pRb is an obesity suppressor in hypothalamus and high-fat diet inhibits pRb in this location

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pRb is frequently inactivated in tumours by mutations or phosphorylation. Here, we investigated whether pRb plays a role in obesity. The Arcuate nucleus (ARC) in hypothalamus contains antagonizing POMC and AGRP/NPY neurons for negative and positive energy balance, respectively. Various aspects of ARC neurons are affected in high-fat diet (HFD)-induced obesity mouse model. Using this model, we show that HFD, as well as pharmacological activation of AMPK, induces pRb phosphorylation and E2F target gene de-repression in ARC neurons. Some affected neurons express POMC; and deleting *Rb1* in POMC neurons induces E2F target gene de-repression, cell-cycle re-entry, apoptosis, and a hyperphagia-obesity-diabetes syndrome. These defects can be corrected by combined deletion of *E2f1*. In contrast, deleting *Rb1* in the antagonizing AGRP/NPY neurons shows no effects. Thus, pRb-E2F1 is an obesity suppression mechanism in ARC POMC neurons and HFD-AMPK inhibits this mechanism by phosphorylating pRb in this location.

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Introduction

The retinoblastoma protein (pRb) and its gene *RB1* (*Rb1* in mouse) were discovered by their absence in most retinoblastomas due to inherited or sporadic inactivating mutations (Weinberg, 1992). Naturally occurring mutations occur at low

frequencies; an *RB1*-deficient cell becomes detectable only when it grows up to become a tumour mass. Since mouse *Rb1*^{-/-} embryos die *in utero*, it is unlikely that an *RB1* null individual would be generated by *RB1* heterozygous parents. It is therefore unknown whether inactivation of pRb also plays roles in diseases other than in cancer.

Although retinoblastoma is a rare cancer, it is believed that pRb function is lost in most tumours since pRb function can also be inactivated by protein phosphorylation chiefly by cyclin-dependent kinases (Sherr, 1996). In theory, phosphorylation of pRb could occur in larger numbers of cells within a local environment that induces pRb phosphorylation and this kind of pRb inactivation could lead to detectable local cellular pathology with or without the tumour-like expansion of pRb-deficient cells. Here, we used this rationale to investigate whether pRb is physiologically involved in neuronal regulation of energy balance.

Anorexigenic POMC neurons and orexigenic AGRP/NPY neurons co-reside in the Arcuate nucleus (ARC) of the mediobasal hypothalamus and play antagonizing roles to maintain energy balance (Schwartz *et al*, 2000). An increase in energy store in the form of adipose tissue elevates blood concentration of adipocyte cytokine leptin. Leptin activates POMC neurons but inhibits AGRP/NPY neurons. Activated POMC neurons secrete more neuropeptide α -melanocyte stimulating hormone (α -MSH), which binds to its receptor (MC4R) on downstream neurons to inhibit feeding and promote energy expenditure. AGRP/NPY neurons secrete neuropeptide AGRP to counter α -MSH, and NPY, a most potent physiological appetite stimulator known in mammals. This combined activation of POMC neurons and inhibition of AGRP/NPY neurons corrects the increase in energy store. Inactivating mutations in leptin (*ob/ob*), its receptor (*db/db*), or the *Pomc* gene (*Pomc*^{-/-}) explain the development of obesity in the most satisfying fashion, but they account for rare cases of obesity in humans.

High-fat diet (HFD)-induced obesity (DIO) provides a mechanistically distinct obesity model since it is associated with, counter-intuitively, elevated levels of leptin. This model suggests the development and presence of leptin resistance as the cause of obesity and mimics the vast majority of human obesity cases. Many studies have provided insights into the causes of leptin resistance; some of them reveal that HFD can affect neurons in ARC (Munzberg and Myers, 2005).

For example, after 16 weeks of HFD, leptin-activated pStat3 signalling was reduced and SOCS3, an inhibitor of leptin receptor signalling, was increased in ARC neurons (Munzberg *et al*, 2004). In another example, HFD led to gliosis as a reaction to neuron injury in ARC (Thaler *et al*, 2012). Increases in ARC neuron apoptosis in response to HFD has also been reported (Moraes *et al*, 2009), which might lead to the observed 25% loss of POMC neurons after 8 months of HFD (Thaler *et al*, 2012). On the other hand, while the DIO model shows leptin resistance, treatment with CNTF (ciliary neurotrophic factor) was able to achieve and maintain

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reduced body weight because it stimulated neurogenesis in ARC (Kokoeva *et al*, 2005). HFD reduced ARC neurogenesis as determined by reduced numbers of BrdU-labelled cells (Li *et al*, 2012; McNay *et al*, 2012) and Sox-2-labelled cells (Li *et al*, 2012), further suggesting hypothalamus neurogenesis being important for negative energy balance. In a similar study, however, HFD increased ARC neurogenesis, and depleting proliferating cells with focal radiation protected the mice from HFD-induced obesity, implicating ARC neurogenesis in promoting positive energy balance (Lee *et al*, 2012). These findings uncovered connections between HFD and ARC neuron homeostasis and, at the same time, demonstrate the pressing need for further studies.

Two best-established mechanisms of tumorigenesis following loss of pRb are de-repression of E2F transcription factors (whose target genes promote proliferation) (Trimarchi and Lees, 2002) and de-repression of Skp2 ubiquitin ligase (which targets p27 for degradation to promote survival of pRb-deficient cells) (Wang *et al*, 2010). Since some of the ARC alterations by HFD relate to cell proliferation and survival, we investigated whether pRb in ARC neurons was preferentially susceptible to effects of HFD. Postnatal neurons are generally postmitotic and pRb is expected to be in the hypophosphorylated form to repress E2F. We hypothesized that HFD may inhibit pRb function by inducing its phosphorylation. If it were functionally inhibitory, then the phosphorylation would be accompanied by de-repression of E2F target gene expression. If these effects occurred to many neurons in ARC, then they would become detectable with or without the expansion of the affected cells. These findings would then indicate that inhibition of pRb function is physiologically involved in HFD DIO. Here, we report that HFD could induce pRb phosphorylation and E2F target gene de-repression in most neurons in ARC.

The co-residence of antagonizing POMC and AGRP/NPY neurons in ARC poses a logistic question about the purpose of pRb phosphorylation in this location. If HFD indiscriminately induces pRb phosphorylation in ARC neurons and POMC and AGRP/NPY neurons were similarly compromised by pRb phosphorylation, which direction would the energy balance be tilted? These questions must be addressed by neuron-specific inactivation of pRb, which has been made possible by previously established *Pomc-Cre* and *Agrp-Cre* lines. Here, we used these lines to delete *Rb1* in POMC and AGRP/NPY neurons, and found that *Rb1* is required for maintaining POMC neurons but is dispensable in AGRP/NPY neurons. Thus, the outcome of widespread phosphorylation and inhibition of pRb in ARC would reflect the impairment of POMC neurons.

