Sexual dimorphism exists in energy balance, but the underlying mechanisms remain unclear. Here we show that the female mice have more pro-opiomelanocortin (POMC) neurons in the arcuate nucleus of hypothalamus than males, and female POMC neurons display higher neural activities, compared to male counterparts. Strikingly, deletion of the transcription factor, TAp63, in POMC neurons confers “male-like” diet-induced obesity (DIO) in female mice associated with decreased POMC neural activities; but the same deletion does not affect male mice. Our results indicate that TAp63 in female POMC neurons contributes to the enhanced POMC neuron functions and resistance to obesity in females. Thus, TAp63 in POMC neurons is one key molecular driver for the sexual dimorphism in energy homeostasis.
Sexual dimorphism exists in various physiological processes, including feeding behavior and energy homeostasis. For example, total daily energy intake in male rats is higher than that in females, even when corrected by their larger lean body mass and metabolic rate. In addition, high-fat diet (HFD) feeding leads to larger body weight gain in male rats/mice than in female counterparts. While sex hormones (e.g., estrogens and testosterone) and the sex chromosome complement have been implicated as major contributors for these sex-related differences, the cellular and molecular mechanisms underlying the sexual dimorphism in body weight balance remains to be fully revealed.

Pro-opiomelanocortin (POMC) neurons in the arcuate nucleus of hypothalamus (ARH) play essential roles in the regulation of feeding and body weight balance, as genetic ablation of POMC neurons in the ARH leads to hyperphagia and obesity in mice. On the other hand, selective activation of ARH POMC neurons results in decreased food intake and body weight loss, indicating that the neural activities of ARH POMC neurons are important for the regulation of energy homeostasis. In addition, transcription of the POMC gene itself is physiologically relevant for body weight control. One of the POMC gene products, α-melanocyte-stimulating hormone (α-MSH), acts upon brain melanocortin 3 and 4 receptors to suppress the food intake and body weight gain. Further, massive obesity is observed in mice with POMC gene deficiency. Consistently, humans carrying loss-of-function mutations in the POMC gene develop obesity.

We observed that female mice have more POMC neurons in the ARH than males, and female POMC neurons display higher neural activities, compared to male counterparts. We generated mice with POMC-specific deletion or overexpression of TAp63, a transcriptionally active variant of p63 (a transcription factor). Systemic characterization of these mutant mouse models revealed that TAp63 contributes to sexual dimorphism in POMC neuron functions and regulates energy homeostasis in a sex-specific manner.

Results

Sexual dimorphism in POMC neurons. To explore the cellular basis for sexual dimorphism in energy balance, we first examined the neural activities of several neural populations that are known to play key roles in the regulation of energy homeostasis. These include ARH neurons co-expressing agouti-related peptide (AgRP) and neuropeptide Y (NPY), stereoiodogenic factor-1 (SF1) neurons in the ventromedial hypothalamic nucleus (VMH), single-minded 1 (SIM1) neurons in the medial amygdala (MeA), and SIM1 neurons in the paraventricular nucleus of the hypothalamus (PVH). We did not observe any sex-specific difference in resting membrane potential or firing rate of AgRP/NPY neurons, VMH SF1 neurons, or MeA SIM1 neurons (Supplementary Figure 1A-1C, 1E-1G). While male and female PVH SIM1 neurons showed similar resting membrane potential (Supplementary Figure 1D), the firing rate of female PVH SIM1 neurons was significantly lower than that of the male counterparts (Supplementary Figure 1H). However, since the majority of PVH SIM1 neurons are anorexigenic neurons that suppress food intake, the decreased firing activity of female PVH SIM1 neurons should not account for the relative low body weight in female mice.

We then compared the neural activities of TOMATO-labeled mature ARH POMC neurons (Fig. 1a) in male vs. female POMC-CreER<sup>12</sup>/Rosa26-tdtOMATO mice. Interestingly, we observed that the firing rate in female POMC neurons was significantly higher, compared to male POMC neurons (Fig. 1b, c). In addition, the resting membrane potential in female POMC neurons was significantly depolarized, compared to that in male POMC neurons (Fig. 1b, d). In other words, female POMC neurons displayed significantly enhanced excitability, compared to male POMC neurons.

It has been reported that female mice express higher POMC mRNAs and protein products in the hypothalamus than male mice. Consistently, here we detected significantly higher POMC mRNA levels in the hypothalamus of female mice than age-matched males (Fig. 1e). In addition, more β-endorphin (one POMC protein product)-positive neurons were observed in female ARH than in males (Fig. 1f,g). Increased number of POMC neurons (as revealed by β-endorphin immunostaining) may reflect higher POMC expression levels in each cell that exceed the detecting threshold by immunostaining; however, these results may also reflect the different POMC neuron numbers in female vs. male mice. Indeed, using POMC-CreER<sup>12</sup>/Rosa26-tdtOMATO mice, in which all POMC neurons are labeled by TOMATO after tamoxifen induction (11 weeks), regardless of the expression levels of POMC gene, we found significantly more POMC neurons in the ARH of female mice than in male mice (Fig. 1b,i).
**Fig. 1** POMC neurons display sexual dimorphism in neural activities and gene transcription. 

