Islet 1 specifies the identity of hypothalamic melanocortin neurons and is critical for normal food intake and adiposity in adulthood

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Food intake and body weight regulation depend on proper expression of the proopiomelanocortin gene (*Pomc*) in a group of neurons located in the mediobasal hypothalamus of all vertebrates. These neurons release POMC-encoded melanocortins, which are potent anorexigenic neuropeptides, and their absence from mice or humans leads to hyperphagia and severe obesity. Although the pathophysiology of hypothalamic POMC neurons is well understood, the genetic program that establishes the neuronal melanocortinergic phenotype and maintains a fully functional neuronal POMC phenotype throughout adulthood remains unknown. Here, we report that the early expression of the LIM-homeodomain transcription factor Islet 1 (ISL1) in the developing hypothalamus promotes the terminal differentiation of melanocortinergic neurons and is essential for hypothalamic *Pomc* expression since its initial onset and throughout the entire lifetime. We detected ISL1 in the prospective hypothalamus just before the onset of *Pomc* expression and, from then on, *Pomc* and *Isl1* coexpress. ISL1 binds in vitro and in vivo to critical homeodomain binding DNA motifs present in the neuronal *Pomc* enhancers nPE1 and nPE2, and mutations of these sites completely disrupt the ability of these enhancers to drive reporter gene expression to hypothalamic POMC neurons in transgenic mice and zebrafish. ISL1 is necessary for hypothalamic *Pomc* expression during mouse and zebrafish embryogenesis. Furthermore, conditional *Isl1* inactivation from POMC neurons impairs *Pomc* expression, leading to hyperphagia and obesity. Our results demonstrate that ISL1 specifies the identity of hypothalamic melanocortin neurons and is required for melanocortin-induced satiety and normal adiposity throughout the entire lifespan.

hypothalamus | melanocortin | obesity | Isl1 | pomc

Regulation of body weight and energy balance in vertebrate animals is controlled by hypothalamic circuits that integrate environmental, peripheral, and central signals to promote food intake or satiety (1). In particular, a set of neurons located in the arcuate nucleus of the hypothalamus expresses *proopiomelanocortin* (*Pomc*), a gene that encodes the anorexigenic melanocortins α-, β- and γ-melanocyte-stimulating hormone (MSH). POMC neurons sense leptin, insulin and glucose levels, and receive multiple synaptic contacts which, together, orchestrate neuronal activity and determine the pattern of melanocortins release (2, 3). The physiological relevance of the central melanocortin system can be readily appreciated in hypothalamic *Pomc*-deficient mice, which are hyperphagic and display early onset severe obesity (4). In addition, humans carrying biallelic null *POMC* variants are also severely obese (5). Although much has been learned about the physiology of central melanocortinergic neurons and the pathological consequences of their malfunction, the genetic programs that determine the phenotypic identity of these neurons and maintain the neuronal POMC phenotype throughout the entire lifespan remain to be explored. A unique feature that specifies the neuronal melanocortinergic phenotype is, in fact, the expression of *Pomc* because other genes necessary for POMC processing and melanocortin release are common to most other neurons expressing neuropeptide brain genes. Cotransmitters such as glutamate, GABA, or cocaine- and amphetamine-regulated transcript (CART) are found only in fractions of hypothalamic POMC neurons (6, 7), as are leptin, insulin, estrogen, and serotonin receptors (8–10).

It has been shown, first in the invertebrate worm *Caenorhabditis elegans*, that the identity of a neuronal type is specified early in development by the expression of a specific transcription factor (TF) that promotes terminal differentiation of postmitotic neurons into their final functional phenotype (11). A TF acting on terminal specification binds to *cis*-acting regulatory motifs present in target genes that determine the neurotransmitter phenotype and may continue later in life maintaining the same postmitotic identity (11). A similar scenario has been found more recently in vertebrates, after the identification of the genetic programs that give birth and maintain serotonergic (12), dopaminergic (13), and noradrenergic neurons (14) in the mouse nervous system. Less is known, however, about the specification of neuronal types expressing neuropeptide genes.

Transcriptional regulation of *Pomc* in the hypothalamus is conveyed by a distal upstream regulatory *cis*-acting module that contains

Significance

Food intake and body weight regulation depend on a group of hypothalamic neurons that release satiety-induced neuropeptides known as melanocortins. Central melanocortins are encoded by the proopiomelanocortin gene (*Pomc*), and mice and humans carrying deleterious mutations in the *Pomc* gene display hyperphagia and severe obesity. Although the importance of these neurons is well understood, the genetic program that establishes hypothalamic melanocortin neurons and maintains normal *Pomc* expression levels remains unknown. Here, we combined molecular neuroanatomical and biochemical analyses with functional genetic studies in transgenic mice and zebrafish and discovered that the transcription factor Islet 1 determines the identity of central melanocortin neurons during early brain development and is critical for melanocortin-induced satiety and normal adiposity throughout the entire lifetime.

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two enhancers, named "neuronal *Pomc* enhancers" (nPE1 and nPE2), which are highly conserved in mammals (15). Expression studies in transgenic mice showed that nPE1 and nPE2 drive completely overlapping spatiotemporal enhancer activities to the ∼3,000 POMC neurons present in the mouse ventromedial hypothalamus during embryogenesis as well as in adulthood (16) and only the concurrent removal of both enhancers from the mouse *Pomc* locus by targeted mutagenesis reduced *Pomc* expression to very low levels, leading to hyperphagia and early-onset obesity (17). Interestingly, nPE1 and nPE2 are unrelated sequences with distinct evolutionary origins; whereas nPE2 is derived from the exaptation (cooption) of a CORE-short interspersed nuclear element (SINE) retroposon in the lineage leading to mammals more than 166 million years ago (Mya) (18), nPE1 is a placental mammal novelty derived from the exaptation of a LTR-containing a mammalian apparent LTR retroposon that occurred between 150 and 90 Mya (16).

We have recently identified conserved C/ATTA motifs that might be recognized by homeodomain-containing transcription factors (HDTFs) within nPE2 and nPE1, which are essential for driving reporter gene expression to POMC neurons (17). Because nPE1 and nPE2 are functional analogs, it is conceivable that these sequences recruit a common, yet unidentified TF able to transactivate *Pomc* in the hypothalamus. *Pomc* expression starts in the ventral lining of the developing mouse hypothalamus at embryonic day 10.5 (E10.5) (15–17), a precise spatiotemporal domain in which multiple HDTFs determine the early patterning of the mouse ventral hypothalamus (19, 20). Therefore, we considered of interest to investigate whether the terminal differentiation of melanocortin-expressing neurons is regulated by a HDTF.

In this study we used a combination of bioinformatic, molecular, genetic, and evolutionary approaches that led to the identification of the LIM-homeodomain TF Islet 1 (ISL1) as a crucial regulator of the terminal specification of melanocortinergic neurons in the vertebrate hypothalamus by interacting with specific ISL1 binding motifs present in two neuronal enhancers of *Pomc*. Furthermore, our results demonstrate that *Isl1* expression is necessary to maintain elevated levels of hypothalamic *Pomc* mRNA in adult mice to assure normal food intake and body weight regulation.

Results

ISL1 Is a Candidate TF to Regulate Hypothalamic *Pomc* Expression. Previously, we found six putative HDTF binding sites in critical regions of nPE2 and three in the 144-bp core region of nPE1 (nPE1core) (16) that are necessary for transgene expression in the mouse hypothalamus (17) and conserved in most mammals (*SI Appendix*, Fig. S1). To identify HDTFs that might recognize these DNA elements, we took advantage of a comprehensive study that determined the preferential DNA binding motifs of mouse homeodomains (21). Based on their preferential binding, we assembled a preliminary list of candidate HDTFs that was further trimmed down by selecting those showing expression patterns compatible with that of *Pomc* in the developing and/or adult ventromedial hypothalamus by searching the mouse gene expression Allen Brain Atlas (22). One particular candidate, the LIM-homeodomain TF *Islet1* (ISL1), immediately stood out from the rest because its expression pattern in this brain region accompanies that of *Pomc* from its early onset and throughout the entire mouse life.