Finally, we investigated the functional mechanism for the required role of *Rb1* in POMC neurons. We show that *E2f1* is not required for the development and maintenance of POMC neurons but must be repressed by *Rb1* for POMC neuron maintenance. Together, these studies suggest that pRb-E2F1 is a uniquely important mechanism in POMC neurons in ARC, is vulnerable to HFD effects in this location, and is an attractive intervention target for the ill effects of HFD in ARC.

Results

Long-term HFD induces pRb phosphorylation in ARC neurons

pRb contains 16 Ser or Thr residues in phosphorylation consensus sequences for cyclin-dependent kinases (CDK,

S/TPxK/R, 'x' is any residue), 8 of them have been shown to modulate pRb-E2F interaction. pRb sequences can be divided into three parts: the N terminus (1–380, human pRb sequences), the Pocket (380–787), and the C terminus (787–928). Mapping experiments and X-ray crystal structures show that pRb Pocket interacts with the transactivation domain (TD) at the C termini of E2F proteins, and pRb C terminus interacts with the Coiled Coil and Marked Box domains (CM) in the centre of E2F proteins. Phosphorylation of the T356/T373 cluster and the S608/S612 cluster dissociates the Pocket–TD interaction, while phosphorylation of the S788/S795 cluster and the T821/T826 cluster destabilizes the C terminus–CM interaction (Rubin *et al*, 2005). Overall, however, phosphorylation of pRb is a progressive and cumulative process through various combinations of phosphorylation sites, resulting in progressive and cumulative disruption of pRb-E2F interactions leading to gradual activation of E2F in cell-cycle progression from G1 to S phase.

We used three male C57BL6 mice that had been on HFD for 11 months to determine whether pRb phosphorylation in ARC was altered compared with three age- and sex-matched mice on Chow diet. BW was 55–60 g for HFD mice and 30–35 g for Chow mice. We used a rabbit antibody specific for Ser608 phosphorylated pRb to perform immunofluorescence staining as previously reported (Kim *et al*, 2007). pRb is a nuclear protein; positive staining by the pRbS608p antibody is nuclear. As shown in Figure 1B, pRbS608p antibody showed widespread nuclear staining in ARC of the HFD mice that merged with most of the co-stained NeuN (a neuronal nuclear marker) positive nuclei. In comparison, control Chow mice showed few positive staining that did not merge with NeuN (Figure 1A). We found that this HFD-associated pRb phosphorylation is enriched in ARC. As an example, the granular zone of dentate gyrus (DG) contains densely packed neurons as shown by NeuN stain; but none of them showed positive co-stain with pRbS608p in the same HFD brain sections (Supplementary Figure S1A). When pRbS608p co-staining was performed with a GFAP antibody (an astrocyte marker), most nuclear pRbS608 staining in HFD brains did not merge with GFAP staining (Supplementary Figure S1B). We conclude that long-term HFD induced pRb phosphorylation in ARC neurons, but not in DG neurons.

Pharmacological activation of AMPK induces pRb phosphorylation and E2F de-repression in ARC

HFD induces a wide spectrum of effects, including locally in ARC such as inflammatory injury (Thaler *et al*, 2012). We considered the possibility that these tissue injuries might result in non-specific binding of the pRbS608p antibody to ARC. We sought to identify a stimulus that could induce pRb phosphorylation in ARC independent of HFD but might mediate pRb phosphorylation downstream of HFD. We considered AMPK as a candidate. In Chow mice, leptin can inhibit AMPK in ARC as one mechanism to inhibit feeding but in HFD-induced obesity, leptin lost this ability resulting in constitutively activated AMPK (Martin *et al*, 2006; Steinberg *et al*, 2006). Conversely in CaMKK2 knockout mice, hypothalamic activation of AMPK is defective, and these mice are resistant to HFD-induced obesity (Anderson *et al*, 2008). AMPK substrates include pRb (Dasgupta and Milbrandt, 2009). AMPK can be specifically activated by

Figure 1 Phosphorylation of pRb and de-repression of cyclin A and E2F1 in ARC neurons in response to HFD or AICAR. (A, B) ARC from C57/Bl wild-type mice fed on HFD for 11 months and matched mice fed on Chow co-stained with antibodies to pRbS608p and NeuN, as marked. (C) ARC from wild-type mice at 2 and 6 h after third ventricle injection of ACSF or AICAR stained with antibodies to pRbS608p, E2F1, or cyclin A, as marked. (D) ARC from *Pomc-Cre; RosaYFP* mice fed on HFD or Chow for 8 weeks following their 1 month birthday were stained with the indicated antibodies. Direct YFP was photographed in green. (E) Quantification of stain-positive cells versus YFP-positive cells in ARC presented as %, from samples in (D) and Supplementary Figure S2C ($n = 3$). Data are expressed as average $\pm$ s.e.m. Unpaired *t*-tests were performed, * $P < 0.05$, ** $P < 0.005$ and *** $P < 0.0005$. Scale bars: 100 μ m.

AICAR, which can be administered directly to the ARC of Chow mice via third ventricle intra-cerebral-ventricular (i.c.v.) injection.

Also, if pRbS608p antibody detected true pRb phosphorylation in ARC, positive staining by pRbS608p antibody might be accompanied by detection of E2F target

gene de-repression as functional consequences of pRb inactivation.

We therefore performed AICAR i.c.v. experiments with Chow mice and examined ARC at 2 and 6 h time points for positive staining with the pRbS608p antibody and with antibodies to cyclin A and E2F1, two established E2F target genes (Figure 1C). The results show that pRbS608p staining was negative in control ACSF injection at both time points and in AICAR injection at 2 h point. At 6 h, AICAR-injected ARC showed positive pRbS608p staining. Further, both negative and positive pRbS608p staining correlated with negative and positive staining with cyclin A and E2F1 antibodies in consecutive sections. We also performed ISH for cyclin A and observed positive staining in ARC at 6 h post AICAR injection but not post ACSF injection, while some positive staining was observed in DG in both AICAR- and ACSF-injected brains (Supplementary Figure S1C). These findings document that AICAR can induce positive staining of ARC with the pRbS608p antibody independent of HFD and this positive staining was associated with positive staining for E2F target genes E2F1 and cyclin A.