- **a** Fluorescence for TOMATO (left) and Lucifer Yellow (middle), and bright-field illumination (right) of the recorded POMC neuron in a brain slice prepared from POMC-CreERT²/Rosa26-tdTOMATO mouse with tamoxifen induction at 11 weeks of age.
- **b** Representative current clamp traces in POMC neurons from chow-fed male or female POMC-CreERT²/Rosa26-tdTOMATO mice at the age of 15–16 weeks. 
  - **c, d** Average firing rate (**c**) and resting membrane potential (**d**) in POMC neurons from male or female POMC-CreERT²/Rosa26-tdTOMATO mice. Data are presented as mean ± SEM. N = 37–78 per group. *P < 0.05 or **P < 0.01 in t-tests.
  - **e** POMC mRNAs levels in the entire hypothalamus from male or female littermates (16 weeks of age), quantified by RT-qPCR. Data are presented as mean ± SEM with individual data points. N = 4 or 6 per group. **P < 0.01 in t-tests.
  - **f** Representative immunostaining images for β-endorphin in the hypothalamus of male or female littermates (16 weeks of age).
  - **g** Quantiﬁcations of β-endorphin-positive neurons in the hypothalamus from 1 out of 5 consecutive brain sections of male or female littermates. Data are presented as mean ± SEM with individual data points. N = 6 per group. **P < 0.01 in t-tests.
  - **h** Representative fluorescent images for TOMATO in the hypothalamus of male or female POMC-CreERT²/Rosa26-tdTOMATO littermates (tamoxifen injected at 16 weeks of age, and perfused at 16 weeks of age). 
  - **i** Quantiﬁcations of TOMATO-positive neurons (POMC neurons) in the hypothalamus of one out of 5 consecutive brain sections of male or female littermates (tamoxifen injected at 11 weeks of age, and perfused at 16 weeks of age). Data are presented as mean ± SEM with individual data points. N = 4 or 7 per group. **P < 0.01 in t-tests.
also located\textsuperscript{35,36}, presumably due to the weak tamoxifen-induced Cre activity in this population. The \textit{TAp63}\textsuperscript{ΔN\beta} allele was also recombined in the pituitary, which contains ACTH cells (Fig. 3b). However, there were no significant differences in pituitary levels of \textit{TAp63}, \textit{ΔNp63}, \textit{FSH β} subunit, \textit{LH β} subunit, or \textit{POMC} (ACTH) between female control and \textit{pomc-TAp63} KO mice (Supplementary Figure 3C-3G); no difference in circulating ACTH was observed (Supplementary Figure 3H). Importantly, the levels of corticosterone were not altered in the \textit{pomc-TAp63} KO mice, compared to control mice, either at the basal condition or in response to the restraint stress (Supplementary Figure 3I). Thus, we suggest that the deletion of \textit{TAp63} in pituitary ACTH cells, if any, should not affect the hypothalamus-pituitary-gonad and the hypothalamus-pituitary-adrenal axes. Further, we examined the effects of tamoxifen injections on estrous cycles in female mice; while tamoxifen transiently disrupted the estrous cyclicity, it recovered in about 3 weeks after tamoxifen induction (Supplementary Figure 4). Thus, most of our studies were carried out at least 4 weeks after tamoxifen induction.

We monitored the body weight balance in male \textit{pomc-TAp63} KO mice and their littermate controls (\textit{TAp63}\textsuperscript{ΔN\beta/ΔN\beta}). Male \textit{pomc-TAp63} KO mice showed comparable body weight as their male controls, when fed on a regular chow diet (from weaning till 30 weeks of age; Fig. 3c). HFD feeding (from 30 weeks till 43 weeks of age) accelerated body weight gain in both \textit{pomc-TAp63} KO mice and control males, but no difference was observed between the two genotypes (Fig. 3c). No differences were observed in fat mass and lean mass, except that a modest increase in lean mass was observed in chow-fed \textit{pomc-TAp63} KO mice at 15 weeks of age (Fig. 3d, e). Thus, deletion of \textit{TAp63} in \textit{POMC} neurons had none to minor effect on body weight balance and adiposity in male mice.

We also monitored the body weight gain in female \textit{pomc-TAp63} KO mice and their littermate controls. Similar to male mutants, \textit{pomc-TAp63} KO female mice showed comparable body weight, fat mass, and lean mass as their control females, when fed on the regular chow (Fig. 3f-h). Interestingly, when challenged with HFD feeding, \textit{pomc-TAp63} KO female mice gained significantly more body weight, compared to controls; at the end of 13-week of HFD feeding, \textit{pomc-TAp63} KO mice were 13 grams heavier than control mice (>30% of the control body weight; Fig. 3f). The body weight gain in female \textit{pomc-TAp63} KO mice was primarily due to a two-fold increase in fat mass, and to a less extent an increase in lean mass (Fig. 3g, h).

