Molecular and functional genetics of the proopiomelanocortin gene, food intake regulation and obesity

Marcelo Rubinstein[1,2,3, *] and Malcolm J. Low[3,4]

[1]Instituto de Investigaciones en Ingeniería Genética y Biología Molecular, Consejo Nacional de Investigaciones Científicas y Técnicas, Buenos Aires, Argentina

[2]Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, 1428 Buenos Aires, Argentina

[3]Department of Molecular and Integrative Physiology, University of Michigan Medical School, Ann Arbor, MI, USA

[4]Department of Internal Medicine, Division of Metabolism, Endocrinology and Diabetes, University of Michigan Medical School, Ann Arbor, MI, USA

*Corresponding author: mrubins@dna.uba.ar (M. Rubinstein)

Abstract

During the last decade, the obesity pandemia has skyrocketed, and the spectre is threatening children, adolescents and lower income families worldwide. Although driven by an increase in the consumption of ultra-processed edibles of poor nutritional value, the obesogenic changes in contemporary human lifestyle affect people differently, revealing that some individuals are more prone to developing

increased adiposity. During the last years, we performed a variety of genetic, evolutionary, biochemical and behavioral experiments that allowed us to understand how a group of neurons present in the arcuate nucleus of the hypothalamus regulate the expression of the proopiomelanocortin (*Pomc*) gene and induce satiety. We disentangled the neuronal transcriptional code of *Pomc* by identifying the cis-acting regulatory elements and primary transcription factors controlling hypothalamic *Pomc* expression and determined their functional importance in the regulation of food intake and adiposity. Altogether, our studies reviewed here shed light on the power and limitations of the mammalian central satiety pathways and may contribute to the development of individual and collective strategies to reduce the debilitating effects of the self-induced obesity pandemia.

Introduction to the central regulation of food intake

Body weight and energy balance are controlled in vertebrate animals by brain circuits that promote foraging, food intake or satiety after integrating multiple metabolic and environmental signals with current and future metabolic demands. Peripheral organs such as the adipose tissue, pancreas, liver and gastrointestinal tract release hormones in response to nutrient flux. This circulating information about the energy status of the organism is then sensed and interpreted by specialized arrays of neurons located in the arcuate nucleus of the ventromedial hypothalamus and in the dorsal medulla. Molecular cloning of the leptin gene[1], mutations of which are responsible for an autosomal recessive form of early-onset extreme obesity, propelled the discovery of hypothalamic circuits and genes directly involved in the central regulation of food intake[2]. Leptin is a circulating hormone mainly secreted by adipocytes and its plasma levels correlate with the amount of triglycerides present in the adipose tissue. Thus, circulating leptin provides a direct readout of the available energy stored as fat in the organism.

Two distinct populations of neurons present in the arcuate nucleus of the hypothalamus express leptin receptors and also respond to other humoral metabolic signals such as ghrelin, glucose and insulin. In simple terms, neurons expressing the agouti-related protein (Agrp) promote food intake and those expressing the proopiomelanocortin (POMC) gene induce satiety to limit food intake. These two interrelated and functionally opposite sets of arcuate neurons are considered the yin and yang of food intake regulation[3]. POMC neurons release melanocortin peptides that induce anorexia upon stimulation of G_s-coupled melanocortin 4 receptors (MC4R), whereas, Agrp released from Agrp neurons promotes feeding by acting as a competitive MC4R antagonist. Stereotaxic application of MC4R antagonists into the cerebral ventricles [4] as well as optogenetic activation of Agrp neurons [5]

promote food intake even in well fed mice, whereas pharmacological stimulation of MC4R in the hypothalamus [4] and optogenetic activation of POMC neurons promote satiety [5]. This general view of POMC and Agrp neurons playing contrasting anorexigenic and orexigenic responses has been challenged recently by novel provocative results based on photometric detection of hypothalamic neuronal activity in freely behaving mice[6]. Just the presentation of food to fasted mice, or even food cues without any consumption, produced an immediate activation of POMC neurons and a concomitant inactivation of Agrp neurons[6]. These opposing activity patterns were maintained during food consumption and then switched back when feeding was interrupted by abrupt removal of the food[6], suggesting that the endogenous release of Agrp and melanocortins anticipate consummatory and satiety behavior, respectively.

The increasing knowledge gained during the last 25 years concerning the genetics and physiology involved in food intake regulation has been ironically mirrored by a dramatic increase in the prevalence of adult and childhood overweight and obesity. This worldwide burden prompted the World Health Organization and Public Health systems of many countries to declare obesity as a pandemia[7]. Pandemic obesity has been driven primarily by pervasive changes in the global food system that instigate people from all countries, ages and socioeconomic status to consume high calorie, ultra-processed, affordable and ubiquitously marketed edibles with poor nutritional value. However, the obesogenic changes in contemporary human lifestyle do not affect all individuals equally, revealing that some individuals have an inherently greater predisposition to develop overweight and increased adiposity. The genetic contribution to body weight determined in family studies, adopted children and twins reared apart [8-9] has consistently been reported at 40–70% [10], placing weight as a highly heritable trait only slightly below that of height [11-12]. As expected, homozygous null mutations in the human leptin[13] and leptin receptor[14] genes lead to early-onset extreme obesity syndromes remarkably similar to the phenotypes found in mice with mutations in the orthologous genes. The rare existing cases of leptin deficiency have responded therapeutically to recombinant leptin replacement treatment[15]. However, leptin treatment is largely ineffective at promoting weight loss in patients with common obesity, probably because they already have high serum leptin levels and develop an as yet incompletely defined resistance to further leptin action. More recent studies suggest that leptin sensitizing agents may be potentially employed in leptin pharmacotherapy[16]. Similarly rare, null allele mutations in the human POMC gene lead to severe hyperphagia, early-onset obesity, and adrenal insufficiency[17], whereas mutations in melanocortin 4 receptor gene (*MC4R*) are the most common monogenic disorders causing obesity[18]. A recently developed MC4R agonist named setmelanotide has

induced significant weight loss in two POMC-deficient patients[19]. Although this drug has potential in broader populations of obese patients, caution is warranted as it may act at other melanocortin receptors involved in autonomic functions and skin pigmentation.

In summary, human monogenic obesity syndromes are rare and most familial cases of obesity appear to be driven by several coexisting low-frequency polymorphic alleles, each of them contributing a small percentage to the overall phenotype. A large number of genome-wide association studies has been performed worldwide during the last several years and identified nearly 100 different loci associated with high body mass index, type II diabetes, increased adiposity or high leptin levels[20-22]. However, the individual contribution of most of these variants is practically irrelevant except for a few like the one reported in the *FTO* locus[23-24]. Another exceptional single locus is *POMC*. Despite the fact that humans deficient in POMC are rare, the *POMC* locus has been strongly associated to obesity. A number of genome-wide studies have found highly significant linkage scores between obesity-related traits and a genomic segment in chromosome 2 near *POMC*[25-27]. Because polymorphisms in *POMC* coding sequences do not appear to account for these associations[28], it is likely that mutations in non-coding regulatory elements may alter *POMC* transcript levels and modify the relative amount of central melanocortins.

Regulation of *Pomc* gene expression

Pomc is expressed at high levels in endocrine cells of the pituitary gland and neurons present in the arcuate nucleus of the hypothalamus. POMC encodes a prohormone that after cell-specific posttranslational processing generates several biologically active peptides that orchestrate the mammalian stress response[29]. In the anterior pituitary, corticotrophs release adenocorticotropic hormone (ACTH) whereas in hypothalamic neurons the POMC prohormone is further processed to generate the melanocortins α-, β-, and γ- melanocyte stimulating hormones (MSH) and the analgesic endogenous opioid β-endorphin[29-30](Fig. 1).

The transcriptional regulation of *Pomc* also follows distinct cell-specific cis/trans codes. By combining *in vitro* transfection assays in immortalized pituitary cell lines[31] and reporter gene expression in transgenic mice it was possible to initiate and advance studies on regulation of POMC expression in pituitary cell types[32-33]. These early studies demonstrated that pituitary-specific expression of rat or mouse *Pomc* is controlled by several cis-acting elements localized within 500 bp proximal to the transcriptional start site[32-33]. A number of transcription factors including Nurr77, NeuroD1, Ascl1,

Pitx1, SP1, and Tpit have been shown to participate in the control of pituitary *Pomc* expression and/or the determination of corticotropic and melanotropic cell lineages[33-38]. In addition to the proximal promoter, a conserved pituitary specific *Pomc* enhancer was identified 7 kb upstream of the mouse *Pomc* gene transcriptional start site[39]. This distal pituitary enhancer recruits the same subset of transcription factors as the proximal promoter, except Pitx1, and is highly dependent on Tpit for activity[39]. When tested in transgenic mice, the -7 kb enhancer was shown to be more active in corticotrophs than in melanotrophs, contrary to what has been observed for the proximal *Pomc* promoter[39]. More recently, the transcription factor Pax7 has been identified as an essential pioneer factor to establish the melanotroph lineage[40]. Early expression of Pax7 remodels the chromatin along specific enhancers allowing access of factors like Tpit to otherwise hidden DNA binding sites. This Pax7-induced epigenetic remodeling drives expression of melanotroph-specific genes, including those encoding the prohormone convertase PC2 and the dopamine D2 receptor[40]. For a recent comprehensive review about the transcriptional regulation of *Pomc* in pituitary cells and related human diseases, see Ref.[41].