Since hypothalamus AMPK activity is regulated by fasting and refeeding (Minokoshi *et al*, 2004), our AICAR injection experiments suggested that ARC of fasted mice should contain more phosphorylated pRb than ARC of refed mice. Experiments shown in Supplementary Figure S1D tested and confirmed this expectation. Positive staining by the pRbS608p antibody of ARC was observed after a 24-h fasting, which was, remarkably, reverted to baseline negative staining following a 3-h refeeding.

Previous studies showed that ARC AMPK is constitutively active in HFD-induced obesity; our findings show that pharmacological activation of AMPK can induce pRb phosphorylation in ARC, suggesting that HFD might induce pRb phosphorylation via AMPK. S804 (S811 in human) of pRb is in a combined CDK and AMPK phosphorylation consensus (IxxSPxxKI, 'P' and 'K' are CDK consensus; 'T' and 'P' are AMPK consensus). Immunopurified AMPK can phosphorylate S804 (S811 in human) of pRb *in vitro* (Dasgupta and Milbrandt, 2009). However, structural studies of pRb-E2F interaction suggested that phosphorylation of S811 did not affect pRb-E2F binding (Rubin *et al*, 2005). It is likely that AMPK initiates pRb phosphorylation, while cyclin-dependent kinases perform most of the phosphorylation on sites that disrupt pRb-E2F interaction to induce de-repression of E2F target gene expression.

HFD induces pRb phosphorylation and E2F de-repression in ARC POMC and AGRP/NPY neurons

We generated *Pomc-Cre;RosaYFP* mice (on mixed strain background) for HFD to determine the identities of ARC neurons with pRb phosphorylation by HFD. *Pomc-Cre;RosaYFP* marks POMC neurons and up to a quarter of AGRP/NPY neurons in ARC (Padilla *et al*, 2010). We also determined the length of HFD necessary to induce pRb phosphorylation in ARC and whether pRb phosphorylation was associated with E2F target gene de-repression. We started HFD when the mice reached the age of 4 weeks. At the end of 8-week treatment, HFD mice gained on average 10% more weight than matched Chow mice. As shown in Figure 1D, positive staining for pRbS608p, E2F1, and cyclin A was detected in ARC at 8 weeks after HFD but not Chow. Similarly to AICAR 3v i.c.v., increased cyclin A

expression in ARC, but not in DG, was also detected by ISH (Supplementary Figure S2A). With another set of wild-type mice on Chow or HFD for 8 weeks, we detected increased E2F1 expression on western blot of their hypothalami using an anti-E2F1 antibody that was validated for its ability to detect increased E2F1 expression following deletion of *Rb1* in *E2f1* WT MEF but not in *E2f1* KO MEF (Supplementary Figure S2B).

Quantification and time course experiments (Figure 1E; Supplementary Figure S2C) showed gradually increased staining for these three antibodies from 1 week of HFD to about half of the *Pomc-Cre;RosaYFP* marked ARC neurons. We noted that positive staining for E2F1 and cyclin A showed faster kinetics than pRbS608p staining, suggesting that de-repression of certain E2F target genes could take place when pRb was partially phosphorylated. These results reveal that pRb function could be progressively inhibited in ARC neurons marked by *Pomc-Cre* in response to HFD in a timeframe of 2 months.

***Rb1* is required for POMC neuron maintenance**

Our finding that HFD induced pRb phosphorylation and E2F target gene de-repression in ARC POMC and some AGRP/NPY neurons suggests that pRb inactivation may be an important causal mechanism for ARC neuron malfunction in obesity development by HFD. Alternatively, pRb phosphorylation and E2F de-repression could be secondary to neuron injuries by HFD as described in Introduction. To distinguish between these two alternatives, we used targeted *Rb1* deletion to directly determine the roles of *Rb1* in defined neurons in ARC of mice on Chow.

Previous studies suggested that pRb and its family member p107 function to activate the *Pomc* promoter (Batsche *et al*, 2005a, b). We first deleted *Rb1* in pan-neuronal progenitors using *Nestin-Cre;Rb1^{lox/lox}*, and found that *Rb1* was not required for the activation of *Pomc* gene expression in embryogenesis (unpublished results). We then generated *Pomc-Cre;Rb1^{lox/lox}* mice, which were born in normal Mendelian frequency. Assuming that POMC expression continued after *Rb1* deletion, we used POMC IHC to visualize POMC neurons in ARC. As shown in Figure 2A, POMC neurons in *Pomc-Cre;Rb1^{lox/lox}* mice appeared normal at 1 week of age. However, POMC neuron numbers in ARC reduced significantly from 4 weeks of age (Figure 2A and B), and the remaining POMC neurons progressively increased their soma sizes (Figure 2A and C). It is of note that anti-POMC IHC experiments do not detect POMC neurons that stopped expressing POMC following *Rb1* deletion. We addressed this issue with *RosaYFP* marking in Supplementary Figure S3G and H and some of the later studies below.

We also used POMC stain to investigate whether *Rb1*-deficient POMC neurons were able to extend axons to arborize PVH, which is located anterior (~2 mm) and rostral (~1 mm) to ARC. Figure 2D shows that the characteristic outlines of PVH, as visualized by POMC stain, were indistinguishable between *Pomc-Cre;Rb1^{lox/lox}* and control mice, but the stain intensity was significantly reduced (Figure 2D and E). Similar results were obtained for aborization to DMH, which is rostral (~1 mm) to ARC. To quantify POMC expression output, we performed RT-qPCR on hypothalamus and found that POMC expression was slightly elevated at 1 week of age but significantly reduced by 5 weeks of age

Figure 2 *Rb1* is required for POMC neuron maintenance. (A) ARC POMC neurons were visualized by anti-POMC stain in indicated mice; ARC sections were from Bregma -1.75 to -1.8 mm. (B) POMC neuron numbers were counted on sections at comparable Bregma positions. ARC1 at about -1.34 , ARC2 about -1.58 , ARC3 about -1.75 ($n=3$). (C) Soma areas of ARC POMC neurons were measured in 1-, 4-, 8-week-old mice ($n=3$). (D) POMC immunoreactive axon fibres in PVH of 8-week-old mice and their quantification based on POMC stain density ($n=3$). (E) Control mice densities were set as 1.0. (F) POMC expression was determined by RT-qPCR of hypothalamus ($n=6$). POMC/Actin ratios in control mice were set as 1.0. (G) Hypothalamus content of POMC pre-peptide, α -MSH, and β -endorphin ($n=6$). Data are expressed as average $\pm$ s.e.m. Unpaired *t*-test was performed, * $P<0.05$, ** $P<0.005$, *** $P<0.0005$. Scale bars: 200 μ m.

