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MOLECULAR METABOLISM

The homeodomain transcription factor *Six3* regulates hypothalamic *Pomc* expression and its absence from POMC neurons induces hyperphagia and mild obesity in male mice

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ABSTRACT

Objective: Proopiomelanocortin (POMC) neurons release potent anorexigenic neuropeptides, which suppress food intake and enhance energy expenditure via melanocortin receptors. Although the importance of central melanocortin in physiological regulation is well established, the underlying genetic mechanisms that define the functional identity of melanocortin neurons and maintain hypothalamic *Pomc* expression remain to be fully determined. In this study, we investigate the functional significance of *Six3,* a transcriptional regulator notably expressed in embryonic and adult mouse POMC neurons, in the regulation of hypothalamic *Pomc* expression and downstream physiological consequences.

Methods: We first evaluated the expression of *Six3* in the developing and adult hypothalamus by double fluorescence *in situ* hybridization. Next, we assessed POMC immunoreactivity in mutant mice selectively lacking *Six3* from *Pomc*-expressing neurons and quantified *Pomc* mRNA levels in a tamoxifen-inducible *Six3* knockout mouse model activated at embryonic E9.5 days. We also determined glucose and insulin sensitivity, daily food intake, body composition and body weight in adult male and female mice lacking *Six3* specifically from POMC neurons. Lastly, we assessed the physiological consequences of ablating *Six3* from POMC neurons in adult mice.

Results: *Six3* and *Pomc* were co-expressed in mouse hypothalamic neurons during development and adulthood. Mouse embryos deficient in *Six3* showed reduced *Pomc* expression in the developing hypothalamus. Targeted deletion of *Six3* specifically from POMC neurons resulted in decreased hypothalamic *Pomc* expression, increased daily food intake, enhanced glucose sensitivity and mild obesity in male but not in female mice. Finally, conditional removal of *Six3* from POMC neurons in adult mice led to a reduction in hypothalamic POMC immunoreactivity with no significant effects in body weight or food intake.

Conclusions: Altogether, our results demonstrate that *Six3* plays an essential role in the early establishment of POMC neuron identity and the maintenance of physiological levels of hypothalamic *Pomc* expression. In addition, our study demonstrates that the functional significance of *Six3* expression in POMC neurons is sexually dimorphic and age-dependent.

Keywords Proopiomelanocortin (*Pomc*); *Six3*; Obesity; High-fat diet; Hypothalamus

1. INTRODUCTION

The regulation of body weight and energy balance is governed by neural brain circuits which orchestrate food intake behavior by integrating multiple central and peripheral signals. The arcuate nucleus of the hypothalamus (ARC) plays crucial roles in the regulation of appetite, energy balance, and metabolism. Specifically, proopiomelanocortin (POMC) neurons release potent anorexigenic melanocortins α-, β-, and γ-melanocyte-stimulating hormones, which suppress food intake and increase energy expenditure through melanocortin receptors [1]. Mice lacking *POMC* specifically from ARC neurons develop early-onset hyperphagia and severe obesity, whereas reinstating *Pomc* expression in these mice normalized their food intake and adiposity [2]. Similarly, humans carrying null-allele mutations in *POMC* exhibit early-onset obesity and significant adrenal insufficiency [3], whereas Labrador Retriever dogs carrying deletion mutations in *POMC* often manifest

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morbid obesity [4]. Of note, null mutations in the melanocortin receptor 4 (MC4R) gene have been identified as the most common monogenic cause of obesity [5,6].

Since the initial characterization of *POMC* in humans and bovines [7], a series of studies have delved into elucidating the function of POMC neurons and the regulatory mechanisms governing *Pomc* expression. Developmental investigations have revealed that *Pomc* expression commences in the tuberal portion of the prospective mouse hypothalamus as early as embryonic day 10.5 (E10.5) [8]. Key transcription factors such as *Mash1* (*Ascl1*) [9], *Isl1* [10], *Nkx2-1* [11], *Shh* [12], *Ngn3* [13], and *Prdm12* [14] play pivotal roles in establishing early POMC neuron identity and maintaining normal *Pomc* expression throughout postnatal life. More recently, the transcription factor *Tbx3* has also been shown to be expressed in POMC neurons from late embryonic stages through adulthood, suggesting its crucial role in maintaining terminal differentiation rather than in establishing the early identity of these neurons [15]. Current progress in single-cell technology (scRNA-seq) is greatly advancing these investigations, allowing for the simultaneous identification of multiple transcripts within individual POMC neurons. In fact, a recent study from our laboratory traced the origin and maturation of arcuate POMC neurons in the developing and early postnatal hypothalamus using scRNA-seq transcriptomics of fluorescently labeled POMC cells at critical embryonic (E11.5, E13.5, E15.5, and E17.5) and early postnatal (P5 and P12) stages [16]. This study unveiled eight distinct POMC clusters based on the differential expression of several transcription factor and neuropeptide genes [16]. Notably, the *Pomc*$^{(high)}$/*Prdm12* cluster, which represents canonical POMC neurons with the greatest abundance of *Pomc* transcripts, also showed high level expression of several transcription factor genes known to play critical roles in the early establishment of POMC neuron identity, such as *Isl1* [16], *Nkx2.1* [16], *Prdm12* [16] and *Tbx3* [16]. Another transcription factor that stood out in this major *Pomc*$^{(high)}$/*Prdm12* cluster for being expressed at high levels at all developmental time points was *Six3* [16]. Thus, the current investigation focused on elucidating the role of *Six3* in the regulation of hypothalamic *Pomc* expression and its significance in central melanocortin function.

The homeodomain transcription factor *Six3* (*Sine oculis 3*) was first identified in 1995 as one of the most anterior homeobox genes reported at that time [17]. *Six3* expression commences as early as embryonic day 6.5 (E6.5) in the anterior neural plate, with increased signaling around E8.5. At this stage, *Six3* expression localizes predominantly in the anterior neural ridge and its derivatives, including the hypothalamic anlage, optic vesicles, ventral forebrain, and neurohypophysis. In humans, *SIX3* mutations contribute to ~1.3% of holoprosencephaly (HPE) cases [18]. In the teleost fish medaka, overexpression of *Six3* induces expanded and ectopic retinal primordia while its absence results in forebrain and eye developmental defects [19]. Consistent with observations in humans and fish, *Six3*-null mouse embryos exhibit perinatal lethality due to the absence of most craniofacial structures [20]. Furthermore, *Six3* expression is implicated in pituitary morphology and function, being considered one of the causative genes for Combined Pituitary Hormone Deficiency [21]. Haploinsufficiency of *Six3* leads to pituitary dysmorphology, and conditional knockout of *Six3* in the hypothalamus of mice results in failed expansion of Rathke's pouch. Recent investigations have also supported a role for *Six3* in metabolism, as evidenced by the deletion of *Six3* from mature neurons resulting in dwarfism with improved glucose tolerance, increased lean mass, and elevated metabolic rates [22].

Given the pivotal role of *Six3* in early hypothalamic development and its abundant mRNA presence in POMC neurons [15], we sought to elucidate the significance of *Six3* in the initial establishment of POMC neuronal identity. Furthermore, we aimed to assess the physiological consequences elicited by the targeted deletion of *Six3* specifically from POMC neurons.

