Inactivating NHLH2 variants cause idiopathic hypogonadotropic hypogonadism and obesity in humans

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Abstract
Metabolism has a role in determining the time of pubertal development and fertility. Nonetheless, molecular/cellular pathways linking metabolism/body weight to puberty/reproduction are unknown. The KNDy (Kisspeptin/Neurokinin B/Dynorphin) neurons in the arcuate nucleus of the hypothalamus constitute the GnRH (gonadotropin-releasing hormone) pulse generator. We previously created a mouse model with a whole-body targeted deletion of nescient helix-loop-helix 2 (Nhlh2; N2KO), a class II member of the basic helix-loop-helix family of transcription factors. As this mouse model features pubertal failure and late-onset obesity, we wanted to study whether NHLH2 represents a candidate molecule to link metabolism and puberty in the hypothalamus. Exome sequencing of a large Idiopathic Hypogonadotropic Hypogonadism cohort revealed obese patients with rare sequence variants in NHLH2, which were characterized by in-silico protein analysis, chromatin immunoprecipitation, and luciferase reporter assays. In vitro heterologous expression studies demonstrated that the variant p.R79C impairs Nhlh2 binding to the Mc4r promoter. Furthermore, p.R79C and other variants show impaired transactivation of the human KISS1 promoter. These are the first inactivating human variants that support NHLH2’s critical role in human puberty and body weight control. Failure to carry out this function results in the absence of pubertal development and late-onset obesity in humans.

Introduction

The “KNDy” (Kisspeptin/NKB/Dynorphin) neurons (also known as kisspeptin neurons) in the arcuate nucleus (ARC) of the hypothalamus, which project to GnRH neurons, represent a central node in the control of GnRH secretion (Lehman et al. 2010). KNDy cells contain receptors sensitive to circulating gonadal steroids and thus convey the feedback influence of sex steroids onto the GnRH system (Lehman et al. 2010). Furthermore, compelling evidence has accumulated in recent years indicating that KNDy neurons constitute the so-called GnRH pulse generator (Herbison 2018). Pulsatile GnRH secretion drives the episodic secretion of pituitary gonadotropins, LH and FSH, that induce the development of the gonads and secretion of the sex steroids responsible for inducing the secondary sexual characteristics. The activation of the GnRH pulse generator has been recognized as the key event underlying pubertal onset in humans and other mammals. Although KNDy neurons are now recognized as the long-sought GnRH pulse generator, the signal that re-activates them after a long, quiescent period (i.e., childhood or juvenile phase) remains unknown.
Several stimuli (environmental, neuronal, gonadal, somatic, epigenetic, or metabolic/nutritional) have been proposed as potential mediators of puberty. It has been well-observed that nutritional or metabolic cues are essential determinants of the initiation of puberty. The “critical bodyweight” hypothesis was first postulated by Frisch and Revelle (Frisch and Revelle 1971) for determining pubertal onset based on historical data. A bodyweight of 47 kg, at which puberty started, was attained by a girl at age 17 years old in the 1840s and at 13 years old in the 1960s, which was probably secondary to the improved nutrition (Tanner 1968). To the other extreme, obesity is often complicated by subfertility in women, typically due to polycystic ovary syndrome (de Zegher et al. 2018). A nutrition/metabolism connection with reproduction has long been observed, but there is a lack of knowledge regarding the exact mechanisms of interactions between these two fundamental physiological compartments. We have been studying a large cohort of IHH patients to determine genetic underpinnings of the timing of puberty. In this cohort obesity is more enriched (14.9%) than in the general population of Turkish children (3.9%) (Ozturk et al. 2008). Therefore, our cohort may harbor yet unidentified mutant genes that have a role in body weight regulation and reproduction.

In this study, we present clinical and human genetic evidence complemented by in silico and in vitro experiments confirming the involvement of nescent helix-loop-helix 2 (NHLH2) in the control of pubertal development as well as energy balance. The phenotype of these patients remarkably complements those of the Nhlh2 knock-out mice (N2KO). The N2KO mouse, similar to its human counterpart, features both hypogonadotropic hypogonadism (HH) and late-onset obesity (Good et al. 1997). These results introduce a potentially critical transcription factor in the metabolic control of pubertal onset and reproduction in humans.