Identification of the enhancers and transcription factors that control hypothalamic *Pomc* expression experienced a relative delay mainly due to the lack of an authentic neuronal cell line able to express *Pomc* with transcriptional fidelity and with which to perform reliable expression studies. In addition, early transgenic mouse expression studies showed that the proximal *Pomc* promoter drives cell-specific expression of reporter genes only to pituitary POMC cells but not to the hypothalamus [32, 42-43]. Therefore, our initial attempts to search for regulatory elements controlling *Pomc* expression in the arcuate nucleus of the hypothalamus in the pregenomic era were performed blindly by chromosome walking upstream and downstream of the *Pomc* gene and testing the isolated flanking fragments in transgenic mouse expression assays. Using this approach we identified a 4 kb fragment located between -13 and -9 kb upstream of the mouse *Pomc* promoter that when included in transgenic constructs allowed authentic neural-specific and developmentally regulated reporter expression in hypothalamic POMC neurons [44] (Fig. 1). The possibility to specifically target POMC neurons prompted us to drive enhanced green fluorescent protein (EGFP) expression to the arcuate nucleus and perform the first electrophysiological recordings in readily identified bright fluorescent POMC neurons in hypothalamic slices[45]. Double immunofluorescence performed in brain slices of *Pomc*-EGFP transgenic mice confirmed coexpression of EGFP and POMC peptides in more than 99% of these neurons[45]. Electrophysiological studies in *Pomc*-EGFP mouse arcuate slices led to the discovery that leptin increases the frequency of action potentials in POMC neurons by two concurrent mechanisms: depolarization

through a leptin-activated non-specific cation channel and reduced inhibition by local orexigenic Agrp/GABA neurons[45]. We also found that melanocortins play an autoinhibitory effect on this circuit. The discovery that leptin activates arcuate POMC neurons revealed a fundamental signaling entry point from the periphery to the brain through the blood-brain barrier. Stimulation of leptin receptors expressed on arcuate POMC neurons increases their activity leading to the release of POMC-encoded peptides. Central melanocortins, in turn, stimulate melanocortin receptors to induce satiety and increase metabolic rate[46][2]. Altogether, the increase in leptin levels due to accumulation of triglyceride stores triggers negative feedback mechanisms that reduce further food consumption. *Pomc*-EGFP transgenic mice have been extensively used during the last 15 years and are the gold standard system for interrogation of the physiological and pharmacological properties of various neurotransmitters and peptides, such as 5-HT and PYY(3-36), that modulate the activity of POMC neurons[47-48]. However, not all hypothalamic POMC neurons express leptin receptors[49]; neither are all POMC neurons involved in the control of food intake. In fact, anatomical, histological, pharmacological, electrophysiological and molecular studies have accumulated extensive evidence supporting the idea that hypothalamic POMC neurons are highly heterogeneous and that several phenotypically and functionally distinctive subpopulations seem to coexist. From the arcuate nucleus POMC neurons project towards a wide array of rostral nuclei including the septum, striatum, thalamus and hypothalamus and, caudally, to the brainstem and medulla[50]. In addition, subpopulations of POMC neurons are distributed along the antero-posterior and medial-lateral axes of the arcuate nucleus and co-release either the fast neurotransmitters GABA or glutamate[51] while partially overlapping populations express the serotonin 5HT2c receptor[52], leptin or insulin receptors[53] and melanocortin autoreceptors MC3R or MC4R. A recent single-cell RNA sequencing study performed on FACS sorted green fluorescent hypothalamic neurons taken from Pomc-EGFP mice revealed the great level of heterogeneity exhibited by this population of around 3,000 neurons expressing *Pomc*[54].

Regulation of hypothalamic *Pomc* expression

The completion of the first vertebrate genome projects in the early 2000´s allowed us to perform phylogenetic footprinting analyses along the 4 kb distal mouse *Pomc* module necessary for reporter gene expression in hypothalamic POMC neurons of transgenic mice[55]. This type of bioinformatic algorithm globally compares multiple short overlapping local alignments to detect high identity cross species sequences. Thus, this strategy can be used as a proxy to identify transcriptional enhancers embedded in intergenic or intronic regions based on the concept that the rate of molecular evolution in

functional sequences is more constrained due to selective pressure and mutations accumulate much slower than in non-functional residues the evolve at neutral rates. Following this strategy, we detected two highly conserved sequences within the 4 kb distal module which are located at -12 and -10 kb of the mouse *Pomc* 5´-flanking region[55]. We named these sequences putative neuronal *Pomc* Enhancers nPE1 and nPE2. To determine whether nPE1 and nPE2 had neuron-specific transcriptional enhancer function, we performed a comprehensive deletional transgenic mouse analysis of the distal module cloned immediately upstream of either the mouse *Pomc* promoter or a heterologous minimal promoter. In addition, we tested a human chromosomal region orthologous to the distal 4 kb mouse *Pomc* module. This study led to the following conclusions: (i) a genomic region located between -13 and -9 kb of mouse *Pomc* is necessary and sufficient to direct authentic reporter gene expression to hypothalamic POMC neurons; (ii) this genomic neuron-specific enhancer region contains two highly conserved short sequences that we named nPE1 and nPE2 which are present in mammalian genomes but not in birds, amphibians or fishes; (iii) either nPE1 or nPE2 are able to drive cell-specific reporter expression to POMC arcuate neurons, whereas the simultaneous deletion of both enhancers abolishes reporter expression in POMC neurons; (iv) a human genomic fragment carrying nPE1 and nPE2 also drives reporter gene expression to POMC hypothalamic neurons of transgenic mice proving that mouse and human orthologs are functionally conserved; (v) brain and pituitary *Pomc* expression are independently controlled by distinct sets of enhancers[55] (Fig. 1A).

The comparative genomic analyses at the *Pomc* locus used in this study also revealed that the teleost fishes Tetraodon, Fugu and zebrafish possess two *POMC* genes, which we called *POMCa* and *POMCb*, unlike the human, mouse, and chicken genomes that have only one POMC gene[56]. We found that these two *POMC* paralogs originated in the whole-genome duplication specific to the teleost lineage over 300 million years ago (Mya). Since *POMCa* has been found to be expressed in the equivalent teleost arcuate nucleus of the hypothalamus and *POMCb* in the preoptic area of the brain, we concluded that the *POMC* paralogs underwent a process of subfunctionalization of their expression domains during teleost evolutionary history[56]. In addition, we found evidence for subfunctionalization of the peptide domains encoded by the two *POMC* paralogs, although it is unknown if these distinct processes were concurrent[56].

Molecular evolution of neuronal *Pomc* enhancers

Although *Pomc* is expressed in the ventromedial hypothalamus of all jawed vertebrates, we failed to find nPE1 and nPE2 genomic sequences in non-mammalian vertebrate Classes. To investigate this

discrepancy, we searched for the evolutionary history of nPE1 and nPE2 sequences. We first found that the enhancer nPE2 is more ancient than nPE1. Whereas nPE2 is highly conserved across Prototheria (monotremes), Metatheria (marsupials) and Eutheria (placental mammals), nPE1 is a placental novelty. To reconstruct the origin of functional novelties we developed an *in silico* paleogenomics strategy based on systematic and progressive searches for nPE1 and nPE2 paralogs in all available genome databases that could reveal evolutionary relics. We did not find any sequence similar to nPE2 in any of the available vertebrate genomes except the opossum, where we identified three short sequences similar to opossum nPE2[57]. When used as queries in further BLAST searches, those four sequences showed to be similar to members of the marsupial MAR1 family of CORE-SINE retroposons[57]. Mobile elements have been shown to be involved in gene and genome evolution by providing a large reservoir of raw sequences that, upon acquiring functional mutations, may become adaptive and fixed in their host genomes, a process called exaptation[58-59]. Thus, our results suggest that an ancient CORE-SINE retroposon was inserted into the *POMC* locus and exapted as a neuronal *Pomc* enhancer in the mammalian lineage, before the marsupial/placental split that occurred around 170 Mya (Fig. 1A). It is impossible to confirm whether or not the CORE-SINE inserted upstream *POMC* functioned immediately as an enhancer, as is assumed for Alu elements carrying potential binding sites for nuclear receptors[60-62], or that the inserted mobile element accumulated mutations until evolving into a novel neuronal *POMC* enhancer that became fixed during mammalian evolution[63].