2. METHODS AND MATERIALS

2.1. Animal care

All animal experiments followed the Public Health Service guidelines for the humane care and use of experimental animals and were conducted according to the Institutional Animal Care and Use Committee (PRO00010383) at the University of Michigan. The mice were housed in ventilated cages under a controlled temperature (~23 °C) and photoperiod (12 h light/dark cycle, lights on from 6:00 am to 6:00 pm). Mice had free access to tap water and laboratory chow (5L0D; LabDiet). Breeding mice were fed with the breeder chow diet (5008, LabDiet). The strains of mice used in these studies were transgenic *Pomc*-TdTomato [23], compound *Pomc-Cre.Six3*$^{fl/fl}$, compound *Pomc-CreERT.Six3*$^{fl/fl}$, and compound *CAAG-CreERT.Six3*$^{fl/fl}$. The *Six3*$^{fl/fl}$ mice were generated by Dr. Guillermo Oliver from Northwestern University [19]. BAC transgenic *Pomc-CreERT* mice were a gift from Dr. Joel Elmquist at UT Southwestern University [24]. *Pomc-Cre* [BAC Tg(Pomc1-cre)1^{Lowl}] (JAX strain # 005965) and *CAAG-CreERT* [Tg(CAG-cre/Esr1*)5^{Amc}/J] (JAX strain # 004682) were obtained from The Jackson Laboratory. Mice were ear tagged and tailed around postnatal day 14 and weaned after 21 days.

2.2. High fat diet (HFD) experiment

After 17 weeks, *Pomc-CreERT.Six3*$^{fl/fl}$ mice were transitioned to a rodent diet containing 60 kcal% fat (Research Diets, D12492), whereas *Pomc-Cre.Six3*$^{fl/fl}$ mice were introduced to the same diet after reaching 4 weeks of age. High-fat diet (HFD) pellets were placed in a small food hopper to prevent spillage. Cages were cleaned at least once a week, or twice a week for mice prone to food wasting. Food hoppers were refilled twice a week, or three times for food-wasting mice. Prior to transitioning to the high-fat diet, mice were weighed weekly on a specific day of the week. Once on the high-fat diet, weekly body weight measurements continued until the mice underwent metabolic tests conducted by the Animal Phenotyping Core at the University of Michigan just prior to euthanasia.

2.3. Tamoxifen injection

Tamoxifen or control vehicle was administered to adult *Pomc*-CreERT.*Six3*$^{fl/fl}$ mice at 9 weeks of age for 5 consecutive days, at a dosage of 50 mg/kg (i.p.) (Sigma—Aldrich, T5648). Metabolic parameters, including body weight, were assessed the week following tamoxifen administration. In pregnancy experiments, breeding females were monitored daily for the presence of vaginal copulation plugs, marking the day of plug detection as embryonic day 0.5 (E0.5). Pregnant CAAG-CreERT females received a single dose of 150 mg/kg tamoxifen (i.p.) when embryos were E9.5 days old. Embryos were harvested for quantitative RT-PCR analysis at E11.5 and for histology at E12.5. Tamoxifen was prepared using a solution of 10% ethanol and 90% sesame oil (Sigma—Aldrich, S3547). A control vehicle consisting of 10% ethanol and 90% sesame oil was also prepared.

2.4. RNA isolation and quantification

Total RNA extraction was carried out from the entire embryonic heads at E11.5 (one day prior to the onset of *Pomc* expression in the embryonic pituitary at E12.5) and adult pituitary samples were isolated from the underlying pituitary. TRIzol™ Reagent (Sigma—Aldrich, 15596026) was employed for RNA extraction following the

manufacturer's protocol. Subsequently, first-strand cDNA synthesis was performed using 1 μg of RNA along with random primers and M-MLV Reverse Transcriptase (Invitrogen, 28025013). Quantitative real-time polymerase chain reaction (qRT-PCR) analysis was conducted on all samples utilizing a CFX Real-Time PCR system (Bio-Rad) with SYBR Green Master Mix (Applied Biosystems). Primer details can be found in Supplementary Table 1, with all primers utilized at a final concentration of 250 nM. Data analysis was carried out using the $2^{-\Delta\Delta CT}$ relative quantification method.

2.5. Glucose and insulin tolerance tests

Oral glucose tolerance tests (OGTT) and corresponding insulin measurements were performed by the Metabolic, Physiological and Behavioral Phenotyping Core at the University of Michigan. Briefly, the mice were fasted for 5 h (8:00 am-1:00 pm) and orally gavaged with 2 g/kg glucose in PBS. Blood was sampled at 0, 15, 30, 60, and 120 min for glucose (Accu-Check glucometer, Roche) and insulin measurements (Millipore rat/mouse insulin ELISA kit, EZRMI-13 K). The HOMA index was calculated using fasting glucose * fasting insulin/22.5. For insulin tolerance tests (ITT), the animals were fasted for 5 h (9:30 am-2:30 pm) and 1 U/kg insulin per body weight was injected intraperitoneally. Blood glucose was measured at 0, 15, 30, 60, and 120 min after insulin injection.

2.6. Fluorescence *in situ* hybridization (RNAscope)

Embryos ranging from E10.5 to E17.5 were fixed in 4% paraformaldehyde in phosphate buffered saline (PBS) at 4 °C for varying durations depending on their developmental stage. Specifically, embryos at E10.5 to E12.5 were fixed for 1 h, while heads from embryos at E15.5 to E16.6 were fixed for 2 h, and those at E17.5 were fixed for 4 h. Tissues were stabilized in 10% sucrose/10% gelatin in PBS at 37 °C for 30 min prior to embedding in OCT compound as described previously [11]. The experiment was done according to the RNAscope Multiplex Fluorescent Reagent Kit V2 Assay (Advanced Cell Diagnostics) with some modifications. The adult brain and embryo samples were sectioned using a cryostat and mounted to SuperFrost Excell microscope slides (Fisher Scientific). Sections were fixed with 4% PFA, washed in PBS, dehydrated in ethanol series, and treated with RNAscope pretreatment agents. Then the hybridized probes were amplified, and fluorophores were attached according to the manufacturer's instructions. Slides were dried overnight and imaged the next day using a Nikon Instruments A1 Confocal Laser Microscope with NIS-Elements Software. Probes used for staining were *Pomc* (Mm-*Pomc*-C2) for native *Pomc* expression and *Six3* (Mm-*Six3*-C3) for detection of endogenous *Six3* expression.