ISL1 Coexpresses with *Pomc* Since Its Early Onset at E10.5 to Adulthood. To compare the expression patterns of *Isl1* and *Pomc*, we performed a detailed double immunofluorescence analysis in the developing mouse brain using a rabbit polyclonal antibody raised against the *Pomc*-derived peptide adrenocorticotropic hormone (ACTH) and a mouse monoclonal antibody raised against ISL1. At E9.5 we did not detect ISL1 or ACTH immunolabeled cells in the developing hypothalamus, although ISL1 immunoreactive cells were evident in the hindbrain (Fig. 1*A*). At E10.0, a few ISL1$^+$ cells are identifiable in the future hypothalamus, together with a strong expression in the future Rathke's pouch and in the first pharyngeal arch, but no ACTH$^+$ neurons are evident yet. Coexpression of *Isl1* and *Pomc* was observed at E10.5 and E12.5, when every single ACTH$^+$ neuron showed ISL1 nuclear labeling (Fig. 1*A*, *Insets*), indicating that *Pomc* expression commences a few hours later than the expression onset of *Isl1* in the developing hypothalamus (Fig. 1*A*). ISL1$^+$ cells accumulate in the mantle zone of the developing hypothalamus where postmitotic neurons are located (Fig. 1*A*), suggesting that *Isl1* expression starts in early postmitotic neurons before terminal differentiation.

Analysis of *Isl1* and *Pomc* expression in the arcuate nucleus of adult mice was performed using brain sections of *Pomc*-EGFP transgenic mice (2) to overcome the high background signal that most mouse monoclonal antibodies yield at this age. Immunofluorescence using a rabbit anti-ISL1 antibody showed that ∼86% of adult *Pomc*-EGFP$^+$ neurons coexpress *Isl1* (Fig. 1*B*). The expression pattern of *Isl1* is much broader than that of *Pomc* in the adult hypothalamus, where only 20% of ISL1$^+$ hypothalamic neurons coexpress *Pomc*-EGFP (Fig. 1*B*).

ISL1 Specifically Binds in Vitro and in Vivo to DNA Elements Present in nPE1 and nPE2. We then examined the in vitro binding properties of ISL1 in electrophoretic mobility shift assays (EMSAs) using a bacterially expressed human ISL1 clone truncated in its LIM domains, because it has been shown that these domains inhibit in vitro DNA binding. We found that DNA probes encompassing the canonical TAAT HDTF-binding site present in nPE1core, nPE2 region 1, or nPE2 region 3 were shifted upon incubation with ΔLIM-ISL1 bacterial extracts (Fig. 2*A* and *SI Appendix*, Fig. S2). We then tested all possible individual ISL1 binding sites with probes carrying inactivating mutations for each HDBS and found

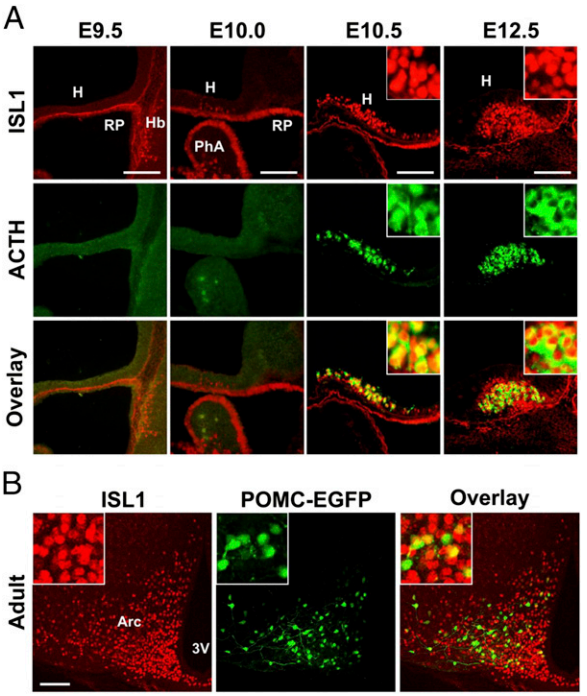

Fig. 1. *Isl1* is coexpressed with *Pomc* in the developing and adult mouse hypothalamus. (*A*) Immunofluorescence analysis of ISL1 and ACTH expression in sagittal cryosections at specified developmental stages. *Insets* are magnified views and show that all ACTH$^+$ cells express ISL1. H, future hypothalamus; RP, future Rathke's pouch; Hb, future hindbrain; PhA, first pharyngeal arch. (Scale bar, 100 μm.) (*B*) *Pomc* coexpresses with *Isl1* in adult mice. Anti-ISL1 immunofluorescence was performed on coronal brain sections of *Pomc*-EGFP transgenic mice. Arc, arcuate nucleus; 3V, third ventricle. (Scale bar, 100 μm.)

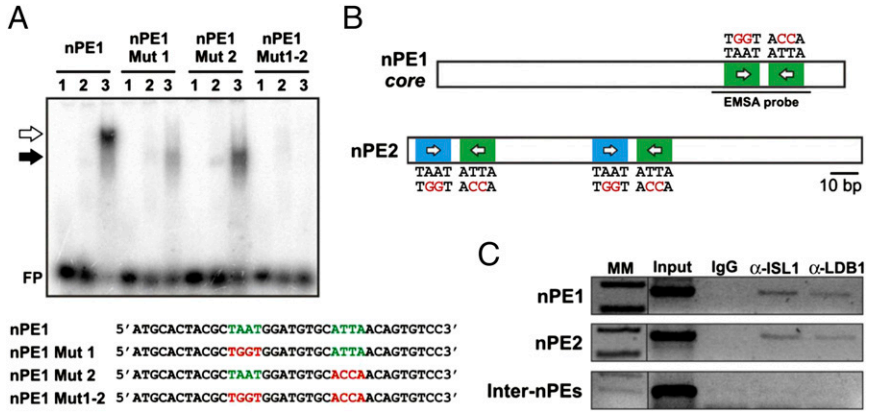

Fig. 2. ISL1 binds to nPE1 and nPE2 sequences in vitro and in vivo. (*A*) EMSA shows that ISL1 binds to both HD-TFBS present in nPE1core. Four EMSA probes were designed, one carrying the wild-type sequences of both HD-TFBS (shown in green), and three carrying either individual or simultaneous mutations of each HD-TFBS (shown in red). Three lanes are shown for each probe: 1, no protein; 2, control bacterial extract; and 3, bacterial extract expressing ΔLIM-ISL1. The white arrow indicates the high molecular weight complex formed by ΔLIM-ISL1 and the nPE1 probe, and the black arrow shows the lower molecular weight complexes formed when only one HD-TFBS remains intact. FP, free probe. A similar experiment with nPE2 is shown in *SI Appendix*, Fig. S2. (*B*) Schematic summary of the in vitro interactions between ISL1 and nPE1core and nPE2 sequences. TAAT sites are represented as boxes and those bound by ISL1 are shown in green. Mutated nucleotides are shown in red for each site, and the region represented by the EMSA probes used in *A* is depicted. (*C*) ChIP assay with adult mouse hypothalamus chromatin for testing in vivo binding of ISL1 and LDB1 to nPE1 and nPE2 sequences and a control region located between both nPEs in the mouse *Pomc* locus (InternPEs). MM, molecular marker.

that ΔLIM-ISL1 binds to both inverted TAAT motifs present in nPE1core (Fig. 2 *A* and *B*). Differently, ΔLIM-ISL1 binds to only one HDBS in region 1 and to another HDBS in region 3 of nPE2 (Fig. 2*B* and *SI Appendix*, Fig. S2).

The coexpression of *Pomc* and *Isl1* prompted us to test whether the interactions observed in vitro between ISL1 and nPE sequences in the EMSA studies are also found in vivo. To this end, we used a mixture of two different anti-ISL1 antibodies in a chromatin immunoprecipitation (ChIP) assay using sheared chromatin harvested from wild-type adult mouse hypothalami. The ISL1 antibodies pulled down nPE1 and nPE2 sequences, but not a region located between the enhancers (Fig. 2*C*). A control IgG antibody, in contrast, failed to immunoprecipitate either nPE (Fig. 2*C*). Interestingly, we observed that LDB1, a LIM-only containing protein often found to be a necessary cofactor of ISL1 in different regulatory contexts (23, 24), also binds in vivo to nPE1 and nPE2, but not to the region between them (Fig. 2*C*).