To discover the evolutionary origin of nPE1, we again performed *in silico* paleogenomics using human nPE1 as a query and detected 15 high identity hits in the human genome, annotated as sequences derived from the family of mammalian-apparent LTR retrotransposons (MaLR) [64]. MaLRs originated before the radiation of eutherians, betwenn 80 to 100 Mya[63], a period that matches well with the hypothesis that nPE1 constitutes a placental novelty. Altogether, the distal hypothalamic enhancer module located upstream of the *Pomc* gene contains two highly conserved, but evolutionarily unrelated, sequences originated after the sequential exaptation of two different types of retroposons[65; Fig. 1A].

Based on the independent and distinct evolutionary history of nPE1 and nPE2 we wanted to determine whether both enhancers drove overlapping or distinct spatio-temporal expression patterns in the ventromedial hypothalamus. To this end, we designed two structurally similar transgenes in which nPE1 and nPE2 drove the expression of the red fluorescent proteins tomato or EGFP, respectively (Fig. 1B). Analysis of nPE1-tomato.nPE2-EGFP compound mice showed more than 85% of co-expression of both fluorescent markers throughout the entire antero-posterior and medial-lateral axes of the arcuate nucleus[65](Fig. 1). The onset of both transgenes was coincidental within the same array of neurons

located at the base of the developing hypothalamus of compound nPE1-tomato,nPE2-EGFP e10.5 embryos, matching the initial spatiotemporal expression of mouse *Pomc*[66]. These results led us to conclude that nPE1 and nPE2 control *Pomc* expression in the entire population of hypothalamic neurons that normally express this gene[65]. The independent evolutionary origin and identical enhancer function of nPE1 and nPE2 indicate that these two regulatory elements are functional analogs that underwent a process of convergent evolution that started during basal mammalian evolution, more than 166 Mya[63], and finished after the marsupial/placental split around 147 Mya[63]. To our knowledge this is the first and probably still the only functionally documented example of authentic convergent molecular evolution of cell-specific enhancers[65] (Fig. 1). It has been reported that around half of the early population of immature POMC neurons that originate in the developing hypothalamus stop expressing *Pomc* at midgestation and adopt different neuropeptide phenotypes including the expression of Agrp/Npy[67] or kisspeptin[68]. The mechanisms by which *Pomc* expression is silenced and whether the enhancers nPE1 and nPE2 play any role during this phenotypic transition are still unknown.

The presence of two apparently redundant enhancers controlling neuronal *Pomc* expression suggested to us that one of them is likely to be under lower selective pressure [69-70]. To challenge this hypothesis we calculated the rate of molecular evolution of nPE1 and nPE2 by performing, first, a long-scale interspecies' mammalian orthologs comparison to assess the sequence variation during the last 100 million years, and, second, a short-scale intraspecies' human genetics population study. The former calculation showed that nPE1 is evolving 2.64 times faster than nPE2 and also faster than sequences coding for bioactive POMC peptides present in exon 3[65]. At the human population level, we found three polymorphic sites at nPE1 and absence of polymorphisms in nPE2. These results showed that the regulatory elements nPE1 and nPE2 are evolving slower than coding sequences, although almost all exon 3 SNPs do not introduce missense mutations in the amino acid sequences of any bioactive POMC peptides processed from the prohormone[65]. Altogether, these interspecies' and intraspecies' comparisons show that the more ancient nPE2 is under stronger selective pressure than the younger nPE1, precluding the hypothesis that the exaptation of nPE1 relaxed the selective pressure on nPE2[65].

The existence of two overlapping enhancers controlling gene expression in the same cell types has been observed in several developmental genes[70-71]. This level of redundancy may be critical to buffer environmental perturbations and control the spatio-temporal expression pattern of developmental genes in a precise and stable manner during embryogenesis. A recent study indicates that overlapping

enhancers are a predominant feature in Drosophila developmental genes[72]. So far, nPE1 and nPE2 appear to be the only reported case of overlapping enhancers controlling expression of a gene that plays important physiological roles throughout the postnatal life. Carrying two apparently redundant enhancers may constitute an adaptation to allow for higher transcriptional rates and/or lower fluctuations. These two possible alternatives may be illustrated by a double-scull rowboat metaphor in which the two rowers act together to attain a faster –transcriptional- speed, and also maintain a lower cruise control level with the participation of only one rower. It is interesting to note that nPE1 and nPE2 are both present in all placental mammalian Orders, indicating that both enhancers coexist upstream of *Pomc* under purifying selection since the radiation of mammals around 90 Mya. This finding strongly suggests that both enhancers play important roles in *Pomc* transcription and mammalian physiology.

To investigate the importance of having two enhancers and the individual contributions of nPE1 and nPE2 to hypothalamic *Pomc* expression, we generated mutant mice lacking nPE1, nPE2 or both enhancers simultaneously[73] (Fig. 2). Quantitative analysis of *Pomc* mRNA levels during various developmental stages and adulthood in wild-type and the different homozygous enhancer mutants showed that: (i) only the concurrent presence of nPE1 and nPE2 in both *Pomc* alleles assures normal hypothalamic *Pomc* mRNA levels; (ii) the simultaneous deletion of nPE1 and nPE2 induces a profound deficit in Pomc mRNA levels that induces hyperphagia and obesity; (iii) at the early start of *Pomc* expression, both enhancers act synergistically to attain typical *Pomc* mRNA levels in the presumptive hypothalamus. The synergism is evident when measuring the much higher *Pomc* mRNA levels present in wild-type e10.5 embryos in comparison with the sum of the levels detected in the individual enhancer mutants; (iv) during adulthood, however, the individual activity of each enhancer seems to be additive with nPE1 and nPE2 contributing with ~80% and ~20% of the total Pomc mRNA levels, respectively; (v) nPE1 ablation revealed its main contribution to *Pom*c mRNA adult levels since its absence impairs satiety and, consequently, induces obesity (Fig. 2); and (vi) deletion of nPE2 does not seem to alter the control of food intake in the presence of nPE1, at least in laboratory conditions. However, ablation of nPE2 in the absence of nPE1 triggers hyperphagia and extreme obesity [73](Fig. 2).

In general, we have observed that once *Pomc* mRNA levels drop below ~35% of its normal values mice exhibit satiety control deficits and excessive fat accumulation. Although lacking nPE2 does not alter food intake regulation, a 20% reduction in *Pomc* mRNA levels may increase the risk of an insufficient control of energy balance mechanisms [73]. Hyperphagia and excessive body weight are highly maladaptive in the wild because a greater body mass and unnecessary foraging increases the likelihood

of encounters with predators while limiting escape efficiency. Therefore, the adaptive value of maintaining *Pomc* expression above a critical threshold seems to be essential for reproductive success. This comprehensive study highlights the importance of evaluating the participation of individual enhancers to gene expression in their native genomic context to then study the effects of each mutant allele in general physiology and fitness [73](Fig. 2).

The cis-trans code controlling hypothalamic *Pomc* expression

An emerging conclusion drawn by the discovery that nPE1 and nPE2 are two evolutionarily convergent enhancers that regulate *Pomc* expression in all POMC of hypothalamic neurons[55,65] is that these two functional analogs are likely to share DNA motifs that recruit similar transcription factors (TFs). In fact, we detected a 21-bp imperfect palindromic motif in nPE1 which is highly similar to a sequence present in nPE2[73]. These sequences carry two TAAT inverted motifs, known to be recognized by homeodomain-TFs (HDTFs). Another sequence present in nPE2 also showed two inverted homeodomain (HD) binding sites. These similar pairs of inverted TAAT motifs concur with a canonical TCAAG/T motif probably recognized by a HD-TF of the NKX family[73]. All these HD-binding sites showed high identity levels between humans, mice and most other mammals. We investigated the putative functional relevance of these shared conserved sequences in expression studies performed in transgenic mice with transition mutations in either nPE1 or nPE2. All transgenic mouse founders carrying mutated nPE1 or nPE2 failed to drive EGFP expression to the arcuate nucleus of the hypothalamus in contrast to the control transgenes carrying *wild-type* nPE1 or nPE2 that drove clear EGFP expression to this brain region[73]. These results suggested to us that a common array of cis-acting motifs, probably recognized by the same TFs, are responsible for the functional analogy between nPE enhancers and prompted us to search for TFs involved in specification of the POMC hypothalamic neuronal lineage.