Given the sex-specific phenotypes observed in HFD-fed \textit{pomc-TAp63} KO mice, we analyzed the HFD-induced body weight gain and body fat gain in male vs. female mice with or without \textit{TAp63} deletion. Consistent with the notion that wild-type female rodents are more resistant to HFD-induced obesity, compared to their male counterparts\textsuperscript{4-8,27}, we found that HFD-induced body weight gain and fat gain in female control mice were significantly lower than those in male control mice (Fig. 3i, j). Strikingly, \textit{pomc-TAp63} KO female mice gained as much body weight and fat as male mice (Fig. 3i, j). In other words, the sex differences in HFD-induced obesity were largely diminished by selective deletion of \textit{TAp63} in \textit{POMC} neurons.

We then analyzed the food intake in chow or HFD-fed mice. We found that male mutants and their controls showed comparable food intake, regardless they were fed on chow or HFD (Fig. 3k S5A). Food intake in chow-fed \textit{pomc-TAp63} KO female mice were also similar to their chow-fed female controls (Supplementary Figure 5B). Importantly, HFD-fed \textit{pomc-TAp63} KO female mice showed increased food intake (Fig. 3i), although these changes only became significant toward the later phase of HFD feeding, which made it difficult to interpret their contributions to obesity. We then performed detailed analyses on energy balance in female \textit{pomc-TAp63} KO mice and their control littermates with comparable body weight, lean mass, and fat mass (Supplementary Figure 6A-6C). These mice were acclimated into the Comprehensive Lab Animal Monitoring System (CLAMS) and subjected to a 3-day chow 3-day HFD feeding protocol. While there was no significant changes in 24-h or dark cycle food intake (either chow or HFD) between the two genotypes, female \textit{pomc-TAp63} KO mice showed significantly increased light-cycle food intake (Fig. 2m), which has been associated with increased body weight gain\textsuperscript{37,38}. On the other hand, we did not observe any significant changes in energy expenditure or ambulation between the two groups during these short chow or HFD-feeding periods (Fig. 2n, o).

\section*{Estrogen-induced anorexigenic effects in female mice.}

The female-specific metabolic deficits observed in \textit{pomc-TAp63} KO mice suggested a potential interaction between the ovarian hormones and \textit{TAp63} functions in the regulation of energy balance. To test this, we subjected the female \textit{pomc-TAp63} KO mice and their control littermates to either ovariectomy (OVX+V) or OVX plus estrogen supplement (17β-estradiol, 0.5 µg per day lasting 90 days; OVX+E), followed by 11-day chow feeding and then 16-day HFD feeding. During the initial chow-feeding period, we did not observe significant differences in the body weight among all groups (Fig. 4a). During the following HFD feeding period, OVX+E treatment induced significant reductions in body weight and food intake, compared to OVX+V treatment, in both control and \textit{pomc-TAp63} KO mice (Fig. 4b, c). No significant differences were observed in body weight and HFD intake between OVX+E-
Deletion of TAp63 in POMC neurons diminishes sexual dimorphism in body weight responses to chronic HFD feeding. a Construction of TAp63fl/fl allele. Two loxP sites flank exon 2 of the p63 gene and Cre-mediated recombination results in disruption of TAp63 expression; ΔNp63 expression starts from exon 3 and is therefore not affected by the recombination. b PCR detection of TAp63fl/fl allele and Cre-induced recombination in various tissues from the control (TAp63fl/fl) or the pomc-TAp63 KO (TAp63fl/fl/POMC-CreERT2) mice. Adrenal, adrenal gland; ARH, arcuate nucleus of the hypothalamus; BAT, brown adipose tissue; hippo, hippocampus; NTS, nucleus of the solitary tract; pitu, pituitary.

Body weight (g)

Chow

HFD

Female

Male

Sexual dimorphism in body weight responses to chronic HFD feeding.

Female body weight

Male body weight

Female fat mass

Male fat mass

Female lean mass

Male lean mass

Body weight gain

Female food intake

Female energy expenditure

Female ambulation

Cumulative HFD intake

Cumulative HFD intake (g)

Weeks after HFD feeding

Weeks after HFD feeding

Fig. 3 Deletion of TAp63 in POMC neurons diminishes sexual dimorphism in body weight responses to chronic HFD feeding. a Construction of TAp63fl/fl mouse allele. Two loxP sites flank exon 2 of the p63 gene and Cre-mediated recombination results in disruption of TAp63 expression; ΔNp63 expression starts from exon 3 and is therefore not affected by the recombination. b PCR detection of TAp63fl/fl allele and Cre-induced recombination in various tissues from the control (TAp63fl/fl) or the pomc-TAp63 KO (TAp63fl/fl/POMC-CreERT2) mice. Adrenal, adrenal gland; ARH, arcuate nucleus of the hypothalamus; BAT, brown adipose tissue; hippo, hippocampus; NTS, nucleus of the solitary tract; pitu, pituitary.

Chow

HFD

Cumulative HFD intake (g)

Weeks after HFD feeding

Sexual dimorphism in body weight responses to chronic HFD feeding.
Fig. 4 Selective deletion of TAp63 in POMC neurons fails to affect the estrogen-induced anorexigenic effects in female mice. a, b Changes in body weight of female control or pomc-TAp63 KO mice that received OVX+V or OVX+E treatment, followed by 11-day chow feeding (a) and 16-day HFD feeding (b). c Cumulative food intake during the HFD feeding period. Data are presented as mean ± SEM. N = 4 or 5 per group. *P < 0.05 in two-way repeated ANOVA followed by post hoc Sidak tests.

