) Basal plasma corticosterone levels in male PDK1$^{fl\Delta neo/fl\Delta neo}$ (white bars, n = 7–10) and PDK1$^{\Delta POMC}$ (black bars, n = 5–9) mice at 3, 4, and 5 weeks of age.

(C) Plasma corticosterone levels in PDK1$^{fl\Delta neo/fl\Delta neo}$ (white bars, n = 5) and PDK1$^{\Delta POMC}$ (black bars, n = 5) mice after intraperitoneal injection of saline or an ACTH analog (Synacthen) at 12 weeks of age.

(D) Immunohistochemistry for GFP from pituitary sections of GFP$^{\Delta POMC}$ and GFP:PDK1$^{\Delta POMC}$ mice at approximately 12 weeks of age. Brown, horseradish peroxidase (GFP). Single corticotrophs are marked by arrows.

(E) Quantification of GFP-positive corticotrophs from pituitary sections of GFP$^{\Delta POMC}$ (white bar, n = 2) and GFP:PDK1$^{\Delta POMC}$ (black bar, n = 3) mice.

(F) Immunohistochemistry for ACTH from pituitary sections of PDK1$^{fl\Delta neo/fl\Delta neo}$ and PDK1$^{\Delta POMC}$ mice at approximately 8 weeks of age. Green, ACTH. At least three mice of each genotype were analyzed. Original magnification, ×100.

(G) Relative pituitary POMC expression in female and male PDK1$^{fl\Delta neo/fl\Delta neo}$ (white bars, n = 5–6) and PDK1$^{\Delta POMC}$ (black bars, n = 4–6) mice on ND or HFD at 18 weeks of age as measured by real-time PCR.

Displayed values are means ± SEM. *p < 0.05; **p < 0.01; ***p < 0.001.

controls expression of proapoptotic genes and has also been implicated in the control of *POMC* transcription (Kim et al., 2005, 2006; Kitamura et al., 2006; Medema et al., 2000). As FOXO1 phosphorylation and exclusion from the nucleus are AKT dependent, cells lacking PDK1 have increased FOXO1 activity. Therefore, we aimed to generate a mouse line with Cre-inducible expression of a dominant-negative FOXO1 (FOXO1$^{\Delta 256}$) mutant that lacks the transactivation domain and the nuclear export signal (Figure S3) (Nakae et al., 2001). Expression of FOXO1$^{\Delta 256}$ leads to its accumulation in the nucleus, where it binds to FOXO1 *cis*-elements and inhibits binding of endogenous FOXO1 protein, thus precluding transactivation. We crossed FOXO1$^{\Delta 256}$ mice with PDK1$^{\Delta POMC}$ mice to generate FOXO1$^{\Delta 256}$:PDK1$^{\Delta POMC}$ mice, which lack PDK1 specifically in POMC-expressing cells but express FOXO1$^{\Delta 256}$ at the same time.

Since the mRNA encoding the FOXO1$^{\Delta 256}$ mutant also codes for GFP protein, which is translated from an internal ribosome entry site (IRES), GFP immunohistochemistry can identify cells expressing the FOXO1$^{\Delta 256}$ mutant (Figure S3). Hypothalamic GFP-positive cell counts were similar between FOXO1$^{\Delta 256}$:PDK1$^{\Delta POMC}$, GFP:PDK1$^{\Delta POMC}$, and GFP $^{\Delta POMC}$ reporter mice at 4 or 12 weeks of age, indicating appropriate expression of the FOXO1$^{\Delta 256}$ mutant protein without an effect on POMC neuron number (Figure 1E; Figures 6A and 6B).

Because young PDK1$^{\Delta POMC}$ mice showed increased body weight and hyperphagia, we next analyzed Cre-negative PDK1$^{fl\Delta neo/fl\Delta neo}$, FOXO1$^{\Delta 256}$:PDK1$^{fl\Delta neo/fl\Delta neo}$, PDK1$^{\Delta POMC}$, and FOXO1$^{\Delta 256}$:PDK1$^{\Delta POMC}$ littermates with regards to energy homeostasis. Body weight of FOXO1$^{\Delta 256}$:PDK1$^{fl\Delta neo/fl\Delta neo}$ and PDK1$^{fl\Delta neo/fl\Delta neo}$ mice was indistinguishable; thus, animals of both genotypes were combined into the control group (called PDK1^{Cre-}). While PDK1$^{\Delta POMC}$ mice showed significantly increased body weight compared to control littermates, expression of FOXO1$^{\Delta 256}$ restored normal body weight in FOXO1$^{\Delta 256}$:PDK1$^{\Delta POMC}$ mice (Figure 6C). Similarly, FOXO1$^{\Delta 256}$ expression prevented hyperphagia in PDK1$^{\Delta POMC}$ mice