2.7. Immunohistochemistry and image quantification

For DAB (3,3′-Diaminobenzidine) staining, adult *Pomc-CreERT.Six3*$^{fl/fl}$ mice were transcardially perfused with 4% paraformaldehyde in PBS. Brains were removed, placed in 4% paraformaldehyde overnight, and dehydrated in 30% sucrose for several days. The brains were then cut into 30 μm sections using a freezing microtome (Leica). Sections were treated sequentially with 3% hydrogen peroxide/0.5% sodium hydroxide, 0.3% glycine, 0.03% sodium dodecyl sulfate, and blocking solution (PBS with 0.1% triton, 3% Normal Goat Serum) for 1 h and incubated with primary antibody (Anti-POMC, H-029-30, Phoenix Pharmaceuticals) overnight. After three times wash with PBS, the biotinylated secondary antibody (Goat Anti-Rabbit, BA-1000-1.5, Vector Lab) was applied to the sections followed by another three times wash with PBS and incubation in avidin-biotin complex (Thermo Scientific, 32050) for 30 min. Freshly made DAB substrate solution (Thermo Scientific, 34002) was used for color development. All slides were sent to the Department of Pathology Aperio Slide Scanning Core at the University of Michigan for image acquisition at 40× magnification. Quantitative analysis was manually performed in anatomically similar sections from 3 to 4 animals at −1.4 and −1.9 mm distance to bregma. The POMC positive cells per hemisection of the hypothalamus were quantified using Image J. The quantification of POMC immunofluorescence intensity in the *Pomc-Cre.Six3*$^{fl/fl}$ embryonic brain was conducted using Image J. This analysis involved comparing three anatomically similar sections from three animals, with replicates for each measurement.

2.8. Daily and hourly food intake

Adult mice were individually housed, and a fresh cage was provided prior to measuring food intake. Hourly food consumption was quantified every 2 h over a 6-hour period following an 18-hour fast. Daily food intake was recorded for five consecutive days at 4:00 pm. Some of the *Six3*$^{fl/fl}$ mice exhibited pronounced food wasting behavior, especially females, these mice were excluded from the food intake experiment.

2.9. Single-cell RNA sequencing and TRAP-seq analysis

The single-cell RNA-sequencing data were analyzed as previously described [16], and violin plots were generated with Seurat (version 2.0). The percentages of cells in each cluster were calculated by identifying the number of cells that had at least one unique molecular identifier (UMI) for *Six3* against the total number of cells in each cluster. The TRAP-seq data [16], presented as counts per million (CPM), were normalized for the counts of *Six3* and *Pomc* against the total counts.

2.10. Data analysis

The data presented are shown as means ± standard error of the mean (SEM) and sample sizes are indicated in the figure legends and the main text. All data were analyzed using GraphPad Prism 9.0 software (GraphPad Software, San Diego, California, USA). When two groups were compared, the unpaired two-tailed Student's t test was applied. One-way or two-way ANOVAs followed by Tukey's or Bonferroni's multiple comparison tests were conducted for pair-wise analyses. A detailed description of the statistical method used for each analysis is noted in each figure legend. $P < 0.05$ was considered significant.

3. RESULTS

3.1. Six3 and Pomc co-express during hypothalamic development

By using single-cell RNA sequencing (scRNA-seq), we have recently characterized the transcriptomic profiles of mouse hypothalamic POMC neurons at various developmental and postnatal stages [16]. In particular, *Six3* emerged as one of the transcription factor coding genes prominently expressed within clusters exhibiting high *Pomc* transcript abundance [16]. Reanalysis in this report of the datasets collected in that study indicate that *Six3* is notably enriched in cluster 1 (*Pomc*^{high}*/Prdm12*); cluster 4 (*Pomc*^{low}*/Otp*) and cluster 7 (*Pomc*^{low}*/Prdm13*) with over 75% of cells within these three clusters expressing *Six3* (Figure 1A). *Six3* transcripts are less abundant in the other five clusters in which *Pomc* is also detected at much lower levels [16]. Further analysis of cluster 1 across four embryonic stages (E11.5, E13.5, E15.5 and E17.5) and two early postnatal periods (P5 and P12) revealed that *Six3* transcripts are present in more than 75% of the highest *Pomc*-expressing neurons at all six developmental stages (Figure 1B). In addition, reanalysis of a different transcriptomic dataset

