Stem Cell Reports
Article

ISSCR

OPEN ACCESS

PC1/3 Deficiency Impacts Pro-opiomelanocortin Processing in Human Embryonic Stem Cell-Derived Hypothalamic Neurons

Liheng Wang,[1,2] Lina Sui,[1] Sunil K. Panigrahi,[2] Kana Meece,[2] Yurong Xin,[3] Jinrang Kim,[3] Jesper Gromada,[3] Claudia A. Doege,[4] Sharon L. Wardlaw,[2] Dieter Egli,[1,5] and Rudolph L. Leibel[1,2,*]

[1]Division of Molecular Genetics, Department of Pediatrics and Naomi Berrie Diabetes Center, College of Physicians and Surgeons, Columbia University, 1150 St. Nicholas Avenue, Room 620A, New York, NY 10032, USA
[2]Department of Medicine and Naomi Berrie Diabetes Center, College of Physicians and Surgeons, Columbia University, New York, NY 10032, USA
[3]Regeneron Pharmaceuticals, Tarrytown, NY 10591, USA
[4]Department of Pathology and Cell Biology and Naomi Berrie Diabetes Center, College of Physicians and Surgeons, Columbia University, New York, NY 10032, USA
[5]New York Stem Cell Foundation Research Institute, 3960 Broadway, New York, NY 10032, USA
*Correspondence: rl232@columbia.edu
http://dx.doi.org/10.1016/j.stemcr.2016.12.021

SUMMARY

We recently developed a technique for generating hypothalamic neurons from human pluripotent stem cells. Here, as proof of principle, we examine the use of these cells in modeling of a monogenic form of severe obesity: *PCSK1* deficiency. The cognate enzyme, PC1/3, processes many prohormones in neuroendocrine and other tissues. We generated *PCSK1* (PC1/3)-deficient human embryonic stem cell (hESC) lines using both short hairpin RNA and CRISPR-Cas9, and investigated pro-opiomelanocortin (POMC) processing using hESC-differentiated hypothalamic neurons. The increased levels of unprocessed POMC and the decreased ratios (relative to POMC) of processed POMC-derived peptides in both *PCSK1* knockdown and knockout hESC-derived neurons phenocopied POMC processing reported in PC1/3-null mice and PC1/3-deficient patients. PC1/3 deficiency was associated with increased expression of melanocortin receptors and *PRCP* (prolylcarboxypeptidase, a catabolic enzyme for α-melanocyte stimulating hormone (αMSH)), and reduced adrenocorticotropic hormone secretion. We conclude that the obesity accompanying *PCSK1* deficiency may not be primarily due to αMSH deficiency.

INTRODUCTION

Proprotein convertase subtilisin/kexin type 1 (*PCSK1*), encoding prohormone convertase 1/3 (PC1/3), was one of the first genes linked to monogenic early-onset obesity (Jackson et al., 1997). Homozygous or compound heterozygous mutations are associated with severe forms of obesity (Farooqi et al., 2007; Jackson et al., 1997; Martin et al., 2013; O'Rahilly et al., 1995). In addition, common nonsynonymous variants in *PCSK1* confer risk of obesity (Benzinou et al., 2008). PC1/3 is essential for processing prohormones and neuropeptides in neuroendocrine tissues encompassing hypothalamus, pituitary, adrenal glands, and pancreatic islets (Jackson et al., 2003; Zhu et al., 2002). PC1/3 substrates include propeptides for pro-opiomelanocortin (POMC), neuropeptide Y (NPY), agouti-related peptide (AGRP), progrowth-hormone releasing hormone (GHRH), prothyrotropin-releasing hormone, proinsulin, and proglucagon (Creemers et al., 2006; Jackson et al., 2003; O'Rahilly et al., 1995; Paquet et al., 1996; Zhu et al., 2002). PC1/3 deficiency results in several endocrinopathies, including growth hormone and adrenal insufficiency, hypogonadism, hypothyroidism, and hyperproinsulinemia in both humans and rodents (Jackson et al., 2003; O'Rahilly et al., 1995; Zhu et al., 2002).

In vitro functional assays of PC1/3 bioactivity suggest that most mutant alleles identified in PC1/3-deficient patients affect the enzymatic activity of PC1/3 by influencing either the stability of *PCSK1* mRNA or the production and secretion of mature PC1/3 (Farooqi et al., 2007; Martin et al., 2013). In the hypothalamus, the prohormone convertases, PC1/3 and PC2, function proximally to another proteolytic enzyme, carboxypeptidase E (CPE), to mediate the processing of POMC and other neuropeptides (Figure 1A). PC1/3 preferentially hydrolyzes the dibasic proteolytic cleavage site, KR, on the POMC propeptide to generate intermediate peptides including pro-adrenocorticotropic hormone (pro-ACTH), ACTH, and β-lipotropin (β-LPH) (Zhou et al., 1993). PC2 and CPE participate in downstream processing of ACTH and β-LPH into active α-melanocyte stimulating hormone (αMSH) and β-endorphin (βEP), respectively (Figure 1A) (Wardlaw, 2011). Importantly, αMSH is the endogenous agonist at the melanocortin-4 receptor (*MC4R*) leading to its activation and reduced food intake (Marsh et al., 1999). In PC1/3-deficient patients, concentrations of circulating POMC and ACTH intermediates are significantly increased, while levels of ACTH are unchanged or decreased, consistent with impaired POMC processing in the pituitary (O'Rahilly et al., 1995). In PC1/3 null mice, the levels of *Pomc* transcript and protein in both hypothalamus and pituitary are significantly increased. Pituitary ACTH is undetectable and αMSH production is unchanged or decreased, consistent with impaired POMC processing in the hypothalamus and pituitary (Pan et al., 2005; Zhu et al., 2002).

This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

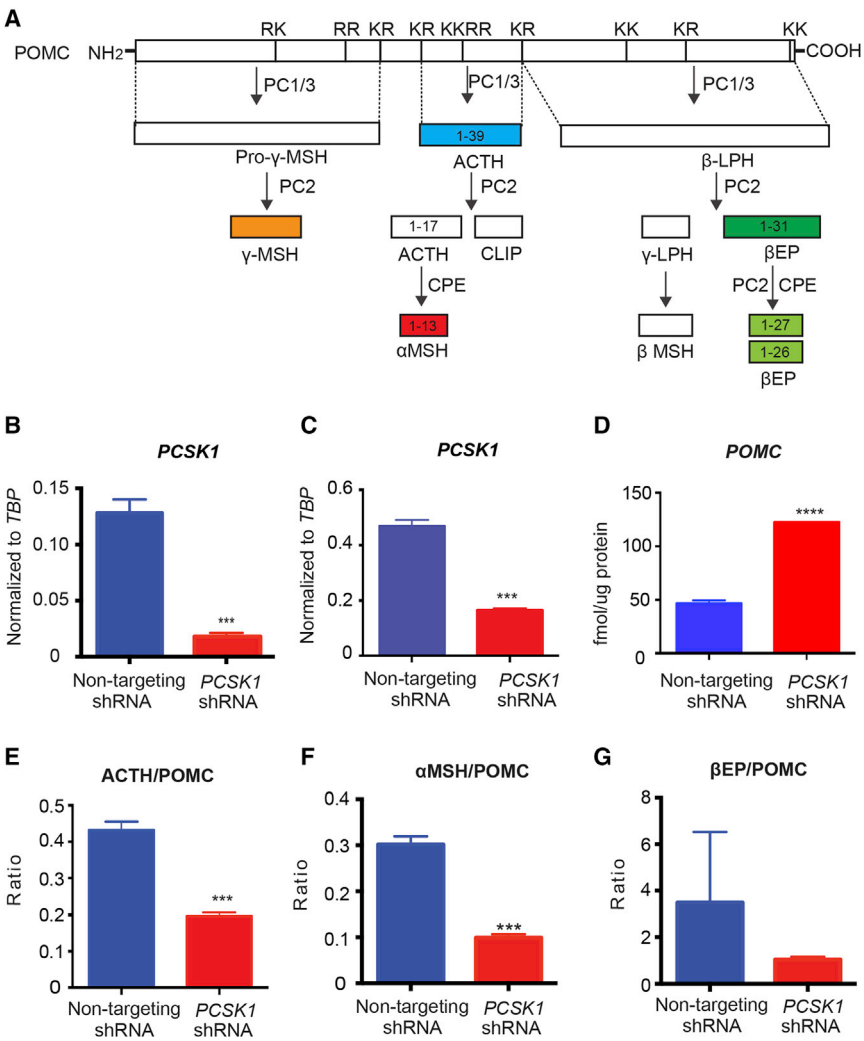

Figure 1. *PCSK1* Knockdown Impairs POMC Processing in hESC-Derived Hypothalamic Neurons

(A) Diagram of hypothalamic POMC processing by sequential cleavage of proteolytic enzymes. The cleavage sites of prohormone convertase 1/3 (PC1/3) and PC2 in hypothalamic POMC neuropeptide are marked above the POMC peptide. PC1/3 preferentially hydrolyzes the dibasic proteolyic cleavage site, KR, while PC2 cleaves dibasic peptides RK, RR, and KK.

LPH, lipotropin; CLIP, corticotropin-like intermediate peptide.

Modified from Zhou et al. (1993) and Wardlaw (2011). Key proteolytic enzymes include PC1/3, PC2, and carboxypeptidase E (CPE). αMSH (1–13) and β-endorphin (βEP, 1–26 and 1–27) are the major products from hypothalamic POMC processing.

(B and C) qPCR analysis of *PCSK1* expression in non-targeting shRNA and *PCSK1* shRNA hESCs (B) and corresponding day 29 hESC-derived hypothalamic neurons (C).

(D) Production of POMC prohormone in lysates of day 29 hESC-derived hypothalamic neurons.

(E–G) The ratios of ACTH/POMC (E), αMSH/POMC (F), and βEP/POMC (G) in lysates of day 29 non-targeting and *PCSK1* shRNA-expressing hESC-derived neurons.