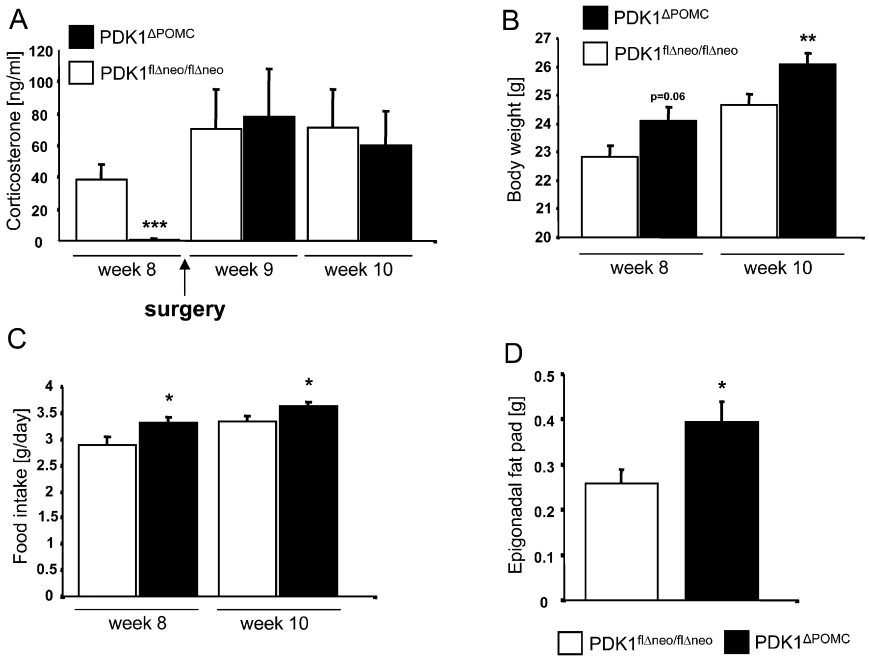

Figure 5. Corticosterone Restoration Maintains Hyperphagia and Increased Body Weight in PDK1$^{\Delta POMC}$ mice.

(A) Plasma corticosterone concentration in PDK1$^{fl\Delta neo/fl\Delta neo}$ (white bars) and PDK1$^{\Delta POMC}$ (black bars) mice before and after implantation of a corticosterone minipump. Animals were bled at 8 weeks of age, and surgery was performed immediately afterwards. Corticosterone levels were measured 1 and 2 weeks after surgery (n = 7–12 per genotype).

(B) Body weight of PDK1$^{fl\Delta neo/fl\Delta neo}$ (white bars) and PDK1$^{\Delta POMC}$ (black bars) mice before and after corticosterone minipump implantation (n = 7–12 per genotype).

(C) Daily food intake of PDK1$^{fl\Delta neo/fl\Delta neo}$ (white bars) and PDK1$^{\Delta POMC}$ (black bars) mice before and after corticosterone minipump implantation (n = 7–12 per genotype).

(D) Epigonadal fat-pad mass of PDK1$^{fl\Delta neo/fl\Delta neo}$ (white bars) and PDK1$^{\Delta POMC}$ (black bars) mice at the end of corticosterone restoration (3 weeks after surgery; n = 7–10).

Displayed values are means ± SEM. *p < 0.05; **p < 0.01; ***p < 0.001.

(Figure 6D). Moreover, while blood glucose concentrations in PDK1$^{\Delta POMC}$ mice were significantly increased compared to PDK1^{Cre-} animals, there was no difference between PDK1^{Cre-} and FOXO1$^{\Delta 256}$:PDK1$^{\Delta POMC}$ mice (Figure S4A). Strikingly, while hypothalamic POMC expression was significantly decreased in PDK1$^{\Delta POMC}$ mice compared to PDK1^{Cre-} mice, there was no significant difference between control and FOXO1$^{\Delta 256}$:PDK1$^{\Delta POMC}$ mice (Figure 6E). Consistent with earlier observations, we noticed a strong tendency toward decreased expression of the anorexigenic neuropeptide cocaine- and amphetamine-related transcript (CART) in PDK1$^{\Delta POMC}$ mice, which was completely rescued in FOXO1$^{\Delta 256}$:PDK1$^{\Delta POMC}$ mice (Figure 6E) (Kim et al., 2006). Overall, these in vivo findings indicate that regulation of FOXO1 activity in hypothalamic POMC neurons is the principal means by which PDK1 signaling controls energy homeostasis.

Corticotroph Loss in PDK1$^{\Delta POMC}$ Mice Is FOXO1 Independent

To investigate the effect of FOXO1$^{\Delta 256}$ expression in corticotrophs, we performed stress tests in FOXO1$^{\Delta 256}$:PDK1$^{\Delta POMC}$ mice. FOXO1$^{\Delta 256}$:PDK1$^{\Delta POMC}$ mice had reduced basal and stress-induced corticosterone levels, similar to PDK1$^{\Delta POMC}$ mice (Figure S1G). GFP and ACTH staining of pituitaries from FOXO1$^{\Delta 256}$:PDK1$^{\Delta POMC}$ mice showed the same reduction in corticotrophs as seen in PDK1$^{\Delta POMC}$ mice, and POMC expression in the pituitaries was again significantly reduced in 8-week-old mice (Figures S4B and S4C).

To gain better insights into the dynamics of corticotroph loss, we analyzed gene expression patterns in pituitaries of 3-week-old mice. POMC mRNA expression was already critically reduced at this age in both PDK1$^{\Delta POMC}$ and FOXO1$^{\Delta 256}$:PDK1$^{\Delta POMC}$ mice (Figure 6F). We also assessed expression of growth hormone-releasing hormone receptor (GHRHR) and thyroid-stimulating hormone β subunit (TSHβ), but we found no difference between the different genotypes, indicating that the thyroid axis as well as the general pituitary architecture is not affected in PDK1$^{\Delta POMC}$ mice (data not shown). Strikingly, we found significantly increased expression of the proapoptotic genes *Bax* and *Bak* in PDK1$^{\Delta POMC}$ mice, but not in FOXO1$^{\Delta 256}$:PDK1$^{\Delta POMC}$ mice (Figure 6F). Moreover, only PDK1$^{\Delta POMC}$ mice, but not FOXO1$^{\Delta 256}$:PDK1$^{\Delta POMC}$ mice, exhibited a tendency toward increased expression of the proapoptotic gene *Zac1*, which has been shown to be under negative control of PI3K in pituitary cells in vitro (Theodoropoulou et al., 2006) (Figure 6F). Taken together, our data present direct in vivo evidence for important roles of PDK1-dependent signaling in control of pituitary corticotroph and melanotroph function. While increased expression of several proapoptotic genes in pituitaries of PDK1$^{\Delta POMC}$ mice can be rescued by expression of FOXO1$^{\Delta 256}$, survival of corticotrophs appears to depend on one or more additional PDK1-dependent, FOXO1-independent pathways.