To detect HDTFs that may recognize these DNA elements, we looked for the preferred DNA binding motifs of each mouse homeodomain obtained in a massive *in vitro* binding study [74]. Using the nPE sequences as queries, we generated an initial list of candidate HDTFs that was later reduced by discarding those showing expression patterns incompatible with that of *Pomc* based on gene expression data obtained from the Allen Brain Atlas at different mouse ages [75]. The final TF candidates were tested in gel mobility shift assays to determine their ability to bind in vitro to particular motifs present in nPE1 and/or nPE2. We selected the LIM-homeodomain TF Islet1 (ISL1) because its expression pattern in the ventromedial hypothalamus is similar to that of *Pomc* since its expression onset and throughout the adult mouse life. After combining molecular, genetic, physiological, and evolutionary approaches, we

demonstrated that ISL1 directly plays a fundamental regulatory role in hypothalamic *Pomc* expression[76]. In this work we concluded that: (i) *Pomc* and *Isl1* co-express during embryonic development, postnatal life and adulthood; (ii) gel-shift and ChIP assays demonstrated that ISL1 binds to precise sequences present in nPE1 and nPE2 which are essential for their enhancer function; (iii) ISL1 plays a critical role for *Pomc* expression during the early stages of hypothalamic development; (iv) after birth, the lack of *Isl1* expression induces a remarkable deficit in hypothalamic *Pomc* expression levels; (v) the absence of *Isl1* expression in POMC neurons of adult mice reduces *Pomc* expression causes an energy balance phenotype including obesity; and (vi) hypothalamic *Pomc* depends on Isl1 in mice and zebrafish demonstrating that this regulation is conserved throughout vertebrate evolution[76]. Altogether, our results indicate that ISL1 acts as a terminal differentiation gene establishing the specific identity of POMC neurons after their birth and accumulation in the mantle zone and before the arcuate nucleus is formed. In addition, Isl1 controls hypothalamic *Pomc* expression in adulthood and therefore plays a fundamental role in food intake and body weight regulation[76]. More recently, it has been found that ISL1 not only plays an essential role in the differentiation of POMC neurons but also in other neuronal subtypes present in the arcuate nucleus such as those expressing *Agrp, Ghrh* and somatostatin[78].

It is conceivable that other TFs and motifs participate in establishing the neuronal-specific expression of *Pomc* so further work is necessary to reveal the identity of the full molecular complex. Other motifs present in the hypothalamic enhancers probably bind TFs related to the hormonal regulation of *Pomc*. For example, we identified a conserved element in nPE2 that can bind TFs of the nuclear receptor family with a zinc-finger DNA binding domain, and a ligand-binding domain that recruits several types of steroid hormones[79]. We found that the estrogen receptor alpha (ESR1) is a candidate nuclear receptor factor to regulate neuronal *Pomc* expression since it binds to this nPE2 motif in vitro and is expressed in POMC neurons during development and adulthood[79]. Thus, it is possible that estrogen exerts its anorectic effect by controlling POMC expression. We have also identified a conserved canonical STAT3 binding site in nPE1 which is likely to participate in leptin-induced activation of *Pomc* expression, although the importance of this site remains to be explored.

Hypothalamic *Pomc* expression in teleosts

Our phylogenetic footprinting analysis showed that nPE1 and nPE2 are highly conserved sequences in mammals but are absent from the genomes of all other Classes of vertebrates despite the fact that

fishes, amphibians, reptiles and birds express *Pomc* in functionally homologous neurons of the ventromedial hypothalamus. This apparent discrepancy raises two intriguing questions: (i) what are the regulatory elements that control the expression of *Pomc* in the hypothalamus of non-mammalian vertebrates? and, (ii) are nPE1 and/or nPE2 able to function as transcriptional enhancers in *Pomc* neurons of non-mammalian vertebrates? We have answered these two questions by studying teleost fishes, the most distant evolutionary Class of vertebrates from mammals that express *Pomc* in the hypothalamus. We found that the proximal promoter sequences of zebrafish *POMCa* (the teleost paralog expressed in the ventromedial hypothalamus) drove reporter gene expression only to the pituitary gland of transgenic zebrafish, similar to what we had previously found for mammalian *Pomc*[80]. However, insertion of the distal mouse *Pomc* module containing nPE1 and nPE2 upstream of the zebrafish promoter construct allowed reporter gene expression in POMC neurons of the zebrafish ventromedial hypothalamus[80]. These data indicate that although the mammalian *Pomc* hypothalamic enhancers are mammalian specific, they nonetheless contain a transcriptional code that has remained conserved for more than 450 million years of vertebrate evolution. Furthermore, when nPE2 and nPE1 originated *de novo* during early mammalian evolution, the newly created cis/trans interactions were similar to the ancestral ones. This result matched our previous finding that hypothalamic Isl1 is essential for *Pomc* expression in the ventromedial hypothalamus of mice and zebrafish[76]. Deletional analysis showed that, as in transgenic mice, the sole presence of nPE1 or nPE2 was able to drive EGFP expression in zebrafish hypothalamic neurons, but the concurrent removal of both enhancers completely inactivated the 4 kb mouse distal *Pomc* module. By performing chromosome walking upstream of zebrafish *POMCa* we identified a 1.2 kb fragment, non-homologous to the mammalian *Pomc* enhancers, similarly capable of targeting EGFP expression to *POMCa* hypothalamic neurons[80]. Within this fragment we found two short motifs containing TAAT sequences that showed 85% identity with a 50 bp critical fragment of nPE2. Strikingly, transgenic zebrafish embryos bearing substitution mutations in the TAAT motifs failed to express EGFP in the brain, indicating that they play a critical role in *POMCa* neuronal expression controlled by the intact 1.2 kb region[80]. The TAAT motifs present in zfnPE are within an annotated hAT Charlie DNA transposon, a family of DNA transposons that is highly abundant in the zebrafish genome. Because the *POMCa* loci of medaka, stickleback, Tetraodon and Fugu are devoid of sequences derived from a hAT Charlie transposon we hypothesized that insertion of this mobile DNA element upstream of zebrafish *POMCa* was a relatively recent event. The completion of additional genome projects will allow to determine whether exaptation of zfnPE from a hAT Charlie DNA transposon occurred only in zebrafish, in Cypriniformes or in the lineage leading to Ostariophysi.

Altogether, this type of comparative genomics sheds light on the molecular evolution of gene expression regulation by showing how enhancers undergo extensive turnover while maintaining the ancestral transcriptional features.

Physiological importance of hypothalamic POMC function in the control of food intake and obesity

Although pituitary POMC is a pan-vertebrate feature, hypothalamic *Pomc* expression is an evolutionary novelty originated in the lineage leading to jawed vertebrates. The functional independence of two different territories and cell types expressing the same *Pomc* gene is supported by the modular architecture of the transcriptional cis-acting domains. Whereas pituitary expression uses the proximal *Pomc* promoter and a -7 kb enhancer, the hypothalamic neurons rely on the distal enhancer module carrying nPE1 and nPE2[39, 55]. To further investigate the functional relevance of this distal module, we generated a strain of mutant mice lacking the ability to express *Pomc* selectively in the arcuate nucleus[81]. The targeted insertion of a heterologous cassette into the neural enhancer domain of the *Pomc* locus prevented hypothalamic *Pomc* expression but had no effect on expression levels in pituitary cells[81]. In mice lacking arcuate *Pomc* expression (Arc*Pomc*KO), hypothalamic POMC neurons develop normally and project to the typical target areas, indicating that POMC-derived peptides are not critical for normal development and maintenance of the neuronal circuitry per se. Arc*Pomc*KO mice were hyperphagic and developed early-onset extreme obesity when consuming a standard low-fat chow, in contrast to high-fat or high-sucrose diet-induced obesity models that produce changes in dopaminergic reward centers[81]. Arc*Pomc*KO mutants also exhibited hyperinsulinemia, hyperleptinemia, hepatic steatosis and decreased locomotor activity. In contrast, heterozygous arc*Pomc*$^{+/-}$ mice expressing only 50% of hypothalamic *Pomc* mRNA levels showed normal body weight and daily food intake, and also all other measured parameters, including leptin and insulin levels, were normal [81].