Student's t test, ***$p < 0.001$, ****$p < 0.0001$. Data are presented as means ± SEM from three independent experiments.

In PC1/3$^{N222D/N222D}$ mice, hypothalamic αMSH peptide is reduced, but POMC protein levels are not changed. And, in these animals, plasma concentrations of mature ACTH are not affected (Lloyd et al., 2006). These findings implicate important functions of PC1/3 in hypothalamic and pituitary POMC processing, with likely mutation- and even species-specific molecular compensations for reductions in the enzyme's activity. Moreover, prolylcarboxypeptidase (PRCP) functions as an enzyme to inactivate αMSH by removing one amino acid from its C terminus and is expressed in the hypothalamic neurons that send efferents to areas where αMSH is released (Wallingford et al., 2009). Inhibition of PRCP activity or deletion of *Prcp* in mice decreases food intake and protects the mice from obesity induced by a high-fat diet (Wallingford et al., 2009).

Recently, we and another group reported in vitro differentiation protocols for the generation of hypothalamic neurons from human pluripotent stem cells (hPSCs) (Merkle et al., 2015; Wang et al., 2015). To investigate the impact of *PCSK1* loss of function in a relevant human cell type, we differentiated *PCSK1* short hairpin RNA (shRNA) knockdown and CRISPR-Cas9 knockout hESCs into neurons that closely resemble those of the arcuate nucleus (ARC) (Schneeberger et al., 2013) of the hypothalamus (Wang et al., 2015, 2016). Here, we assess POMC processing-specific cellular and molecular phenotypes in PC1/3-deficient cells and further examine the neuromolecular physiology of *PCSK1* hypomorphic mutations. We find that the molecular phenotypes of hESC-derived hypothalamic neurons recapitulate those seen in the mouse. Interestingly, the upregulation of POMC production and consequences of downstream processing enzymes appear to compensate for the loss of *PCSK1* in hESC-derived hypothalamic neurons, maintaining the production of αMSH and βEP.

RESULTS

Decreased *PCSK1* Expression Impairs POMC Processing in hESC-Derived Hypothalamic Neurons

To create PC1/3-deficient cells, we knocked down *PCSK1* with lentiviral shRNA in an NKX2.1 GFP/W-hESC line (Goulburn et al., 2011). In stable shRNA-transfected hESC lines, *PCSK1* mRNA was reduced by 80%–90% versus control (Figure 1B). Both *PCSK1* knockdown and control (non-targeting shRNA-transfected) hESC lines were differentiated into hypothalamic neurons with our efficient protocol (>95% cells are ARC-type neurons) to investigate the effects of PC1/3 deficiency on neuropeptide processing (Wang et al., 2015).

In day 29 differentiated hypothalamic neurons, levels of *PCSK1* transcripts were reduced by 60% versus the control line treated with non-targeting shRNA (Figure 1C). Immunostaining for POMC and PC1/3 indicated that the percentage of POMC-positive neurons was not affected in the *PCSK1* knockdown line (Figures S1A and S1B), suggesting that reduced *PCSK1* expression did not affect differentiation of hESCs into *POMC*-expressing neurons. As anticipated, the number of PC1/3-positive cells was significantly decreased in the *PCSK1* knockdown neurons (Figure S1C), confirming the effective knockdown of *PCSK1* in hESC-derived hypothalamic neurons.

To assess POMC processing, we measured the total amount of POMC, ACTH, αMSH, and βEP proteins in cell lysates of day 29 differentiated hypothalamic neurons by ELISA and radioimmunoassay (RIA) (Crosby et al., 1988; Wardlaw, 1986). Levels of POMC peptide were significantly increased in *PCSK1* knockdown neurons (Figure 1D). In addition, ratios of ACTH/POMC (Figure 1E), αMSH/POMC (Figure 1F), and βEP/POMC (Figure 1G) were significantly decreased compared with non-targeted shRNA knockdown neurons. Furthermore, the ratios of ACTH/POMC (Figure S1D), αMSH/POMC (Figure S1E), and βEP/POMC (Figure S1F) were also significantly reduced in media of *PCSK1* knockdown lines. Hence, POMC processing was impaired in the *PCSK1* shRNA knockdown hESC-derived hypothalamic neurons. Surprisingly, the total amounts of products of POMC processing, including ACTH, αMSH, and βEP, were unaffected in *PCSK1* knockdown neurons (Figure S1G).

PC1/3 Deficiency in CRISPR-Cas9-Generated *PCSK1* Mutant Lines

We asked whether complete loss of PC1/3 protein would cause a more severe impairment of POMC processing. To knock out *PCSK1* in hESCs, we utilized the genome-editing tool CRISPR-Cas9. We designed a guide RNA (gRNA) that binds specifically to exon 10 of *PCSK1* and recruits Cas9 to induce DNA cleavage (Figures 2A and S2). Four isogenic hESC clones representing wild-type (WT), knockout (KO1, KO2), and heterozygous (Het) lines were selected for study (Figure 2B). Both KO1 and KO2 contain one frameshift mutation in each allele, each resulting in a premature stop codon in the transcribed *PCSK1* mRNA.

PC1/3 expression was assessed in these four isogenic lines over the time course of differentiation. *PCSK1* transcripts were greatly reduced in both day 12 neuron progenitors (Figure 2C) and day 28 differentiated hypothalamic neurons (Figure 2D). Western blot analysis of day 28 neurons confirmed the complete absence of the active isoform of PC1/3 (67 kDa) in the two knockout lines (Figure 2E). We performed single-cell transcriptome sequencing of day 27 differentiated hypothalamic neurons; day 12 neuron progenitors; and undifferentiated hESCs (Figure 2F). *PCSK1* was only expressed in *MAP2*- and *CPE*-expressing neurons, indicating the respective neuronal and neurosecretory status of those cells; 73.1% of *PCSK1*-expressing neurons also expressed *POMC*. Some *PCSK1*-expressing neurons also expressed *NPY* (21.2%) or *SST* (30.8%). From immunostaining, we found that PC1/3-positive cells could not be detected in KO1 and KO2 hESC-derived hypothalamic neurons (Figure 2G). Thus, PC1/3 was eliminated in CRISPR-Cas9-generated compound heterozygous KO1 and KO2 hESC lines. All four CRISPR-Cas9-generated *PCSK1* hESC lines had normal karyotypes and showed no changes in genomic sequence in the exonic "off-target" sites that were screened (four sites) (Figure S3). These results indicate that these lines can reasonably be considered as isogenic in the experimental contexts reported here.

POMC Processing Is Impaired in *PCSK1* Knockout hESC-Derived Hypothalamic Neurons

Immunostaining of day 28 neurons using anti-POMC and anti-αMSH showed no differences in the percentage of POMC neurons in *PCSK1* KO1 and KO2 lines compared with WT and Het lines (Figures 3A and 3B), suggesting that PC1/3 deficiency does not affect the differentiation efficiency of POMC neurons. To assess POMC processing, we measured the amounts of POMC, ACTH, αMSH, and βEP in neuronal lysates of day 28 hESC-differentiated hypothalamic neurons. The amount of intracellular POMC was significantly increased in Het, KO1, and KO2 neurons (Figure 3C). The ratios of ACTH/POMC (Figure 3D), αMSH/POMC (Figure 3E), and βEP/POMC (Figure 3F) were greatly reduced in KO1 and KO2 mutant neurons compared with WT neurons. Thus, the processing of POMC was impaired in the two *PCSK1* knockout cell lines. The ratios of ACTH/POMC and αMSH/POMC were reduced by 50% in Het compared with WT cells, suggesting that there is a dose effect of *PCSK1* allele on POMC processing. The higher ACTH/POMC ratios in hESC-derived hypothalamic