DISCUSSION

The results of the current study reveal multiple, differential, and important roles for PDK1 signaling in POMC cells. They demonstrate in vivo that PI3K/PDK1/FOXO1-dependent signaling is required for hypothalamic *POMC* transcription and that impaired activation of this pathway results in hyperphagia and increased body weight (Figure 7). Indeed, we demonstrate that mice exposed to HFD exhibit a significant reduction in POMC expression compared to ND-exposed mice (Figure 3H). In late stages of obesity, hypothalamic leptin and insulin resistance due to increased SOCS3 expression and JNK activation have been reported (Bjorbaek et al., 1998; De Souza et al., 2005; Kievit et al., 2006); moreover, direct inhibition of hormone signaling by nutrients such as fatty acids has also been reported (Pocai et al., 2006). This would suggest that, in the presence of inhibited insulin and/or leptin stimulation and thus AKT activation, FOXO1 may constantly repress POMC expression, reducing the ability of

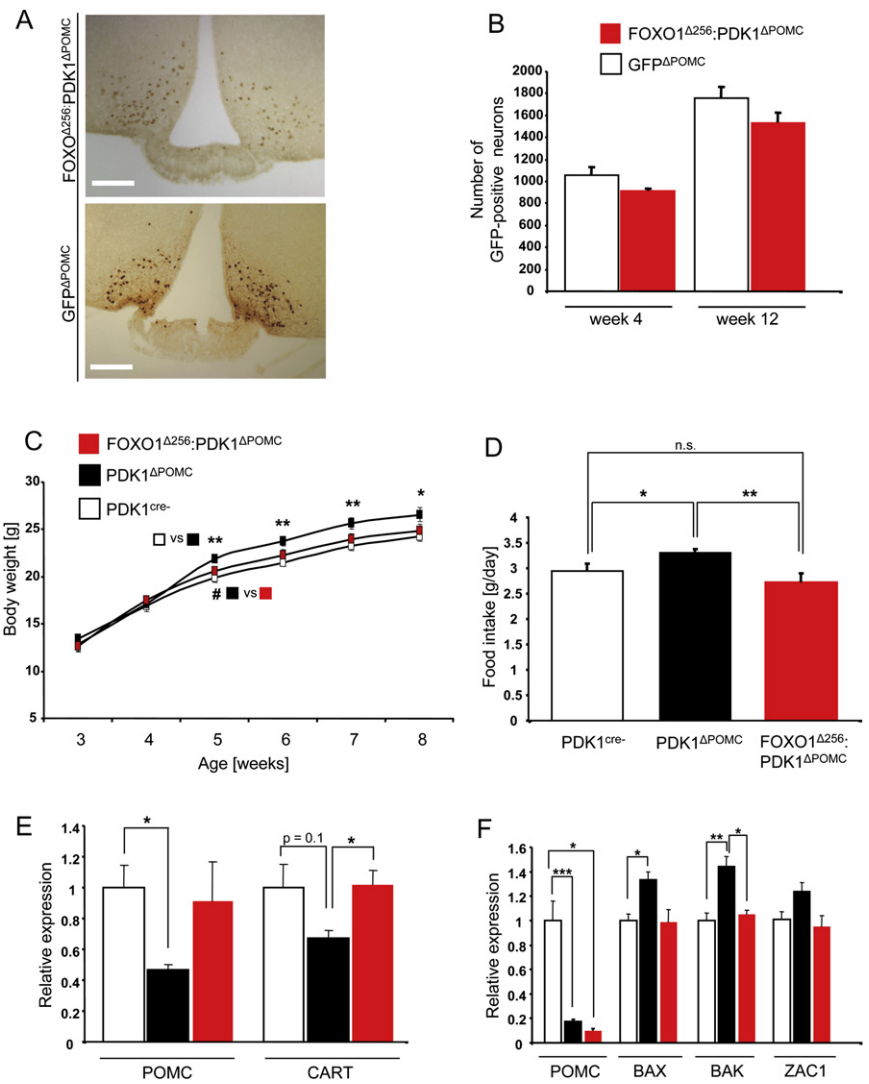

Figure 6. Expression of FOXO1$^{\Delta 256}$ Rescues the Hypothalamic Phenotype of PDK1$^{\Delta POMC}$ Mice

(A) Cre-mediated recombination was visualized using immunohistochemistry for GFP in brains of GFP$^{\Delta POMC}$ and FOXO1$^{\Delta 256}$:PDK1$^{\Delta POMC}$ mice. Brown, horseradish peroxidase (GFP). Scale bar = 100 μm.

(B) POMC cell counts in the arcuate nucleus of GFP reporter mice showed no difference between GFP$^{\Delta POMC}$ (white bars) and FOXO1$^{\Delta 256}$:PDK1$^{\Delta POMC}$ reporter (red bars) mice at 4 and 12 weeks of age (n = 3 per genotype at each age). Results are expressed as number of neurons staining positive for GFP.