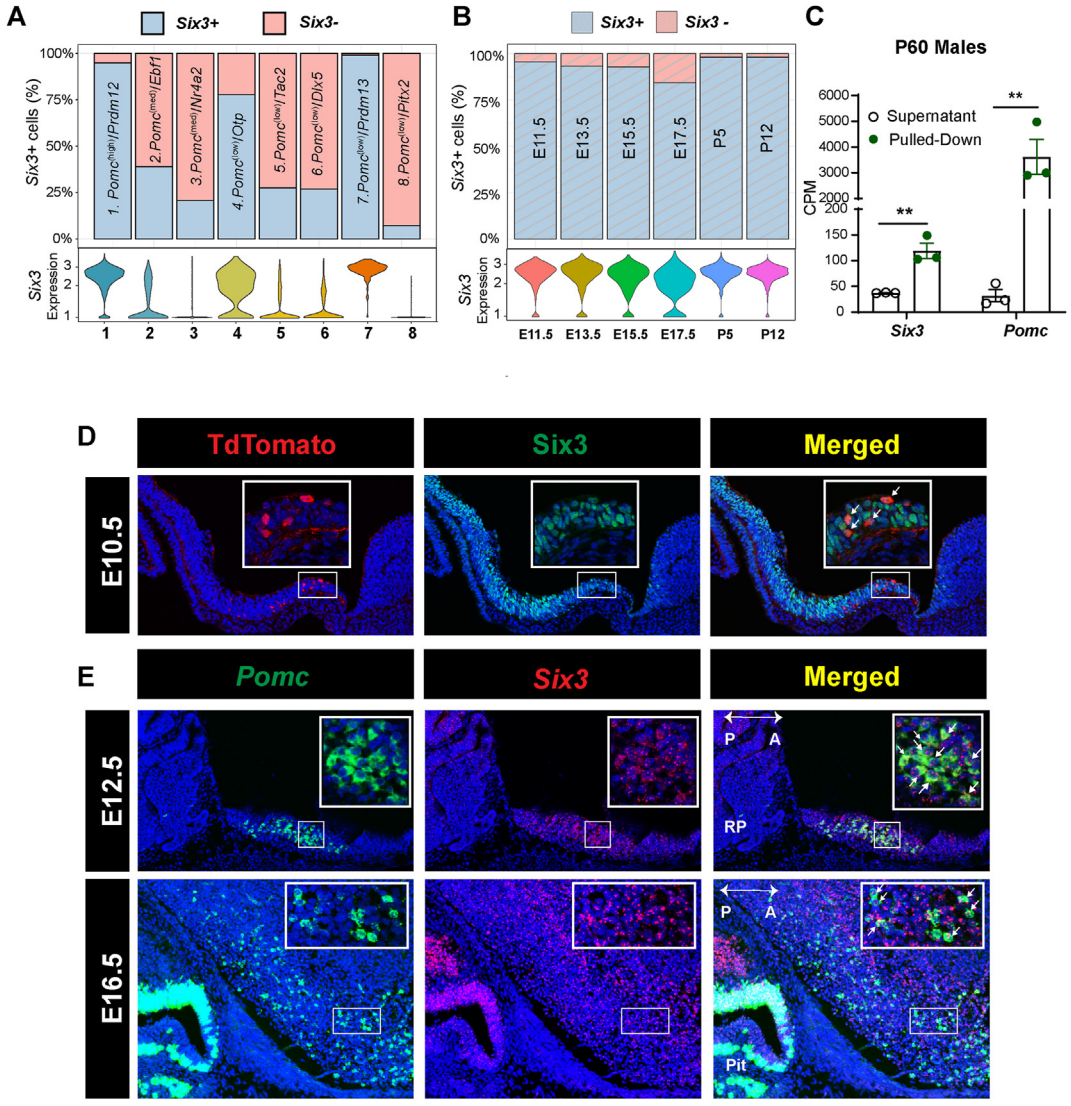

Figure 1: *Six3* is expressed in *Pomc*-expressing cells during development and adulthood. (A) Percentage of *Six3*-positive cells across the eight cell clusters from previous single-cell RNAseq study [16]; (B) Percentage of *Six3*-positive cells across six developmental stages in the *Pomc*^{high}/*Prdm12* cluster [16]; (C) The expression levels of *Six3* and *Pomc* are both significantly elevated in pulled-down samples from a previous TRAP-seq study performed on male mice at postnatal day 60 [16]; (D) Double immunofluorescence on sagittal cryosections of *Pomc*-TdTomato E10.5 embryos showing the co-localization of TdTomato (red) and SIX3 (green). Double fluorescence *in situ* hybridization showing the co-localization of *Pomc* (green) and *Six3* (red) at E12.5 and at E16.5. CPM: Counts Per Million; TRAP-seq: Translating ribosome affinity purification with RNA-sequencing; Pit: Pituitary; 3V: third ventricle; RP: Rathke's pouch; Sagital image orientation: left, posterior (P); right, anterior (A). Scale Insets are magnified views of the indicated boxes. Arrows indicate co-expressing neurons in the merged panels. Scale bar: 50 μm.

collected from adult (P60) male mice hypothalamic POMC neurons obtained by performing a Translating Ribosome Affinity Purification (TRAP)-seq study [16] also showed that *Six3* is significantly enriched in the ribosomal fraction associated with high *Pomc* expression (Figure 1C). To further verify that *Six3* and *Pomc* coexpress in arcuate hypothalamic neurons we conducted double immunofluorescence on sagittal sections taken from *Pomc*-TdTomato transgenic E10.5 mouse embryos (Figure 1D, top panels). In addition, we performed double fluorescence *in situ* hybridization (RNAscope) on sagittal mouse brain sections taken at E12.5 and E16.5 (Figure 1E) and on coronal E14.5 and adult mouse hypothalamic sections (Supplementary Fig. 1). Our findings show that *Six3* is expressed in POMC neurons since the onset of *Pomc* expression in the basal hypothalamic region at E10.5 and maintained throughout development and adulthood.

3.2. Deletion of Six3 from POMC neurons reduced Pomc expression

To elucidate the potential molecular regulation of *Six3* in hypothalamic *Pomc* expression, we generated mice specifically lacking *Six3* from POMC cells (POMC*Six3*KO) by crossing POMC cell-specific BAC transgenic *Pomc*-Cre mice with conditional *Six3* mutant (*Six3*$^{fl/fl}$) mice [19]. We found that immunofluorescence intensity in POMC+ hypothalamic neurons from E12.5 and E16.5 POMC*Six3*KO embryos decreased by 33% and 48% respectively, in comparison with same age wild-type embryos (Figure 2A), indicating a role for *Six3* in hypothalamic POMC immuno-expression during development. To explore whether *Six3* is involved in the early establishment of the hypothalamic POMC lineage, we generated a temporal-specific conditional mutant mouse strain allowing for *Six3* ablation at different developmental

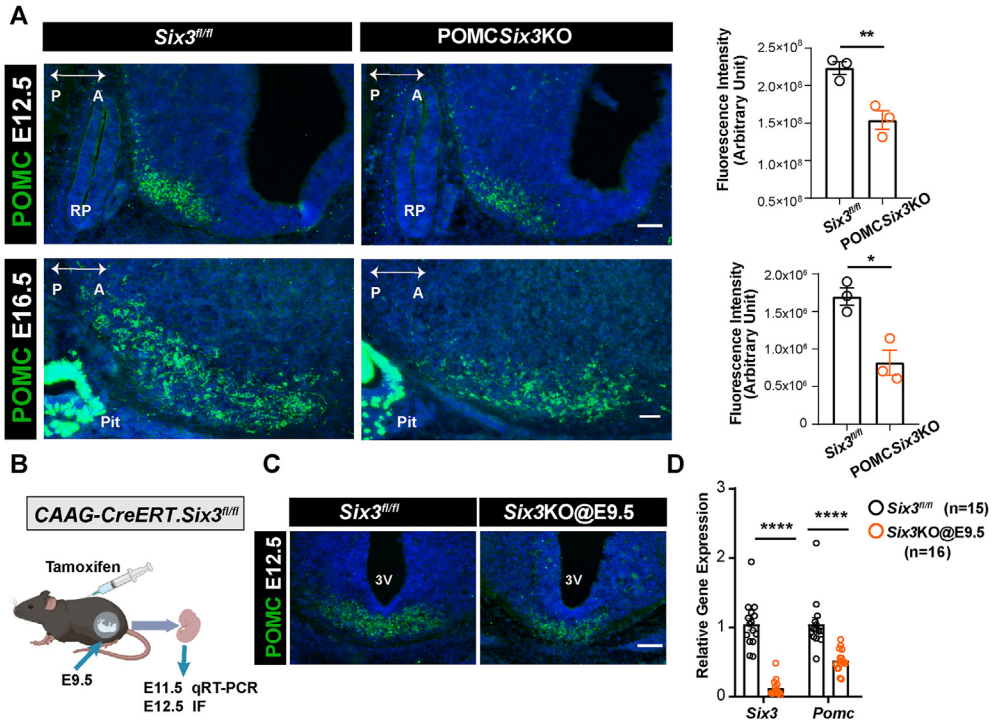

Figure 2: *Six3* ablation reduced *Pomc* expression during development. (A) Comparison of POMC (green) immunofluorescence intensity on sagittal cryosections of *Six3*$^{fl/fl}$ and POMC*Six3*KO E12.5 and E16.5 embryos. Intensity (right) was quantified on anatomically similar sections from three embryos. (B) Schematic diagram showing the overall experimental procedure for panels C and D. (C) Reduced POMC (green) immunofluorescence intensity in *Six3*KO@E9.5 embryos at E12.5. (D) Quantitative PCR showing significantly decreased *Six3* and *Pomc* transcripts abundance in *Six3*KO@E9.5 embryos analyzed at E11.5. Pit: Pituitary; 3V: third ventricle; RP: Rathke's pouch; Image orientation: left, posterior (P); right, anterior (A). Scale Insets are magnified views of the indicated boxes. Arrows indicate co-expressing neurons in the merged panels. Scale bar: 50 μm. Figure 2B was generated from Biorender.