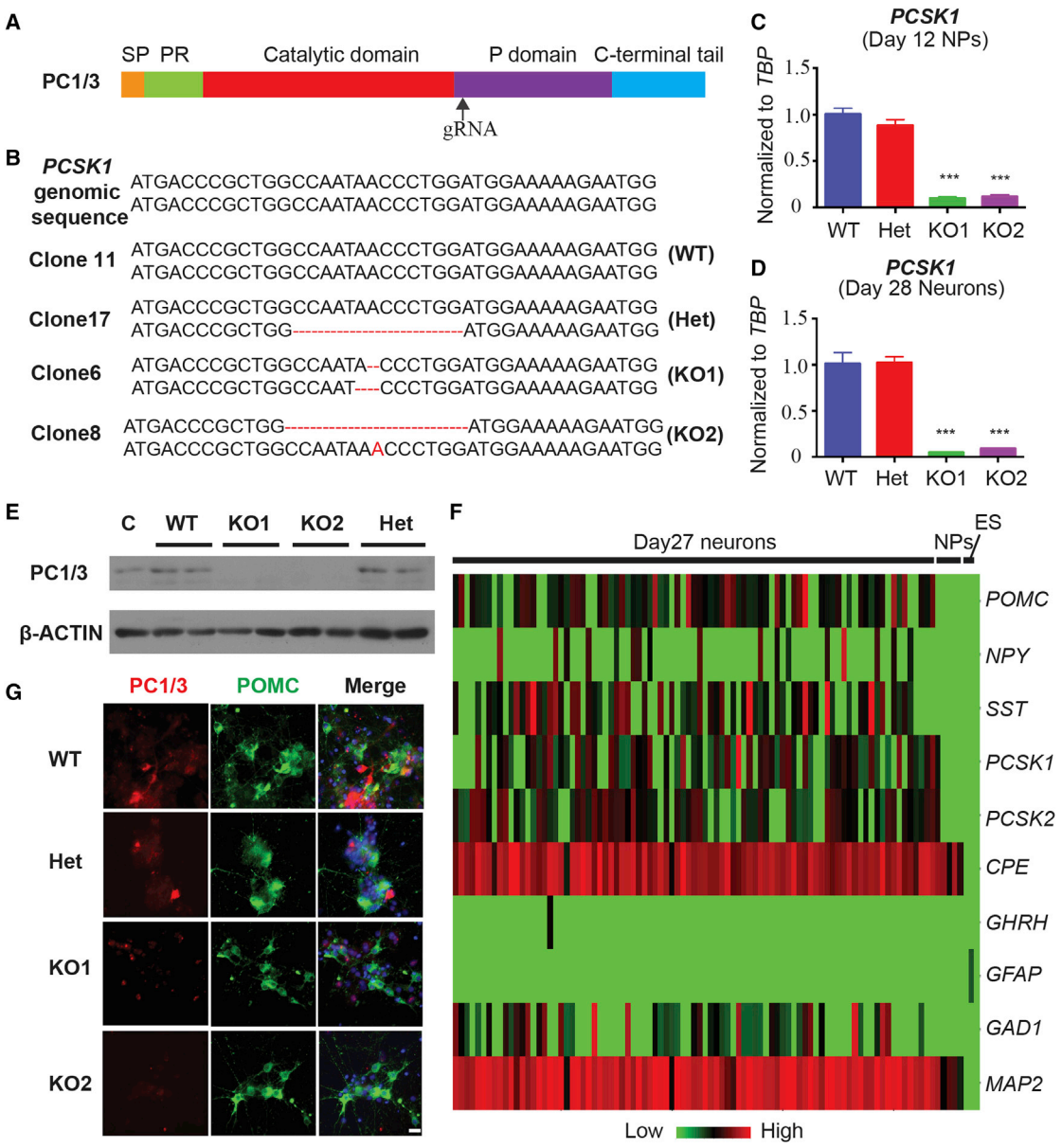

Figure 2. Generation of PC1/3 Mutant hESC Lines Using CRISPR-Cas9

(A) Diagram of the protein structure of PC1/3. PC1/3 contains the following elements: signal peptide (SP), prosegment (PR), catalytic domain, P domain, and C-terminal tail. The guide RNA (gRNA) targeted the P domain of PC1/3.

(B) Sequencing results of both alleles of *PCSK1* at gRNA targeted sites from four CRISPR-Cas9-generated hESC lines: *PCSK1* WT, Het, and two mutant lines (KO1, KO2). The deletion and insertion of nucleotides are marked in red.

(C and D) Expression of *PCSK1* in day 12 neuron progenitors (C) and day 28 hypothalamic neurons (D) derived from four CRISPR-generated hESC lines as indicated; ***$p < 0.001$. Student's t test. Data are represented as means ± SEM from three independent experiments.

(E) Western blot analysis of PC1/3 (67 kDa) in day 27 differentiated neurons derived from control NKX2.1: GFP/W-hESC line (C), *PCSK1* WT, Het, KO1, and KO2. β-Actin was used as a loading control.

(F) Heatmap of hypothalamic transcripts from single-cell transcriptome sequencing of 88 day 27 neurons; four day 12 neuron progenitors (NPs); and three ESCs from Nkx2.1 GFP/W-hESC line. Among 52 neurons that expressed *PCSK1* (RPKM>1), all of them expressed *MAP2* and *CPE*; 38 cells also expressed *POMC* (RPKM>1), 16 cells also expressed *SST* (RPKM>10), and 11 cells also expressed *NPY* (RPKM>10).

(G) Immunostaining of PC1/3 and POMC in day 28 neurons from four hESC lines as indicated. Cell nuclei were stained with Hoechst. Scale bar represents 20 μm.

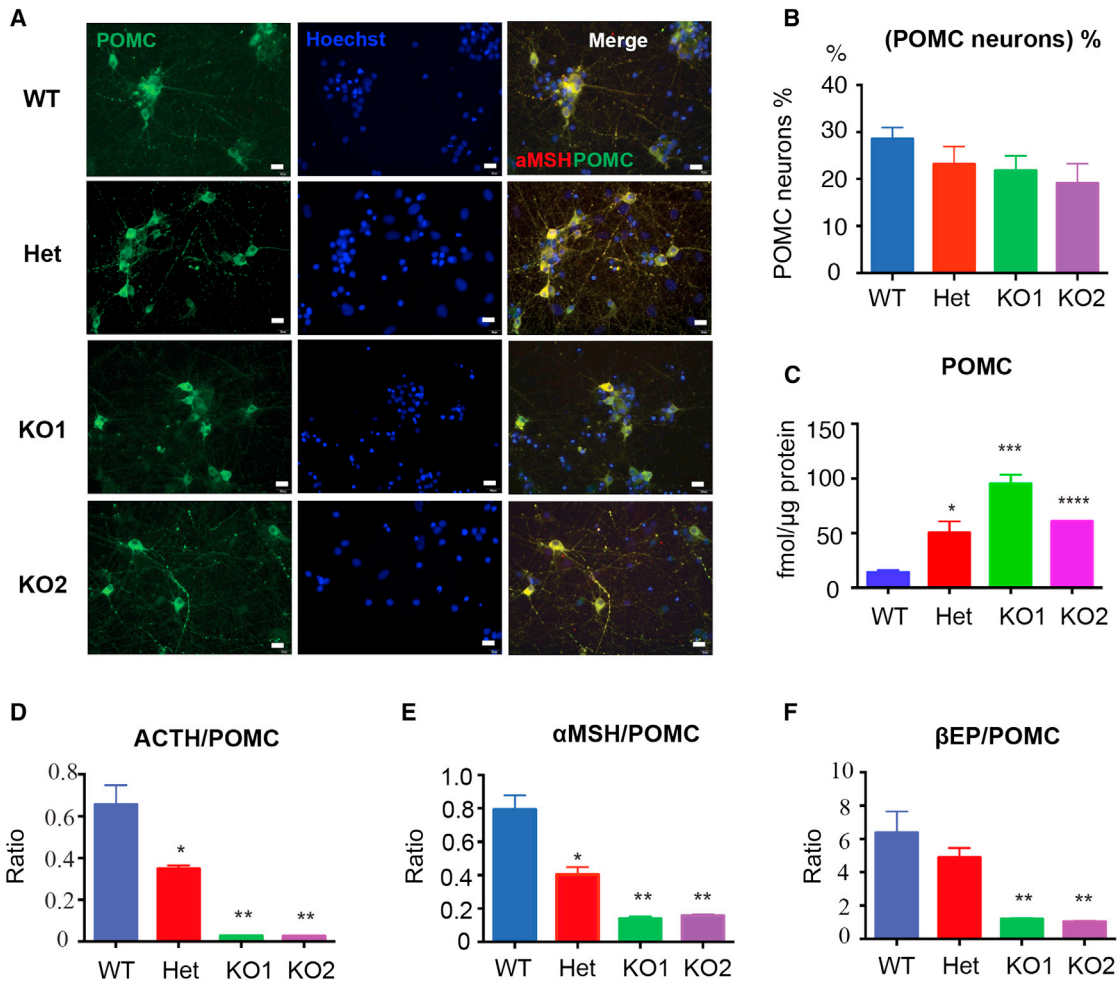

Figure 3. Impaired POMC Processing in *PCSK1* Knockout hESC-Derived Hypothalamic Neurons

(A) Immunocytochemistry for POMC and αMSH in day 28 hypothalamic neurons derived from four CRISPR-generated hESC lines: PC1/3 WT, Het, KO1, and KO2. Scale bar represents 20 μm; Hoechst was used as a nuclear marker.

(B) Quantification of percentage of POMC neuron in (A). The proportion of POMC neurons was determined as (number of POMC neurons)/(number of Hoechst-positive cells) × 100%.

(C) The amount of POMC prohormone produced in day 28 differentiated neurons.

(D–F) The ratios of ACTH/POMC (D), αMSH/POMC (E), and βEP/POMC (F) in day 28 hypothalamic neurons derived from hESC lines are indicated.

Student's t test, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$. Data are presented as means ± SEM from three independent experiments.

neurons in comparison with ratios in rat hypothalamic extracts or human cerebrospinal fluid, probably reflect the relative developmental immaturity of the hESC-derived neurons (Angelogianni et al., 2000; Pritchard et al., 2003; Tsigos et al., 1993; Wang et al., 2015).

Compensatory Responses to PC1/3 Deficiency in Hypothalamic Neurons

PC1/3 participates in the initial processing of POMC propeptide into ACTH and β-LPH, which are further processed into αMSH and βEP, respectively (Figure 1A). Intracellular ACTH was significantly decreased in *PCSK1* knockout neurons (Figure 4A). We anticipated proportional reductions of levels of αMSH and βEP proteins. However, the amounts of αMSH and βEP in the KO1 and KO2 hypothalamic neurons were unchanged compared with WT neurons (Figures 4B and 4C). By qPCR analysis, we found that expression of *POMC* mRNA was significantly elevated in KO1 and KO2 cells compared with WT neurons, a finding consistent with the increased levels of POMC protein (Figure 4D). Transcript levels of *MAP2*, a neuronal marker, were comparable among four *PCSK1* lines, further indicating that