The genetic cassette inserted in the *Pomc* locus that led to the generation of arc*Pomc*KO mice was flanked by two loxP sites. The use of this type of genetic switch allows the reactivation of hypothalamic *Pomc* expression using a temporally controlled cre recombinase and allowed us to answer a fundamental question: is established obesity reversible? This question is particularly timely given the alarming prevalence of obesity in young and adult people, most of which fail to achieve a normal weight after dieting, probably due to compensatory reductions in metabolic rate[82-84]. Diet-induced obesity in rodents also showed to permanently tune-up the body weight set point once animals return to normal food consumption[85-87]. By using a tamoxifen activable cre transgene, we rescued hypothalamic *Pomc* expression in mice carrying different levels of overweight and found a remarkable improvement in food

intake, body weight and fat deposits, including cases of extreme obesity. However, we also observed that when overweight levels at the time of genetic rescue were higher, the ability of mice to regain a normal body weight was progressively reduced. To evaluate whether obesity itself was directly involved in the resistance to regain normal body weight after *Pomc* restoration in older mice, we food restricted arc*Pomc*KO mice from weaning until age P60. After hypothalamic *Pomc* rescue the mice were given free access to chow. Interestingly, we found that food-restricted normoweight arc*Pomc*KO mice completely maintained their normal body weight once *Pomc* expression was rescued even when eating *ad libitum* [81]. In contrast, non-tamoxifen rescued arc*Pomc*KO mice acquired hyperphagic state that promoted an accelerated weight gain similar to that of naive arc*Pomc*KO mice. These results indicated that the historical body weight at the time of arcuate *Pomc* rescue, and not the age of the mice, determines the body weight set point and, therefore, the food intake level necessary to achieve those values [81]. This finding is in agreement with clinical [82,84] and rodent data[85-87] obtained during high-fat diet–induced obesity in which chronic overweight has led to secondary metabolic adaptations that act to maintain obesity despite caloric intake reductions. Because in these experiments we controlled for genetic and environmental factors including standard chow of low hedonic value, our results indicate that the onset of obesity may cause a permanent change in the body weight set point by promoting a maladaptive allostatic state that will defend a greater than necessary body weight.

What condition prevents obese arc*Pomc*KO mice to recover a normal body weight even when eating normal chow? Is it possible to reprogram their body weight set point? To address these questions, we exposed obese adult arc*Pomc*KO mice weighing almost 60 g to a calorie restricted diet for 8-12 weeks, a period sufficient for all the mice to reach normal body weight (~28 g). At that point we activated the Cre-inducible genetic switch that rescues hypothalamic *Pomc* expression and found that the mice had reprogrammed their body weight set point to normal values and were able to sustain a normal body weight for a prolonged time eating regular chow *ad libitum*[88]. The readjustment of body weight set point was prevented by long-lasting PASylated leptin given days before and during the hypothalamic rescue by tamoxifen treatment, indicating that leptin resistance is a key factor preventing normalization of the body weight set point[88]. These results led us to the conclusion that restoration of hypothalamic leptin sensitivity is a necessary condition, together with normal *Pomc* expression, for obese mice to achieve and sustain normal metabolic homeostasis; whereas deficits in either parameter set a maladaptive allostatic balance that defends increased adiposity and body weight[88].

Different to autonomic and endocrine homeostatic mechanisms that work to maintain a physiological variable close to a fixed set point, body weight and energy storage follow an allostatic regulation that includes voluntary actions like foraging and food intake which may be activated in anticipation of future caloric needs. The allostatic control of body growth starts during early fetal development and continues postnatally until sexual maturation is achieved, and only beyond this point body weight is maintained as a relatively fixed parameter[89]. Exceptional periods occur during particular physiological conditions such as pregnancy, lactation, or hibernation in which allostatic mechanisms regain the scene to induce short-term adaptive hyperphagia that promotes fat accumulation and body weight gain. However, these parameters revert to normal once environmental conditions become less demanding. Unlike allostatic hyperphagia triggered by pregnancy or hibernation to induce "viability through change" [90], when chronic overfeeding followed by obesity is detached from physiological or environmental needs, it generates a state that is maladaptive to the health of an organism.

In conclusion, during the last 25 years we have generated extensive genetic, evolutionary and functional evidence that has contributed to a better understanding of the role of hypothalamic *Pomc* in the control of food intake and body weight.

Acknowledgments

References

1 Zhang Y, Proenca R, Maffei M, Barone M, Leopold L & Friedman JM (1994) Positional cloning of the mouse obese gene and its human homologue. *Nature* **372**, 425–432.

2 Schwartz MW, Woods SC, Porte D, Seeley RJ & Baskin DG (2000) Central nervous system control of food intake. *Nature* **404**, 661–71.

3 Seeley RJ & Berridge KC (2015) The hunger Games. *Cell* **160**, 805–806.

4 Fan W, Boston BA, Kesterson RA, Hruby VJ & Cone RD (1997) Role of melanocortinergic neurons in

feeding and the agouti obesity syndrome. *Nature* **385**, 165–168.

5 Aponte Y, Atasoy D & Sternson SM (2011) AGRP neurons are sufficient to orchestrate feeding behavior rapidly and without training. *Nat. Neurosci.* **14**, 351–355.

6 Chen Y, Lin Y-C, Kuo T-W & Knight Z (2015) Sensory Detection of Food Rapidly Modulates Arcuate Feeding Circuits. *Cell* **160**, 829–841.

7 James WPT (2008) WHO recognition of the global obesity epidemic. *Int. J. Obes.* **32**, S120–S126.

8 Maes HH, Neale MC & Eaves LJ (1997) Genetic and environmental factors in relative body weight and human adiposity. *Behav. Genet.* **27**, 325–51.

9 Stunkard AJ, Harris JR, Pedersen NL & McClearn GE (1990) The body-mass index of twins who have been reared apart. *N. Engl. J. Med.* **322**, 1483–7.

10 Barsh GS, Farooqi IS & O'Rahilly S (2000) Genetics of body-weight regulation. *Nature* **404**, 644–51.

11 Silventoinen K, Kaprio J & Lahelma E (2000) Genetic and environmental contributions to the association between body height and educational attainment: a study of adult Finnish twins. *Behav. Genet.* **30**, 477–85.

12 Carmichael CM & McGue M (1995) A cross-sectional examination of height, weight, and body mass index in adult twins. *J. Gerontol. A. Biol. Sci. Med. Sci.* **50**, B237–44.

13 O'Rahilly S, Montague CT, Farooqi IS, Whitehead JP, Soos MA, Rau H, Wareham NJ, Sewter CP, Digby JE, Mohammed SN, Hurst JA, Cheetham CH, Earley AR, Barnett AH & Prins JB (1997) Congenital leptin deficiency is associated with severe early-onset obesity in humans. *Nature* **387**, 903–908.

14 Froguel P, Clément K, Vaisse C, Lahlou N, Cabrol S, Pelloux V, Cassuto D, Gourmelen M, Dina C, Chambaz J, Lacorte J-M, Basdevant A, Bougnéres P, Lebouc Y & Guy-Grand B (1998) A mutation in the human leptin receptor gene causes obesity and pituitary dysfunction. *Nature* **392**, 398–401.

15 Farooqi IS, Matarese G, Lord GM, Keogh JM, Lawrence E, Agwu C, Sanna V, Jebb SA, Perna F, Fontana S, Lechler RI, DePaoli AM & O'Rahilly S (2002) Beneficial effects of leptin on obesity, T cell hyporesponsiveness, and neuroendocrine/metabolic dysfunction of human congenital leptin deficiency. *J. Clin. Invest.* **110**, 1093–103.

16 Quarta C, Sánchez-Garrido MA, Tschöp MH & Clemmensen C (2016) Renaissance of leptin for obesity therapy. *Diabetologia* **59**, 920–927.

17 Krude H, Biebermann H, Luck W, Horn R, Brabant G & Grüters A (1998) Severe early-onset obesity, adrenal insufficiency and red hair pigmentation caused by POMC mutations in humans. *Nat. Genet.* **19**, 155–7.

18 Coll AP, Farooqi IS & O'Rahilly S (2007) The Hormonal Control of Food Intake. *Cell* **129**, 251–262.

19 Kühnen P, Clément K, Wiegand S, Blankenstein O, Gottesdiener K, Martini LL, Mai K, Blume-Peytavi U, Grüters A & Krude H (2016) Proopiomelanocortin Deficiency Treated with a Melanocortin-4 Receptor Agonist. *N. Engl. J. Med.* **375**, 240–246.

20 Wheeler E, Huang N, Bochukova EG, Keogh JM, Lindsay S, Garg S, Henning E, Blackburn H, Loos RJF, Wareham NJ, O'Rahilly S, Hurles ME, Barroso I & Farooqi IS (2013) Genome-wide SNP and CNV analysis identifies common and low-frequency variants associated with severe early-onset obesity. *Nat. Genet.* **45**, 513–517.