Fig. 5 Selective overexpression of TAp63 in POMC neurons reduces the body weight of male HFD-fed mice. a Schematic construction of AAV vector, AAV-FLEX-TAp63-2A-GFP. b Immunofluorescent images showing GFP (left panel), TOMATO (middle panel), and merge (right panel) in POMC neurons of POMC-Cre/Rosa26-ttdTOMATO mice receiving AAV-FLEX-TAp63-2A-GFP stereotaxically injected in the ARH bilaterally. The majority of GFP-labeled neurons are TOMATO-positive; only a few double-labeled neurons are pointed by arrowheads for a better view. 3V, 3rd ventricle; ARH, arcuate nucleus of the hypothalamus. c Changes in body weight in HFD-fed male mice. Data are presented as mean ± SEM. N = 7 or 12 in control or OE group; N = 4 in one side group. *P < 0.05 between groups and #P < 0.05 between the control and pomc-TAp63 OE groups at each time point in two-way ANOVA followed by post hoc Sidak tests. e Cumulative HFD intake in HFD-fed male mice. Data are presented as mean ± SEM. N = 4 in one side group.
treated controls and pomc-TAp63 KO mice (Fig. 4b, c). Thus, the lack of TAp63 in POMC neurons did not affect the anorexigenic effects of estrogens in female mice. Interestingly, we noted that OVX+V-treated pomc-TAp63 KO mice showed significantly less body weight gain and HFD intake, compared to OVX+V-treated control mice (Fig. 4b, c). Collectively, deletion of TAp63 from POMC neurons blunted the OVX-induced body weight gain in female mice, a phenotype associated with decreased food intake.

Overexpression of TAp63 prevents DIO in male mice. To further investigate the function of TAp63 in POMC neurons, we tested the effects of TAp63 overexpression only in POMC neurons. To this end, we constructed an AAV vector, namely AAV-EF1-FLEX-TAp63-2A-GFP, which encodes loxP-flanked TAp63 cDNA in an opposite orientation that overexpresses TAp63 and GFP only in Cre-positive cells (Fig. 5a). We stereotaxically injected this virus in the two sides (bilaterally) of ARH in POMC-Cre mice, resulting in TAp63 overexpression only in POMC neurons (referred as pomc-TAp63 OE; Fig. 5b).

When challenged with HFD feeding, male pomc-TAp63 OE mice showed significantly less body weight gain than controls (Fig. 5c), mainly reflected by reduced fat mass gain (Fig. 5d). Food intake of male pomc-TAp63 OE mice were significantly reduced, compared to controls (Fig. 5e). Interestingly, in male mice with only one side of the ARH hit by the virus (Supplementary Figure 7A), we observed partial and nonsignificant reductions in body weight, fat mass, and food intake (Fig. 5c–e). These data indicate that enhanced TAp63 expression in POMC neurons can partially prevent the development of DIO in male mice. In HFD-fed female mice, overexpression of TAp63 in POMC neurons did not appear to significantly change body weight, fat mass, and food intake (Fig. 5f–h). However, it is worth noting that we started HFD feeding 3 days after the virus injections. Thus, potential effects of overexpression in female mice may have been confounded by the short “incubation period” after the virus injections, together with the limited sample size in certain groups and big variations among individual animals. Further, TAp63 overexpression in POMC neurons did not significantly alter the body weight and the fat mass in chow-fed male or female mice, although a significant decrease in lean mass was observed in female mice with TAp63 overexpression (Supplementary Figure 7B–7E).

Effects of TAp63 on POMC firing activities. In order to reveal the mechanisms by which TAp63 regulates POMC neurons, we analyzed the effects of TAp63 deletion on POMC neural activities in HFD-fed male and female mice. While the firing rate and resting membrane potential of POMC neurons were not significantly different between the male control and the male mutant mice, TAp63 deletion led to a significant reduction in firing rate and resting membrane potential in female POMC neurons (Fig. 6a, b). Importantly, the sex differences in firing rate and resting membrane potential of male vs. female POMC neuron were diminished by TAp63 deletion (Fig. 6a, b). Thus, these data indicate that TAp63 deletion diminished the sex differences in the excitability of POMC neurons.

We also compared the post-synaptic currents in male vs. female POMC neurons. No significant difference in glutamate-mediated miniature excitatory post-synaptic current (mEPSC) was observed (Supplementary Figure 8). Interestingly, we found that the amplitude of GABA-mediated miniature inhibitory post-synaptic current (mIPSC) was significantly lower in female mice compared to male mice (Fig. 6f-h). These data indicate that enhanced TAp63 expression in POMC neurons can significantly alter the post-synaptic currents in POMC neurons.
POMC neurons than in male POMC neurons, while the mIPSC frequency was comparable between the sexes (Fig. 6c–e). More importantly, female POMC neurons lacking TAp63, displayed increased mIPSC amplitude to the level of male POMC neurons (Fig. 6c, d). Together, these data suggest that TAp63 in female POMC neurons suppresses their responsiveness to the inhibitory GABAergic inputs, a potential mechanism that contributes to enhanced excitability.