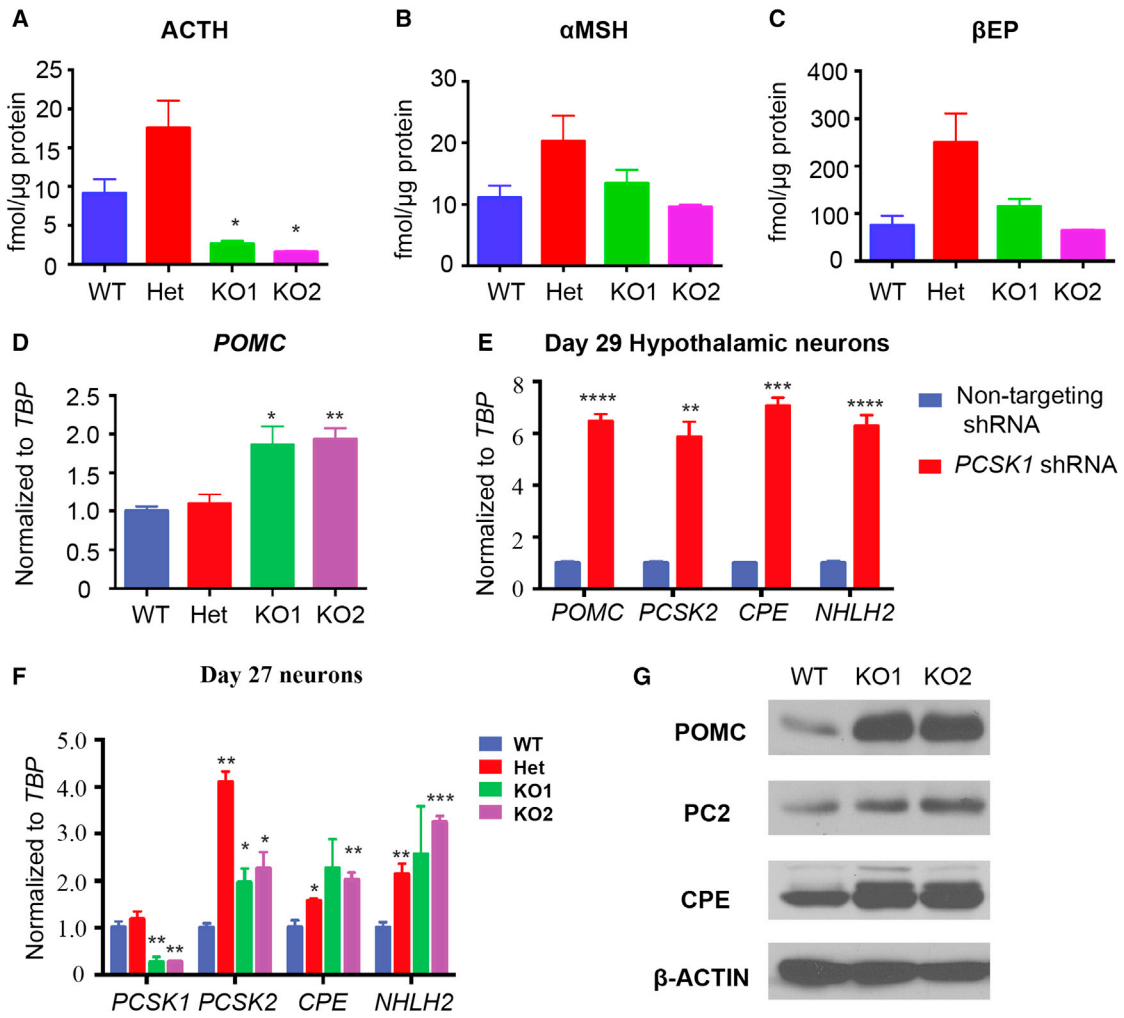

Figure 4. Compensatory Changes in *PCSK1* Knockout hESC-Derived Hypothalamic Neurons that Maintain Production of αMSH and βEP

(A–C) Amounts of ACTH (A), αMSH (B), and βEP (C) present in lysates of day 28 differentiated hypothalamic neurons in four lines as indicated; ACTH and βEP levels were determined by ELISA, αMSH was measured by RIA.

(D) qPCR analysis of the expression of *POMC* in day 28 neurons derived from four *PCSK1* hESC lines.

(E) qPCR analysis of *POMC*, *PCSK2*, *CPE*, and nescient helix-loop-helix 2 (*NHLH2*) in day 29 differentiated neurons derived from *PCSK1* knockdown line and non-targeting shRNA expressed hESC line.

(F) qPCR analysis of the transcript levels of *PCSK1*, *PCSK2*, *CPE*, and *NHLH2* in day 27 neurons derived from CRISPR-generated WT, Het, KO1, and KO2 hESC lines.

(G) Western blot analysis of POMC, PC2, and CPE in day 30 differentiated hypothalamic neurons derived from WT, KO1, and KO2 hESC lines. β-Actin was used as a loading control.

Student's t test, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$; $n = 3$ for each bar. Data are presented as means ± SEM from three independent experiments.

neuronal differentiation was not affected by PC1/3 deficiency (Figure S4A). The level of *CART* transcript, the other key functional peptide produced in POMC neurons and processed by PC1/3, was also greatly increased (Figure S4B). However, the levels of orexigenic transcripts including *NPY* and *AGRP* were not altered in KO1 and KO2 neurons (Figures S4C and S4D), suggesting that PC1/3 deficiency has differential effects on the processing of canonical ARC neuropeptides.

In addition, the expression of the downstream processing enzymes, *PCSK2* and *CPE*, and the upstream transcription factor, *NHLH2*, were significantly increased in *PCSK1* knockdown neurons, while *POMC* transcript levels were greatly increased (Figure 4E). Consistent with these

findings, the expression of these genes also increased in Het, KO1, and KO2 neurons compared with WT lines (Figure 4F). Levels of PC2, CPE, and POMC proteins in day 30 hESC-derived hypothalamic neurons were consistently elevated in the KO1 and KO2 lines (Figure 4G). These apparent adaptive changes observed in the PC1/3-deficient hypothalamic ARC neurons may account in part for the unaltered production of some of the end products of POMC processing in the mutant cells.

PC1/3 Deficiency Increases αMSH Degradation and Reduces ACTH Secretion

PC1/3-deficient patients segregating for compound heterozygous hypomorphic mutations of *PCSK1* present with early-onset severe obesity. As noted above, we find that the amounts of αMSH and βEP in *PCSK1* knockout hypothalamic POMC neurons are unchanged, although the processing of POMC to ACTH is impaired in these neurons. If αMSH production is not reduced in hypothalamic neurons of patients with hypomorphic mutations of *PCSK1*, why are these individuals obese?

To address this question, we first investigated the secretion of POMC neuropeptides in the hypothalamic neurons derived from the four *PCSK1* hESC lines by measuring the amounts of POMC-related peptides in their culture media. However, the amounts of secreted αMSH and βEP were unaffected in *PCSK1* knockout neurons, whereas decreased levels of αMSH and βEP were observed in the *PCSK1* Het line (Figure 5A). The secretion of unprocessed POMC was greatly increased in *PCKS1* knockout lines but not in the Het (Figure 5A), thereby decreasing the ratio of αMSH/POMC and βEP/POMC (Figure 5B). These findings confirmed that POMC processing in the two *PCSK1* knockout lines was impaired. We also assessed expression of melanocortin receptors that are specifically expressed in the hypothalamic ARC including melanocortin-3 receptor (*MC3R*) and *MC4R*. MC3R is the major form of melanocortin receptors expressed in the ARC, while MC4R is only expressed in a small proportion of ARC neurons but widely expressed in the so-called second-order neurons in the paraventricular nucleus (PVN) to regulate food intake (Balthasar et al., 2005; Chen et al., 2000). The levels of *MC3R* and *MC4R* transcripts were greatly increased in *PCSK1* knockout neurons compared with WT cells (Figure 5C). Moreover, the expression of αMSH-degrading enzyme, *PRCP*, was increased in PC1/3 deficient neurons. The upregulation of melanocortin receptors and *PRCP* might reflect functional hypoactivity of αMSH (Figure 5D) (Wallingford et al., 2009).

In *PCSK1* knockout hypothalamic neurons, secretion of ACTH was also dramatically reduced (Figure 5E). But knockdown of *PCSK1* did not affect the secreted ACTH/POMC ratio (Figure S1D). These findings are in agreement with differential effects on the plasma ACTH level of hypomorphic versus null alleles of *PCSK1* in human subjects and rodent models (O'Rahilly et al., 1995; Zhu et al., 2002). ACTH administered directly into the hypothalamus is anorexigenic in rats and not influenced by intracerebroventricular infusion of αMSH antibody (Schulz et al., 2010). Although the production and secretion of αMSH are unchanged in PC1/3 knockout hypothalamic neurons, increased αMSH degradation and reduction of ACTH in hypothalamic neurons are potential mediators of the hyperphagia in PC1/3-deficient subjects.

PC1/3 Deficiency Does Not Induce ER Stress or Apoptosis in hESC-Derived Hypothalamic Neurons

As an additional mechanism by which PC1/3 deficiency might affect the functional integrity of hypothalamic mechanisms for control of body weight, we examined whether the neuronal accumulation of POMC propeptide might induce endoplasmic reticulum (ER) stress, leading to cell death. A recent study suggested that mutant PC1/3 proteins interact directly with ER stress protein BiP, accelerating their degradation (Stijnen et al., 2016). To this point, we examined the expression of the ER stress and apoptotic markers in the *PCSK1* WT and mutant neurons. The transcript levels of *ATF4*, *ATF6*, but not *sXBP1* or *GRP78*, were decreased in the *PCSK1* knockout lines (Figure 6A). Expression of apoptosis genes *CASP3*, *CASP8* was also decreased in KO1 and KO2 neurons (Figure 6B). We also challenged these neurons with an ER stressor (tunicamycin (3 μg/mL) for 4 and 8 hr) and investigated ER stress responses by western blot. The WT and Het neurons showed time-dependent increases of GRP78 and p-eIF2α with tunicamycin treatment, while KO1 and KO2 neurons did not respond (Figure 6C). Interestingly, CHOP levels were elevated in KO1 and KO2 mutant lines while levels of caspase-3 protein were decreased compared with WT neurons (Figure 6C).

We further investigated proliferation and cell death of differentiated neurons by Ki67 and TUNEL staining, respectively. We did not find any Ki67-positive (Figure S5) or TUNEL-positive POMC neurons in any of the four lines (Figure 6D), suggesting that PC1/3 deficiency does not affect either proliferation or apoptosis of hESC-derived hypothalamic neurons under basal circumstances. These data suggest that PC1/3 deficiency does not increase ER stress or apoptosis in hESC-derived hypothalamic neurons.