(C) Average body weight of male PDK1^{Cre-} (white boxes, n = 19), PDK1$^{\Delta POMC}$ (black boxes, n = 15) and FOXO1$^{\Delta 256}$:PDK1$^{\Delta POMC}$ (red boxes, n = 8) mice on ND until 8 weeks of age. *p < 0.05 between control and PDK1$^{\Delta POMC}$; **p < 0.01 between control and PDK1$^{\Delta POMC}$; #p < 0.05 between PDK1$^{\Delta POMC}$ and FOXO1$^{\Delta 256}$:PDK1$^{\Delta POMC}$.

(D) Food intake of male PDK1^{Cre-} (white bar, n = 9), PDK1$^{\Delta POMC}$ (black bar, n = 10), and FOXO1$^{\Delta 256}$:PDK1$^{\Delta POMC}$ (red bar, n = 7) mice on ND at 8 weeks of age.

(E) Relative expression of hypothalamic POMC and CART mRNA in male PDK1^{Cre-} (white bars, n = 17), PDK1$^{\Delta POMC}$ (black bars, n = 15), and FOXO1$^{\Delta 256}$:PDK1$^{\Delta POMC}$ (red bars, n = 7) mice on ND at 8 weeks of age as measured by real-time PCR.

(F) Relative expression of *POMC* and proapoptotic genes in pituitary extracts from male PDK1^{Cre-} (white bars, n = 8), PDK1$^{\Delta POMC}$ (black bars, n = 11), and FOXO1$^{\Delta 256}$:PDK1$^{\Delta POMC}$ (red bars, n = 4) mice at 3 weeks of age as determined by real-time PCR.

Displayed values are means ± SEM. Unless stated otherwise: *p < 0.05; **p < 0.01; ***p < 0.001; n.s., not significant.

POMC neurons to release α-MSH during obesity. In fact, Enriori et al. (2007) have demonstrated impaired α-MSH release in HFD-exposed mice.

On the other hand, in the earlier stages of obesity, insulin and leptin levels are increased, leading to initially increased PI3K activation. Our previous work has revealed that enhanced activation of PI3K signaling specifically in POMC cells results in hyperphagia, due to neuronal silencing as a consequence of K_{ATP} channel opening in the presence of increased *POMC* transcription. Here we demonstrate that this effect is directly mediated by PIP_3-dependent, PDK1-independent K_{ATP} channel activation (Figure 7). Taken together, these studies demonstrate a tightly regulated dynamic range of the PI3K signaling pathway in control of energy homeostasis: both initially enhanced insulin action as occurs early in overfeeding and also insulin and leptin resistance as present later in the development of obesity lead to POMC cell dysfunction. Although mechanistically different, the biological outcome, namely hyperphagia and weight gain is the same. Thus, pharmacological manipulation of the PI3K pathway in POMC cells must carefully restore signaling in the optimal range (Figure 7).

Moreover, we clearly demonstrate a pivotal role for PDK1 signaling in POMC cell survival in the pituitary. The mechanism underlying this phenomenon appears to be complex. Previous in vitro experiments on cultivated adenoma cells had already indicated a role for PI3K/PDK1-dependent regulation of Zac1 in control of somatotroph and corticotroph cell survival (Pagotto et al., 1999; Theodoropoulou et al., 2006). This seems not to be the only PI3K/PDK1-dependent pathway responsible for POMC cell survival in the pituitary in vivo, since increased expression of proapoptotic genes such as *Bax*, *Bak*, and *Zac1* can be restored by expression of FOXO$^{\Delta 256}$ in vivo without rescuing pituitary POMC cell survival. Further experiments unraveling the exact nature of this pathway may help to design novel therapeutic interventions for pituitary corticotroph adenomas.

The integrative view of the hypothalamic and pituitary phenotype of PDK1$^{\Delta POMC}$ mice also highlights the impact of *POMC* gene dosage on energy homeostasis. While humans and mice null for both *POMC* alleles are hyperphagic and obese in the absence of circulating cortisol/corticosterone, humans heterozygous for one *POMC* null allele exhibit a predisposition for increased body weight and obesity (Farooqi et al., 2006; Krude