21 Thorleifsson G, Walters GB, Gudbjartsson DF, Steinthorsdottir V, Sulem P, Helgadottir A, Styrkarsdottir U, Gretarsdottir S, Thorlacius S, Jonsdottir I, Jonsdottir T, Olafsdottir EJ, Olafsdottir GH, Jonsson T, Jonsson F, Borch-Johnsen K, Hansen T, Andersen G, Jorgensen T, Lauritzen T, Aben KK, Verbeek AL, Roeleveld N, Kampman E, Yanek LR, Becker LC, Tryggvadottir L, Rafnar T, Becker DM, Gulcher J, Kiemeney LA, Pedersen O, Kong A, Thorsteinsdottir U & Stefansson K (2009) Genome-wide association yields new sequence variants at seven loci that associate with measures of obesity. *Nat. Genet.* **41**, 18–24.

22 Gaulton Kj, et al. (2015) Genetic fine mapping and genomic annotation defines causal mechanisms at type 2 diabetes susceptibility loci. *Nat. Genet.* **47**, 1415–1425.

23 Scuteri A, Sanna S, Chen W-M, Uda M, Albai G, Strait J, Najjar S, Nagaraja R, Orrú M, Usala G, Dei M, Lai S, Maschio A, Busonero F, Mulas A, Ehret GB, Fink AA, Weder AB, Cooper RS, Galan P, Chakravarti A, Schlessinger D, Cao A, Lakatta E & Abecasis GR (2007) Genome-Wide Association Scan Shows Genetic Variants in the FTO Gene Are Associated with Obesity-Related Traits. *PLoS Genet.* **3**, e115.

24 Loos RJF & Bouchard C (2008) FTO: the first gene contributing to common forms of human obesity. *Obes. Rev.* **9**, 246–250.

25 Comuzzie AG, Hixson JE, Almasy L, Mitchell BD, Mahaney MC, Dyer TD, Stern MP, MacCluer JW & Blangero J (1997) A major quantitative trait locus determining serum leptin levels and fat mass is located on human chromosome 2. *Nat. Genet.* **15**, 273–276.

26 Rotimi CN, Comuzzie AG, Lowe WL, Luke A, Blangero J & Cooper RS (1999) The quantitative trait locus on chromosome 2 for serum leptin levels is confirmed in African-Americans. *Diabetes* **48**, 643–4.

27 Delplanque J, Barat-Houari M, Dina C, Gallina P, Clément K, Guy-Grand B, Vasseur F, Boutin P & Froguel P (2000) Linkage and association studies between the proopiomelanocortin (POMC) gene and obesity in caucasian families. *Diabetologia* **43**, 1554–7.

28 Hixson JE, Almasy L, Cole S, Birnbaum S, Mitchell BD, Mahaney MC, Stern MP, MacCluer JW, Blangero

J & Comuzzie AG (1999) Normal Variation in Leptin Levels Is Associated with Polymorphisms in the Proopiomelanocortin Gene, *POMC* [1]. *J. Clin. Endocrinol. Metab.* **84**, 3187–3191.

29 Raffin-Sanson ML, de Keyzer Y & Bertagna X (2003) Proopiomelanocortin, a polypeptide precursor with multiple functions: from physiology to pathological conditions. *Eur. J. Endocrinol.* **149**, 79–90.

30 Rubinstein M, Mogil JS, Japón M, Chan EC, Allen RG & Low MJ (1996) Absence of opioid stress-induced analgesia in mice lacking beta-endorphin by site-directed mutagenesis. *Proc. Natl. Acad. Sci. U. S. A.* **93**, 3995–4000.

31 Newell-Price J (2003) Proopiomelanocortin gene expression and DNA methylation: implications for Cushing's syndrome and beyond. *J. Endocrinol.* **177**, 365–72.

32 Liu B, Hammer GD, Rubinstein M, Mortrud M & Low MJ (1992) Identification of DNA elements cooperatively activating proopiomelanocortin gene expression in the pituitary glands of transgenic mice. *Mol. Cell. Biol.* **12**, 3978–90.

33 Liu B, Mortrud M & Low MJ (1995) DNA elements with AT-rich core sequences direct pituitary cell-specific expression of the pro-opiomelanocortin gene in transgenic mice. *Biochem. J.* **312 (Pt 3)**, 827–32.

34 Lamolet B, Pulichino AM, Lamonerie T, Gauthier Y, Brue T, Enjalbert A & Drouin J (2001) A pituitary cell-restricted T box factor, Tpit, activates POMC transcription in cooperation with Pitx homeoproteins. *Cell* **104**, 849–59.

35 Philips A, Maira M, Mullick A, Chamberland M, Lesage S, Hugo P & Drouin J (1997) Antagonism between Nur77 and glucocorticoid receptor for control of transcription. *Mol. Cell. Biol.* **17**, 5952–9.

36 Poulin G, Turgeon B & Drouin J (1997) NeuroD1/beta2 contributes to cell-specific transcription of the proopiomelanocortin gene. *Mol. Cell. Biol.* **17**, 6673–82.

37 Pulichino A-M, Vallette-Kasic S, Tsai JP-Y, Couture C, Gauthier Y & Drouin J (2003) Tpit determines alternate fates during pituitary cell differentiation. *Genes Dev.* **17**, 738–47.

38 Tremblay JJ, Lanctôt C & Drouin J (1998) The pan-pituitary activator of transcription, Ptx1 (pituitary homeobox 1), acts in synergy with SF-1 and Pit1 and is an upstream regulator of the Lim-homeodomain gene Lim3/Lhx3. *Mol. Endocrinol.* **12**, 428–41.

39 Langlais D, Couture C, Sylvain-Drolet G & Drouin J (2011) A Pituitary-Specific Enhancer of the POMC Gene with Preferential Activity in Corticotrope Cells. *Mol. Endocrinol.* **25**, 348–359.

40 Budry L, Balsalobre A, Gauthier Y, Khetchoumian K, L'Honore A, Vallette S, Brue T, Figarella-Branger D, Meij B & Drouin J (2012) The selector gene Pax7 dictates alternate pituitary cell fates through its pioneer action on chromatin remodeling. *Genes Dev.* **26**, 2299–2310.

41 Drouin J (2016) 60 YEARS OF POMC: Transcriptional and epigenetic regulation of *POMC* gene expression. *J. Mol. Endocrinol.* **56**, T99–T112.

42 Hammer GD, Fairchild-Huntress V & Low MJ (1990) Pituitary-Specific and Hormonally Regulated Gene Expression Directed by the Rat Proopiomelanocortin Promoter in Transgenic Mice. *Mol. Endocrinol.* **4**, 1689–1697.

43 Rubinstein M, Mortrud M, Liu B & Low MJ (1993) Rat and mouse proopiomelanocortin gene sequences target tissue-specific expression to the pituitary gland but not to the hypothalamus of transgenic mice. *Neuroendocrinology* **58**, 373–80.

44 Young JI, Otero V, Cerdán MG, Falzone TL, Chan EC, Low MJ & Rubinstein M (1998) Authentic cell-specific and developmentally regulated expression of pro-opiomelanocortin genomic fragments in hypothalamic and hindbrain neurons of transgenic mice. *J. Neurosci.* **18**, 6631–40.

45 Cowley MA, Smart JL, Rubinstein M, Cerdán MG, Diano S, Horvath TL, Cone RD & Low MJ (2001) Leptin activates anorexigenic POMC neurons through a neural network in the arcuate nucleus. *Nature* **411**, 480–4.

46 Bjørbaek C & Kahn BB (2004) Leptin signaling in the central nervous system and the periphery. *Recent Prog. Horm. Res.* **59**, 305–31.

47 Heisler LK, Cowley MA, Tecott LH, Fan W, Low MJ, Smart JL, Rubinstein M, Tatro JB, Marcus JN, Holstege H, Lee CE, Cone RD & Elmquist JK (2002) Activation of Central Melanocortin Pathways by Fenfluramine. *Science (80-.).* **297**, 609–611.

48 Batterham RL, Cowley MA, Small CJ, Herzog H, Cohen MA, Dakin CL, Wren AM, Brynes AE, Low MJ, Ghatei MA, Cone RD & Bloom SR (2004) Physiology: Does gut hormone PYY3–36 decrease food intake in rodents? (reply). *Nature* **430**, 650–4.

49 Lam DD, Attard CA, Mercer AJ, Myers MG, Rubinstein M & Low MJ (2015) Conditional expression of Pomc in the Lepr-positive subpopulation of POMC neurons is sufficient for normal energy homeostasis and metabolism. *Endocrinology* **156**, 1292–302.

50 Cone RD (2005) Anatomy and regulation of the central melanocortin system. *Nat. Neurosci.* **8**, 571–578.

51 Jarvie BC & Hentges ST (2012) Expression of GABAergic and glutamatergic phenotypic markers in hypothalamic proopiomelanocortin neurons. *J. Comp. Neurol.* **520**, 3863–3876.