Functional Analysis of ISL1 Binding Sites in Transgenic Zebrafish and Mice. To determine whether the TAAT motifs recognized by ISL1 have functional implications, we took advantage of our recent finding that nPE1 as well as nPE2 can independently drive reporter gene expression specifically to hypothalamic POMC neurons in transgenic zebrafish embryos (Fig. 3*A*) (25). To this end, we built a series of transgenes carrying either wild-type or ISL1 BS mutant versions (Isl1Mut) of nPE1 or nPE2, upstream of the zebrafish *pomca* proximal promoter followed by EGFP coding sequences (Tg1–Tg4; Fig. 3*B*). The zebrafish *pomca* proximal promoter was used because it drives strong EGFP expression exclusively to the pituitary and allows rapid selection of live zebrafish embryos that integrated and expressed the transgenes 72 h postfertilization (hpf), as we have recently reported (25). Using this transient expression assay we found that 36 of 119 (30.2%) nPE1*pomca*-EGFP zebrafish embryos showing a bright green pituitary (Pit^+) also showed hypothalamic EGFP expression (Tg1; Fig. 3*B*). In contrast, only 10 of 120 (8.3%) Pit^+ transgenic zebrafish carrying nPE1(Isl1Mut)*pomca*-EGFP showed hypothalamic EGFP expression (Tg2; Fig. 3*B*), indicating that the four point mutations that disrupt the ISL1 BS present in nPE1 significantly reduced the enhancer's ability to drive reporter gene expression to hypothalamic neurons. Similarly, 112 of 161 (69.5%) Pit^+ nPE2*pomca*-EGFP transgenic embryos showed positive neuronal enhancer activity (Tg3; Fig. 3*B*), whereas hypothalamic EGFP expression significantly dropped to 41% (66 of 159) in nPE2(Isl1Mut)*pomca*-EGFP transgenic embryos, indicating that the two ISL1 binding sites present in nPE2 contribute to the transcriptional strength of this enhancer in the fish (Tg4; Fig. 3*B*).

To further test the importance of these ISL1 binding sites, we generated transgenic mice carrying either the wild-type or Isl1Mut versions of nPE1 or nPE2, upstream of the mouse proximal *Pomc* promoter driving EGFP expression. The mouse proximal promoter is included in these constructs because it has been shown to drive reporter gene expression exclusively to POMC pituitary cells and allowed us to discard transgenic pups carrying silent integrations (15, 16). We found that the transgenes nPE1*Pomc*-EGFP and nPE2*Pomc*-EGFP drove specific expression to POMC hypothalamic neurons, as previously reported (16), whereas the mutant versions nPE1(Isl1Mut)*Pomc*-EGFP and nPE2(Isl1Mut)*Pomc*-EGFP completely failed to express EGFP in hypothalamic neurons (Fig. 3 *C* and *D*) in three independent transgenic founder mice that showed EGFP expression in the pituitary gland. Altogether, our transgenic studies in zebrafish and mice indicate that nPE1 as well as nPE2 function depend on the ISL1 binding sites to fully drive reporter gene expression in hypothalamic neurons and suggest that ISL1 is involved in neuronal *Pomc* expression.

Early Hypothalamic *Pomc* Expression Depends on ISL1. We have shown above that the onset of *Isl1* expression in the prospective hypothalamus precedes that of *Pomc* by half a day, suggesting that ISL1 participates in the initial transactivation of *Pomc*. To examine this possibility, we sought to analyze *Pomc* expression in mice deficient in *Isl1*. Because $Isl1^{-/-}$ mouse embryos die at E9.5 due to impaired vascular development (26), we used $Isl1^{loxP/loxP}$ mice that carry two loxP sites flanking the critical exon 2 (27) in combination with mice ubiquitously expressing a tamoxifen (TAM)-inducible Cre recombinase (CAAG-CreERT) (28). Pregnant $Isl1^{loxP/loxP}$ dams previously crossed with $Isl1^{loxP/loxP}$.CAAG-CreERT male mice were injected at E9.5 with TAM to generate *Isl1*KO@E9.5 embryos that were analyzed 2 d later (Fig. 4*A*). Analysis of *Isl1*KO@E9.5 embryos at E11.5 showed a normal overt morphology and size and no evidence of tissue necrosis. Immunofluorescence performed on sagittal sections of these embryos showed absence of *Pomc* expression (Fig. 4*A*) in contrast to what was observed in control sibling embryos. Despite the lack of *Pomc* expression in *Isl1*KO@E9.5 embryos, the anatomy of the future hypothalamic and pituitary areas remained unaltered (Fig. 4*A*). Specifically, no changes were observed in the thickness of the hypothalamic neuroepithelium, the morphology of the infundibulum, Rathke's pouch, ventral mesenchyme, or other neighboring regions as found in other reported HDTF null mice, such as $Nkx2.1^{-/-}$ or $Rax^{-/-}$ (29, 30). This observation suggests that the lack of ISL1 does not alter the expression of genes involved in neurogenesis or general specification in the prospective hypothalamus. To further confirm this hypothesis, we followed the expression of *Neurogenin 3* (*Ngn3*) and *achaete-scute*

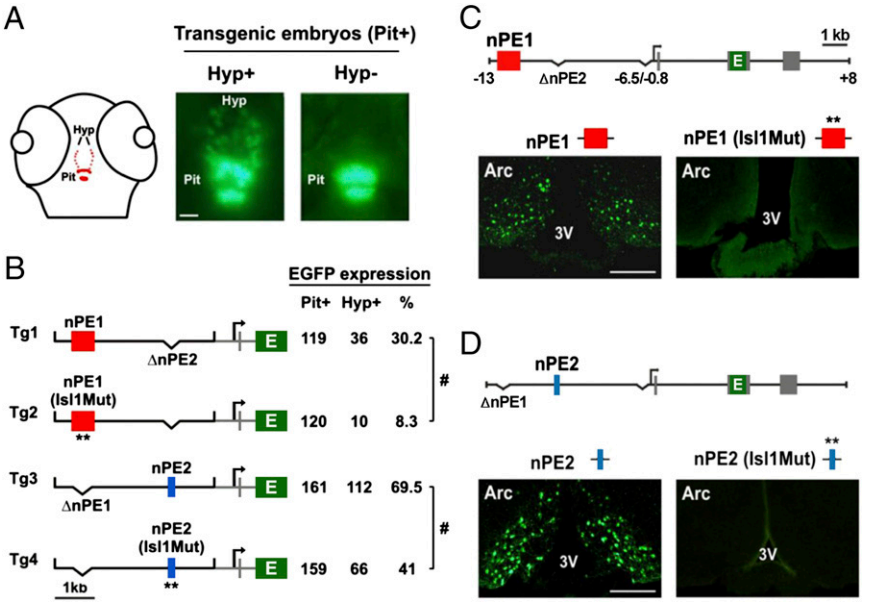

Fig. 3. ISL1 binding sites in nPE1 and nPE2 are important for enhancer function. (*A*) Schematic of a 72-hpf zebrafish head showing the distribution of *pomca*-expressing cells (red dots) in the pituitary (Pit) and hypothalamus (Hyp). For transient reporter gene expression analyses, transgenic embryos showing strong pituitary EGFP expression (Pit^+) at 72 hpf are selected and then sorted as expressing (Hyp^+) or not expressing (Hyp^-) EGFP in the hypothalamus. (Scale bar, 50 μm.) (*B*) Expression analyses of transgenes driving EGFP under the control of zebrafish proximal *pomca* sequences (from −0.6 to +0.4 kb, in gray) and a neuronal mouse *Pomc* distal module carrying either wild-type or Isl1Mut enhancers nPE1 (red box) or nPE2 (blue box) in transgenic zebrafish. Seventy-two hours postfertilization, transgenic embryos were selected based on pituitary EGFP expression (Pit^+), and the number of Pit^+ embryos also showing $EGFP^+$ neurons in the hypothalamus (Hyp^+) was quantified. $^{\#}P < 0.0001$, χ^2 test. (*C*) Expression analysis of transgenic mice carrying nPE1 or nPE1 (Isl1Mut) sequences driving EGFP on coronal sections at the hypothalamic arcuate (Arc) level. (*D*) Expression analysis of transgenic mice carrying nPE2 or nPE2 (Isl1Mut) sequences driving EGFP on coronal sections at the hypothalamic arcuate (Arc) level. 3V, third ventricle. (Scale bar, 500 μm.)