(Figure 2F). We measured the amounts of POMC pre-peptide and its proteolytically processed products, α -MSH and β -endorphin, in the hypothalamus. Figure 2G shows that the amounts of these peptides were reduced, but remained proportional to the reduction in POMC pre-peptide therefore suggesting that these proteolytic processing steps were preserved in the absence of *Rb1*. The consequences of *Rb1* deletion in AGRP/NPY neurons were examined in Figure 6 below.

The *Pomc-Cre* line expresses Cre in a number of sites in addition to ARC, including the pituitary gland, the NTS, and the DG. We used *Pomc-Cre;RosaYFP* to examine the effects of *Rb1* deletion in these additional sites and found that deleting *Rb1* in pituitary melanotrophs is tumorigenic as expected, while POMC neurons in NTS and newborn neurons in DG appear unaffected by *Rb1* deletion (please see Supplementary Figure S3 for more details). Thus, the observed effects of *Rb1* deletion in POMC neurons are neuron type specific.

We conclude that *Rb1* is not required for ARC POMC neuron development but plays important maintenance roles in them starting at a young age. Without *Rb1*, POMC neurons undergo progressive loss while maintaining the ability to express POMC, to process POMC pre-peptide, and to arborize distant sites. Nevertheless, due to reduction in POMC neuron numbers, POMC output after 1 week of age was significantly reduced.

***Pomc-Cre;Rb1^{lox/lox}* mice develop a hyperphagia-obesity-diabetes syndrome**

We determined the effects of *Pomc-Cre* mediated deletion of *Rb1* on energy balance. Figure 3A shows that *Pomc-Cre;Rb1^{lox/lox}* mice were significantly heavier than control mice from 1 month of age. Since increases in fat % (Figure 3B) can mostly account for the increases in body weight, these mice were obese, although they were also larger

Figure 3 *Pomc-Cre;Rb1^{lox/lox}* mice developed a hyperphagia-obesity-diabetes syndrome, and lost melanocortinergic tone in the hypothalamus. (A) Body weights of *Rb1^{lox/lox}* and *Pomc-Cre;Rb1^{lox/lox}* mice ($n = 5-12$ for various age points). (B) Fat % of the mice in (A). (C) Nasoanal length. (D) Daily food intake during light and dark cycles ($n = 4$). (E) Following an overnight fasting, tail vein blood was collected to measure leptin levels ($n = 5$). (F) Fasting serum insulin concentration ($n = 4-8$). (G) Fasting blood glucose concentration ($n = 6-8$). (H) Glucose tolerance test was performed at 4 months of age ($n = 3$). (I) Cumulative food intake measured for 6 h following i.c.v. administration of ACSF or SHU9119 ($n = 5-6$). (J) Cumulative food intake measured for 3.5 h following i.c.v. administration of ACSF or PYY ($n = 5-6$). (K) Cumulative food intake in response to i.c.v. administration of ACSF or MTII ($n = 5-6$). Data are expressed as average $\pm$ s.e.m. Unpaired t -tests were performed, $*P < 0.05$, $**P < 0.005$ and $***P < 0.0005$. For (I-K), # refers to differences in the same genotypes performed with paired t -test, $^{\#}P < 0.05$ and $^{\#\#}P < 0.005$.

as shown by nasoanal length (Figure 3C). Food intake was significantly increased in these mice (Figure 3D), providing an explanation for body weight gain. Serum leptin concentrations were greatly increased (Figure 3E), consistent with their high fat % and indicating a leptin-resistant state. Obesity is often associated with diabetes; insulin concentrations were increased starting at 2 months of age (Figure 3F). By 4 months, *Pomc-Cre;Rb1^{lox/lox}* mice exhibited frank diabetes with high blood glucose levels (Figure 3G) and glucose intolerance (Figure 3H). These characteristics of *Pomc-Cre;Rb1^{lox/lox}* mice suggest that targeted deletion of *Rb1* in ARC POMC neurons could essentially reproduce HFD DIO.

Although the above body phenotypes of *Pomc-Cre;Rb1^{lox/lox}* mice were consistent with POMC neuron defects by *Rb1* deletion in them, we cannot be certain that these defects

were completely responsible for the body phenotypes since defects in other cells that experienced *Pomc-Cre* expression (see Supplementary Figure S3) could be involved. In Supplementary Figure S4, we show that *Pomc-Cre;Rb1^{lox/lox};p27^{T187A/T187A}* mice and *Pomc-Cre;Rb1^{lox/lox};Skp2^{+/-}* mice did not develop pituitary tumours but still show POMC neuron defects and obesity, indicating that POMC neuron defects, rather than pituitary tumours, caused obesity in *Pomc-Cre;Rb1^{lox/lox}* mice.

We determined the functional status of the POMC axis in hypothalamus regulation of food intake in *Pomc-Cre;Rb1^{lox/lox}* mice. To do so, we measured the responses of *Pomc-Cre;Rb1^{lox/lox}* mice to third ventricle i.c.v. of melanocortin receptor antagonist SHU9119. We found that injection of SHU9119 increased food intake in control mice (Figure 3I),

demonstrating that there is normally a melanocortin-mediated inhibition of food intake (i.e., presence of a melanocortinergic tone). In comparison, same amount of SHU9119 did not increase food intake in *Pomc-Cre;Rb1^{lox/lox}* mice, indicating a lack of melanocortinergic tone in them. To test whether the mutant mice could still be stimulated to increase food intake, we injected the orexigenic peptide YY (PYY) (Hagan, 2002) and observed a significant increase in food intake in both groups (Figure 3J). These findings demonstrate that the central melanocortin system was functioning inadequately in *Pomc-Cre;Rb1^{lox/lox}* mice.

To test the functions of α -MSH target neurons in *Pomc-Cre;Rb1^{lox/lox}* mice, we injected melanocortin receptor agonist MT-II. Since MT-II administration significantly suppressed food intake in both genotypes (Figure 3K), melanocortin target neurons in *Pomc-Cre;Rb1^{lox/lox}* mice remained functional. These findings, together with well-established roles of POMC neurons for negative energy balance, built a compelling case that reduction in α -MSH levels in the hypothalamus, as a result of POMC neurons defects by *Rb1* deletion, caused obesity of *Pomc-Cre;Rb1^{lox/lox}* mice.