We have previously shown that ERα signals can trigger rapid activations in a portion of POMC neurons, which in turns produces anorexigenic effects in female mice. Here, we examined the effects of a selective ERα agonist, propyl pyrazole triol (PPT), on neural activities of POMC neurons from either control or pomc-TAp63 KO mice. While 10 out of 19 (53%) POMC neurons from control female mice were activated by PPT (>20% increase in firing rate), only 4 out of 20 (20%) POMC neurons from pomc-TAp63 KO female mice were activated by PPT (P < 0.05 in χ²-test, Fig. 6i). A similar trend was also observed when PPT-induced depolarization (>2 mV) was compared in control vs. pomc-TAp63 KO mice (P = 0.09 in χ²-test, Fig. 6g). Thus, these results indicate that TAp63 deletion reduced the percentage of POMC neurons that respond to the ERα agonist.

To further test the responsiveness of POMC neurons to estrogenic signals, we crossed a recently generated Rosa26-ZsGreen mouse allele with the control (POMC-CreER<sup>122</sup>/Rosa26-tdTOMATO) and mutant (POMC-CreER<sup>122</sup>/TAp63<sup>fl/fl</sup>/Rosa26-tdTOMATO) mice, and performed electrophysiological recordings in ERα-positive POMC neurons (double labeled by ZsGreen and TOMATO, Fig. 6h). We found that PTT activated all these ERα-positive POMC neurons, but the deletion of TAp63 significantly attenuated the PPT-induced depolarization and increases in firing rate (Fig. 6j, k). These results indicate that TAp63 deletion impaired the responsiveness of ERα-positive POMC neurons to the ERα agonist.

**Discussion**

One major finding of the current study is that female POMC neurons have higher neural activities, compared to male POMC neurons, while such sexual dimorphism was not observed in most of the other neural populations we tested (e.g., AgRP/NPY neurons, SF1 neurons, and SIM1 neurons). Given the well-established anorexigenic nature of POMC neurons, these enhanced POMC neuron firing in female brains may contribute to the relatively lower body weight in female animals, compared to male counterparts. Interestingly, deletion of TAp63 in POMC neurons increased the susceptibility to DIO in female mice associated with decreased POMC neural activities; but the same deletion did not affect male mice. Although we could not fully exclude the possibility that TAp63 was not fully deleted from male POMC neurons, our results suggest that TAp63 in POMC neurons is one critical molecule that underlies, at least partly, sexual dimorphism in energy balance.

The mechanisms by which TAp63 in POMC neurons regulates energy balance, clearly involves its effects on POMC firing activities. We showed that TAp63 deletion resulted in decreased firing rate only in female POMC neurons, but not in male POMC neurons. The decreased POMC firing activities likely contribute to the increased food intake and increased susceptibility to DIO in these mutant mice. More importantly, while female POMC neurons had increased firing rates associated with decreased mIPSC amplitude, compared to their male counterparts, this sexual dimorphism in POMC firing activities and mIPSC amplitude was abolished by TAp63 deletion. Thus, we suggest that TAp63 in female POMC neurons is required to suppress the responsiveness to GABAergic inputs and to maintain high firing rate of these neurons, which therefore distinguishes them from male POMC neurons. Although firing activities recorded from ex vivo brain slices may not fully reconstruct the impacts of physiological environments of the male vs. female animals on POMC neurons, our results supported the notion that TAp63 has cell-autonomous effects on POMC neuron activities in a sexually dimorphic fashion.

It is quite surprising that we detected increased body weight only during HFD feeding, but not during chow feeding. The lack of body weight gain in chow-fed pomc-TAp63 KO females suggest that redundant mechanisms may exist at the physiological condition to compensate for the impaired POMC neuron functions in these mutant mice. Similarly, loss of SIRT1 in POMC neurons, while resulting in robust body weight gain in HFD-fed female mice, causes no body weight gain in chow-fed females.

In addition, deletion of LKB1 in POMC neurons results in about 50% reduction in α-MSH levels in female mice, but fails to affect the body weight balance. Further, simultaneous deletion of the insulin receptor and the leptin receptor in POMC neurons leads to profound decreases in POMC gene expression, but does not affect the body weight in chow-fed mice. These observations raise the possibility that impaired POMC neuron functions in chow-fed mice could be compensated by redundant mechanisms, and therefore fail to produce detectable body weight phenotypes. On the other hand, impaired POMC neuron functions combined with HFD feeding may be sufficient to cause energy imbalance, and thus result in detectable body weight gain. It is also worth noting that TAp63 has been shown to prevent skin aging by regulating cellular senescence and genomic stability. Thus, the profound body weight gain during HFD feeding (starting from 30 weeks of age) may also be attributed, at least partly, to a combined impact of TAp63 deletion and aging.