complex like 1 (*Ascl1*) as molecular markers of early neurogenesis; β-*tubulin III* (TUBIII), and *calbindin 28-K* (CALB) as markers of differentiated neurons and *Cleaved caspase 3* (CASP3) as a marker of cell apoptosis, in *Isl1*KO@E9.5 embryos collected at E11.5 (Fig. 4*A* and *SI Appendix*, Fig. S3). We found that expression of the proneuronal markers NGN3 and ASCL1 in the developing hypothalamus at E11.5 was indistinguishable between control and *Isl1*KO@E9.5 embryos, suggesting that the lack of ISL1 does not affect neurogenesis in this brain region. Likewise, we did not find significant differences in the expression of TUBIII or CALB between control and *Isl1*KO@E9.5 embryos, showing that lack of ISL1 since E9.5 does not affect general differentiation of hypothalamic neurons at E11.5. Regarding apoptotic activity, we detected an increase in the amount of $CASP3^+$ cells in *Isl1*KO@E9.5 embryos relative to controls (*SI Appendix*, Fig. S3). However, this increase is not specific to the hypothalamus because it was observed throughout the embryo, and even in mesenchymal tissue, probably a sign of a defective vascular system in *Isl1* mutant embryos (26). To further test whether ISL1 is involved in the development of other neuronal types, we studied the expression of *Orthopedia* (*Otp*), a HDTF expressed in a small group of developing hypothalamic neurons that coexpresses *Isl1* but not *Pomc* (*SI Appendix*, Fig. S4) in *Isl1*KO@E9.5 embryos. Consistent with our hypothesis, we found that *Otp* expression is absent in the hypothalamus of *Isl1*KO@E9.5 embryos but not in the hindbrain, where these two HDTFs do not coexpress (*SI Appendix*, Fig. S4). Altogether, this molecular marker analysis indicates that *Isl1* ablation from the early steps of embryonic development prevents the onset of *Pomc* expression without affecting the general development of the future hypothalamus.

ISL1 Regulates Hypothalamic *Pomc* Expression Across Different Ages. TFs that specify neuronal-subtype identities during embryonic development may continue to drive cell-specific gene expression and maintain postmitotic neuronal identities later in life (11). This may be the case for ISL1 acting on POMC neurons because *Isl1* and *Pomc* coexpress in the ventral hypothalamus across the mouse lifespan. To investigate whether *Pomc* expression depends on ISL1 at different ages, we treated $Isl1^{loxP/loxP}$.CAAG-CreERT mice with TAM at various time points and analyzed hypothalamic *Pomc* expression. We first gave TAM to pregnant dams carrying E11.5 embryos, a time point in which hypothalamic *Pomc* expression has already started and analyzed *Isl1*KO@11.5 embryos at E15.5. Although this TAM treatment produced a drastic, but incomplete, reduction in ISL1 levels, hypothalamic *Pomc* expression was not detected in *Isl1* knockdown E15.5 embryos (Fig. 4*B*). Interestingly, *Pomc* expression in the pituitary remained intact (Fig. 4*B*). Lack of hypothalamic *Pomc* expression does not seem to be a consequence of a drop in the number of mature neurons, because CALB and TUBIII are normally expressed in these embryos (Fig. 4*B*). These results demonstrate that hypothalamic *Pomc* expression completely depends on ISL1 during the first stages of mouse development, from its early onset at E10.5 to at least E15.5.

To investigate the importance of ISL1 in *Pomc* expression after birth, we treated $Isl1^{loxP/loxP}$.CAAG-CreERT mice with TAM for 5 consecutive days, starting at postnatal day 21 (P21) or at 3 mo old (P90). A month later, we measured the levels of hypothalamic *Isl1* and *Pomc* mRNAs by qRT-PCR and found that our injection protocol was highly efficient, yielding a reduction of ∼90% of hypothalamic *Isl1* mRNA. *Isl1*@P21 and *Isl1*@P90 knockdown mice exhibited around 40% of hypothalamic *Pomc* mRNA levels relative to control $Isl1^{loxP/loxP}$ mice (Fig. 4*C*). In summary, these results indicate that ISL1 is required for maintaining normal levels of hypothalamic *Pomc* expression during pre- and postnatal life.

Role of *Ascl1* and *Ngn3* in the Ontogeny of Hypothalamic ISL1/POMC Neurons. During neurogenesis, the progenitor cells that become committed to a neuronal fate express TFs of the basic helix–loop–helix

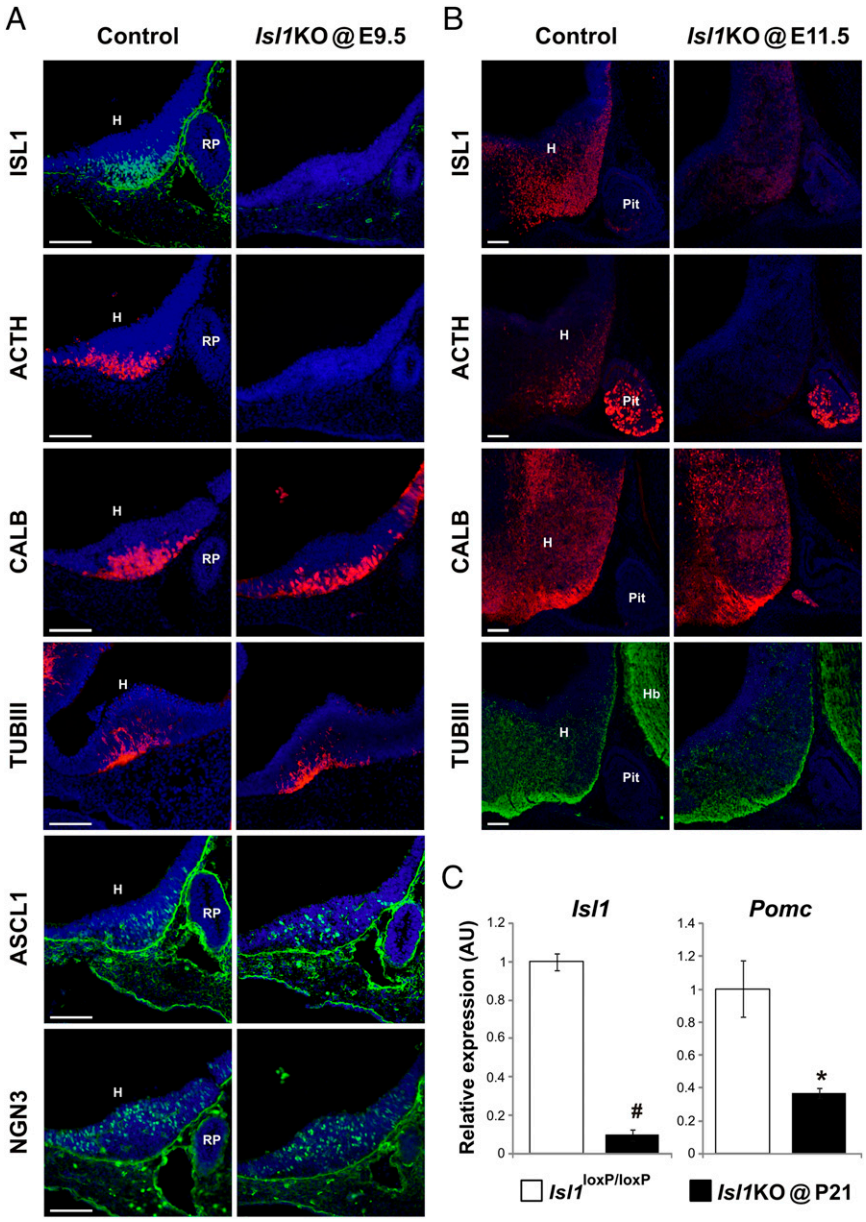

Fig. 4. Hypothalamic *Pomc* expression is impaired in *Isl1* conditional knockout mice. (*A*) Immunofluorescence analysis of the specified molecular markers in sagittal cryosections of *Isl1*KO@E9.5 and control embryos at E11.5. *Isl1* deficiency prevents hypothalamic *Pomc* expression onset without severely affecting the expression of neurogenin 3 (NGN3), achaete-scute complex like 1 (ASCL1), calbindin 28-K (CALB), or β-tubulin III (TUBIII). H, future hypothalamus; RP, future Rathke's pouch. DAPI staining is shown in blue. (Scale bar, 100 μm.) (*B*) *Isl1* function is required for maintaining hypothalamic *Pomc* expression. Immunofluorescence analysis of the specified molecular markers in sagittal cryosections of *Isl1*KO@E11.5 and control embryos at E15.5. ISL1 deficiency prevents *Pomc* expression in the hypothalamus but not in the pituitary (Pit). H, future hypothalamus; Hb, hindbrain. DAPI staining is shown in blue. (Scale bar, 100 μm.) (*C*) *Isl1* is necessary for hypothalamic postnatal *Pomc* expression. qRT-PCR of *Isl1* and *Pomc* mRNAs obtained from *Isl1*KO@P21 mouse hypothalami at 7 wk old, relative to control group ($Isl1^{loxP/loxP}$). AU, arbitrary units. $n = 3$ for each group. Error bars correspond to SEM. $^{\#}P < 0.0001$, $^{*}P < 0.05$, Student's *t* test.