DISCUSSION

We used hESC-derived hypothalamic neurons to examine possible molecular mechanisms for the obesity associated with congenital deficiency of *PCSK1*. This gene encodes

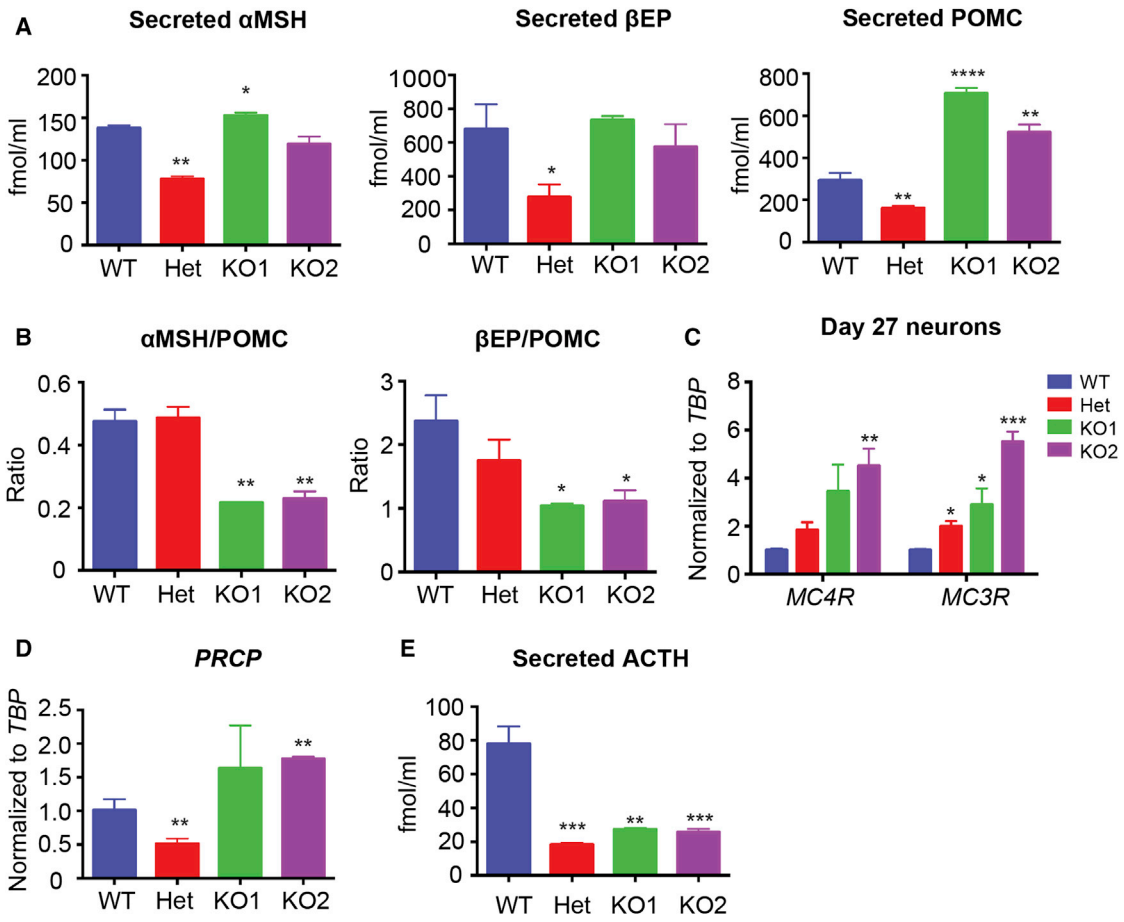

Figure 5. PC1/3 Deficiency Increases the Transcript Levels of Melanocortin Receptors and PRCP and Reduces ACTH Secretion in hESC-Derived Hypothalamic Neurons

(A) Secretion of POMC-derived neuropeptides from cultured hESC-derived hypothalamic neurons. After 16 hr incubation of day 28 hESC-derived hypothalamic neurons, medium samples were collected from each well from 12-well plate to determine the amounts of αMSH, βEP, and POMC in indicated lines.

(B) The ratios of αMSH/POMC and/POMC in secreted medium samples (16 hr incubation) from lines as indicated.

(C) qPCR analysis of the transcript levels of melanocortin receptors (*MC2R*, *MC3R*) that expressed in arcuate nucleus neurons in day 27 differentiated hypothalamic neurons.

(D) qPCR analysis of the expression of *PRCP* in day 27 hypothalamic neurons.

(E) Secretion of ACTH from day 28 hypothalamic neuron in lines as indicated.

Student's t test, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$; n = 3 for each bar. Data are presented as means ± SEM from three independent experiments.

PC1/3, an enzyme mediating a critical initial step in the processing of propeptides for POMC, AgRP, NPY, GHRH, insulin, glucagon, and other molecules that mediate aspects of appetitive behavior, somatic growth, and energy expenditure. We created PC1/3-deficient neurons using two strategies: knocking down *PCSK1* with shRNA and generating *PCSK1* null alleles in hESC lines with CRISPR-Cas9. The significant reductions of *PCSK1* mRNA and PC1/3 active protein and a decrease in the number of neurons expressing PC1/3 confirmed the functional PC1/3 deficiency in hESC-derived hypothalamic neurons. As anticipated, the ratios of ACTH/POMC, αMSH/POMC, and βEP/POMC were decreased in both PC1/3 knockdown and knockout neurons, indicating that POMC processing was impaired as a result of PC1/3 insufficiency. In apparent compensation for the reduction of PC1/3 activity, total amounts of *POMC* mRNA and POMC peptides were significantly increased in the PC1/3 hypomorphs. Levels of POMC-derived peptides are not reduced in hypothalami of PC1/3 null mice, and levels of PC2 protein are unaffected, suggesting compensatory mechanisms are invoked that maintain the production of processed POMC peptides

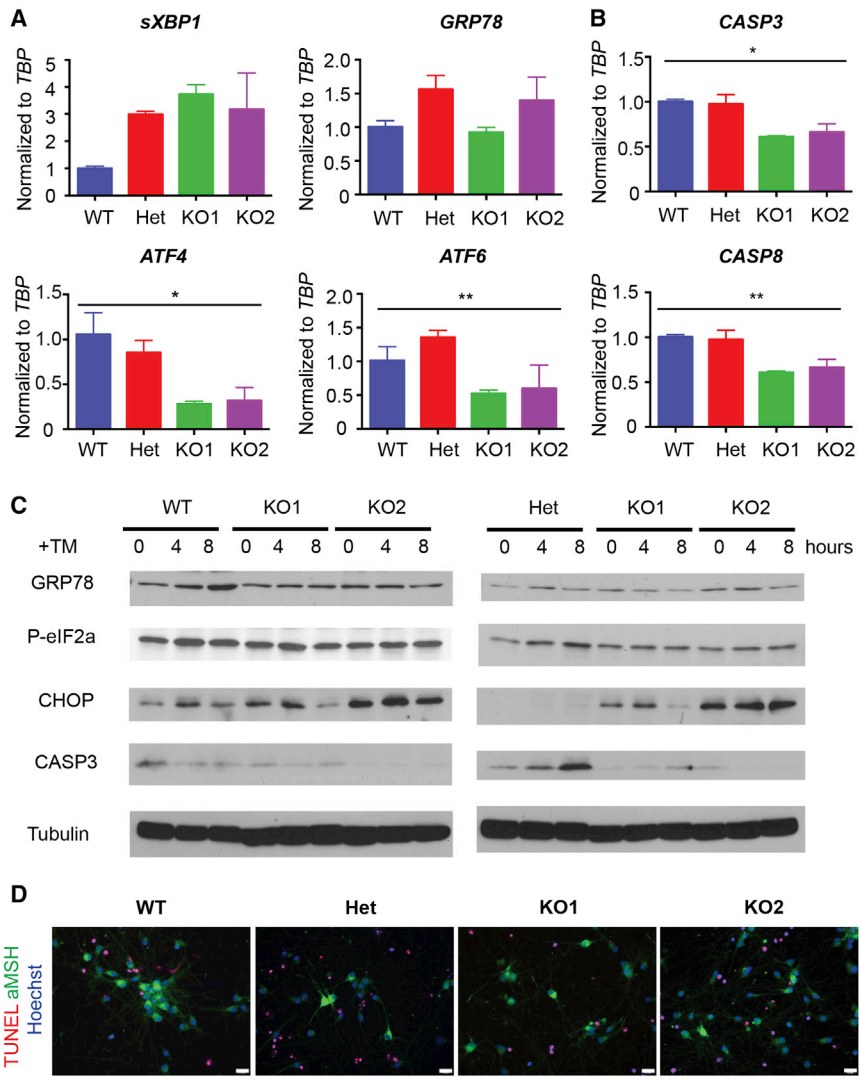

Figure 6. PC1/3 Deficiency Does Not Increase ER Stress or Apoptosis in hESC-Derived Hypothalamic Neurons

(A and B) qPCR analysis of the expression of ER stress (A) and apoptosis markers (B) in day 27 neurons. *sXBP1*, *GRP78*, *ATF4*, and *ATF6* were used as ER stress markers, *CASP3* and *CASP8* were used as apoptotic markers. One-way ANOVA. *$p < 0.05$; **$p < 0.01$. Data are presented as means ± SEM from three independent experiments.

(C) Western blot analysis of ER stress and apoptosis in response to tunicamycin (TM) treatment in differentiated hypothalamic neurons. Day 30 neurons were treated with 3 μg/mL tunicamycin for 0, 4, and 8 hr. GRP78, P-eIF2a, CHOP, and caspase-3 (CASP3) were used as ER stress and apoptosis markers. Tubulin was used as the loading control.

(D) TUNEL staining of day 27 differentiated hypothalamic neurons. POMC neurons were marked by αMSH antibody. Cell nuclei were stained with Hoechst. TUNEL-594 was used for identifying apoptotic cells. Scale bar represents 20 μm.

(Zhu et al., 2002). In hESC-derived hypothalamic neurons, we found that both mRNA and protein levels of POMC and the downstream POMC processing enzymes PC2 and CPE were increased in the PC1/3-deficient lines, perhaps accounting for the apparent maintenance of levels of αMSH and βEP despite reduction of PC1/3 activity. The increased expression of PC2 and CPE might result from feedback effects related to decreased *PCSK1* expression in these neurons.