The female sex hormone, estrogen, has been long believed to largely contribute to the sexual dimorphism in energy balance. Indeed, we previously showed that ERα expressed by POMC neurons suppresses the food intake only in female mice, but not in male mice. Thus, an important question is whether and how TAp63 interacts with estrogenic actions in POMC neurons. Our results suggest that the functional interactions between TAp63 and estrogen is complex. On one hand, certain functions of TAp63 and estrogen are independent of each other. For example, TAp63 deletion enhanced GABA-induced mIPSC in female POMC neurons. In addition, TAp63 can directly bind to the POMC promoter and stimulate its transcription. Notably, POMC expression in female hypothalamus is not affected by the estrous cycles or by ERα deletion. Thus, both these functions of TAp63 do not appear to involve estrogenic actions. In parallel, TAp63 deletion in POMC neurons does not recapitulate some phenotypes seen in mice lacking ERα in POMC neurons, including changes in energy expenditure. Thus, at least a portion of ERα actions in POMC neurons are unrelated to TAp63 functions.

On the other hand, TAp63 may be required for some estrogenic actions in POMC neurons. For example, the ERα-evoked neural activities in a portion of POMC neurons were reduced by TAp63 deletion. Notably, TAp63 deletion failed to significantly affect the effects of exogenous estrogen to reduce body weight and food intake in OVX females. Similar phenotypes were observed in female mice lacking ERα in POMC neurons, which develop hyperphagia, but respond to exogenous estrogen with comparable reductions in body weight as their controls. We speculate that this may be due to the compensations in other estrogen-responsive neurons, including those in the ventromedial hypothalamic nucleus, and the medial amygdala, which also mediate estrogenic actions to suppress the body weight.
Interestingly, we found that female pomc-TAp63 KO mice were less sensitive to OVX-induced body weight gain, suggesting that TAp63 in POMC neurons is, at least partly, required for effects of endogenous ovarian hormones (estrogens and/or progesterone). These possibilities warrant future investigations.

In summary, we show that female POMC neurons display higher neural activities, compared to male counterparts. We further identified the transcription factor, TAp63, as one key molecule that underlies this sexually dimorphic feature of POMC neurons. Our results indicate that TAp63 regulates POMC neuron functions via both ERα-dependent and ERα-independent mechanisms. Importantly, TAp63, in POMC neurons, is required to prevent diet-induced obesity only in female mice, but not in male mice. Thus, these results provide important mechanistic insights onto the cellular and molecular basis for the sex differences in body weight control, and may facilitate the development of novel therapeutic interventions for obesity and associated metabolic disorders.

Methods

Mice

Wt, pomc-TAp63 KO mice (referred as pomc-TAp63 KO), and TAp63 OE mice (referred as controls) littermates. In parallel, we crossed the transgenic mice with WT littermates, and some mice with POMC-CreERT2 mice to generate SIM1-Cre/Rosa26-tdTOMATO mice. These mice were used for electrophysiological recordings in SIM1 neurons in the PVH and the MeA.

In tamoxifen-inducible POMC-CreERT2 transgenic allele was crossed with TAp632A-GFP mice to generate TAp632A-POMC-CreERT2 (referred as pomc-TAp63 KO) and TAp63 OFF mice (referred as controls) littermates. In parallel, we also crossed the transgenic mice with WT littermates, and some mice with POMC-CreERT2 mice to generate TAp63 mRNAs in POMC neurons.

Validation of TAp63 recombination in POMC neurons. Control and pomc-TAp63 KO mice (referred as controls) littermates. In parallel, we crossed the transgenic mice with WT littermates, and some mice with POMC-CreERT2 mice to generate TAp63 mRNAs in POMC neurons.

Body weight, body composition and food intake. Male and female pomc-TAp63 KO and control littermates were weaned on chow. The body weight was monitored weekly till the end of the study. Body composition was determined using quantitative magnetic resonance (QMR) at the time, as indicated in the figures. Mice were singly housed in order to measure the food intake in home cages.

Ovx–V and Ovx–E To examine whether TAp63 mediates the effect of estrogen on body weight control and glucose homeostasis, 12-week-old female pomc-TAp63 KO mice and control mice were anesthetized with intraperitoneal 3% pentobarbital sodium (50 mg/kg, ip). Plasma was obtained by centrifugation and stored at −80 °C. Total RNAs were extracted using TRIzol Reagent (Invitrogen), and cDNA was synthesized using random primers. The expression of TAp63 and ERα in the pituitary was measured by real-time PCR using SYBR green PCR master mix (Applied Biosystems). The promoter activity was measured by luciferase assay as described previously.

Quantitative PCR in pituitary. After deep anesthesia, mice were decapitated at 9:00 am and the trunk blood was collected to measure stress levels of corticosterone, mice were restrained for 60 min with a plastic restraint cone, and blood was collected from the tail vein. Plasma was obtained by centrifugation and stored at −80 °C. Total RNAs were extracted using TRIzol Reagent (Invitrogen). Quantitative PCR (qPCR) was performed, as described previously. Primer sequences were listed in Supplementary Table 1.