***Rb1* is required to maintain ARC POMC neurons postmitotic postnatally**

To study the cellular mechanisms of pRb function in POMC neurons, we performed Ki67 staining to determine the proliferation status of *Pomc-Cre;RosaYFP* marked ARC neurons. In newborns (P 0), we detected a few Ki67-positive cells in control and *Pomc-Cre;RosaYFP;Rb1^{lox/lox}* mice, some of which merged with YFP-positive cells (Figure 4A and B). These results indicate that most cells in ARC have exited cell cycle at birth and deletion of *Rb1* did not prevent cell-cycle exit of POMC neurons. Remarkably, a burst of Ki67-positive cells emerged in the ARC of 1-week-old *Pomc-Cre;RosaYFP;Rb1^{lox/lox}* mice, while control littermates remained mostly negative for Ki67 in this region. The majority of Ki67-positive cells merged with YFP-positive cells, and amounted to >40% of YFP-positive cells (Figure 4C, D, and I). This finding reveals that *Rb1* is required for maintaining ARC POMC neurons in a postmitotic state postnatally. This Ki67-positive status persisted at 3 and 6 weeks of age (Figure 4E–I).

We next examined postnatal born neurons in DG, which enter and exit the cell cycle postnatally. Results in Figure 4J and K revealed Ki67-positive cells in the SGZ (these cells are being born and *Pomc-Cre* is not yet expressed in them). After they were born, *Pomc-Cre* is expressed in them and the newborn neurons are permanently labelled with *RosaYFP* in *Pomc-Cre;RosaYFP* mice (Supplementary Figure S3K). Since, unlike *RosaYFP* labelling, there are no Ki67-positive cells in GCL of *Pomc-Cre;RosaYFP;Rb1^{lox/lox}* mice, *Rb1* is not required for maintaining the postmitotic status of these postnatal born neurons. Thus, cell-cycle re-entry is also a neuron type-specific effect of *Rb1* deletion with *Pomc-Cre*.

We performed staining for pHH3 to determine whether cell-cycle re-entry of ARC neurons progressed to mitosis but did not detect any (data not shown). We observed with *Pomc-Cre;RosaYFP* and DAPI staining that the enlarged neurons in *Pomc-Cre;RosaYFP;Rb1^{lox/lox}* mice contained enlarged nuclei (Figure 4L and M). These two types of results suggest that cell-cycle re-entry of ARC POMC neurons failed to progress to cell division.

We performed staining for Caspase3a to determine whether the reduction in ARC POMC neurons was associated with apoptosis. Results shown in Figure 4P–R reveal increases in Caspase3a-positive cells (overlapping with *RosaYFP*-positive cells) in the ARC of 1- to 2-week-old *Pomc-Cre;RosaYFP;Rb1^{lox/lox}* mice. Since Caspase3a-positive cells were not detected in P0 *Pomc-Cre;RosaYFP;Rb1^{lox/lox}* mice, neuron apoptosis at 1 week of age was associated with the abnormal and blunted cell-cycle re-entry. We conclude from these experiments that *Rb1* is required for maintaining ARC POMC neurons postmitotic postnatally. However, cell-cycle re-entry of *Rb1*-deficient POMC neurons does not lead to neuron proliferation but to cell-cycle block in G2/M and apoptosis.

Repression of *E2F1* is a major function of pRb in POMC neurons

A large number of pRb targets have been identified. When a pRb target is physiologically relevant, its deregulation following *Rb1* loss would be responsible for the defects caused by *Rb1* deletion. Here, we investigated whether deregulation of the two best-established *Rb1* targets, pRb-E2F and pRb-Skp2-p27, are the underlying mechanisms for POMC neuron defects. We introduced the p27^{T187A/T187A} knockin mutation (p27^{T187A/T187A}) or deleted one allele of *Skp2* (*Skp2^{+/-}*) in *Pomc-Cre;Rb1^{lox/lox}* mice and found that pituitary tumorigenesis was blocked as expected (Supplementary Figure S4Ba and Ca, compared with Aabc). The pituitary IL was mostly ablated in *Pomc-Cre;Rb1^{lox/lox};p27^{T187A/T187A}* mice, but was mostly retained at the WT sizes in *Pomc-Cre;Rb1^{lox/lox};Skp2^{+/-}* mice. In both cases, the POMC neuron losses (Supplementary Figure S4Bbc, D, Cbc and G) and obesity phenotypes (Supplementary Figure S4E, F, H, and I) were not corrected. These findings indicate that *Skp2* deregulation was not the mechanism for, and pituitary tumours was not the cause of, obesity of *Pomc-Cre;Rb1^{lox/lox}* mice.

E2F is a family of eight members. pRb primarily binds and represses the ‘activator’ sub-family of E2F1, 2, and 3, whose target genes include those encoding for DNA replication enzymes and cyclin-dependent kinases. Figure 5A, B, D–F, and H shows that expression of the DNA replication licensing factor MCM3 and cyclin A, both are E2F target genes, was not detectable in ARC of 1-week-old control mice but became positive in about half of *RosaYFP* marked cells in *Pomc-Cre;RosaYFP;Rb1^{lox/lox}* mice, demonstrating that E2F target genes were postnatally de-repressed when *Rb1* was absent.

E2F1 is special in being able to induce apoptosis in addition to S-phase entry. Since POMC neurons in *Pomc-Cre;Rb1^{lox/lox}* mice exhibited S-phase re-entry and apoptosis, we generated *Pomc-Cre;Rb1^{lox/lox};E2f1^{-/-}* mice to test the hypothesis that repression of E2F1 is the major functional mechanism of pRb in POMC neurons. Figure 5C, D, G and H shows that ectopic expression of the E2F target genes following *Rb1* deletion was largely (MCM3) or completely (cyclin A) corrected with combined deletion of *E2f1*. We further show that cell-cycle re-entry of POMC neurons at 1 week of age in *Pomc-Cre;Rb1^{lox/lox}* mice was completely prevented by combined deletion of *E2f1* (Figure 5I–L). Caspase3a, which was increased in ARC POMC neurons following *Rb1* deletion (Figure 4P–R) was undetectable in the ARC of *Pomc-Cre;Rb1^{lox/lox};E2f1^{-/-}* mice (data not shown). When POMC neurons were examined directly with

Figure 5 pRb maintained POMC neurons postmitotically postnatally by repressing E2F1. ARC sections with YFP (pictured in green) and anti-MCM3 (A–C) or anti-cyclin A (E–G) stain of the indicated mice at 1 week of age. Consecutive sections of the same brains were stained with anti-Ki67 (I–K). (D, H, L) Quantification of stain-positive cells as ratio of YFP-positive cells ($n = 3$). (M–P) ARC POMC neurons of the indicated mice were visualized by POMC stain and counted ($n = 3$) (Q). Scale bar: 100 μ m. Data are expressed as average $\pm$ s.e.m. Unpaired t -test was performed * $P < 0.05$, *** $P < 0.0005$.