52 Heisler LK, Cowley MA, Tecott LH, Fan W, Low MJ, Smart JL, Rubinstein M, Tatro JB, Marcus JN, Holstege H, Lee CE, Cone RD & Elmquist JK (2002) Activation of Central Melanocortin Pathways by Fenfluramine. *Science (80-.).* **297**, 609–611.

53 Williams KW, Margatho LO, Lee CE, Choi M, Lee S, Scott MM, Elias CF & Elmquist JK (2010) Segregation of acute leptin and insulin effects in distinct populations of arcuate proopiomelanocortin neurons. *J. Neurosci.* **30**, 2472–9.

54 Lam BYH, Cimino I, Polex-Wolf J, Nicole Kohnke S, Rimmington D, Iyemere V, Heeley N, Cossetti C, Schulte R, Saraiva LR, Logan DW, Blouet C, O'Rahilly S, Coll AP & Yeo GSH (2017) Heterogeneity of hypothalamic pro-opiomelanocortin-expressing neurons revealed by single-cell RNA sequencing. *Mol. Metab.* **6**, 383–392.

55 de Souza FSJ, Santangelo AM, Bumaschny V, Avale ME, Smart JL, Low MJ & Rubinstein M (2005) Identification of Neuronal Enhancers of the Proopiomelanocortin Gene by Transgenic Mouse Analysis and Phylogenetic Footprinting. *Mol. Cell. Biol.* **25**, 3076–3086.

56 de Souza FSJ, Bumaschny VF, Low MJ & Rubinstein M (2005) Subfunctionalization of expression and peptide domains following the ancient duplication of the proopiomelanocortin gene in teleost fishes. *Mol. Biol. Evol.* **22**, 2417–2427.

57 Santangelo AM, De Souza FSJ, Franchini LF, Bumaschny VF, Low MJ & Rubinstein M (2007) Ancient exaptation of a CORE-SINE retroposon into a highly conserved mammalian neuronal enhancer of the proopiomelanocortin gene. *PLoS Genet.* **3**, 1813–1826.

58 Brosius J (2003) The contribution of RNAs and retroposition to evolutionary novelties. *Genetica* **118**, 99–116.

59 Kazazian HH (2004) Mobile Elements: Drivers of Genome Evolution. *Science (80-.).* **303**, 1626–1632.

60 Norris J, Fan D, Aleman C, Marks JR, Futreal PA, Wiseman RW, Iglehart JD, Deininger PL & McDonnell DP (1995) Identification of a new subclass of Alu DNA repeats which can function as estrogen receptor-dependent transcriptional enhancers. *J. Biol. Chem.* **270**, 22777–82.

61 Laperriere D, Wang T-T, White JH & Mader S (2007) Widespread Alu repeat-driven expansion of consensus DR2 retinoic acid response elements during primate evolution. *BMC Genomics* **8**, 23.

62 Babich V, Aksenov N, Alexeenko V, Oei SL, Buchlow G & Tomilin N (1999) Association of some potential hormone response elements in human genes with the Alu family repeats. *Gene* **239**, 341–9.

63 Bininda-Emonds ORP, Cardillo M, Jones KE, MacPhee RDE, Beck RMD, Grenyer R, Price SA, Vos RA, Gittleman JL & Purvis A (2007) The delayed rise of present-day mammals. *Nature* **446**, 507–512.

64 Smit AF (1993) Identification of a new, abundant superfamily of mammalian LTR-transposons. *Nucleic Acids Res.* **21**, 1863–72.

65 Franchini LF, Lopez-Leal R, Nasif S, Beati P, Gelman DM, Low MJ, de Souza FJS & Rubinstein M (2011)

Convergent evolution of two mammalian neuronal enhancers by sequential exaptation of unrelated retroposons. *Proc. Natl. Acad. Sci.* **108**, 15270–15275.

66 Japón MA, Rubinstein M & Low MJ (1994) In situ hybridization analysis of anterior pituitary hormone gene expression during fetal mouse development. *J. Histochem. Cytochem.* **42**, 1117–1125.

67 Padilla SL, Carmody JS & Zeltser LM (2010) Pomc-expressing progenitors give rise to antagonistic neuronal populations in hypothalamic feeding circuits. *Nat. Med.* **16**, 403–405.

68 Sanz E, Quintana A, Deem JD, Steiner RA, Palmiter RD & McKnight GS (2015) Fertility-Regulating Kiss1 Neurons Arise from Hypothalamic Pomc-Expressing Progenitors. *J. Neurosci.* **35**, 5549–5556.

69 Hong J-W, Hendrix DA & Levine MS (2008) Shadow Enhancers as a Source of Evolutionary Novelty. *Science* **321**, 1314–1314.

70 Perry MW, Boettiger AN, Bothma JP & Levine M (2010) Shadow enhancers foster robustness of Drosophila gastrulation. *Curr. Biol.* **20**, 1562–7.

71 Frankel N, Davis GK, Vargas D, Wang S, Payre F & Stern DL (2010) Phenotypic robustness conferred by apparently redundant transcriptional enhancers. *Nature* **466**, 490–3.

72 Cannavò E, Khoueiry P, Garfield DA, Geeleher P, Zichner T, Gustafson EH, Ciglar L, Korbel JO & Furlong EEM (2016) Shadow Enhancers Are Pervasive Features of Developmental Regulatory Networks. *Curr. Biol.* **26**, 38–51.

73 Lam DD, de Souza FSJ, Nasif S, Yamashita M, López Leal R, Otero-Corchon V, Meece K, Sampath H, Mercer AJ, Wardlaw SL, Rubinstein M LM (2014) Partially Redundant Enhancers Cooperatively Maintain Mammalian Pomc Expression Above a Critical Functional Threshold. *PLoS Genet.* 11(4):e1005133.

74 Berger MF, Badis G, Gehrke AR, Talukder S, Philippakis AA, Peña-Castillo L, Alleyne TM, Mnaimneh S, Botvinnik OB, Chan ET, Khalid F, Zhang W, Newburger D, Jaeger SA, Morris QD, Bulyk ML & Hughes TR (2008) Variation in homeodomain DNA binding revealed by high-resolution analysis of sequence preferences. *Cell* **133**, 1266–76.

75 Thompson CL, Ng L, Menon V, Martinez S, Lee C-K, Glattfelder K, Sunkin SM, Henry A, Lau C, Dang C, Garcia-Lopez R, Martinez-Ferre A, Pombero A, Rubenstein JLR, Wakeman WB, Hohmann J, Dee N, Sodt AJ, Young R, Smith K, Nguyen T-N, Kidney J, Kuan L, Jeromin A, Kaykas A, Miller J, Page D, Orta G, Bernard A, Riley Z, Smith S, Wohnoutka P, Hawrylycz MJ, Puelles L & Jones AR (2014) A High-Resolution Spatiotemporal Atlas of Gene Expression of the Developing Mouse Brain. *Neuron* **83**, 309–323.

76 Nasif S, de Souza FSJ, González LE, Yamashita M, Orquera DP, Low MJ & Rubinstein M (2015) Islet 1

specifies the identity of hypothalamic melanocortin neurons and is critical for normal food intake and adiposity in adulthood. *Proc. Natl. Acad. Sci.* **112**, E1861–E1870.

77 Pearson CA & Placzek M (2013) Development of the medial hypothalamus: forming a functional hypothalamic-neurohypophyseal interface. *Curr. Top. Dev. Biol.* **106**, 49–88.

78 Lee B, Lee S, Lee S-K & Lee JW (2016) The LIM-homeobox transcription factor Isl1 plays crucial roles in the development of multiple arcuate nucleus neurons. *Development* **143**, 3763–3773.

79 de Souza FSJ, Nasif S, López-Leal R, Levi DH, Low MJ & Rubinstein M (2011) The estrogen receptorα colocalizes with proopiomelanocortin in hypothalamic neurons and binds to a conserved motif present in the neuron-specific enhancer nPE2. *Eur. J. Pharmacol.* **660**, 181–187.

80 Domené S, Bumaschny VF, de Souza FSJ, Franchini LF, Nasif S, Low MJ & Rubinstein M (2013) Enhancer turnover and conserved regulatory function in vertebrate evolution. *Philos. Trans. R. Soc. Lond. B. Biol. Sci.* **368**, 20130027.

81 Bumaschny VF, Yamashita M, Casas-Cordero R, Otero-Corchón V, de Souza FSJ, Rubinstein M & Low MJ (2012) Obesity-programmed mice are rescued by early genetic intervention. *J. Clin. Invest.* **122**, 4203–4212.