stages by crossing *Six3*$^{fl/fl}$ conditional mutants with mice expressing a tamoxifen-inducible Cre recombinase transgene (*CAAG-CreERT*) [20]. Tamoxifen (150 mg/kg, i.p.) was administered to compound *CAAG-CreERT.Six3*$^{fl/fl}$ pregnant dams at post-coitum day 9.5, one day before the initiation of *Pomc* expression in the embryonic hypothalamus. The embryos were subsequently collected either at E11.5 for quantitative RT-PCR analysis or at E12.5 for immunofluorescence detection of POMC, respectively (Figure 2B). A great reduction (nearly 90%) of *Six3* transcript levels was found in brain samples from E11.5 CAAG-*CreERT.Six3*$^{fl/fl}$ embryos (*Six3*KO@9.5) confirming a successful tamoxifen-inducible deletion of *Six3* in these compound mutant mice (Figure 2D). Interestingly, the significant drop in *Six3* mRNA levels found in *Six3*KO@9.5 embryos was paralleled by ∼50% decrease in the level of *Pomc* transcripts at E11.5 (Figure 2C), underscoring the functional significance of *Six3* for optimal arcuate *Pomc* expression in early development. This significant reduction in *Pomc* expression was observed in both sexes (Supplementary Fig. 2). We also assessed in E11.5 *Six3*KO@9.5 embryos the expression levels of several additional transcription factor genes known to regulate *Pomc* expression, including *Nkx2-1* [11], *Mash1* [9], *Ngn3* [13], *Prdm12* [14], *Isl1* [10], and *Shh* [12] (Supplementary Fig. 3) and found that deletion of *Six3* at E9.5 did not alter the expression of those genes, suggesting a direct independent effect of *Six3* in the transcriptional regulation of arcuate *Pomc* expression. Given that *Pomc* is also abundantly expressed in corticotrophs and melanotrophs of the pituitary, we quantified *Pomc* transcript levels in the pituitary glands of adult POMC*Six3*KO mice. Our findings revealed a 36% reduction in *Pomc* expression with the deletion of *Six3* (Supplementary Fig. 4). We also evaluated the expression levels of *Pitx1*, an important regulator in the development and maintenance of the normal function of the pituitary [25]. Our results showed deletion of *Six3* from *Pomc*-expressing cells does not affect the expression of *Pitx1* (Supplementary Fig. 4).

3.3. Selective deletion of Six3 from POMC neurons induced hyperphagia and mild obesity in males

Given that *Six3* and *Pomc* coexpress at all developmental and postnatal ages, we investigated whether deletion of *Six3* specifically from POMC neurons throughout life utilizing the compound *Pomc-Cre.Six3*$^{fl/fl}$ mouse strain alters melanocortin function. To this end, we evaluated several metabolic parameters, food intake and body weight in adult compound POMC*Six3*KO mice. When tested at 16–17 weeks of age, POMC*Six3*KO male mice showed a 13% increase ($P = 0.0512$) in daily food intake, with no further increase after 18 h fasting (Figure 3A). Consequently, body growth curves revealed that POMC*Six3*KO males are mildly obese from 14 weeks of age onward, relative to their *wild*-type siblings (Figure 3B). Consistent with body weight, body composition measured at 8 weeks of age showed no significant difference (Figure 3C) whereas at 16 weeks of age, POMC*Six3*KO males exhibited a 33% increase in body fat composition, although without reaching statistical significance (*Six3*$^{fl/fl}$: 12.80 ± 1.59 % vs. POMC*Six3*KO: 17.04 ± 1.58 %; P = 0.068) (Figure 3D). Given the association between obesity and impaired glucose metabolism, we conducted glucose tolerance tests on these mice. Our results indicated that POMC*Six3*KO males exhibited significantly improved glucose tolerance (Figure 3E) and no significant changes in circulating insulin levels (Figure 3F), HOMA-IR values (Figure 3G) or insulin tolerance (Figure 3H). Different from what we found in males, POMC*Six3*KO females displayed normal body weight curves (Supplementary Fig. 5A),

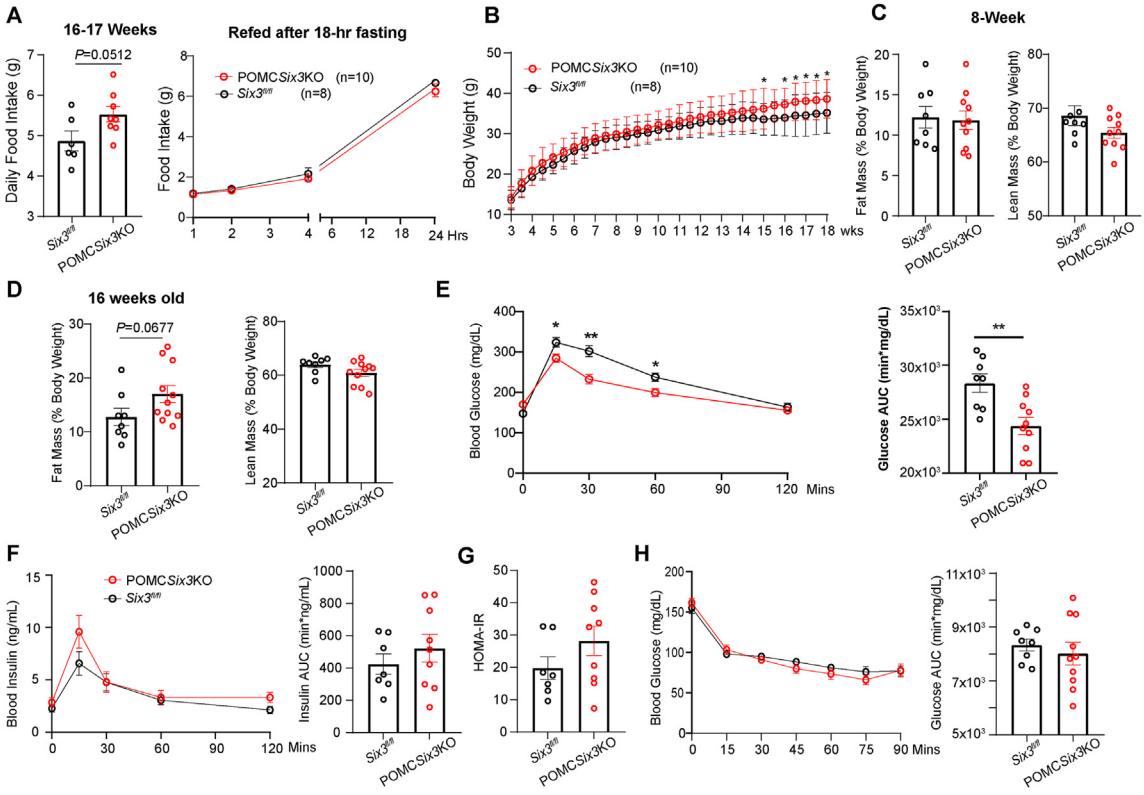

Figure 3: *Six3*-specific deletion from POMC neurons during embryogenesis induced mild obesity in adult male mice. (A) Average daily food intake at 16–17 weeks of age and food intake measurement after 18 h fasting. (B) Body weight comparisons between POMC*Six3*KO and *Six3*$^{fl/fl}$ mice from 3 to 24 weeks of age. (C–D) Body composition of POMC*Six3*KO and *Six3*$^{fl/fl}$ mice at 8 (C) and 16 weeks of age (D). (E) Oral glucose tolerance test with corresponding blood insulin measurements (F) and HOMA-IR (G). (H) Insulin tolerance tests in POMC*Six3*KO and *Six3*$^{fl/fl}$ mice. Two-tailed unpaired Student's t-tests were used to compare the genotype effects. Data shown are the means ± s.e.m of biologically independent samples. *P < 0.05, **P < 0.01.

normal body fat composition when measured at 8 and 16 weeks of age (Supplementary Figs. 5B and 5C) and no clear signs of hyperphagia when fed *ad libitum* or after an 18-h fasting (Supplementary Fig. 5D).