(bHLH) family, known as proneural genes (31). In the mouse hypothalamus, it has been shown that the proneural genes *Ascl1* and *Ngn3* are necessary for hypothalamic neurogenesis and, more specifically, $Ascl1^{-/-}$ as well as $Ngn3^{-/-}$ mutant mice have a reduced number of hypothalamic POMC neurons (32, 33). During development, we observed by immunofluorescence that NGN3 and, specially, ASCL1 are mainly found near the ventricular zone of the future hypothalamus, where neuronal progenitors reside, whereas ISL1 is expressed mainly in the mantle zone, where postmitotic differentiated neurons accumulate (*SI Appendix*, Fig. S5). We observed that ASCL1 and ISL1 colocalize in cells present within a stripe located between the ventricular and mantle zones, whereas the colocalization between NGN3 and ISL1 in this region is complete (*SI Appendix*, Fig. S5).

To study the role of these proneural genes in *Isl1* expression, we analyzed *Ascl1*- and *Ngn3*-deficient mice at E11.5 by immunofluorescence and found that both $Ascl1^{-/-}$ and $Ngn3^{-/-}$ embryos have only a few POMC neurons in the hypothalamus, as previously described (32, 33). The number of $ISL1^{+}$ neurons is also greatly reduced in $Ascl1^{-/-}$ embryos, reaching 10% of wild-type levels. Interestingly, however, $Ngn3^{-/-}$ embryos still have normal numbers of $ISL1^{+}$ neurons (Fig. 5*A*). $Ascl1^{-/-}.Ngn3^{-/-}$ double mutant E11.5 embryos showed a complete absence of POMC immunoreactivity, although the number of $ISL1^{+}$ cells was similar to that

found in *Ascl1*$^{-/-}$ embryos (Fig. 5*A*). In summary, our results show that *Isl1* is epistatically downstream of *Ascl1* but not of *Ngn3* (Fig. 5*B*), and that in the absence of *Ngn3*, *Isl1* is unable to sustain normal *Pomc* expression during hypothalamic development.

Selective Elimination of ISL1 in POMC Neurons Causes Early-Onset Obesity. The ubiquitous loss of ISL1 function during development produces a lethal phenotype that prevents further physiological studies in adult mice. To circumvent this limitation we sought to abolish *Isl1* expression exclusively from POMC neurons by crossing *Isl1*$^{loxP/loxP}$ mice with *Pomc*-Cre mice (34), a BAC transgenic line expressing Cre recombinase from mouse *Pomc* regulatory regions. Because *Isl1* is not expressed in pituitary POMC cells, compound Pomc*Isl1*KO mice lack *Isl1* expression exclusively in POMC arcuate neurons starting at day E10.5. Analysis of hypothalamic *Pomc* mRNA levels in Pomc*Isl1*KO and control *Isl1*$^{loxP/loxP}$ mice by qRT-PCR showed that Pomc*Isl1*KO mice have ∼25% of hypothalamic *Pomc* mRNA levels in comparison with control mice (Fig. 6*A*). An immunofluorescence study performed in coronal mouse brain sections confirmed a marked reduction in ACTH immunoreactivity in the arcuate nucleus, but not in the pituitary, of Pomc*Isl1*KO mice in comparison with their control *Isl1*$^{loxP/loxP}$ siblings (Fig. 6*B*). A distinctive feature of central *Pomc* deficiency is early-onset obesity induced by hyperphagia (4). Analysis of food intake and body weight curves of Pomc*Isl1*KO mice fed ad libitum with standard low-fat chow showed that male and female Pomc*Isl1*KO mice are hyperphagic and display early-onset obesity compared with their *Isl1*$^{loxP/loxP}$ siblings (Fig. 6 *C* and *D*), which, in turn, showed identical growth curves to wild-type *Isl1*$^{+/+}$ mice (*SI Appendix*, Fig. S6). We also observed an increased linear growth in Pomc*Isl1*KO male mice (*SI Appendix*, Fig. S6). Body fat distribution in Pomc*Isl1*KO mice at 5 mo of age showed that the increased body weight is largely due to an exaggerated fat deposition in visceral and s.c. fat pads (Fig. 6 *E*–*G*). As a result of increased adiposity, fast serum leptin levels increased considerably, as observed in 4-mo-old Pomc*Isl1*KO mice compared with controls (Fig. 6*H*). The phenotypes observed in Pomc*Isl1*KO mice are reminiscent of those previously reported in *Pomc*-deficient mice (4, 35). Chronic and excessive accumulation of adipose tissue may lead to a generalized health disorder known as metabolic syndrome, which includes the development of a fatty liver and type II diabetes. In fact, liver size was increased in Pomc*Isl1*KO mice of both sexes (Fig. 6*I*) although fast blood glucose and serum insulin levels were normal in these mice (*SI Appendix*, Fig. S6). To confirm that the phenotypes observed in Pomc*Isl1*KO mice are solely due to the loss of ISL1 in POMC neurons, we sought to rule out the possible ectopic expression of cre in *Pomc*-Cre mice, especially in pancreatic islets where *Isl1* regulates the maturation, proliferation, and survival of the endocrine pancreas (36). To this end, we crossed *Pomc*-Cre mice with the Cre reporter strain Ai14 that activates the expression of a red fluorescent protein upon Cre-mediated recombination. In contrast to the numerous bright red cells observed in the hypothalamus and the pituitary, we did not find any red cell in pancreatic islets of compound *Pomc*-Cre.Ai14 mice (*SI Appendix*, Fig. S7), demonstrating that *Pomc*-Cre mice are indeed a selective tool for this study. Overall, our results demonstrate that *Isl1* expression in POMC hypothalamic neurons plays a fundamental role in normal body weight homeostasis by controlling hypothalamic *Pomc* expression in adulthood.

The regulation of *Pomc* by *Isl1* Is an Ancient Feature in Vertebrates. Because ISL1 is found in the zebrafish hypothalamus, we decided to investigate whether the regulation of *Pomc* expression by *Isl1* observed in the mouse is conserved in an evolutionary distant vertebrate. Teleost fishes have two *Pomc* paralogs named *pomca* and *pomcb* (25). Whereas *pomca* is expressed in the pituitary and in hypothalamic neurons of the nucleus lateralis tuberis (NLT), the homolog structure to the mammalian arcuate nucleus, *pomcb* is expressed in the brain preoptic area. Using double immunofluorescence, we observed that 92% of POMC hypothalamic neurons located in the zebrafish NLT also express ISL1 at 72 hpf (Fig. 7*A*). As we observed in the mouse hypothalamus (Fig. 1), the expression territory of ISL1 in the zebrafish NLT is much broader than that of *pomca*. To study the role of ISL1 in *pomca* transcription, we