CHIP–PCR. Hypothalamic samples from fed C57Bl6 mice were used to assess TAp63 binding at the POMC promoter. Anti-TAp63 antibody (ab18901, Biologend) or IgG was used for immunoprecipitation. p63 binding sites were scanned within 4000 bp upstream of the S-UTR and intron 1, using Genomatix Region Miner release 3.2. The primers used for CHIP-PCR are listed in Supplemental Table 1. SYBR green PCR master mix (Applied Biosystems) was used for quantitative real-time PCR.

Luciferase assay. A fragment of the mouse POMC promoter (~2292 to +589, relative to the transcription start site) was generated by PCR and was inserted into the PGL-3 basic vector (Promega), upstream of the luciferase reporter gene that express firefly, pS3/p63 binding sites 2, 3, and 4 were deleted using QuickChange II Site-Directed Mutagenesis Kit (Agilent) from the WT promoter luciferase reporter, referred to D2, D3, and D4 POMC promoter. All the POMC promoter luciferase reporters (WT, D2, D3, and D4) were co-transfected with the pRL-TK plasmid vectors that express Renilla together with pcDNA3.1-TAp63β or pcDNA3.1 vector as control to the neuronal cell line N46 (purchased from Cells line) using Lipo- transfection (3% Goat–anti-rabbit serum for 1 h), incubated with rabbit anti-β-endorphin antiserum (1:10,000; HI-022-33, Phoenix Peptides) on shaker at room temperature for 24 h and followed by biotinylated anti-rabbit secondary antibody (Vector) for 2 h. Sections were stained in the avidin-biotin complex (1:500, ABC; Vector Elite Kit) and incubated in 0.04% 3,3′-diaminobenzidine and 0.01% hydrogen peroxide. After dehydration through graded ethanol, the slides were then immersed in xylene and cover slipped. Images were analyzed using a bright-field microscope.

The numbers of TOMATO-positive or β-endorphin-positive neurons in the ARH were counted by blinded investigators in all brain sections containing the ARH (1/5 of the whole brain), and the total number was used to reflect the data value for that mouse. Several mice were included in each group for statistical analyses.

Validation of TAp63 recombination in POMC neurons. Control and pomc-TAp63 KO mice (referred as tamoxifen inductions) were anesthetized with intraperitoneal 3% pentobarbital sodium and killed. Various tissues, as detailed in the figures, were collected. Genomic DNAs were extracted using the REDExtract-N-Amp Tissue PCR Kit (OxfordNATs Sigma-Aldrich, St Louis, MO). Following PCR amplification of the flanking sequences of Cre recombinase (310bp), the amplicons were collected into a PCR clean-up kit (Qiagen Inc, Valencia, CA) and sent to Vanderbilt University Medical Center Hormone and Analytical Services Core to measure ACTH levels. The numbers of TOMATO-positive or β-endorphin-positive neurons in the ARH were counted by blinded investigators in all brain sections containing the ARH (1/5 of the whole brain), and the total number was used to reflect the data value for that mouse. Several mice were included in each group for statistical analyses.

Histology. Male and female Rosa26-tdTOMATO/POMC-CreERT2 mice received tamoxifen treatment at 11 weeks of age (0.2 g kg−1, ip.). At 16 weeks of age, these mice were anesthetized with intraperitoneal 3% pentobarbital sodium, and the brains were perfused with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). The brains were sectioned at 25 μm and collected into 5% sodium azide solution. The sections were dehydrated through graded ethanol, and the slides were then immersed in xylene and cover slipped. Images were analyzed using a bright-field microscope.
RiboTag IP. Experiments involving female mice were performed at unknown estrous stage. Hypothalamic punches containing the entire ARH region (6-8 mg) were homogenized in 350 μl buffer, as described previously. After centrifugation, the estrous stage. Hypothalamic punches containing the entire ARH region (6-8 mg) were homogenized in 350 μl buffer, as described previously. After centrifugation, the RNA from inputs and IP treatments were used to extract the RNA quality. RIN higher than 8 indicates good quality. For IP sample, we used RT-qPCR to quantify transcripts of interest (e.g., TAp63 and ΔNp63, etc.) and the house-keeping gene β-actin. We normalized the level of each transcript to the house-keeper level in the same IP sample to obtain a ratio, which was used to reflect the relative level of the transcript in that sample.

Electrophysiology. Briefly, mice were deeply anesthetized with isoflurane and then decapitated at 9:00 am, and the entire brain was removed and immediately submerged in ice-cold sucrose-based cutting solution. Using a Microm HM 650 vibrotome (Thermo Scientific), the brains were cut into coronal slices (without trimming at other dimension) at the thickness of 250 μm each. Usually, 3–4 consecutive brain slices from mouse, ranging from premaga – 2.54 to 3.00 mm, contained the ARH and adjacent nuclei (the ventromedial hypothalamic nucleus, the dorsal medial hypothalamus, and the lateral hypothalamus) were used for recordings. The slices were recovered for 1 h at 34 °C and then maintained at room temperature in artificial cerebrospinal fluid (aCSF, adjusted to pH 7.3) containing (in mM) 126 NaCl, 2.5 KCl, 2.4 CaCl2, 1.2 NaH2PO4, 1.2 MgCl2, 11.1 glucose, and 21.4 NaHCO3 saturated with 95% O2 and 5% CO2 before recording.