3.4. High-fat diet does not exacerbate obesity in POMCSix3KO mice

Given that POMC*Six3*KO males developed mild obesity when fed *ad libitum* with normal chow, we investigated whether a high-fat diet (HFD; 60% fat content) initiated at 4 weeks of age and onward would exacerbate obesity. Similar to what we observed with the chow diet, POMC*Six3*KO males fed on a HFD exhibited increased body weight (~5 g in average) after 14 weeks of age, in comparison to their wild-type brothers (Figure 4A) with no significant changes in body fat composition (Figure 4B) or daily food intake when either fed *ad libitum* (Figure 4C, left) or refed after an 18-h fast, respectively (Figure 4C, right). Glucose tolerance (Figure 4D), serum insulin levels (Figure 4E), HOMA-IR (Figure 4F) and insulin tolerance (Figure 4G) were also normal in POMC*Six3*KO males. Like with the chow diet, POMC*Six3*KO females fed a HFD did not develop obesity (Supplementary Fig. 6A) nor showed changes in body fat content (Supplementary Fig. 6B), glucose tolerance (Supplementary Fig. 6C), corresponding insulin levels during the oral GTT (Supplementary Fig. 6D), HOMA-IR (Supplementary Fig. 6E), and insulin sensitivity (Supplementary Fig. 6F).

3.5. Deletion of Six3 from POMC neurons in adult mice does not induce obesity

Given the co-expression of *Six3* and *Pomc* in the adult arcuate nucleus (Supplementary Fig. 1, bottom panel), we investigated whether the specific ablation of *Six3* from POMC neurons in adult mice affects *Pomc* expression and melanocortin function. To this end, we administered tamoxifen or vehicle to 12-week-old (postnatal day 84) *Pomc-CreERT.Six3*$^{fl/fl}$ (POMC*Six3*KO@P84) mice and their corresponding *Six3*$^{fl/fl}$ control littermates [24]. We performed immunohistochemistry in coronal brain sections from POMC*Six3*KO@P84 mice and found 23% fewer POMC immunolabeled hypothalamic neurons in comparison with vehicle treated control POMC*Six3*-KO@P84 mice (Figure 5A).

To investigate the physiological consequences of this reduction, we measured body growth curves in the POMC*Six3*KO@P84 receiving a chow diet until 19 weeks of age, then switched to a HFD from 19 to 27 weeks of age (Figure 5B). Body weight was recorded weekly before and after tamoxifen or vehicle injections. Two-way ANOVA mixed-effects analysis revealed no significant differences among all groups (Figure 5C). No differences were observed either in body composition or food intake at 25 weeks of age (Figure 5D,E). Glucose tolerance tests performed on 27-week-old POMC*Six3*KO@P84 males fasted for 5 h indicated similar glucose clearance from their blood compared to vehicle-treated *Pomc*-CreERT.*Six3*$^{fl/fl}$ control mice (Figure 5F), but significantly worsened compared to vehicle-treated *Six3*$^{fl/fl}$ mice. We found no changes in insulin sensitivity among the four groups (Figure 5G). When similar comparisons were conducted in females, we found no significant changes in body weight (Supplementary Fig. 7A), fat and lean mass composition (Supplementary Fig. 7B), food intake (Supplementary Fig. 7C), glucose or insulin tolerance (Supplementary Figs. 7D and 7E) in any of the experimentally tested groups. In summary, although *Six3* ablation from mature POMC neurons led to a

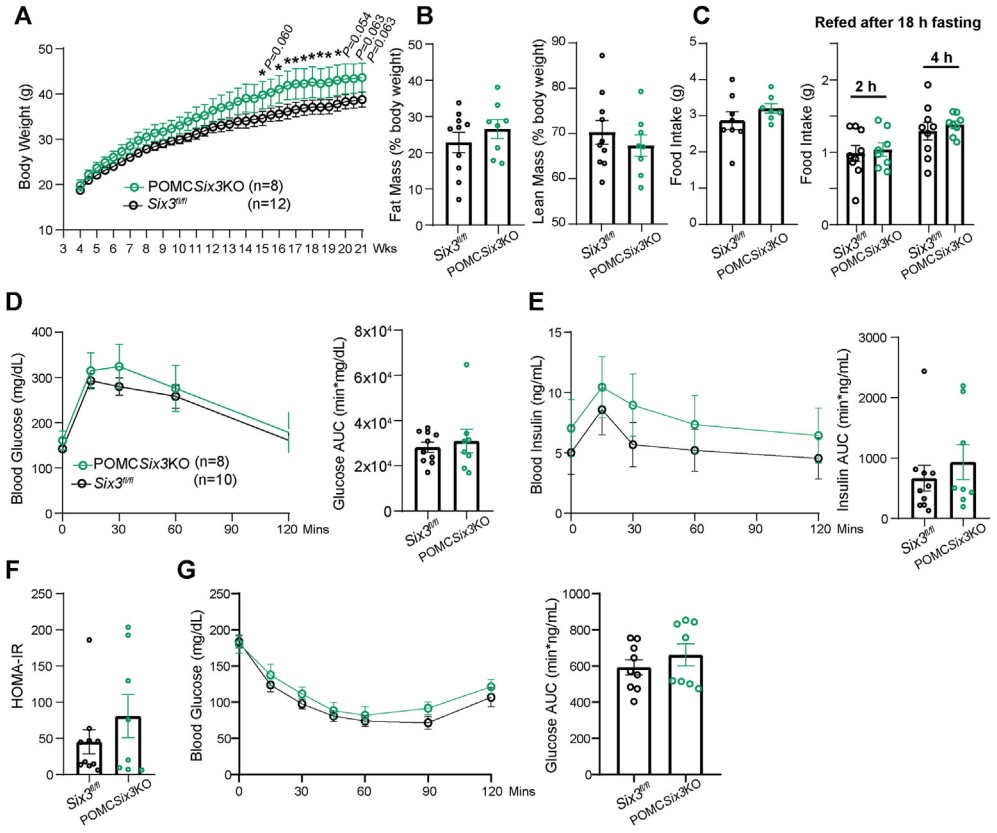

Figure 4: Deletion of *Six3* specifically from POMC neurons led to modest obesity in male mice subjected to a high-fat diet. (A) Body weight comparisons from 4 to 21 weeks of age. (B) Body composition. (C) Average daily food intake and refeeding intake after 18 h fasting. (D) Glucose tolerance test and corresponding blood insulin measurements. (E) HOMA-IR (F) and insulin tolerance test (G) in POMC*Six3*KO and *Six3*$^{fl/fl}$ mice subjected to HFD. Data shown are the means ± s.e.m of biologically independent samples. *P < 0.05.

significant reduction in arcuate POMC expression, this deficit did not result in any overt physiological changes.