The secretion of αMSH and βEP was unchanged in PC1/3 knockout neurons. And PC1/3 insufficiency did not induce ER stress or apoptosis in hESC-derived hypothalamic neurons. However, the upregulation of transcript levels of ARC-related melanocortin receptors 3 and 4 and *PRCP* implicates possible increased αMSH degradation in *PCSK1* knockout neurons. These changes, together with great reduction of ACTH production and secretion, may explain, at least partially, the hyperphagia in PC1/3-deficient subjects.

Plasma ACTH levels in individuals with PC1/3 deficiency are either decreased or unchanged (Jackson et al., 2003; Martin et al., 2013). In mice, a hypomorphic mutation of *PCSK1* (N222D) results in a decreased αMSH production, mild reduction of ACTH, and unchanged *POMC* mRNA expression in the hypothalamus (Lloyd et al., 2006). Congenital loss of PC1/3 in mice leads to a severe impairment of pituitary ACTH production, increased POMC, and pro-ACTH levels and unaltered αMSH levels (Zhu et al., 2002). Our hESC-derived hypothalamic neurons also displayed POMC processing molecular phenotypes that were specific to the degree of PC1/3 deficiency (Table S1); ACTH levels were unaffected in *PCSK1* knockdown lines but were decreased in *PCSK1* knockout lines. The differential effects of these manipulations are consistent with

the distinct in vivo consequences of hypomorphic and null mutations in PC1/3-deficient patients. In this context, it is important to note that the hESC-derived neurons used in these experiments do not express molecular markers of pituitary cells (Wang et al., 2015). The processing of POMC to ACTH is mainly dependent on PC1/3 activity (Wardlaw, 2011). These characteristics suggest that other prohormone convertases or enzymes can partially, but not fully, compensate for the reduction of PC1/3 activity in *PCSK1* knockout neurons. In addition to *POMC*, *CART* transcript levels were also increased in these neurons, while transcripts for the orexigenic peptides NPY and AGRP were not altered in PC1/3-deficient neurons, suggesting a differential effect of PC1/3 deficiency on neuropeptide processing in different types of neurons.

Taken together, our studies have demonstrated cellular and molecular phenotypes in a PC1/3-deficient and obesity-relevant human cell type: hESC-derived ARC-type hypothalamic neurons. These cellular phenotypes recapitulate many of those previously described in rodent models of PC1/3 deficiency and are in agreement with clinical data from patients segregating for inactivating mutations in *PCSK1*. Moreover, a recent paper by our group suggests that the hyperphagia and neuroendocrine disorders in Prader-Willi syndrome are due in part to PC1/3 deficiency (Burnett et al., 2016). Such studies further support the utility of stem cell-derived hypothalamic neurons in understanding the molecular neurobiology of body weight regulation and in the provision of tools for the screening of drugs that may be of therapeutic benefit.

In addition to the processing of neuropeptides, PC1/3 also plays a critical role in hormone processing in the pancreas and gut. PC1/3 participates in the processing of proinsulin to insulin in pancreatic β cells and proglucagon to glucagon-like peptide-1 (GLP-1) in L cells in the gut and in a subset of neurons in the nucleus of the solitary tract (NTS). PC1/3-deficient individuals also misprocess propeptides for insulin and vasopressin, leading to diabetes mellitus and diabetes insipidus (Martin et al., 2013; O'Rahilly et al., 1995). Insulin has anorexigenic effects via inhibition of NPY expression in the ARC (Schwartz et al., 1991). Plasma GLP-1 concentrations are reduced in PC1/3-deficient human subjects (Bandsma et al., 2013). GLP-1 receptor agonists can induce weight loss in both human and rodents (de Boer et al., 2016; Nuffer and Trujillo, 2015; Secher et al., 2014). GLP-1 neurons in the NTS project directly to the hypothalamus, ventral tegmental area, and nucleus accumbens to suppress food intake (Alhadeff et al., 2012; Larsen et al., 1997). Thus, impaired insulin processing and/or reduced GLP-1 levels (circulating and brain-endogenous), may also contribute to obesity in PC1/3-deficient subjects.

CRISPR-Cas9 is a widely used technology for genome editing. However, low-frequency off-target sequence changes have been reported (Smith et al., 2014; Veres et al., 2014). When there is a low level of aneuploid cells (1%) in hPSCs, karyotyping would not reliably detect such an aberration (Baker et al., 2016). Hence, for absolute confidence, whole-genome sequencing should be performed to exclude any potential off-target effects in stem cell lines generated from CRISPR-Cas9.

EXPERIMENTAL PROCEDURES

The studies described here were reviewed and approved by the Columbia University Institutional Review Board (protocol number IRB-AAAI1347).

Knockdown of *PCSK1* with shRNA

PCSK1 shRNA lentivirus particles were purchased from Sigma (SHCLNV-NM_000439). The sequence of this PCSK1 shRNA was: CCG GGC ACT AAA TCT CTT CAA TGA TCT CGA GAT CAT TGA AGA GAT TTA GTG CTT TTT G (TRCN0000051496). The non-targeting shRNA (SHC002 V) was used as a negative control. Nkx2.1 GFP/W-hESCs were cultured in 24-well mouse embryonic fibroblast (MEF) feeder cells until >90% confluency. Ten microliters of shRNA lentivirus particles was added to each well filled with 0.5 mL of hESC medium (500 mL of knockout DMEM, 90 mL of knockout serum, 6.5 mL of GlutaMAX, 6.5 mL of non-essential amino acids [NEAA], 6.5 mL of penicillin/streptomycin, 0.65 mL of β-mercaptoethanol, and 10 ng/mL basic fibroblast growth factor [bFGF]). All reagents were from Life Technologies (http://www.lifetechnologies.com). After incubation with the virus for 48 hr, virus-containing medium was removed and new hESC medium containing 1 μg/mL puromycin (Life Technologies) was added. After 24 hr, medium was aspirated and new MEFs were added to each well. Once the new colonies formed, the selection steps were repeated until stable puromycin-resistant hESCs were generated. *PCSK1* shRNA knocking down and non-targeting shRNA-generated hESCs lines were used.

Generation of *PCSK1* Mutant hESC Lines with CRISPR

The two *PCSK1* gRNAs were designed using software from Zhang Feng's laboratory at the Massachusetts Institute of Technology (http://crispr.mit.edu). The sequence of *PCSK1* gRNA was GACC CGC TGG CCA ATA ACC C. Individual hESC colonies, derived from single stem cells, were picked for clonal expansion. Thus, cells in each hESC clone carried the same mutations imposed using CRISPR-Cas9. These clones were screened with Sanger sequencing of the PCR products amplified around the targeted genomic loci. To enable DNA sequencing of both alleles around the target site in each CRISPR-Cas9-generated hESC line that derived from a single ESC, PCR products amplified from each line were inserted into the TOPO blunt-end cloning vector (Thermo Fisher Scientific). After screening several bacteria colonies, the DNA sequences for both alleles in each clone were determined. Four isogenic hESC clones were selected for further hypothalamic neuron differentiation and functional studies: clone 6

and clone 8 (compound heterozygous for inactivating mutations of PC1/3, referred to as KO1, KO2); clone 17 (heterozygous mutation, referred to as Het); clone 11 (wild-type, referred to as WT) were selected for further hypothalamic neuron differentiation and functional studies. Experimental details for CRISPR-Cas9 transfection and screening of off-target sites are included in the Supplemental Information.

Hypothalamic Neuron Differentiation

Hypothalamic neurons were generated from *PCSK1* shRNA knockdown and CRISPR-generated mutant hESC lines using a protocol described previously (Wang et al., 2016). hESCs were cultured overnight on Matrigel plates (1 million cells/well for 6-well plates) in hESC medium (500 mL of knockout DMEM, 90 mL of knockout serum, 6.5 mL of GlutaMAX, 6.5 mL of NEAA, 6.5 mL of penicillin/streptomycin, 0.65 mL of β-mercaptoethanol, and 10 ng/mL bFGF; all reagents were from Life Technologies) with 10 μM ROCK inhibitor (Y27632, Stemgent) and then differentiated in EB medium: hESC medium without bFGF, supplemented with sonic hedgehog (100 ng/mL, R&D), purmorphamine (2 μM, Stemgent), 10 μM SB-431542 (Selleckchem), and 2.5 μM LDN 193289 (Selleckchem). From days 4–8, EB medium was gradually replaced with N2 medium (25) (500 mL of DMEM/F12 supplemented with 5.5 mL of N2 supplement, 0.2 mM ascorbic acid, 5.5 mL of GlutaMAX, 5.5 mL of penicillin/streptomycin, and 5.5 mL of 16% glucose). At day 8, cells were switched to N2 medium supplemented with B27 and 10 μM DAPT (Stemgent) until day 12. Cells were harvested with trypsin LE (Life Technology) at 37°C for 4 min and washed twice with N2 medium; the pellet was then resuspended with N2 medium plus B27 and 10 μM ROCK inhibitor and passed through a Falcon tube with cell-strainer cap (Fisher Scientific) to make a single-cell suspension. Cells were counted and aliquots plated on poly-L-ornithine (0.01%, Sigma-Aldrich) and laminin-coated (4 μg/mL, Life Technology) plates (150,000 cells/well for 24-well plate, 300,000 cells/well for 12-well plate, 1 million cells/well for 6-well plate). After cells completely attached to the plate, medium was changed to N2 medium plus B27 and 10 μM DAPT. Four days later, cells were switched to N2 medium supplemented with B27 and 20 ng/mL brain-derived neurotrophic factor (R&D) until analysis. Medium was changed every 2 days until analysis.

qPCR Analysis

Differentiated neurons were lysed with TRIzol RNA reagent (Thermo Fisher Scientific) or RLT buffer. RNA was isolated by isopropanol precipitation or RNeasy micro kit (QIAGEN). cDNA was made with SuperScript VILO Master Mix (Thermo Fisher Scientific) using 1 μg RNA for each sample. GoTaq qPCR Master Mix was used for qPCR (Promega). See Table S2 for qPCR primers.