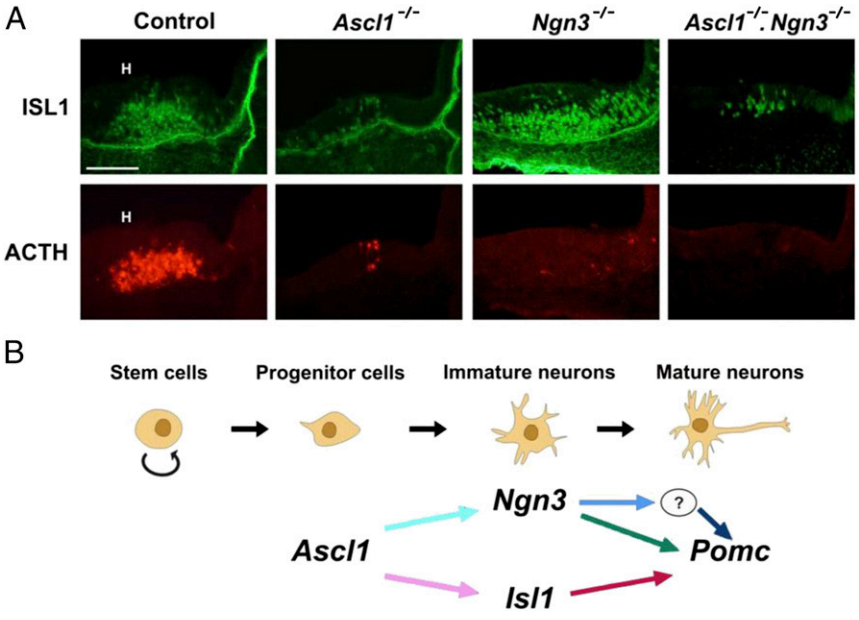

Fig. 5. Role of bHLH TFs on *Isl1* hypothalamic expression during development. (*A*) Immunofluorescence analysis of ISL1 (green) and *Pomc*-derived ACTH (red) expression in *Ascl1*$^{-/-}$, *Ngn3*$^{-/-}$, and *Ascl1*$^{-/-}$.*Ngn3*$^{-/-}$ mutant E11.5 embryos. H, future hypothalamus. (Scale bar, 100 μm.) (*B*) Schematic of regulatory interactions of TFs involved in *Pomc* expression in the developing hypothalamus. ASCL1 is required in early steps of hypothalamic neurogenesis to activate *Ngn3* and *Isl1* expression. These two TFs may independently activate *Pomc* expression. NGN3 may directly transactivate *Pomc* as a pioneer factor (green arrow) and/or by activating an intermediate TF of unknown identity, represented by a question mark.

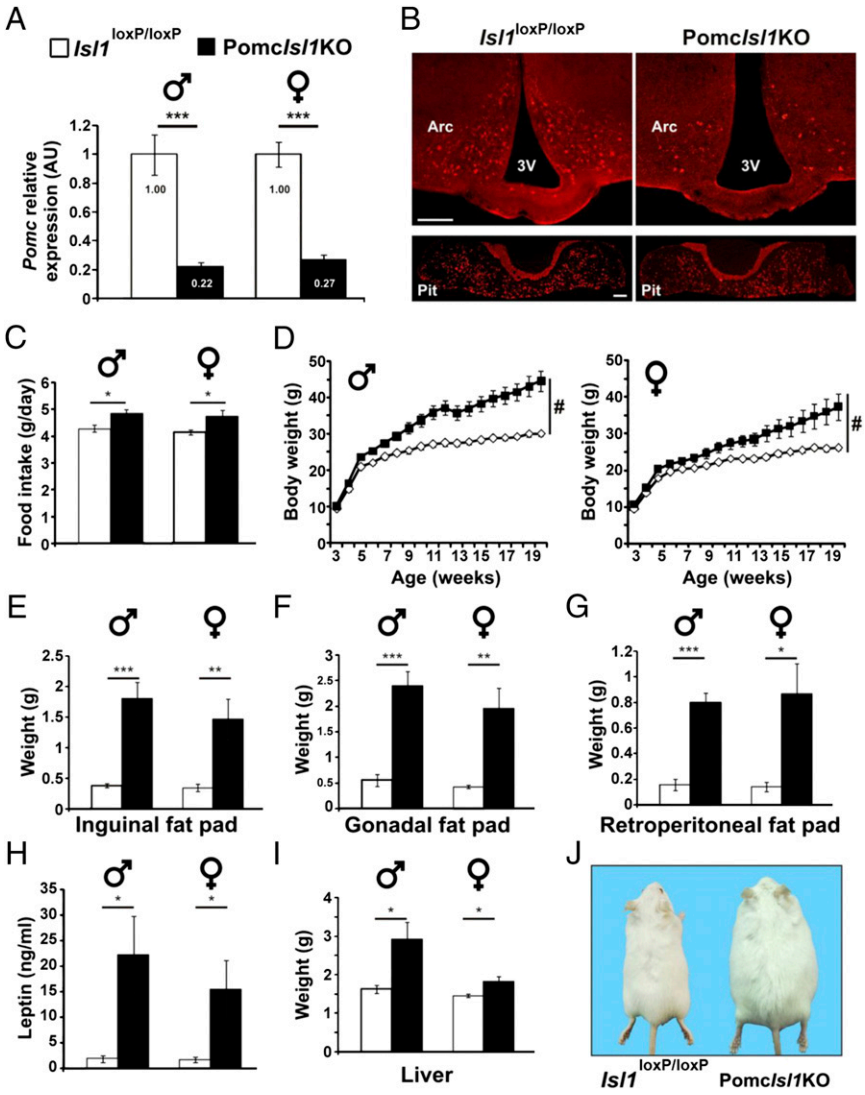

Fig. 6. ISL1-specific deletion in POMC neurons causes early onset obesity. (*A*) Hypothalamic *Pomc* mRNA expression normalized to 18S rRNA, relative to *Isl1*$^{loxP/loxP}$, in arbitrary units (AU). (*B*) Immunofluorescence analysis of *Pomc* expression (ACTH, red) in coronal sections of adult hypothalamus. Arc, arcuate nucleus; Pit, pituitary; 3V, third ventricle. (Scale bars, 100 μm.) (*C*) Average daily food intake measured during 3 consecutive weeks at 3 mo of age. (*D*) Body weight curves of *Isl1*$^{loxP/loxP}$ and Pomc*Isl1*KO male and female mice. Pomc*Isl1*KO mice are obese (RMA, genotype × time effect. $^{\#}P < 0.0001$ for Pomc*Isl1*KO vs. *Isl1*$^{loxP/loxP}$ for both sexes). (*E–G*) Determination of inguinal, gonadal, and retroperitoneal fat pad and liver (I) weights in *Isl1*$^{loxP/loxP}$ and Pomc*Isl1*KO 5-mo-old mice. (*H*) Fasting serum leptin levels were determined in 4-mo-old mice. (*J*) A representative obese Pomc*Isl1*KO male mouse and a normal *Isl1*$^{loxP/loxP}$ sibling at 5 mo of age. Values represent mean ± SEM (n = 5–8). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ (Student's t test).

microinjected zebrafish embryos with antisense morpholino oligonucleotides (MOs) designed to block *isl1* mRNA translation (37) and the results were compared with embryos microinjected with vehicle or with a heterologous morpholino (het-MO) of a similar length and nucleotide composition to that of the Isl1-MO. At 72 hpf, *Isl1* morphant embryos (Isl1-MO) have reduced body size, curled down tail, and defective heart development (Fig. 7*A*), in agreement with phenotypes previously reported for *isl1* mutant zebrafish lines (38). Analysis of *isl1* expression by immunofluorescence showed a great reduction of ISL1 in Isl1-MO embryos in comparison with both control groups, although the overall anatomy of the ventral hypothalamus remained normal (Fig. 7*B*). In addition, ISL1 knockdown zebrafish embryos showed an almost complete loss of *pomca* expression in the hypothalamus but not in the pituitary, as shown by immunofluorescence and whole mount in situ hybridization (Fig. 7 *B* and *C*). The effect of ISL1 on *pomca* expression is specific because the levels of *pomcb* mRNA in the preoptic area and those of the hypothalamic neuropeptide gene *agrp* remained unaltered in Isl1-MO microinjected zebrafish embryos (Fig. 7*B*).

Previously in this work, we have shown that the ability of mouse nPE1 and nPE2 sequences to drive EGFP expression to hypothalamic POMC neurons in transgenic zebrafish is impaired when the ISL1 binding sites are mutated (Fig. 3*B*), suggesting that zebrafish ISL1 binding to the mouse enhancers plays an important role for transactivating the reporter gene in this specific neuronal population. To further test this hypothesis, we microinjected Isl1-MO in embryos obtained from a stable line of transgenic zebrafish harboring a 4-kb mouse sequence encompassing nPE1 and nPE2 upstream of the *pomca* pituitary promoter followed by EGFP (25). We found that transgenic embryos receiving Isl1-MO were unable to express EGFP in the hypothalamus, but retained bright green fluorescent cells in the pituitary in contrast to zebrafish embryos microinjected with vehicle or the het-MO, which showed EGFP expression in POMC neurons as well (*SI Appendix*, Fig. S8). Altogether, these results indicate that ISL1 is a positive regulator of