POMC staining, the results show that the reduction in POMC neuron numbers (Figure 5M–Q), in POMC neuron axon projection to PVH (Supplementary Figure S5A–D), and in POMC expression in hypothalamus (Supplementary Figure S5E) were all completely or nearly completely corrected by combined deletion of *E2f1*.

Deletion of *E2f1* delayed pituitary tumorigenesis in *Rb1^{+/-}* mice (Yamasaki *et al*, 1998). In *Pomc-Cre; Rb1^{lox/lox}; E2f1^{-/-}* mice, pituitary IL was of WT sizes at 8 weeks of age (Supplementary Figure S5Fb, compare Supplementary Figure S4Aabc). When examined at 6 months of age, however, *Pomc-Cre; Rb1^{lox/lox}; E2f1^{-/-}* mice still developed macroscopic IL tumours. These findings together demonstrate that *Rb1* represses *E2f1* to suppress tumorigenesis in melanotrophs in

the pituitary and to maintain POMC neurons postnatally in the hypothalamus. Importantly, *E2f1* KO mice show normal pituitary (Supplementary Figure S5Fa), normal POMC expression in hypothalamus (Supplementary Figure S5E), and normal POMC neuron appearance and numbers in ARC (Figure 5O and Q). Thus, *E2f1* is not required for the development and maintenance of melanotrophs and POMC neurons, but must be repressed by pRb to prevent melanotroph tumorigenesis and to maintain POMC neurons.

Combined *E2f1* deletion also prevented obesity development in *Pomc-Cre; Rb1^{lox/lox}* mice (Supplementary Figure S5G). This corrective effect was nearly complete for body weight and fat % in 1-month-old mice. Although *Pomc-Cre; Rb1^{lox/lox}; E2f1^{-/-}* mice still showed significant fat

Figure 6 *Rb1* is dispensable in AGRP/NPY neurons. The role of *Rb1* in AGRP/NPY neurons was studied in three lines of mice. (A–C) NPY immunoreactive axons in PVH of 4-month-old *Pomc-Cre;Rb1^{lox/lox}* and control mice, and quantification of density of NPY stained axon fibres in PVH and DMH ($n = 3$). (D) AGRP expression in hypothalamus was measured by RT-qPCR in 1- and 5-week-old *Pomc-Cre;Rb1^{lox/lox}* and control mice ($n = 3$). Values in control mice were set as 1.0. (E, F) ARC sections of the indicated genotypes. AGRP/NPY neurons were visualized with direct YFP (pictured in green). (G–I) NPY immunoreactive axons in PVH of 6-month-old mice of the indicated genotypes and quantification of density of NPY stained axon fibres in PVH and DMH ($n = 3$). (J) AGRP and NPY expression was determined by RT-qPCR ($n = 6$). Values in control mice were set as 1.0. (K) Body weight and fat % of 2-month-old male mice on Chow. (L) Body weight and fat % of 3-month-old male mice that were on HFD for the last 2 months. (M) Body weight and fat % of 7-week-old female mice of the indicated genotypes ($n = 3–6$). (N) Single cell RT-PCR detected *Rb1* expression in POMC neurons and AGRP/NPY neurons. Data are expressed as average $\pm$ s.e.m. ** $P < 0.005$, *** $P < 0.0005$. Scale bars: 100 μ m.

accumulation compared to control mice when they grew to 2 and 3 months of age, the correction from *Pomc-Cre;Rb1^{lox/lox}* mice remained significant at 2 months and just missed significance ($P = 0.056$) at 3 months. The correction of body weight gain appeared more durable in these periods. The exact mechanisms for these body phenotype changes, however, could be multi-fold, since *E2f1* deletion also delayed pituitary tumorigenesis and *E2f1^{-/-}* mice showed low adiposity on Chow feed and resisted obesity development on HFD (Fajas et al, 2002).

Deletion of *Rb1* in AGRP/NPY neurons does not cause neuron defects or body weight changes

Since a quarter of AGRP/NPY neurons originate from *Pomc*-expressing progenitors (Padilla et al, 2010) and therefore also

incurred *Rb1* deletion in *Pomc-Cre;Rb1^{lox/lox}* mice, we first examined the status of AGRP/NPY neurons in *Pomc-Cre;Rb1^{lox/lox}* mice. Figure 6A–C shows that NPY stain in the projection sites in PVH was not different between testing and control mice. Merge of immunofluorescence co-staining for POMC and NPY demonstrated reduction of POMC stain, but not NPY stain, in the ARC of *Pomc-Cre;Rb1^{lox/lox}* mice (Supplementary Figure S6A). RT-qPCR revealed that expression of NPY and AGRP was not different in *Pomc-Cre;Rb1^{lox/lox}* mice compared to control mice (Figure 6D; Supplementary Figure S6B). These findings provided the first indications that *Rb1* deletion by *Pomc-Cre* affected POMC neurons but spared AGRP/NPY neurons.

We next used an *Agpr-Cre* line to delete *Rb1* in AGRP neurons. In Figure 6E and F, *RosaYFP* showed no cell number

reduction or size enlargement in the ARC of *Agrp-Cre;RosaYFP;Rb1^{lox/lox}* mice. NPY stain did not decrease in PVH of *Agrp-Cre;Rb1^{lox/lox}* mice (Figure 6G–I), and RT-qPCR did not reveal any reduction in expression levels of NPY or AGRP in the hypothalamus of *Agrp-Cre;Rb1^{lox/lox}* mice (Figure 6J). AGRP/NPY neurons maintained their postmitotic state in the absence of *Rb1* (data not shown). Body weight and fat % were normal in *Agrp-Cre;Rb1^{lox/lox}* mice on Chow (Figure 6K), and they gained similar amount of body weight and fat on HFD (Figure 6L). Thus, *Rb1* is dispensable for AGRP/NPY neuron maintenance and, consistently, its loss in AGRP/NPY neurons had no disruptive effects on energy balance.

To further determine the role of *Rb1* in body weight control by the antagonizing POMC neurons and AGRP/NPY neurons, we deleted *Rb1* in both of them; and found that *Pomc-Cre;Agrp-Cre;Rb1^{lox/lox}* mice were as obese as *Pomc-Cre;Rb1^{lox/lox}* mice (Figure 6M). These results suggest that if pRb was inactivated in ARC indiscriminately of anorexigenic POMC neurons and orexigenic AGRP/NPY neurons, such as by HFD induced pRb phosphorylation (Figure 1) the consequence would be obesity.