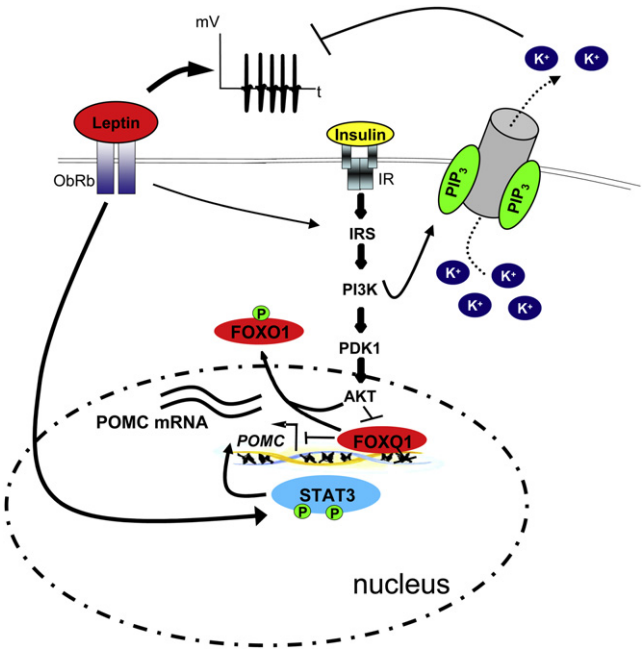

Figure 7. Model of PI3K- and Stat3-Dependent Pathways in POMC Neurons

Activation of phosphatidylinositol-3 kinase (PI3K) in POMC neurons results in moderate AKT activation leading to phosphorylation of the transcription factor FOXO1. Phosphorylated FOXO1 is excluded from the nucleus, allowing for *POMC* transcription. At the same time, normal activation of PI3K results in the opening of only a few K_{ATP} channels, which enables leptin-dependent firing of the POMC neuron and leptin-dependent stimulation of *POMC* transcription by signal transducer and activator of transcription 3 (Stat3), as FOXO1 is excluded from the nucleus and cannot block *POMC* transcription. IR, insulin receptor; IRS, insulin receptor substrate; PDK1, 3-phosphoinositide-dependent protein kinase 1; FOXO1, forkhead box O1; PIP_3, phosphatidylinositol 3,4,5-trisphosphate; K^+, potassium cation.

et al., 1998; Yaswen et al., 1999). Moreover, we show that the relatively small effect of severely reduced hypothalamic POMC expression in PDK1$^{\Delta POMC}$ mice on food intake and obesity is caused by the pronounced loss of circulating corticosterone, as restoration of corticosterone leads to sustained hyperphagia, weight gain, and increased fat mass at an age at which unrestored PDK1$^{\Delta POMC}$ mice do not show any difference in food intake and body weight compared to control mice. Nonetheless, the notion that β-endorphin and other POMC neuropeptides in the pituitary may affect the overall phenotype of PDK1$^{\Delta POMC}$ mice cannot be completely ruled out at this point.

It is also conceivable that POMC neurons use compensatory signals to decrease food intake and energy expenditure independently of POMC expression and α-MSH release, for example the neurotransmitters glutamate and GABA (Collin et al., 2003; Hentges et al., 2004). Although we noticed a significant reduction in POMC neuron membrane potential by an as yet unknown mechanism, it is unlikely that this has an effect on overall neuron function, as all other electrophysiological parameters, including firing rate, were unchanged. Additionally, residual PDK1 expression in a very small percentage of POMC neurons may also attenuate the effect of PDK1 loss in the majority of POMC neurons. Moreover, we observed a small subpopulation of POMC neurons that do not seem to express PDK1 at all. Previous studies reported that leptin and insulin fail to stimulate a small percentage of POMC neurons, possibly because these neurons lack the leptin and insulin receptors or downstream signal cascade members, indicating that there are several subpopulations of POMC neurons (Choudhury et al., 2005). Thus, the present study underlines the complex regulation of cellular and molecular pathways in control of energy balance and reveals important, cell-type-specific roles of PDK1 as a determinant of energy homeostasis and stress response.

EXPERIMENTAL PROCEDURES

Animal Care

All animal care was within University of Cologne institutional animal care committee guidelines. All animal procedures were conducted in compliance with protocols approved by local government authorities (Bezirksregierung Köln, Cologne, Germany) and were in accordance with National Institutes of Health guidelines. Mice were housed in groups of three to five at 22°C–24°C with a 12 hr light/12 hr dark cycle. Animals were fed either a normal chow diet (ND; Teklad Global Rodent 2018, Harlan) containing 53.5% carbohydrate, 18.5% protein, and 5.5% fat (12% of calories from fat) or a high-fat diet (HFD; C1057, Altromin) containing 32.7% carbohydrate, 20% protein, and 35.5% fat (55.2% of calories from fat). Animals were given ad libitum access to water at all times, and food was only withdrawn if required for an experiment. Body weight was measured once per week, and nasoanal body length was measured before sacrifice. Genotyping was performed by PCR using genomic DNA isolated from tail tips as described previously (Lawlor et al., 2002). Mice were sacrificed using CO_2.

Generation of PDK1$^{\Delta POMC}$ Mice

PDK1$^{\Delta POMC}$ mice were generated as described in Results. PDK1$^{fl\Delta neo/fl\Delta neo}$ mice were backcrossed three times onto a C57BL/6 background before crossing with POMC-Cre mice. Male PDK1$^{\Delta POMC}$ mice were bred with female PDK1$^{fl\Delta neo/fl\Delta neo}$ mice. The background was unchanged throughout all experiments. Littermates were used for all analyses except reporter mouse studies. The stated phenotypes of the PDK1$^{\Delta POMC}$ mice were stable for more than five generations.

Generation of FOXO1$^{\Delta 256}$ Mice

We generated a *ROSA26* locus targeting vector in which *CAGS* promoter-driven expression of the FOXO1$^{\Delta 256}$ mutant and IRES-GFP is inhibited by a loxP-flanked stop cassette. This vector was transfected into F1 embryonic stem (ES) cells, which were screened for correct integration by standard Southern blot methods. Correctly targeted ES cells were used to generate chimeras, which were backcrossed on a C57/BL6 background and examined for germline transmission. All FOXO1$^{\Delta 256}$:PDK1$^{\Delta POMC}$ mice were heterozygous for the FOXO1$^{\Delta 256}$ transgene.

Analytical Procedures

Blood glucose values were determined from whole venous blood using an automatic glucose monitor (GlucoMen, A. Menarini Diagnostics). Serum insulin, leptin, and plasma corticosterone were measured by ELISA using mouse standards according to the manufacturer's guidelines (Rat Insulin ELISA, #INSKR020, Crystal Chem Inc.; Mouse Leptin ELISA, #90030, Crystal Chem Inc.; Corticosterone EIA Kit, #900-097, Assay Designs).