4. DISCUSSION

In this study, we combined developmental, molecular, cellular, and functional genetics approaches to explore the participation of the transcriptional regulator *Six3* in the establishment of melanocortin neuron identity and the initiation and maintenance of *Pomc* expression within the arcuate nucleus. Specifically, our findings indicate the following key observations: 1) *Six3* and *Pomc* co-localize in arcuate hypothalamic neurons from the onset of *Pomc* expression at E10.5 and throughout the entire lifespan; 2) Selective deletion of *Six3* from POMC neurons reduces hypothalamic *Pomc* expression during development; 3) *Six3* expression in the developing ventral hypothalamus is necessary for the onset of arcuate *Pomc* expression as demonstrated in temporally conditioned *Six3* null allele mutant mice; 4) *Six3* early ablation does not impair transcript levels of other transcription factors known to regulate the onset of *Pomc* expression, suggesting a direct participation of *Six3* as a transactivator of arcuate *Pomc*; 5) the expression pattern of *Six3* in the hypothalamus extends beyond that of *Pomc*, suggesting that *Six3* is necessary but not sufficient for arcuate *Pomc* expression; 6) Selective deletion of *Six3* from POMC neurons induces mild obesity and improved glucose tolerance in adult male mice; 7) Conditional deletion of *Six3* from POMC neurons of adult mice leads to a ∼23% reduction in hypothalamic *Pomc* expression without critically altering any measured metabolic parameter. Together, these results indicate that *Six3* plays important roles in the regulation of hypothalamic *Pomc* expression in age dependent and sexually dimorphic patterns.

The Sine Oculis Homeobox Homolog (SIX) proteins represent a family of evolutionarily conserved transcription factors that play crucial roles in regulating multiple cellular processes, including proliferation, differentiation, apoptosis, adhesion, and migration [26]. The protein SIX3 was initially found at the anterior border of the developing neural plate and within the eye, underscoring its significance in forebrain and ocular development, respectively [17]. *Six3* is also expressed early in the ventral hypothalamic anlage, and a few studies have documented its important role in the maturation of *GnRH* and *kisspeptin* expressing neurons [27,28].

Our recent scRNA-seq study performed with POMC neurons at different developmental ages revealed that *Six3* is also present in arcuate POMC neurons [15]. To study the functional roles of *Six3* in arcuate POMC neurons, we first generated *Pomc-Cre.Six3*$^{fl/fl}$ mice and demonstrated that the selective deletion of *Six3* from POMC neurons leads to a 40% reduction in *Pomc* expression, resulting in a mild obesity phenotype in adult males (Figure 2A-B and Figure 3). This finding supports the threshold theory, which suggests that below certain incrementally reduced levels of *Pomc* expression mice develop from mild to extreme hyperphagia and obesity, respectively. Evidence supporting this theory is based on the fact that a ∼20% reduction of *Pomc* mRNA found in mice lacking the *Pomc* neuronal enhancer 2 (nPE2) does not cause obesity, while a 70% reduction in *Pomc* mRNA resulting from the absence of *Pomc* neuronal enhancer 1 (nPE1) leads to an appreciable

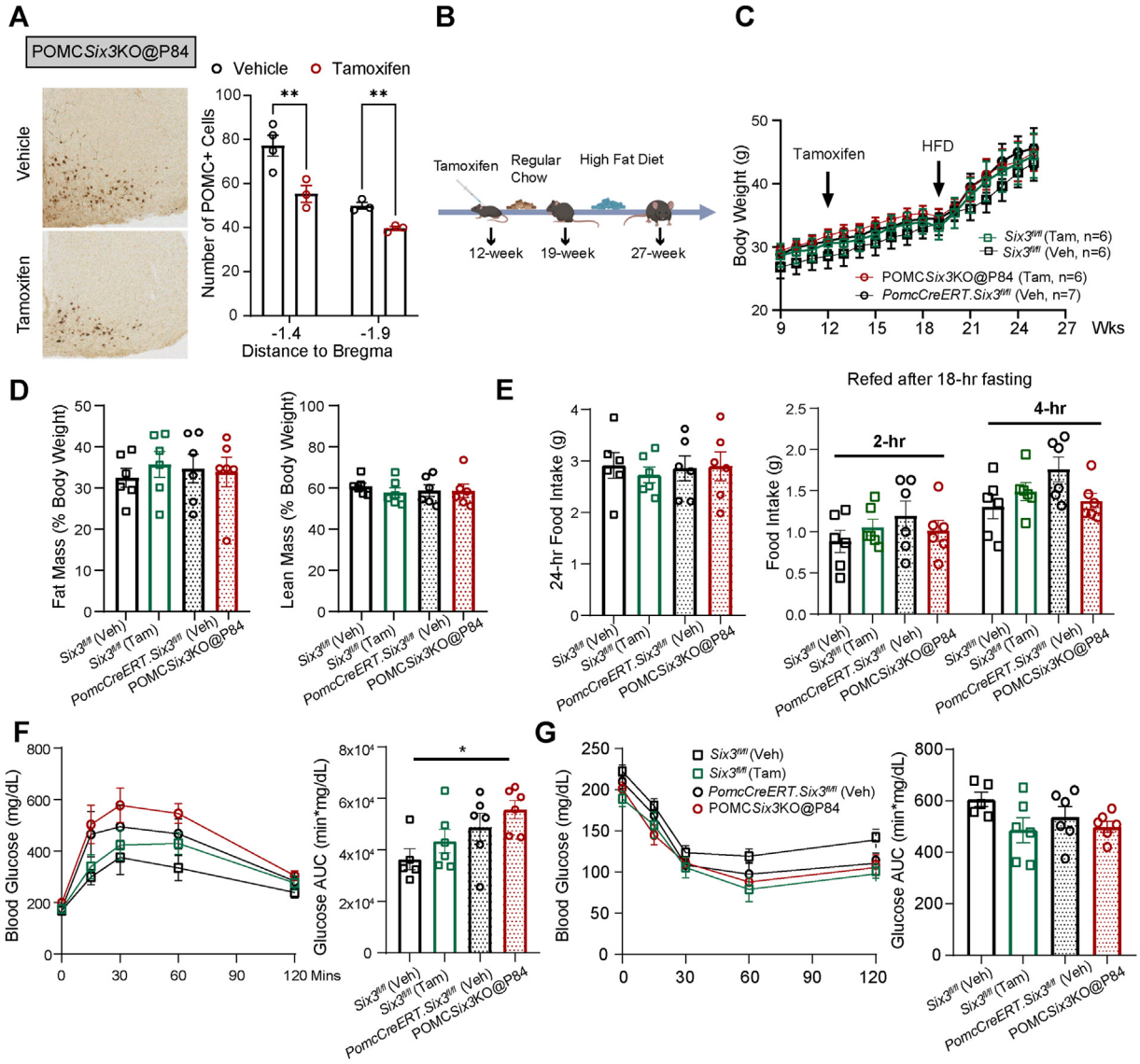

Figure 5: Conditional deletion of *Six3* from POMC neurons in adult male mice did not induce obesity. (A) Immunostaining illustrates the reduction in the number of POMC neurons following tamoxifen injection compared to vehicle injection in POMCSix3KO@P84 mice. (B) Schematic diagram outlining the overall experimental design. (C) Body weight, (D) body composition, (E) daily food intake and refeeding intake after 18 h fasting, (F) glucose tolerance and (G) insulin tolerance in *Pomc*-CreERT.*Six3*$^{fl/fl}$ and *Six3*$^{fl/fl}$ mice treated either with tamoxifen or vehicle. Data shown are the means ± s.e.m of biologically independent samples. *P < 0.05, **P < 0.01. Figure 5B was generated from Biorender.

but mild increase in body weight, whereas the simultaneous ablation of nPE1 and nPE2 enhancers reduces *Pomc* levels by 90% resulting in extreme obesity [29]. These results have led us to propose that food intake and body weight are visibly impaired once hypothalamic *Pomc* levels drop beyond ~40% [29].