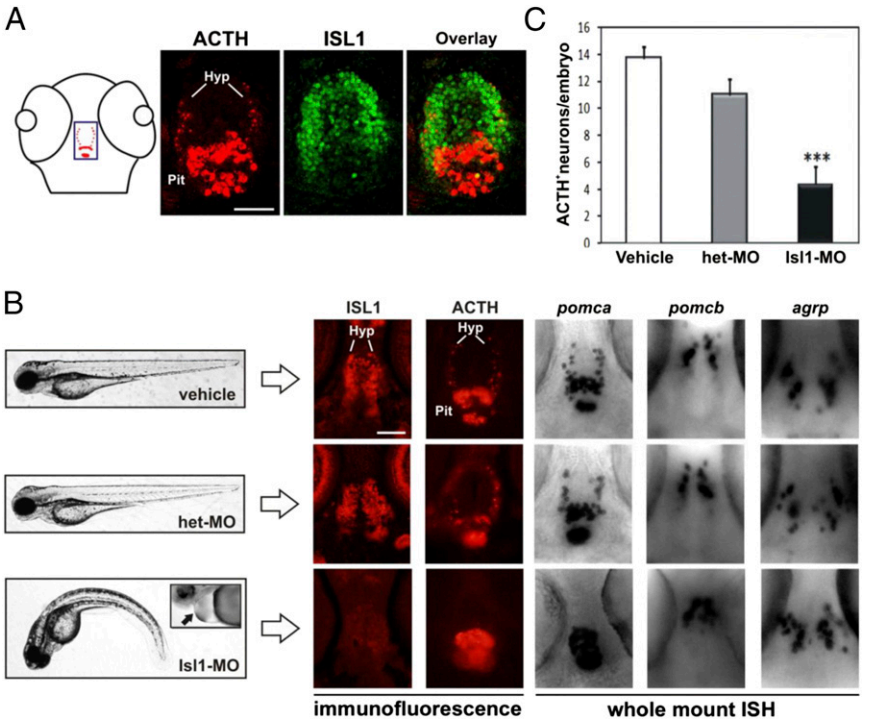

Fig. 7. ISL1 regulates hypothalamic *pomca* expression in zebrafish. (*A*) Schematic of a 72-hpf zebrafish head in a horizontal plane showing the distribution of *pomca*-expressing cells in the pituitary and hypothalamus (red dots). The blue rectangle depicts the magnified area shown in the other panels. Double immunofluorescence analysis showing *pomca* (ACTH, red) and *isl1* (green) coexpression in horizontal sections of 72-hpf zebrafish brain. (*B*, *Left*) Phenotypic characterization of 72-hpf Isl1-MO, het-MO, and vehicle-injected zebrafish. Isl1-MO larvae have reduced body size, curved down tail, and develop cardiac edema (indicated with an arrow in magnified *Inset*). (*Right*) Molecular characterization of the hypothalamus in Isl1-MO, het-MO, and vehicle-injected zebrafish embryos. Immunofluorescence (IF) analysis in horizontal brain sections of 72-hpf zebrafish shows that ISL1 knockdown completely eliminates *pomca* expression (ACTH) in the hypothalamus, but not in the pituitary. Whole-mount in situ hybridization (ISH) further confirms the lack of *pomca* expression in the brain and shows that other hypothalamic markers, *pomcb* and *agrp*, remain unchanged in Isl1-MO relative to control embryos. Hyp, hypothalamus; Pit, pituitary. (Scale bars, 50 μm.) (*C*) Quantification of the total number of ACTH$^+$ neurons per embryo in Isl1-MO ($n = 16$)-, het-MO ($n = 15$)-, and vehicle ($n = 28$)-injected zebrafish. Bars correspond to mean + SEM. ***$P < 0.001$, Student's *t* test vs. vehicle.

hypothalamic *Pomc* transcription in zebrafish and mice and reveal that at least part of this regulation is ancestral to all jawed vertebrates, having been established before the evolutionary divergence of tetrapods and teleost fishes, around 450 Mya.

Discussion

In this study we combined molecular, genetic, physiological, and evolutionary approaches to demonstrate that the LIM-homeodomain transcription factor ISL1 directly regulates the neuronal expression of *Pomc* in the vertebrate hypothalamus. Specifically, we show that: (*i*) *Isl1* is expressed in POMC hypothalamic neurons during embryonic development and adulthood; (*ii*) ISL1 binds in vitro and in vivo to DNA elements present in the *Pomc* neuronal enhancers nPE1 and nPE2, which are important for enhancer function; (*iii*) *Isl1* expression is necessary for hypothalamic *Pomc* expression during embryogenesis; (*iv*) *Isl1* deficiency in the adult hypothalamus causes reduced *Pomc* expression and an energy balance phenotype including obesity; and (*v*) the regulation of *Pomc* by *Isl1* is an ancient feature in vertebrates.

Several transcription factors are known to confer regional identity to the early hypothalamic anlage, regulate neurogenesis, and determine neuronal identity in specific hypothalamic nuclei (39). ISL1 is neither expressed during early forebrain patterning nor in hypothalamic progenitors, but rather in maturing and fully mature hypothalamic neurons. This and our conditional knockout results point to ISL1 being a terminal differentiation gene that is necessary to establish the specific identity of POMC neurons after these cells are born and accumulate in the mantle zone, giving rise to the arcuate nucleus. Recent work has shown that *Isl1* plays pivotal roles in determining the cell fate of GABAergic medium spiny striatofugal neurons (40), cholinergic magnocellular striatal interneurons (41), and cholinergic spinal and cranial motor neurons (42). Interestingly, these different ISL1-dependent neuronal types participate in pyramidal and extrapyramidal circuits that coordinate body movement and locomotion. Besides its importance in the motor system, *Isl1* also participates in the differentiation and maintenance of cell types involved in the physiology of metabolism. For example, *Isl1* is expressed in pancreatic islets where it is required for the maturation, proliferation, and survival of the endocrine pancreas (36). Our results showing that *Isl1* is essential for the neuronal expression of the satiety gene *Pomc* in the ventromedial hypothalamus adds another neuronal type to the category of ISL1-dependent cells involved in the regulation of metabolism and energy balance. In this work, we show that ISL1 is also necessary for the expression of the hypothalamic TF *orthopedia* (*Otp*) in the future arcuate nucleus, indicating that the function of ISL1 in this brain region is not restricted to the POMC lineage (*SI Appendix*, Fig. S4).

Consistent with its terminal specification function, ISL1 does not control general neuronal differentiation, because β-tubulin and calbindin expression appear to be normal in conditional *Isl1* mutants. This is also consistent with the fact that *Isl1* is epistatically downstream of one hypothalamic proneural gene, namely ASCL1. The proneural bHLH factors ASCL1 and NGN3 are expressed in neuronal progenitors in the developing hypothalamus since E9.5 and play a role in general neurogenesis as well as in the specification of some hypothalamic neuronal populations, including those that express POMC (32, 33). In contrast to ISL1, both bHLH factors are expressed only transiently during hypothalamic neurogenesis and are not present in mature POMC neurons

(32, 33). Here we show that *Isl1* expression depends on ASCL1, but not on NGN3. Thus, it can be envisaged that the importance of ASCL1 in the specification of POMC neurons is due, at least in part, to its role in the expression of ISL1. In contrast to what has been observed in pancreatic islets (43), NGN3 is not necessary for the expression of ISL1 in the hypothalamus, even though it is also downstream of ASCL1 (33). Thus, in the hypothalamus, NGN3 and ISL1 seem to act in parallel to regulate *Pomc* expression. The proposed relationship between ISL1 and the bHLH factors is depicted in Fig. 5*B*. Because NGN3 is only transiently expressed in the POMC lineage (33), it is possible that this bHLH factor is necessary for the expression of another TF which, in its turn, is required for full *Pomc* expression. Alternatively, NGN3 could act as, or corecruit, a pioneer transcription factor that might render the *Pomc* gene transcriptionally competent.

In recent years, it has become evident that cell-specific expression of some metazoan genes may be controlled by two or more regulatory regions displaying similar transcriptional activities (44, 45). Such apparently redundant enhancers have been best studied in *Drosophila*, where enhancers with similar activities recruit similar sets of TFs (46). This phenomenon of enhancer redundancy, however, has not been characterized in mammals at the molecular level. nPE1 and nPE2 drive identical reporter expression patterns in transgenic mice (16) and, by targeted mutagenesis in mice, we have recently shown that both enhancers are necessary for full endogenous *Pomc* expression in the hypothalamus (17). Similar to what has been found for redundant enhancers in *Drosophila*, we found that ISL1 binds to DNA elements present in both nPE1 and nPE2, and that these elements are needed for hypothalamic enhancer function. Thus, the similar regulatory activities of nPE enhancers are conferred by the binding of at least one common TF. This observation is even more striking as nPE1 and nPE2 are not the result of a segmental duplication but have originated as separate events of cooption ("exaptation") of retroposon sequences during mammalian evolution (16, 18), implying that the exaptation process involved the independent acquisition of similar TF binding sites by each transposon.