Slices were transferred to the recording chamber and allowed to equilibrate for at least 10 min before recording. The slices were superfused at 34 °C in oxygenated aCSF at a flow rate of 1.8-2.1 ml per min. POMC neurons (for ERα-expressing POMC neurons) in the ARH were visualized using epifluorescence and IR-DIC imaging on an upright microscope (Eclipse FN-1, Nikon) equipped with a moveable stage (MP-285, Sutter Instrument). Patch pipettes with resistances of 3–5 MΩ were backfilled with 0.4 M sodium cacodylate, pH 7.2. Signals were acquired with an Axon Instruments patch-clamp amplifier (Axon Instrument), sampled using Digidata 1440 A, and analyzed offline with pClamp 10.3 software (Axon Instrument). Series resistance was monitored during the recording, and the values were generally <10 MΩ. For the electrical recordings were done using perforated patch. Briefly, the patch pipette solution for gramicidin perforated patch recording contained (in mM): KCl 150 and HEPES 10, pH 7.2 adjusted with Tris-OH. The gramicidin was first dissolved in DMSO in a stock solution of 60 mg ml−1 and then diluted to a final concentration of 30 μg ml−1. The gramicidin-free pipette solution was Backfilled in the pipette first and then the gramicidin-containing pipette solution was then added into the pipette. After 10–15 min of cell-attached formation, series resistance (Rs) decreased and stabilized at around 10 to 50 MΩ. The resting membrane was then recorded in the current clamp model. Action potential (AP) firing rate and resting membrane potential were measured using the clamp fit 10.3 software (Axon Instrument). For the miniature excitatory post-synaptic current (mEPSC) recordings, the internal solution contained recording solution containing 125 mM GlcH3SO3, 10 mM CaCl2, 5 mM NaCl, 2 mM MgCl2, 1 mM EGTA, 10 mM HEPES, 5 mM (Mg)ATP, and 0.3 mM (Na)GTP (pH 7.3 with NaOH). mEPSC in AGNP neurons was measured in the voltage clamp mode with a holding potential of −60 mV. mEPSCs were detected using pClamp 10 software. The miniature inhibitory post-synaptic current (mIPSC) in POMC neurons was measured in the voltage clamp mode with a holding potential of −60 mV in the presence of 1 μM TTX and 0.5 μM bicuculline. Frequency and peak amplitude were measured using the Mini Analysis program (Synaptosoft, Inc.).

Statistics. The minimal sample size was predetermined by the nature of the experiments. For most of physiological readouts (body weight, food intake, etc.), at least 6 mice per group were included. For gene expression, 3–5 mice were included in each group. For electrophysiological studies, 20–40 neurons in each genotype or condition were included. The data are presented as mean ± SEM. Statistical analyses were performed using GraphPad Prism to evaluate normal distribution and variations within and among groups. Methods of statistical analyses were chosen based on the design of each experiment and are indicated in figure legends. P < 0.05 was considered to be statistically significant.

Study approval. Care of all animals and procedures were performed for the Guide and Care of Use of Laboratory Animals of the US National Institutes of Health and were approved by the Animal Subjects Committee of Baylor College of Medicine.

Data availability. All relevant data are available from the authors.

Received: 31 May 2017 Accepted: 13 March 2018 Published online: 18 April 2018

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Acknowledgements
This work was supported by grants from the NIH (R01DK093587, R01DK101379, and R01DK117281 to Y.X.; R01DK107008 to P.X.; R01DK109194 and R56DK109194 to Q.W.; R01DK092605 to Q.T.; and NCI R35CA197452 to E.F.). USDA/CRIS (3092-5-001-059 to Y.X. and Q.W.), American Diabetes Association (1-17-PDF-138 to Y.H.; 7-13-JF-61 to Q.W.; 1-15-BS-184 to Q.T.) and American Heart Association awards (17GRNT3296003 to Y.X.; 16POST2726025 to C.W. and 16B1A26420102 to W.R.L.). Measurements of body composition were performed in the Mouse Metabolic Research Unit (MMRU) at the USDA/ARS Children’s Nutrition Research Center, which is supported by funds from the USDA ARS (www.bcm.edu/cnrc/mmr). We acknowledge the assistance of Firoz Vohra and the MMRU Core Director, Marta Fiorotto. We acknowledge Drs. Joel K. Elmquist and Chen Liu for providing POMC-CreER transgenic mice.

Author contributions
C.W. designed and performed most of the experiments. Y.H. performed physiologic studies. P.X. performed the stereotactic injection of virus. Y.Y. helped in the design and construct of plasmids. K.S., Y.X., Y.H., A.J. Jr., C.Y., H.D., L.Y. and G.S. assisted in experiments. W.R.L. and R.G. assisted in the design and packaging of the AAV-FLEX-Tap63 construct. Q.T. and Q.W. assisted in the design of the study. E.F. provided the Tap63off mice and provided technical support for using this line. Y.X. conceived, designed, and supervised the project.

Additional information
Supplementary Information accompanies this paper at https://doi.org/10.1038/s41467-018-03796-7.

Competing interests: The authors declare no competing interests.

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