Immunocytochemistry of Cultured Neurons

Cultured neurons were fixed with 4% paraformaldehyde at room temperature for 15 min, then washed three times with PBS for 5 min per wash. Cells were blocked with 10% donkey serum (Jackson ImmunoResearch) in PBST (PBS + 0.1% Triton X-100) for 30 min, then switched to primary antibody (diluted with blocking buffer) solution and incubated at room temperature for 2 hr. Cells were washed three times with PBST (5 min each wash), then exposed to secondary antibodies (Life Technology). Alexa Fluor secondary antibodies, goat or donkey anti-mouse, rabbit, goat or chicken 488, 555, and 647 were used at 1:400 dilution in PBST and incubated at room temperature for 1 hr. Following three washes with PBST, cells were incubated with Hoechst (1 μg/mL, diluted in PBST) for 5 min and switched to PBS for fluorescent imaging. A fluorescence microscope was used to acquire images. See Table S3 for antibody information. To investigate the apoptosis of differentiated neurons, a CF 594 TUNEL assay apoptosis detection kit (Biotium) was used following the immunostaining step. Images were quantified manually with "cell count" in Photoshop CS4 software. Cell number was counted in each channel from at least three independent experiments. The percentages of POMC neurons or $PC1^+$ cells were calculated as: (number of POMC/$PC1^+$ neurons)/(number of Hoechst-positive cells) × 100%.

Western Blots

Each protein sample was prepared from one well of neurons cultured in the 6-well plate (start from 1 million cells on day 12). After aspirating medium, each well was rinsed with cold PBS. Lysis buffer (250 μL) was then added to each well, and a cell scraper was used to collect all neuron lysates. The lysis buffer contained: 20 mM Tris (pH 7.4), 150 mM NaCl, 2% Nonidet P-40, 1 mM EDTA (pH 8.0), 10% glycerol, 0.5% sodium deoxycholate, 0.2% semi-dehydroascorbate supplemented with phosphatase, and proteinase inhibitors (Boston Bioproducts). Cell lysates were then transferred into 1.7 mL Eppendorf tubes for sonication. After centrifugation at 10,000 × *g* for 10 min at 4°C, the supernatant was transferred to a new Eppendorf tube. Protein concentration was determined using Pierce BCA Protein Assay Kit (Thermo Fisher Scientific). Proteins (30 μg) were loaded to each lane on an 8% PAGE gel. Anti-β-actin and anti-α-tubulin antibodies were used as loading control. See Table S2 for antibody information.

Neuropeptide Assays

All these neurons were cultured in 12-well plates and initiated with 300,000 cells at day 12. For neuron lysate samples, medium was aspirated and the differentiated neurons rinsed once with PBS, after which 250 μL of 0.1 N HCl was added to each well. Cells were harvested with a cell scraper and the cell lysates were transferred into 1.7 mL Eppendorf tubes. These samples were sonicated for 5 min. After centrifugation at 10,000 × *g* for 10 min at 4°C, the supernatants were collected for POMC, ACTH, αMSH, and βEP assays. For medium samples, 1 mL of neuron culture medium was collected and centrifuged at 8,000 × *g* for 5 min. Nine volumes of the supernatant (720 μL) were mixed with 1 volume of 1 N HCl (80 μL) to prepare a final 0.1 N HCl sample mixture. Neuropeptide assays were performed as described earlier, and all the values are presented in femtomoles (fmol). In brief, POMC was measured using a two-site ELISA with monoclonal antibodies (gifts from Dr. Anne White, University of Manchester, UK) against ACTH (10–18) as capture antibody and the detection antibody against γMSH (Crosby et al., 1988). This assay detects 22 K pro-ACTH but has no cross-reactivity with ACTH, αMSH, γMSH, or βEP. Affinity-purified human 31 K POMC was used for standards. The assay sensitivity for POMC was 8 fmol/mL. ACTH was measured with a

two-site chemiluminescent ELISA kit (Siemens) using the Immulite 1000 Immunoassay Analyzer (Siemens). There was no cross-reactivity with αMSH, βEP, or POMC (Scott-Moncrieff et al., 2003). αMSH and βEP were measured by RIA (Wallingford et al., 2009; Wardlaw, 1986). αMSH was measured with a rabbit polyclonal RIA antiserum (from Dr. Sharon Wardlaw's laboratory) (Wardlaw, 1986). The αMSH RIA cross-reacted fully with des-acetyl-a-MSH but not with ACTH, corticotropin-like intermediate-lobe peptide, βEP, POMC, or αMSH 1–12 (free acid). βEP was measured with a rabbit polyclonal antiserum (from Dr. Sharon Wardlaw's laboratory) that cross-reacted 30% on a molar basis with β-LPH and 3% with POMC (Wardlaw, 1986). The βEP RIA did not cross-react with ACTH or αMSH; there was 30% cross-reactivity on a molar basis with βLPH and 3% with POMC. αMSH and βEP peptides from Phoenix Pharmaceuticals were used as standards.

Single-Cell Transcriptome Sequencing

Experimental details are provided in Supplemental Information.

ACCESSION NUMBERS

All original RNA-seq data have been deposited in the NCBI's Gene Expression Omnibus (GEO: GSE89503).

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, five figures, and three tables and can be found with this article online at http://dx.doi.org/10.1016/j.stemcr.2016.12.021.

AUTHOR CONTRIBUTIONS

L.W. and R.L. designed these studies; D.E. and S.L.W. provided advice on experimental design. L.W. performed experiments, analyzed data, and drafted the manuscript; D.E. and R.L. helped with editing of the manuscript; L.S. provided help with cell cultures and cell line analyses; S.K.P. and K.M. performed the neuropeptide assays for the neuron lysates and incubation medium samples; S.L.W. supervised the neuropeptide assays; Y.X., J.K., and J.G. performed single-cell sequencing experiments and helped with relevant data analyses; C.D. helped with the initial CRISPR experiments and provided helpful suggestions for these experiments. All authors reviewed drafts of manuscript.

ACKNOWLEDGMENTS

We thank Andrew G. Elefanty (Monash University, Melbourne, Australia) for providing the NKX2.1-GFP/W-hESC line; Dr. Anne White (University of Manchester, UK) for providing the antibodies for the POMC neuropeptide assay; Bryan González and Lisa Cole Burnett for helpful suggestions; This research was supported by RO1 DK52431-20, P30 DK26687-35 from NIH, a C026184 award from New York State Stem Cell Science, 7T32DK007559-25 training grant to the New York Obesity Nutrition Research Center, the Foundation for Prader-Willi Research, the Russell Berrie Foundation, the Rudin Foundation, and RO1 DK 093920 (S.L.W.). D.E. is an NYSCF-Robertson Investigator. Y.X., J.K., and J.G. are employees and stockholders of Regeneron Pharmaceuticals.

Received: August 5, 2016
Revised: December 20, 2016
Accepted: December 21, 2016
Published: January 26, 2017

REFERENCES

Alhadeff, A.L., Rupprecht, L.E., and Hayes, M.R. (2012). GLP-1 neurons in the nucleus of the solitary tract project directly to the ventral tegmental area and nucleus accumbens to control for food intake. Endocrinology *153*, 647–658.

Angelogianni, P., Li, H.L., and Gianoulakis, C. (2000). Ontogenesis of proopiomelanocortin and its processing to beta-endorphin by the fetal and neonatal rat brain. Neuroendocrinology *72*, 231–241.

Baker, D., Hirst, A.J., Gokhale, P.J., Juarez, M.A., Williams, S., Wheeler, M., Bean, K., Allison, T.F., Moore, H.D., Andrews, P.W., et al. (2016). Detecting genetic mosaicism in cultures of human pluripotent stem cells. Stem Cell Rep. *7*, 998–1012.

Balthasar, N., Dalgaard, L.T., Lee, C.E., Yu, J., Funahashi, H., Williams, T., Ferreira, M., Tang, V., McGovern, R.A., Kenny, C.D., et al. (2005). Divergence of melanocortin pathways in the control of food intake and energy expenditure. Cell *123*, 493–505.

Bandsma, R.H., Sokollik, C., Chami, R., Cutz, E., Brubaker, P.L., Hamilton, J.K., Perlman, K., Zlotkin, S., Sigalet, D.L., Sherman, P.M., et al. (2013). From diarrhea to obesity in prohormone convertase 1/3 deficiency: age-dependent clinical, pathologic, and enteroendocrine characteristics. J. Clin. Gastroenterol. *47*, 834–843.

Benzinou, M., Creemers, J.W., Choquet, H., Lobbens, S., Dina, C., Durand, E., Guerardel, A., Boutin, P., Jouret, B., Heude, B., et al. (2008). Common nonsynonymous variants in PCSK1 confer risk of obesity. Nat. Genet. *40*, 943–945.

Burnett, L.C., LeDuc, C.A., Sulsona, C.R., Paull, D., Rausch, R., Eddiry, S., Carli, J.F., Morabito, M.V., Skowronski, A.A., Hubner, G., et al. (2016). Deficiency in prohormone convertase PC1 impairs prohormone processing in Prader-Willi syndrome. J Clin. Invest. http://dx.doi.org/10.1172/JCI88648.

Chen, A.S., Marsh, D.J., Trumbauer, M.E., Frazier, E.G., Guan, X.M., Yu, H., Rosenblum, C.I., Vongs, A., Feng, Y., Cao, L., et al. (2000). Inactivation of the mouse melanocortin-3 receptor results in increased fat mass and reduced lean body mass. Nat. Genet. *26*, 97–102.

Creemers, J.W., Pritchard, L.E., Gyte, A., Le Rouzic, P., Meulemans, S., Wardlaw, S.L., Zhu, X., Steiner, D.F., Davies, N., Armstrong, D., et al. (2006). Agouti-related protein is posttranslationally cleaved by proprotein convertase 1 to generate agouti-related protein (AGRP)83-132: interaction between AGRP83-132 and melanocortin receptors cannot be influenced by syndecan-3. Endocrinology *147*, 1621–1631.