To investigate the possibility that *Rb1* was not expressed in AGRP/NPY neurons, as an explanation for its dispensability in them, we used *Pomc-GFP* and *Npy-GFP* mice to mark and isolate POMC and AGRP/NPY neurons, respectively, for single cell RT-PCR to detect *Rb1* expression. This experiment detected *Rb1* expression in both types of neurons (Figure 6N). Another possibility for further study is that pRb family member p107/p130 may express highly in AGRP/NPY neurons to compensate for the loss of *Rb1*.

Discussion

DIO mouse model mimics the majority of human obesity cases in that the obesity is caused by over-nutrition and increased leptin is unable to produce an anorexigenic effect. Recent research has begun to connect these two aspects by showing that HFD can cause injuries to ARC neurons. Since ARC neurons play important roles in the regulation of food intake and energy expenditure, a better understanding of how HFD causes injuries to them may hold the key to a better understanding of how HFD induces obesity. Our discovery that HFD can inhibit pRb and de-repress E2F target genes in ARC neurons links HFD to perhaps the most fundamental mechanism for maintaining cellular homeostasis. The distinctly different roles of *Rb1* in anorexigenic POMC neurons (essential for maintenance) and orexigenic AGRP/NPY neurons (not required for maintenance) provide a straightforward basis on which HFD's induction of pRb phosphorylation in ARC could promote obesity.

The tumour suppressor role of pRb in pituitary melanotrophs provides a contrast for us to study the non-tumour suppressor role of pRb in ARC neurons in the same *Pomc-Cre;Rb1^{lox/lox}* mice. In the absence of *Rb1*, POMC neurons still properly differentiated and exited the cell cycle at birth. However, these cells were unable to maintain their postmitotic state and re-entered the cell cycle within 1 week after birth. This defect is consistent with the tumour suppressor function of *Rb1*. The events following cell-cycle re-entry in POMC neurons are opposite from the tumour suppressor role of pRb. Instead of progressing to cell division

and expansion, *Rb1*-deficient POMC neurons show enlarged nuclei and apoptosis.

Further studies using the 'rescue-by-combined-deletion' approach help shed light on the outcomes of *Rb1* deletion in POMC neurons. Since E2F1, 2, and 3 (the three members in the activating E2F subgroup) share overlapping functions and similar physical interactions with pRb, the near complete correction of POMC neuron defects by *E2f1* deletion would suggest that *E2f2* and *E2f3* might not be expressed in POMC neurons and/or might not increase their expression to compensate for the loss of *E2f1*. *E2f1* therefore is the major source of activating E2F activity in POMC neurons. However, *E2f1* KO mice do not show detectable defects in ARC POMC neurons, indicating that *E2f1* is not required for POMC neuron development and maintenance, or *E2f1*'s role in these aspects of POMC neurons can be compensated by other E2F members. It is therefore possible that, when E2F1 is de-repressed by the loss of *Rb1*, the de-repressed E2F1 may have an abnormally activated function that is not shared with other E2Fs, and is disruptive to POMC neuron homeostasis.

Indeed, apoptotic activity is generally regarded as unique to E2F1. When E2F1, 2, and 3 were ectopically expressed at similar protein levels in quiescent REF52 cells, only E2F1 could induce apoptosis (Hallstrom and Nevins, 2003). pRb contains a binding domain (792 to the C terminus) specifically for E2F1 (the binding sequences are in the N terminal 374 aa of the 437 full-length E2F1) to inhibit E2F1's apoptotic function (Dick and Dyson, 2003). We therefore propose that E2F1 might be highly expressed in POMC neurons and repression of E2F1 by pRb is a critical mechanism for maintaining POMC neurons postnatally. A notable component within this maintenance mechanism is that E2F1 is not required for POMC neuron development and maintenance. Since this pRb-E2F1 pathway appears to be vulnerable to inhibition by HFD's effects in ARC, an activated E2F1 is potentially causative of central positive energy imbalance and therefore an attractive target for the prevention and treatment of DIO.

AMPK regulates metabolism based on the cell's fuel levels. In the hypothalamus, AMPK is activated by fasting and deactivated by subsequent refeeding. Activation of AMPK in hypothalamus promotes food intake, and at least part of leptin's anorexigenic effects are mediated by inhibiting hypothalamus AMPK (Andersson *et al*, 2004; Minokoshi *et al*, 2004). In HFD-induced obesity, leptin loses this effect, leaving AMPK constitutively activated (Martin *et al*, 2006; Steinberg *et al*, 2006). Since our data show that pharmacological activation of AMPK in ARC can induce pRb phosphorylation and E2F target gene de-repression, hypothalamus AMPK might be the upstream kinase for pRb in HFD fed mice, likely function in an HFD-AMPK-pRb-E2F1 pathway in POMC neurons to promote positive energy imbalance.

Using overexpression of E2F1 in REF53 cells, the AMPK α 2 subunit was identified as an E2F1 target gene (Hallstrom *et al*, 2008). Such a link between E2F1 and AMPK, if proven in hypothalamus, together with the HFD-AMPK-pRb-E2F1 pathway discussed above, would suggest a feed-forward HFD-AMPK-pRb-E2F1-AMPK loop. AMPK could be activated to initiate this loop by low energy status signals such as fasting or by resisting downregulation under HFD. Once initiated, this loop may autonomously enhance itself

leading to higher AMPK activity to promote eating and higher E2F1 activity to disrupt POMC neuron homeostasis, both of which can contribute to DIO. In the opposite direction, inhibition of E2F1 may have synergistic effects in dampening this feed-forward loop with inhibition of AMPK.

Materials and methods

Animals and genotyping

All procedures were reviewed and approved by Einstein Animal Care Committee, conforming to accepted standards of humane animal care. Mice were maintained on PicoLab chow 5053 (minimum protein 20%, minimum fat 4.5%; PMI Nutrition International). *Rb1^{lox/lox}* mice (Sage *et al*, 2003), *Nestin-Cre* mice (Betz *et al*, 1996), *Pomc-Cre* mice (Balthasar *et al*, 2004), *Agrp-Cre* mice (Tong *et al*, 2008), *Rosa26EYFP* mice (Srinivas *et al*, 2001), *Skp2^{-/-}* mice (Nakayama *et al*, 2000), *p27^{T187A/T187A}* mice (Malek *et al*, 2001), *E2f1^{-/-}* mice (Yamasaki *et al*, 1998), *Pomc-GFP* mice (The Jackson Lab, MGI Ref ID J:120992), and *Npy-GFP* mice (The Jackson Lab, MGI Ref ID J:120993) have been described. Genotyping primers are listed in Supplementary data.