Western Blotting

Indicated tissues were dissected and homogenized in homogenization buffer with a polytron homogenizer (IKA Werke), and western blot analyses were performed by standard methods with antibodies raised against IR-β (#sc-711, Santa Cruz) and PDK1 (#611070, BD Biosciences) as described previously (Gropp et al., 2005; Janoschek et al., 2006).

Corticosterone Replacement

Before surgery, dexamethasone (5 mg/kg body weight) was injected intraperitoneally to prevent death as a consequence of stress arising from surgery in

PDK1$^{\Delta POMC}$ mice. Osmotic minipumps (model 2004, Alzet) prefilled with 10 μg/μl corticosterone were implanted in anaesthetized mice according to the manufacturer's instructions. Blood was taken every week between 2 and 4 p.m. Mice that did not show a significant increase in plasma corticosterone were dismissed from the study.

Statistical Methods

Data were analyzed for statistical significance by two-tailed unpaired Student's t test unless indicated otherwise. Neuronal firing-rate data were analyzed for statistical significance by Mann-Whitney rank sum test.

SUPPLEMENTAL DATA

Supplemental Data include Supplemental Experimental Procedures and four figures and can be found with this article online at http://www.cellmetabolism.org/cgi/content/full/7/4/291/DC1/.

ACKNOWLEDGMENTS

We thank G. Schmall for excellent secretarial assistance and S. Irlenbusch, J. Baumgartl, and H. Wratil for outstanding technical assistance. We also thank F. Schwenk and J. Seibler (Artemis Pharmaceuticals) for kindly providing ROSA26 β-gal mice; N. Balthasar, J.K. Elmquist, R. Coppari, and B.B. Lowell for kindly providing POMC-Cre mice; and A.F. Parlow from the NIDDK National Hormone and Peptide Program for kindly providing the ACTH antibody. This work was supported by grants from the Deutsche Forschungsgemeinschaft (DFG) (KL 762/2-2) to P.K., the Center for Molecular Medicine Cologne (TV2) and the European Union (LSHM-CT-2003-503041) to J.C.B., the Fritz Thyssen Stiftung (Az. 10.04.1.153/Az. 10.06.2.175) to J.C.B., the European Foundation for the Study of Diabetes/Lilly European Diabetes Research Programme to J.C.B., and the DFG (Br. 1492/7-1) to J.C.B.

Received: September 20, 2007
Revised: December 14, 2007
Accepted: January 17, 2008
Published: April 8, 2008

REFERENCES

Alessi, D.R., James, S.R., Downes, C.P., Holmes, A.B., Gaffney, P.R., Reese, C.B., and Cohen, P. (1997). Characterization of a 3-phosphoinositide-dependent protein kinase which phosphorylates and activates protein kinase Balpha. Curr. Biol. *7*, 261–269.

Appleyard, S.M., Hayward, M., Young, J.I., Butler, A.A., Cone, R.D., Rubinstein, M., and Low, M.J. (2003). A role for the endogenous opioid beta-endorphin in energy homeostasis. Endocrinology *144*, 1753–1760.

Balthasar, N., Coppari, R., McMinn, J., Liu, S.M., Lee, C.E., Tang, V., Kenny, C.D., McGovern, R.A., Chua, S.C., Jr., Elmquist, J.K., and Lowell, B.B. (2004). Leptin receptor signaling in POMC neurons is required for normal body weight homeostasis. Neuron *42*, 983–991.

Bjorbaek, C., Elmquist, J.K., Frantz, J.D., Shoelson, S.E., and Flier, J.S. (1998). Identification of SOCS-3 as a potential mediator of central leptin resistance. Mol. Cell *1*, 619–625.

Bornstein, S.R., and Chrousos, G.P. (1999). Clinical review 104: Adrenocorticotropin (ACTH)- and non-ACTH-mediated regulation of the adrenal cortex: neural and immune inputs. J. Clin. Endocrinol. Metab. *84*, 1729–1736.

Choudhury, A.I., Heffron, H., Smith, M.A., Al-Qassab, H., Xu, A.W., Selman, C., Simmgen, M., Clements, M., Claret, M., Maccoll, G., et al. (2005). The role of insulin receptor substrate 2 in hypothalamic and beta cell function. J. Clin. Invest. *115*, 940–950.

Coll, A.P., Farooqi, I.S., Challis, B.G., Yeo, G.S., and O'Rahilly, S. (2004). Proopiomelanocortin and energy balance: insights from human and murine genetics. J. Clin. Endocrinol. Metab. *89*, 2557–2562.

Collin, M., Backberg, M., Ovesjo, M.L., Fisone, G., Edwards, R.H., Fujiyama, F., and Meister, B. (2003). Plasma membrane and vesicular glutamate transporter mRNAs/proteins in hypothalamic neurons that regulate body weight. Eur. J. Neurosci. *18*, 1265–1278.

Cowley, M.A., Smart, J.L., Rubinstein, M., Cerdan, M.G., Diano, S., Horvath, T.L., Cone, R.D., and Low, M.J. (2001). Leptin activates anorexigenic POMC neurons through a neural network in the arcuate nucleus. Nature *411*, 480–484.

Dallman, M.F. (1984). Control of adrenocortical growth in vivo. Endocr. Res. *10*, 213–242.