Crosby, S.R., Stewart, M.F., Ratcliffe, J.G., and White, A. (1988). Direct measurement of the precursors of adrenocorticotropin in human plasma by two-site immunoradiometric assay. J. Clin. Endocrinol. Metab. *67*, 1272–1277.

de Boer, S.A., Lefrandt, J.D., Petersen, J.F., Boersma, H.H., Mulder, D.J., and Hoogenberg, K. (2016). The effects of GLP-1 analogues in obese, insulin-using type 2 diabetes in relation to eating behaviour. Int. J. Clin. Pharm. *38*, 144–151.

Farooqi, I.S., Volders, K., Stanhope, R., Heuschkel, R., White, A., Lank, E., Keogh, J., O'Rahilly, S., and Creemers, J.W. (2007). Hyperphagia and early-onset obesity due to a novel homozygous missense mutation in prohormone convertase 1/3. J. Clin. Endocrinol. Metab. *92*, 3369–3373.

Goulburn, A.L., Alden, D., Davis, R.P., Micallef, S.J., Ng, E.S., Yu, Q.C., Lim, S.M., Soh, C.L., Elliott, D.A., Hatzistavrou, T., et al. (2011). A targeted NKX2.1 human embryonic stem cell reporter line enables identification of human basal forebrain derivatives. Stem Cells *29*, 462–473.

Jackson, R.S., Creemers, J.W., Ohagi, S., Raffin-Sanson, M.L., Sanders, L., Montague, C.T., Hutton, J.C., and O'Rahilly, S. (1997). Obesity and impaired prohormone processing associated with mutations in the human prohormone convertase 1 gene. Nat. Genet. *16*, 303–306.

Jackson, R.S., Creemers, J.W., Farooqi, I.S., Raffin-Sanson, M.L., Varro, A., Dockray, G.J., Holst, J.J., Brubaker, P.L., Corvol, P., Polonsky, K.S., et al. (2003). Small-intestinal dysfunction accompanies the complex endocrinopathy of human proprotein convertase 1 deficiency. J. Clin. Invest. *112*, 1550–1560.

Larsen, P.J., Tang-Christensen, M., Holst, J.J., and Orskov, C. (1997). Distribution of glucagon-like peptide-1 and other preproglucagon-derived peptides in the rat hypothalamus and brainstem. Neuroscience *77*, 257–270.

Lloyd, D.J., Bohan, S., and Gekakis, N. (2006). Obesity, hyperphagia and increased metabolic efficiency in Pc1 mutant mice. Hum. Mol. Genet. *15*, 1884–1893.

Marsh, D.J., Hollopeter, G., Huszar, D., Laufer, R., Yagaloff, K.A., Fisher, S.L., Burn, P., and Palmiter, R.D. (1999). Response of melanocortin-4 receptor-deficient mice to anorectic and orexigenic peptides. Nat. Genet. *21*, 119–122.

Martin, M.G., Lindberg, I., Solorzano-Vargas, R.S., Wang, J., Avitzur, Y., Bandsma, R., Sokollik, C., Lawrence, S., Pickett, L.A., Chen, Z., et al. (2013). Congenital proprotein convertase 1/3 deficiency causes malabsorptive diarrhea and other endocrinopathies in a pediatric cohort. Gastroenterology *145*, 138–148.

Merkle, F.T., Maroof, A., Wataya, T., Sasai, Y., Studer, L., Eggan, K., and Schier, A.F. (2015). Generation of neuropeptidergic hypothalamic neurons from human pluripotent stem cells. Development *142*, 633–643.

Nuffer, W.A., and Trujillo, J.M. (2015). Liraglutide: a new option for the treatment of obesity. Pharmacotherapy *35*, 926–934.

O'Rahilly, S., Gray, H., Humphreys, P.J., Krook, A., Polonsky, K.S., White, A., Gibson, S., Taylor, K., and Carr, C. (1995). Brief report: impaired processing of prohormones associated with abnormalities of glucose homeostasis and adrenal function. N. Engl. J. Med. *333*, 1386–1390.

Pan, H., Nanno, D., Che, F.Y., Zhu, X., Salton, S.R., Steiner, D.F., Fricker, L.D., and Devi, L.A. (2005). Neuropeptide processing profile in mice lacking prohormone convertase-1. Biochemistry *44*, 4939–4948.

Paquet, L., Zhou, A., Chang, E.Y., and Mains, R.E. (1996). Peptide biosynthetic processing: distinguishing prohormone convertases PC1 and PC2. Mol. Cell Endocrinol. *120*, 161–168.

Pritchard, L.E., Oliver, R.L., McLoughlin, J.D., Birtles, S., Lawrence, C.B., Turnbull, A.V., and White, A. (2003). Proopiomelanocortin-derived peptides in rat cerebrospinal fluid and hypothalamic extracts: evidence that secretion is regulated with respect to energy balance. Endocrinology *144*, 760–766.

Schneeberger, M., Dietrich, M.O., Sebastian, D., Imbernon, M., Castano, C., Garcia, A., Esteban, Y., Gonzalez-Franquesa, A., Rodriguez, I.C., Bortolozzi, A., et al. (2013). Mitofusin 2 in POMC neurons connects ER stress with leptin resistance and energy imbalance. Cell *155*, 172–187.

Schulz, C., Paulus, K., Lobmann, R., Dallman, M., and Lehnert, H. (2010). Endogenous ACTH, not only alpha-melanocyte-stimulating hormone, reduces food intake mediated by hypothalamic mechanisms. Am. J. Physiol. Endocrinol. Metab. *298*, E237–E244.

Schwartz, M.W., Marks, J.L., Sipols, A.J., Baskin, D.G., Woods, S.C., Kahn, S.E., and Porte, D., Jr. (1991). Central insulin administration reduces neuropeptide Y mRNA expression in the arcuate nucleus of food-deprived lean (Fa/Fa) but not obese (fa/fa) Zucker rats. Endocrinology *128*, 2645–2647.

Scott-Moncrieff, J.C., Koshko, M.A., Brown, J.A., Hill, K., and Refsal, K.R. (2003). Validation of a chemiluminescent enzyme immunometric assay for plasma adrenocorticotropic hormone in the dog. Vet. Clin. Pathol. *32*, 180–187.

Secher, A., Jelsing, J., Baquero, A.F., Hecksher-Sorensen, J., Cowley, M.A., Dalboge, L.S., Hansen, G., Grove, K.L., Pyke, C., Raun, K., et al. (2014). The arcuate nucleus mediates GLP-1 receptor agonist liraglutide-dependent weight loss. J. Clin. Invest. *124*, 4473–4488.

Smith, C., Gore, A., Yan, W., Abalde-Atristain, L., Li, Z., He, C., Wang, Y., Brodsky, R.A., Zhang, K., Cheng, L., et al. (2014). Whole-genome sequencing analysis reveals high specificity of CRISPR/Cas9 and TALEN-based genome editing in human iPSCs. Cell Stem Cell *15*, 12–13.

Stijnen, P., Ramos-Molina, B., O'Rahilly, S., and Creemers, J.W. (2016). PCSK1 mutations and human endocrinopathies: from obesity to gastrointestinal disorders. Endocr. Rev. *37*, 347–371.

Tsigos, C., Crosby, S.R., Gibson, S., Young, R.J., and White, A. (1993). Proopiomelanocortin is the predominant adrenocorticotropin-related peptide in human cerebrospinal fluid. J. Clin. Endocrinol. Metab. *76*, 620–624.

Veres, A., Gosis, B.S., Ding, Q., Collins, R., Ragavendran, A., Brand, H., Erdin, S., Cowan, C.A., Talkowski, M.E., and Musunuru, K. (2014). Low incidence of off-target mutations in individual CRISPR-Cas9 and TALEN targeted human stem cell clones detected by whole-genome sequencing. Cell Stem Cell *15*, 27–30.

Wallingford, N., Perroud, B., Gao, Q., Coppola, A., Gyengesi, E., Liu, Z.W., Gao, X.B., Diament, A., Haus, K.A., Shariat-Madar, Z., et al. (2009). Prolylcarboxypeptidase regulates food intake by inactivating alpha-MSH in rodents. J. Clin. Invest. *119*, 2291–2303.

Wang, L., Meece, K., Williams, D.J., Lo, K.A., Zimmer, M., Heinrich, G., Martin Carli, J., Leduc, C.A., Sun, L., Zeltser, L.M., et al. (2015). Differentiation of hypothalamic-like neurons from human pluripotent stem cells. J. Clin. Invest. *125*, 796–808.

Wang, L., Egli, D., and Leibel, R.L. (2016). Efficient generation of hypothalamic neurons from human pluripotent stem cells. Curr. Protoc. Hum. Genet. *90*, 21.5.1–21.5.14.

Wardlaw, S.L. (1986). Regulation of beta-endorphin, corticotropin-like intermediate lobe peptide, and alpha-melanotropin-stimulating hormone in the hypothalamus by testosterone. Endocrinology *119*, 19–24.

Wardlaw, S.L. (2011). Hypothalamic proopiomelanocortin processing and the regulation of energy balance. Eur. J. Pharmacol. *660*, 213–219.

Zhou, A., Bloomquist, B.T., and Mains, R.E. (1993). The prohormone convertases PC1 and PC2 mediate distinct endoproteolytic cleavages in a strict temporal order during proopiomelanocortin biosynthetic processing. J. Biol. Chem. *268*, 1763–1769.

Zhu, X., Zhou, A., Dey, A., Norrbom, C., Carroll, R., Zhang, C., Laurent, V., Lindberg, I., Ugleholdt, R., Holst, J.J., et al. (2002). Disruption of PC1/3 expression in mice causes dwarfism and multiple neuroendocrine peptide processing defects. Proc. Natl. Acad. Sci. USA *99*, 10293–10298.