82 Rosenbaum M, Goldsmith R, Bloomfield D, Magnano A, Weimer L, Heymsfield S, Gallagher D, Mayer L, Murphy E & Leibel RL (2005) Low-dose leptin reverses skeletal muscle, autonomic, and neuroendocrine adaptations to maintenance of reduced weight. *J. Clin. Invest.* **115**, 3579–86.

83 Bray GA & Greenway FL (2007) Pharmacological Treatment of the Overweight Patient. *Pharmacol. Rev.* **59**, 151–184.

84 Tremblay A & Chaput J-P (2009) Adaptive reduction in thermogenesis and resistance to lose fat in obese men. *Br. J. Nutr.* **102**, 488–92.

85 Rolls BJ, Rowe EA & Turner RC (1980) Persistent obesity in rats following a period of consumption of a mixed, high energy diet. *J. Physiol.* **298**, 415–27.

86 Levin BE & Dunn-Meynell AA (2000) Defense of body weight against chronic caloric restriction in obesity-prone and -resistant rats. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **278**, R231–7.

87 Guo J, Jou W, Gavrilova O & Hall KD (2009) Persistent diet-induced obesity in male C57BL/6 mice resulting from temporary obesigenic diets. *PLoS One* **4**, e5370.

88 Chhabra KH, Adams JM, Jones GL, Yamashita M, Schlapschy M, Skerra A, Rubinstein M & Low MJ (2016) Reprogramming the body weight set point by a reciprocal interaction of hypothalamic leptin sensitivity and Pomc gene expression reverts extreme obesity. *Mol. Metab.* **5**, 869–881.

89 Leibel RL (2008) Molecular physiology of weight regulation in mice and humans. *Int. J. Obes.* **32**, S98–

S108.

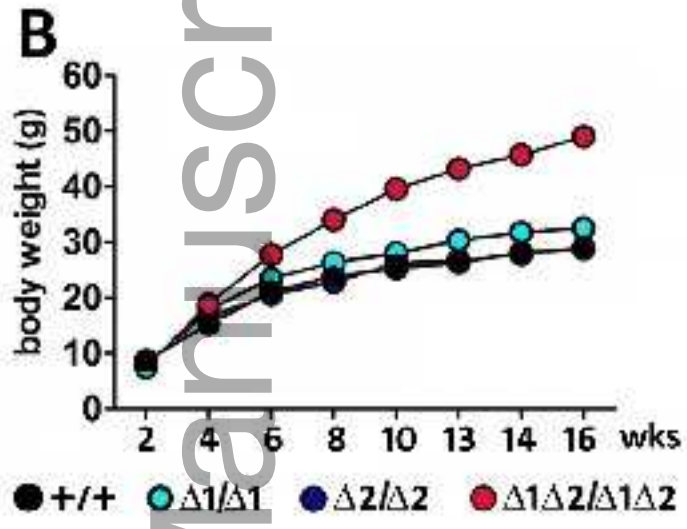90 Schulkin J (2003) Allostasis: a neural behavioral perspective. *Horm. Behav.* **43**, 21–7; discussion 28–30.
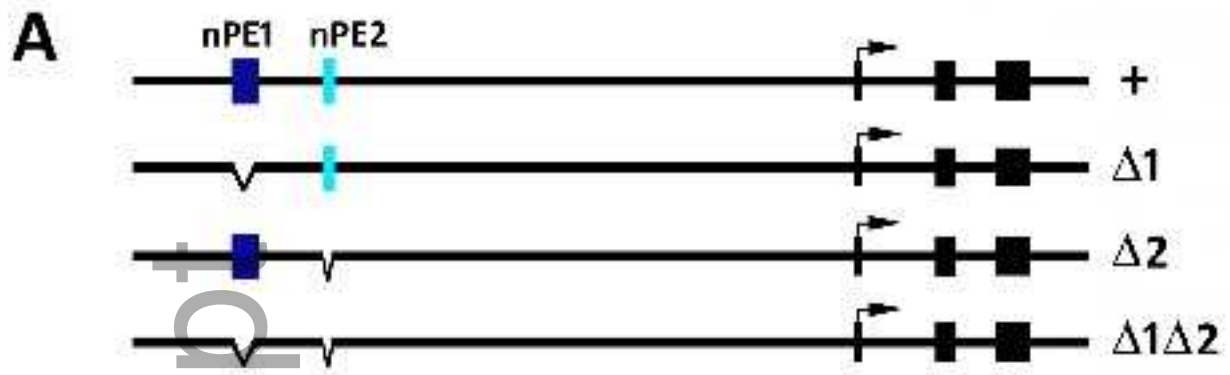
Figure Legends

Figure 1. (A) Schematic of *Pomc* gene expression and peptide profiles in the rodent brain and pituitary. Pituitary expression (Pit, green color) is driven by the proximal promoter (mainly to intermediate lobe melanotrophs) and the -7 kb enhancer (mainly to anterior lobe corticotrophs). *Pomc* expression in the arcuate nucleus of the hypothalamus (Arc, blue color) is controlled by the distal neuronal enhancers nPE1 and nPE2 that were exapted from a Mammalian-apparent LTR and a CORE-SINE retroposon, respectively, at different time points along the lineage leading to mammals. *Pomc* exons are in black boxes. Coding sequences for the POMC prohormone and the peptides obtained after endoproteolytic cleavage are indicated in colored boxes. The asterisk next to β-MSH denotes that this peptide is not released in mice. (B) Schematic of the transgenes nPE1Pomc–tomato and nPE2Pomc-EGFP described in Ref. 75. Expression of tomato (left), EGFP (center), and a merged photograph (right) in the arcuate nucleus of an adult compound transgenic mouse showing extensive colocalization of both reporter fluorescent proteins.

Figure 2. (A) Schematic of the mouse *Pomc* and nPE mutant *Pomc* alleles as shown in Ref. 73. (B) Body weight of wild-type, and homozygous mutants for nPE1, nPE2 and for both enhancers along the first 16 weeks. nPE1 mutants are mildly overweight and nPE1/nPE2 mutants are obese[73]. (C) Comparison of a wild-type and a homozygous adult mutant male lacking both nPE1 and nPE2[73].
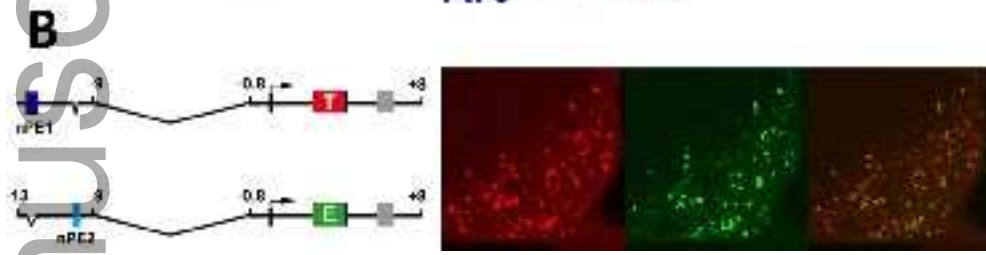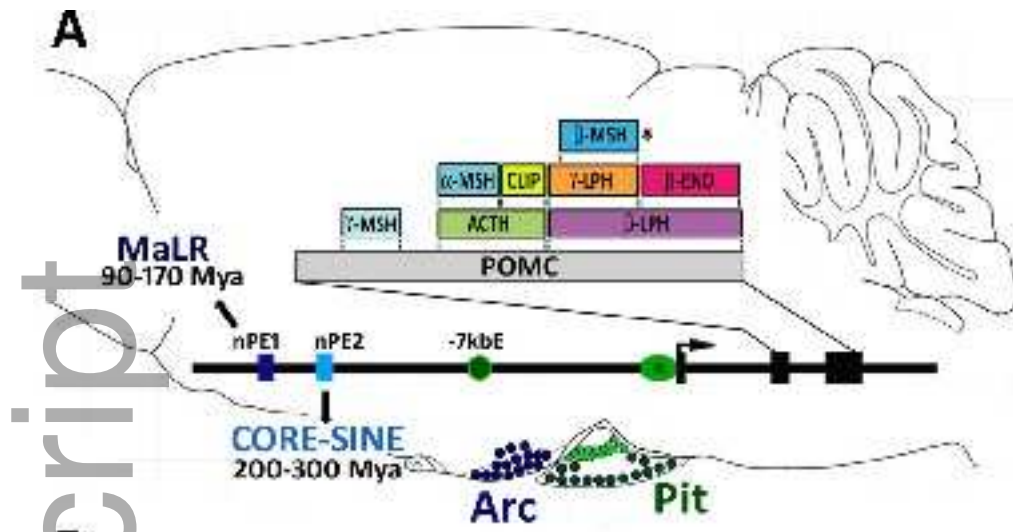
feb2_12776_f1.tif

feb2_12776_f2.tif