Isl1 is an ancient gene, present in the genomes of basal metazoans like cnidarians, ctenophores, and placozoans (47). In some systems, like second heart field and somatosensory neurons, *Isl1* expression is part of a transcriptional signature conserved in invertebrates and vertebrates (48). *Pomc*, on the other hand, is only found in vertebrates, being expressed in the hypothalamus of all jawed vertebrates. Our observation that *Isl1* is expressed in the zebrafish hypothalamus and that it is necessary for *pomca* expression indicates that ISL1 has been part of the POMC neuron transcriptional code since the appearance of this neuronal subtype in the ancestor of teleosts and tetrapods, around 450 Mya. This is reinforced by the fact that mouse nPEs can drive expression to zebrafish *pomca* neurons (25) and that zebrafish ISL1 is also necessary for this activity. Interestingly, we observed that the expression of the zebrafish paralog *pomcb* in the preoptic area does not depend on ISL1. Thus, our results show that the expression of the two zebrafish *Pomc* paralogs depend on a different *cis*/*trans*-transcriptional code and indicate that only the primitive tuberal hypothalamic POMC population was controlled by ISL1 in the vertebrate ancestor, which is consistent with the fact that mouse nPE enhancers can drive expression to the *pomca* territory, where zebrafish ISL1 is abundantly expressed, but not to the *pomcb* territory in the preoptic area (25).

POMC-derived melanocortins and their central receptors play a crucial role in the regulation of food intake and energy balance in adult vertebrates, including humans (1). Moreover, mice and humans lacking POMC or the melanocortin 4 receptor (MC4R) are extremely hyperphagic and obese (5, 35, 49, 50). Despite the fact that genetic factors explain up to two-thirds of body weight and fat mass variation in the human population, monogenic cases of obesity are rarely seen in the clinic. Recent genome-wide association studies (GWASs) suggest that obesity follows a polygenic continuous trait model in which many frequent polymorphic alleles, each providing a very small effect, act in combination to increase the predisposition to gain more fat. Alternatively, the risk for obesity may increase after the concomitant occurrence of a few low-frequency variants, each providing strong or intermediate effects. Our findings showing that depletion of ISL1 specifically in POMC neurons induces obesity may be of biomedical relevance because polymorphisms in regulatory regions leading to low levels of *Isl1* expression in the ventral hypothalamus may impair *Pomc* expression and compromise the normal control of food intake and energy balance.

Materials and Methods

Animal Care. Mice were housed in ventilated cages under controlled temperature and photoperiod (12-h light/12-h dark cycle, lights on from 7:00 AM to 7:00 PM), with tap water and laboratory chow containing 28.8% protein, 5.33% fat, and 65.87% carbohydrate available ad libitum. All mouse procedures followed the *Guide for the Care and Use of Laboratory Animals* (51) and in agreement with the Institutional Animal Care and Use Committee of Instituto de Investigaciones en Genética y Biología Molecular (INGEBI) and the University Committee on the Care and Use of Animals of the University of Michigan.

Breeding of Mice. Animals carrying the $Isl1^{loxP}$ allele (27) were obtained from L. Gan, University of Rochester, Rochester, NY, and maintained in our laboratory as homozygotes on a mixed background. CAAG-CreERT mice (28) were obtained from The Jackson Laboratory (B6.Cg-Tg[CAG-cre/Esr1]5Amc/J) and were intercrossed with $Isl1^{loxP/loxP}$ mice to generate the TAM-inducible *Isl1* conditional knockout strain CAG-CreERT.$Isl1^{loxP/loxP}$. *Pomc*-cre mice (34) were obtained from G. S. Barsh, Stanford University School of Medicine, Stanford, CA, and were maintained in our laboratory as heterozygotes on a mixed background. Animals with POMC-specific *Isl1* deletion (Pomc*Isl1*KO) were generated by intercrossing $Isl1^{loxP/loxP}$ with *Pomc*-cre mice. The cre reporter line Ai14 (52) and $Ascl1^{-/-}$ (53) and $Ngn3^{-/-}$ (43) embryos were a gift from G. Lanuza, Instituto de Investigaciones Bioquímicas de Buenos Aires, Buenos Aires, Argentina.

Transgenes and Transgenic Mice Production. Transgenes nPE1*Pomc*-EGFP and nPE2*Pomc*-EGFP were previously reported (15, 16). Transgenes nPE1 (Isl1Mut)*Pomc*-EGFP and nPE2 (Isl1Mut)*Pomc*-EGFP are identical to the ones above except they carry four point mutations that disrupt the ATTA HDBS recognized by ISL1. These mutations were introduced in the enhancer sequence by a standard megaprimer PCR protocol and confirmed by DNA sequencing (primer information is available in *SI Appendix*, Table S1). More details are provided in *SI Appendix, SI Materials and Methods*.

Reporter Gene Expression in Transgenic Mice. Transgenic pups were killed, heads were fixed overnight in paraformaldehyde 4% (wt/vol) in PBS and then cryoprotected in sucrose 10% (wt/vol) in PBS for another 24 h. Tissue was embedded in a solution of 10% (wt/vol) sucrose with 10% (wt/vol) gelatin in PBS, snap frozen in isopentane cooled to −60 °C in dry ice and stored at −80 °C. Coronal 20-μm-thick sections were cut in a cryostat (Leica 1510S) and collected on Super-Frost Plus slides (Fisher Scientific). Sections were directly visualized or incubated with primary rabbit polyclonal antibody anti-GFP (ab290, Abcam).

Immunofluorescence. Sagittal (14 μm for E9.5–E12.5 and 18 μm for E15.5) sections were obtained in a cryostat (Leica 1510S) and collected on Super-Frost Plus slides (Fisher Scientific). More experimental details are provided in *SI Appendix, SI Materials and Methods* and for additional information of antibodies used in this study see *SI Appendix*, Table S2.

Tamoxifen Treatment. TAM (T56648, Sigma-Aldrich) was dissolved in sesame oil (S3547, Sigma-Aldrich) at 10 $mg{\cdot}mL^{-1}$. For treatment in the embryo, one single dose of TAM (130 μg per gram of body weight) was given to the mother by i.p. injection. For treatment in juvenile and adult mice, five single daily doses of TAM (70 μg per gram of body weight) were given by i.p. injection during 5 consecutive days.

Hypothalamic mRNA Quantification. Hypothalamic total RNA was prepared with TRIzol (Invitrogen) and then treated with RNase-free DNaseI (Ambion). First-strand cDNA synthesis was generated with random primers using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). *Pomc* mRNA was identified by relative quantitative RT-PCR using TaqMan gene expression assay (Mn00435874_M1, Applied Biosystems) and normalized to 18S

rRNA (HS99999901_S1, Applied Biosystems) (4). *Isl1* mRNA was identified by relative qRT-PCR using SYBR Select Master Mix (Applied Biosystems) and normalized to mouse *cyclophilin B* mRNA. Primer information is available in *SI Appendix*, Table S1. Samples were run on a Rotor-Gene 6000 instrument (Corbett Life Science) or Opticon2 (MJ Research), and results were analyzed by the 2-ΔΔCT relative quantitation method (54).

Statistics. All data presented are the mean ± SEM and were analyzed by repeated measures ANOVA or Student's *t* test unless otherwise stated using Statistica. Post hoc pairwise comparisons between groups were performed by Fisher's least significant difference post hoc test. *P* values less than 0.05 were considered significant.

Zebrafish Husbandry. Adult AB zebrafish stocks (Zebrafish International Resource Center) were raised and maintained at 28.5 °C under a 14:10-h light: dark cycle. Embryos were collected soon after natural spawning and raised in zebrafish embryonic E3 media (5 mM NaCl, 0.17 mM KCl, 0.33 mM $CaCl_2$, 0.33 mM $MgSO_4$, 10^{-5}% methylene blue) in Petri dishes kept at 28.5 °C. Larvae to be used for in vivo screenings or whole-mount in situ hybridization were grown in E3 buffer containing 0.2 mM 1-phenyl-2-thiourea (PTU) from 1 d postfertilization.

Further detailed descriptions of all materials and methods are provided in *SI Appendix, SI Materials and Methods*.

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