De Souza, C.T., Araujo, E.P., Bordin, S., Ashimine, R., Zollner, R.L., Boschero, A.C., Saad, M.J., and Velloso, L.A. (2005). Consumption of a fat-rich diet activates a proinflammatory response and induces insulin resistance in the hypothalamus. Endocrinology *146*, 4192–4199.

Drazen, D.L., Wortman, M.D., Schwartz, M.W., Clegg, D.J., van Dijk, G., Woods, S.C., and Seeley, R.J. (2003). Adrenalectomy alters the sensitivity of the central nervous system melanocortin system. Diabetes *52*, 2928–2934.

Enriori, P.J., Evans, A.E., Sinnayah, P., Jobst, E.E., Tonelli-Lemos, L., Billes, S.K., Glavas, M.M., Grayson, B.E., Perello, M., Nillni, E.A., et al. (2007). Diet-induced obesity causes severe but reversible leptin resistance in arcuate melanocortin neurons. Cell Metab. *5*, 181–194.

Farooqi, I.S., Yeo, G.S., Keogh, J.M., Aminian, S., Jebb, S.A., Butler, G., Cheetham, T., and O'Rahilly, S. (2000). Dominant and recessive inheritance of morbid obesity associated with melanocortin 4 receptor deficiency. J. Clin. Invest. *106*, 271–279.

Farooqi, I.S., Drop, S., Clements, A., Keogh, J.M., Biernacka, J., Lowenbein, S., Challis, B.G., and O'Rahilly, S. (2006). Heterozygosity for a POMC-null mutation and increased obesity risk in humans. Diabetes *55*, 2549–2553.

Gropp, E., Shanabrough, M., Borok, E., Xu, A.W., Janoschek, R., Buch, T., Plum, L., Balthasar, N., Hampel, B., Waisman, A., et al. (2005). Agouti-related peptide-expressing neurons are mandatory for feeding. Nat. Neurosci. *8*, 1289–1291.

Hentges, S.T., Nishiyama, M., Overstreet, L.S., Stenzel-Poore, M., Williams, J.T., and Low, M.J. (2004). GABA release from proopiomelanocortin neurons. J. Neurosci. *24*, 1578–1583.

Jacobson, L. (1999). Glucocorticoid replacement, but not corticotropin-releasing hormone deficiency, prevents adrenalectomy-induced anorexia in mice. Endocrinology *140*, 310–317.

Janoschek, R., Plum, L., Koch, L., Munzberg, H., Diano, S., Shanabrough, M., Muller, W., Horvath, T.L., and Bruning, J.C. (2006). gp130 signaling in proopiomelanocortin neurons mediates the acute anorectic response to centrally applied ciliary neurotrophic factor. Proc. Natl. Acad. Sci. USA *103*, 10707–10712.

Kievit, P., Howard, J.K., Badman, M.K., Balthasar, N., Coppari, R., Mori, H., Lee, C.E., Elmquist, J.K., Yoshimura, A., and Flier, J.S. (2006). Enhanced leptin sensitivity and improved glucose homeostasis in mice lacking suppressor of cytokine signaling-3 in POMC-expressing cells. Cell Metab. *4*, 123–132.

Kim, M.S., Pak, Y.K., Jang, P.G., Namkoong, C., Choi, Y.S., Won, J.C., Kim, K.S., Kim, S.W., Kim, H.S., Park, J.Y., et al. (2006). Role of hypothalamic Foxo1 in the regulation of food intake and energy homeostasis. Nat. Neurosci. *9*, 901–906.

Kim, S.J., Winter, K., Nian, C., Tsuneoka, M., Koda, Y., and McIntosh, C.H. (2005). Glucose-dependent insulinotropic polypeptide (GIP) stimulation of pancreatic beta-cell survival is dependent upon phosphatidylinositol 3-kinase (PI3K)/protein kinase B (PKB) signaling, inactivation of the forkhead transcription factor Foxo1, and down-regulation of bax expression. J. Biol. Chem. *280*, 22297–22307.

Kitamura, T., Feng, Y., Kitamura, Y.I., Chua, S.C., Jr., Xu, A.W., Barsh, G.S., Rossetti, L., and Accili, D. (2006). Forkhead protein FoxO1 mediates Agrp-dependent effects of leptin on food intake. Nat. Med. *12*, 534–540.

Könner, A.C., Janoschek, R., Plum, L., Jordan, S.D., Rother, E., Ma, X., Xu, C., Enriori, P., Hampel, B., Barsh, G.S., et al. (2007). Insulin action in AgRP-expressing neurons is required for suppression of hepatic glucose production. Cell Metab. *5*, 438–449.

Krude, H., Biebermann, H., Luck, W., Horn, R., Brabant, G., and Gruters, A. (1998). Severe early-onset obesity, adrenal insufficiency and red hair pigmentation caused by POMC mutations in humans. Nat. Genet. *19*, 155–157.

Lawlor, M.A., Mora, A., Ashby, P.R., Williams, M.R., Murray-Tait, V., Malone, L., Prescott, A.R., Lucocq, J.M., and Alessi, D.R. (2002). Essential role of PDK1 in regulating cell size and development in mice. EMBO J. *21*, 3728–3738.

Medema, R.H., Kops, G.J., Bos, J.L., and Burgering, B.M. (2000). AFX-like Forkhead transcription factors mediate cell-cycle regulation by Ras and PKB through p27kip1. Nature *404*, 782–787.