Initial studies of pRb phosphorylation in ARC in response to HFD (Figure 1A and B; Supplementary Figure S1A and B) used WT C57BL/6 mice. All transgenic mice used in later studies were maintained and mated as mixed background hybrids, including C57BL/6, 129Sv, and FBV. We did not select for or against any particular strain background in the course of our studies. Pituitary tumours, POMC neuron loss, and obesity in *Pomc-Cre;Rb1^{lox/lox}* mice and, in contrast, lack of AGRP/NPY neuron defects, and lack of obesity in *Agrp-Cre;Rb1^{lox/lox}* mice are robust phenotypes among littermates and among large numbers of litters. The block of pituitary tumours, the non-correction of POMC neuron defects and obesity by *Skp2^{+/-}* and *p27^{T187A/T187A}* are also robust phenotypes. The delay in pituitary tumour development and correction of POMC neuron defects by *E2f1^{-/-}* are robust, while the correction of obesity by *E2f1^{-/-}* is less robust and subject to complex interpretations as discussed in the text.

HFD treatment

Regular chow (3.75 kcal/g, 22% calories from fat, 23% calories from protein, 55% calories from carbohydrates) was from Lab Diet (5058) and HFD (5.6 kcal/g, 58% kcal from fat, 16% calories from protein, 26% calories from carbohydrate) was from Research Diets (D-12331). Male C57BL/6 mice were treated with Chow and HFD for 11 months. Male mixed strain background mice were divided into two cohorts at 4 weeks of age. One cohort was fed with Chow and other with HFD, as described previously (McNay *et al*, 2012). Mice were sacrificed and their brains were harvested after 1, 4, and 8 weeks on the respective diets.

i.c.v. injection of AICAR

Male mice were anaesthetized with isoflurane before i.c.v. injection of AICAR (6 µg) in 2 µl of ACSF (Artificial CerebroSpinal Fluid), the control cohort was injected with the vehicle (ACSF). i.c.v. injections were delivered into the third ventricle with a calibrated 10 µl Hamilton syringe 1 h before the start of the dark cycle. Two hours and six hours after i.c.v. injection, hypothalami were harvested (Kim *et al*, 2004; Hu *et al*, 2005).

Measurement of food intake after i.c.v. injection

Cannulae were stereotactically implanted into the third cerebral ventricle. After 7 days of recovery and acclimatization to single housing, mice were challenged by MC3R/MC4R agonist and antagonist. Mice eating *ad libitum* were administered i.c.v. with 1 µl of ACSF (basal condition), 1 nmol of SHU9119 (Calbiochem) or 0.1 nmol of MT-II (Calbiochem) at 1300 h (compounds were delivered in 1 µl of ACSF). Cumulative food intake (Bio-Serv, precision dustless food pellet, #F0163) was measured for 6 h after i.c.v. injection. In all, 5 µg of PYY in 1 µl of ACSF (Phoenix Pharmaceuticals) was administered i.c.v. and cumulative food intake was measured for 3.5 h after i.c.v. injection.

Body composition analysis

Body composition was measured by magnetic resonance spectroscopy (MRS) using an EchoMRI (Echo Medical Systems).

Immunostaining

Mice were anaesthetized and perfused through the left ventricle with PBS followed by 4% paraformaldehyde (PFA) in PBS or 10% formalin. Brains were post-fixed at 4°C for 2 h to overnight and cryoprotected with 30% sucrose for 48 h or longer. Antibodies and detailed protocols used in this study are listed in Supplementary data.

Western blotting

Freshly dissected hypothalamus was homogenized in RIPA buffer to obtain total protein extract, which was separated on SDS-PAGE, blotted, and probed with anti-E2F1 antibody (C20, Santa Cruz Biotechnology) and tubulin. Mouse embryo fibroblasts were isolated from E13.5 embryos and used at p5.

Fluorescent in situ hybridization

Mice were anaesthetized and perfused with 1 × PBS for 5 min, followed by another 5 min with 4% PFA. Dissected brain was post-fixed in 4% PFA for 12 h (O/N) in 4°C and cryoprotected with 30% sucrose for 48 h. Tissues were embedded in OCT gel (Tissue Tek), 20 µm thick coronal sections were collected on Superfrost Plus slides.

Hybridization steps were modified as previous described (Thisse and Thisse, 2008; Draper *et al*, 2010; Padilla *et al*, 2010). Briefly, sections were permeabilized for 30 min at 37°C with TE buffer containing either 1 µg/ml Proteinase K and post-fixed with 4% PFA for 10 min at 4°C. Antisense digoxigenin-labelled riboprobes were generated from plasmids containing PCR fragments of *Cyclin A2* using the following primers set: 5'-GACGTCGACGAATGTCAA CCCCAGAAA-3'/5'-GCTGAATTCCTTCTGCTTCATCCACAT-3. Brain sections were hybridized with *Cyclin A2* riboprobes (15 ng/µl) at 55°C for 20 h, followed by stringent wash with 2 × SSC and 0.2 × SSC at 55°C for 30 min each. After that, HRP was introduced by Anti-Digoxigenin-POD (Roche 11207733910). Signal amplification steps were carried out based on the TSA Plus Cy3 System manual (Perkin-Elmer).

Quantification of POMC neurons number and cell size determination

POMC-stained cells were counted in about three ARC sections per mouse and an average of POMC neuron number was determined for each mouse. Brain slices were chosen at equivalent anatomical position in the ARC. Measurement of the soma area of the POMC neurons was performed with the Image J software with 300–350 POMC-stained neurons for each genotypes.

Projection density quantification

Projection density in the DMH and the PVH was measured according to Bouret *et al* (2008). For each animal, two sections through the PVH or DMH were acquired. Image analysis was performed using Image J software. Each image was binarized and the integrated intensity was then calculated for each image.

Hypothalamic neuropeptide assays and reverse-phase high-performance liquid chromatography

Peptides were extracted from hypothalamus in 0.1N HCl for measuring β-Endorphin and α-MSH by radioimmunoassay (Wardlaw, 1986). POMC precursor was measured by ELISA with reagents provided by Anne White at the University of Manchester (Tsigos *et al*, 1993).

Statistical analysis

Results are shown as average with s.e.m. Paired and unpaired Student's *t*-tests were performed as indicated in figure legends. Statistical analysis was performed with GraphPad Prism 5 and significance was accepted at *P* < 0.05.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

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Conflict of interest

The authors declare that they have no conflict of interest.

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