Our results showed improved glucose tolerance when *Six3* was developmentally absent in POMC neurons. Given that a subset of POMC neurons expressing the *leptin receptor* (*Lepr*) directly regulate glucose homeostasis [30], we hypothesized that the deletion of *Six3* in POMC neurons could impact *Lepr*-expressing neurons and thus might contribute to the improved glucose tolerance observed in our animal model. To investigate this, we assessed the percentage of cells expressing both *Pomc* and *Lepr* cells that also express *Six3* using single-cell RNAseq data from a previous study [27]. Our findings indicated that approximately 85% of cells expressing both *Pomc* and *Lepr* also express *Six3*, suggesting that the deletion of *Six3* in POMC neurons could potentially influence the expression of *Lepr* and the function of cells co-expression of *Pomc* and *Lepr*. However, it is worth noting that this study [30] identified only a small population (9%) of POMC neurons expressing *Lepr*, which is consistent with another single-cell RNA-seq study showing that only 12% of POMC neurons express *Lepr* [31]. Another possible explanation for the improved glucose tolerance is the potential increase in glycosuria, although this was not examined in the current study. This phenotype has previously been reported by our lab in obese hypothalamic POMC-deficient mice with improved glucose tolerance [32].

Consistent with these findings, several studies have demonstrated that restoring *Pomc* expression in specific POMC neuronal subpopulations can mitigate some obesity-related phenotypes in *Pomc*-deficient mice. For example, Burke et al. found that *Pomc* re-expression selectively in 5-hydroxytryptamine 2c receptor neurons, which comprise 40% of all arcuate POMC neurons, prevents hyperphagia in both sexes but

mitigates obesity only in male mice [33]. Similarly, Lam et al. (2015) reported that conditional *Pomc* re-expression in leptin receptor-positive POMC neurons restored POMC immunoreactivity by 67% and normalized energy expenditure, glucose levels, and locomotor activity [34]. A more recent study highlighted that inducing *Pomc* expression in approximately 23–25% of GABAergic POMC neurons was sufficient to normalize food intake and body weight [35]. Conversely, a nonspecific *Pomc* rescue in the same study, which increased *Pomc* expression by 32–35% in *Pomc*-deficient mice, did not improve food intake but resulted in a slight weight loss, suggesting that the optimal *Pomc* dose for maintaining satiety signals depends on the specific neuronal subpopulation [35]. It is worth noting that the above-mentioned studies utilized a reversible strain of *Pomc*-deficient mice, which are constitutively predisposed to hyperphagia and obesity [2]. Thus, it is possible that the threshold for restoring *Pomc* expression in these extremely obese mice differs from the threshold of *Pomc* mRNA levels below which obesity becomes apparent in mice. Overall, the comparison of our current study with prior research emphasizes that a threshold of ~40% of arcuate *Pomc* expression is necessary to maintain proper satiety signals, at least in laboratory mice fed *ad libitum*. Thus, our study presents a novel role for SIX3, as a cell-autonomous transcription factor necessary to maintain high expression levels of *Pomc* in the developing and adult arcuate nucleus. SIX3 adds to the repertoire of transcription factors, which include ISL1 [9], NKX2.1 [10] and PRDM12 [13], that dictate the early specification of melanocortin neuron identity in the developing hypothalamus. Each of these four transcription factors plays a critical role -rather than partially redundant functions- in the regulation of hypothalamic *Pomc* expression since the individual conditional mutants of each of these four genes specifically in POMC cells showed severely impaired hypothalamic *Pomc* expression [9,10,13, and this study].

Our study found that male mice lacking *Six3* specifically from POMC neurons develop mild obesity (Figures 3 and 4). Although this phenotype has not been matched in females (Supplementary Figs. 5 and 6) we did observe excessive food wasting behavior in female mice. Our qRT-PCR study separated by sex showed deletion of *Six3* leads to a significant reduction in *Pomc* expression in both sexes (Supplementary Fig. 2) precluding a sexually dimorphic dependence of *Six3* in arcuate *Pomc* expression (Figure 2A-D). Moreover, given the expression of estrogen receptor alpha (*Esr1*) in POMC neurons, we examined the *Esr1* transcript levels in the absence of *Six3*. Our results indicate that the deletion of *Six3* does not affect *Esr1* expression (data not shown). However, sexual dimorphism in feeding behavior and energy homeostasis is well documented in several mutant mouse models. For example, deletion of the transcription factor *Tap63* specifically from POMC neurons increased diet-induced obesity (DIO) in female but not in male mice [36]. Similar sex-specific regulatory mechanisms have been reported in male mice lacking *Sirt3* (*Sirtuin 3*) in POMC neurons, which showed decreased body weight and adiposity together with an increase in energy expenditure, all effects that were not observed in females [37]. Another example has been found after restoring *Pomc* expression specifically in 5HT2cR+ (5-hydroxytryptamine 2c receptor) POMC neurons of male mice which, in contrast to females, normalized physical activity and energy expenditure [33]. Our own previous study reported that reactivating neuronal *Pomc* expression in arcuate *Pomc*-deficient mice normalized food intake in females whereas males maintained residual hyperphagia [2].

In summary, our findings demonstrate that *Six3* plays a crucial role in the regulation of hypothalamic *Pomc* expression, and that its absence from *Pomc*-expressing cells leads to mild obesity in male mice. Therefore, SIX3 integrates a unique repertoire of TFs, including ISL1, NKX2.1 and PRDM12 which, together, dictate the early identity of central melanocortin neurons and control arcuate *Pomc* expression from development to adulthood.

CREDIT AUTHORSHIP CONTRIBUTION STATEMENT

Hui Yu: Writing — review & editing, Writing — original draft, Visualization, Validation, Supervision, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Angelika Chiang:** Writing — original draft, Validation, Methodology, Formal analysis, Data curation. **Marcelo Rubinstein:** Writing — review & editing, Investigation, Funding acquisition, Formal analysis. **Malcolm J. Low:** Writing — review & editing, Supervision, Investigation, Funding acquisition, Formal analysis, Data curation.

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DECLARATION OF COMPETING INTEREST

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

DATA AVAILABILITY

Data will be made available on request.

APPENDIX A. SUPPLEMENTARY DATA

Supplementary data to this article can be found online at https://doi.org/10.1016/j.molmet.2024.101993.

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