Mercer, J.G., Hoggard, N., Williams, L.M., Lawrence, C.B., Hannah, L.T., and Trayhurn, P. (1996). Localization of leptin receptor mRNA and the long form splice variant (Ob-Rb) in mouse hypothalamus and adjacent brain regions by in situ hybridization. FEBS Lett. *387*, 113–116.

Mora, A., Komander, D., van Aalten, D.M., and Alessi, D.R. (2004). PDK1, the master regulator of AGC kinase signal transduction. Semin. Cell Dev. Biol. *15*, 161–170.

Muglia, L.J., Jacobson, L., Luedke, C., Vogt, S.K., Schaefer, M.L., Dikkes, P., Fukuda, S., Sakai, Y., Suda, T., and Majzoub, J.A. (2000). Corticotropin-releasing hormone links pituitary adrenocorticotropin gene expression and release during adrenal insufficiency. J. Clin. Invest. *105*, 1269–1277.

Nakae, J., Kitamura, T., Silver, D.L., and Accili, D. (2001). The forkhead transcription factor Foxo1 (Fkhr) confers insulin sensitivity onto glucose-6-phosphatase expression. J. Clin. Invest. *108*, 1359–1367.

Niswender, K.D., Morton, G.J., Stearns, W.H., Rhodes, C.J., Myers, M.G., Jr., and Schwartz, M.W. (2001). Intracellular signalling. Key enzyme in leptin-induced anorexia. Nature *413*, 794–795.

Niswender, K.D., Morrison, C.D., Clegg, D.J., Olson, R., Baskin, D.G., Myers, M.G., Jr., Seeley, R.J., and Schwartz, M.W. (2003). Insulin activation of phosphatidylinositol 3-kinase in the hypothalamic arcuate nucleus: a key mediator of insulin-induced anorexia. Diabetes *52*, 227–231.

Pagotto, U., Arzberger, T., Ciani, E., Lezoualc'h, F., Pilon, C., Journot, L., Spengler, D., and Stalla, G.K. (1999). Inhibition of Zac1, a new gene differentially expressed in the anterior pituitary, increases cell proliferation. Endocrinology *140*, 987–996.

Parton, L.E., Ye, C.P., Coppari, R., Enriori, P.J., Choi, B., Zhang, C.Y., Xu, C., Vianna, C.R., Balthasar, N., Lee, C.E., et al. (2007). Glucose sensing by POMC neurons regulates glucose homeostasis and is impaired in obesity. Nature *449*, 228–232.

Pinto, S., Roseberry, A.G., Liu, H., Diano, S., Shanabrough, M., Cai, X., Friedman, J.M., and Horvath, T.L. (2004). Rapid rewiring of arcuate nucleus feeding circuits by leptin. Science *304*, 110–115.

Plum, L., Ma, X., Hampel, B., Balthasar, N., Coppari, R., Munzberg, H., Shanabrough, M., Burdakov, D., Rother, E., Janoschek, R., et al. (2006). Enhanced PIP3 signaling in POMC neurons causes KATP channel activation and leads to diet-sensitive obesity. J. Clin. Invest. *116*, 1886–1901.

Plum, L., Rother, E., Munzberg, H., Wunderlich, F.T., Morgan, D.A., Hampel, B., Shanabrough, M., Janoschek, R., Könner, A.C., Alber, J., et al. (2007). Enhanced leptin-stimulated pi3k activation in the CNS promotes white adipose tissue transdifferentiation. Cell Metab. *6*, 431–445.

Pocai, A., Lam, T.K., Obici, S., Gutierrez-Juarez, R., Muse, E.D., Arduini, A., and Rossetti, L. (2006). Restoration of hypothalamic lipid sensing normalizes energy and glucose homeostasis in overfed rats. J. Clin. Invest. *116*, 1081–1091.

Rees, J.L. (2003). Genetics of hair and skin color. Annu. Rev. Genet. *37*, 67–90.

Simpson, E.R., and Waterman, M.R. (1988). Regulation of the synthesis of steroidogenic enzymes in adrenal cortical cells by ACTH. Annu. Rev. Physiol. *50*, 427–440.

Smart, J.L., Tolle, V., and Low, M.J. (2006). Glucocorticoids exacerbate obesity and insulin resistance in neuron-specific proopiomelanocortin-deficient mice. J. Clin. Invest. *116*, 495–505.

Theodoropoulou, M., Zhang, J., Laupheimer, S., Paez-Pereda, M., Erneux, C., Florio, T., Pagotto, U., and Stalla, G.K. (2006). Octreotide, a somatostatin analogue, mediates its antiproliferative action in pituitary tumor cells by altering phosphatidylinositol 3-kinase signaling and inducing Zac1 expression. Cancer Res. *66*, 1576–1582.

Williams, M.R., Arthur, J.S., Balendran, A., van der Kaay, J., Poli, V., Cohen, P., and Alessi, D.R. (2000). The role of 3-phosphoinositide-dependent protein kinase 1 in activating AGC kinases defined in embryonic stem cells. Curr. Biol. *10*, 439–448.

Xu, A.W., Kaelin, C.B., Takeda, K., Akira, S., Schwartz, M.W., and Barsh, G.S. (2005). PI3K integrates the action of insulin and leptin on hypothalamic neurons. J. Clin. Invest. *115*, 951–958.

Yaswen, L., Diehl, N., Brennan, M.B., and Hochgeschwender, U. (1999). Obesity in the mouse model of pro-opiomelanocortin deficiency responds to peripheral melanocortin. Nat. Med. *5*, 1066–1070.