Results
Identification of NHLH2 rare sequence variants in patients with IHH

By exome screening of 354 well-phenotyped IHH patients, we identified missense variants encoding phylogenetically highly conserved residues in NHLH2, HGNC:7818, (NM_001111061.1) from three patients in three independent families (Fig. 1). Molecular and clinical characteristics of associated with the variants are shown in Table 1. Sanger sequencing confirmed the presence and pedigree segregation of these variants. These variants were novel and not found in 100 healthy Turkish adult controls. None were seen in Clinvar. These variants were classified as variants of uncertain significance (VUS) by ACMG/AMP classification (Richards et al. 2015). These variants were not seen or extremely rare in the most extensive reference population, GnomAD, or a regional database, GME. Pathogenicity predictions based on four in silico methods (PP2, PolyPhen-2; SIFT, Sorting Intolerant From Tolerant; MT, Mutation Taster; LRT, Likelihood Ratio Test) were performed. The variants p.R79C and p.A83P were predicted deleterious by all four methods. p.A9L was predicted deleterious by two (SIFT and LRT) while p.V31M by none of the methods. Our participation in two major online gene-matching programs has revealed no matches, suggesting an exceptional rarity of NHLH2 variants.
Patients and their NHLH2 variants

The NHLH2 variant in case 1 (p.R79C) was found in a 22.7 MB long (97.8–120.5) homozygous region on Chromosome 1 identified in the exome data (Carr et al. 2013). Case 1 presented first at age 14 years with the complaint of absent pubertal development. At presentation, his body mass index (BMI, kg/m²) was 27.2, which denotes obesity by Turkish pediatric BMI percentile charts (Ozturk et al. 2008). As a child, he had not been obese. He started developing obesity around 12–13 years of age. His obesity progressively worsened over the years to reach the BMI of 35.1 at age 19 years. The patient does not describe hyperphagia. His daily physical activity level is noticeably low. His sense of smell is intact. His heterozygous parents and younger sister started puberty in time, and they are not obese. His testicular volumes were 2 ml bilaterally. His lab results showed prepubertal serum testosterone (10 ng/dl) along with low LH (0.1 mIU/mL) and FSH (0.5 mIU/mL). A GnRH stimulation test revealed a maximum LH level of 0.9 mIU/mL, which is
severely abnormally low. This clinical presentation is consistent with IHH.

Case 2 with the heterozygous p.A9L variant (due to c.25G > T and c.26C > T, likely to have arisen as a deletion/duplication event) presented first at age 17 years with complaints of absent pubertal development and obesity. At presentation, his BMI was 27.8, which denotes obesity (Ozturk et al. 2008). As a child, he had not been obese. He started developing obesity around 14 years of age. His obesity significantly worsened by age 19. The patient does not describe hyperphagia. His daily physical activity level is low. His sense of smell is intact. His testicular volumes were 4 ml bilaterally. The proband’s lab results showed prepubertal serum testosterone (< 2.5 ng/dl) along with low LH (0.1 mIU/mL) and FSH (0.6 mIU/mL). A GnRH stimulation test revealed a maximum LH level of 0.1 mIU/mL, which is abnormally low. This clinical presentation is consistent with IHH. His heterozygous mother and sister started puberty in time, and they are not obese. This patient also had coloboma and intellectual disability and was also diagnosed with Kabuki syndrome. His diagnosis was confirmed with a de novo mutation in KMT2D (c.13102dupA:p.T4368fs). KMT2D is not known to be IHH or obesity related. An international group of experts recently created consensus diagnostic criteria for Kabuki syndrome. Neither obesity nor IHH was found in 449 molecularly confirmed Kabuki syndrome cases (Adam et al. 2019). Moreover, recently, a comprehensive systematic analysis of height, weight, and BMI of Kabuki syndrome patients was reported (Ruault et al. 2020). The results indicated lower (but not higher) weight and BMI values in Kabuki syndrome patients than in the normative French population. On the other hand, Kabuki syndrome is heterogeneous in clinical presentation; early or delayed puberty and obesity have been occasionally and variably reported in cases with KMT2D mutations (Ito et al. 2013; Moon et al. 2018). Therefore, it is unclear whether Case 2’s phenotype, i.e., obesity and IHH, is solely due to his NHLH2 variant. It may be that both KMT2D and NHLH2 variants have contributed to his phenotype.

Case 3 with heterozygous p.V31M presented first at age 17 years with the complaints of micropenis and absent pubertal development. He has never been obese; his BMI was 20 (50th percentile) at presentation and 22.5 at age 25. His testicular volumes were 4 ml bilaterally along with a stretched penile length of 7 cm. The proband’s lab results showed prepubertal serum testosterone (< 20 ng/dl) along with low LH (0.1 mIU/mL) and FSH (0.3 mIU/mL). A GnRH stimulation test revealed a maximum LH level of 5.9 mIU/mL, which is abnormally low. This clinical presentation is consistent with IHH. The proband is heterozygous for the NHLH2. He also carries heterozygous variants in two known IHH genes, KISS1 and PROKR2; both inherited from her unaffected mother. His NHLH2 variant is either inherited from his deceased father (from whom no DNA sample is available) or occurred de novo. His KISS1 variant is novel and should not by itself cause IHH in heterozygosity. Although his PROKR2 variant (p.L173R) was reported to cause Kallmann syndrome with in vitro functional evidence (Cole et al. 2008), conflicting interpretations of pathogenicity as Likely Benign or Uncertain Significance were equally recorded by participating labs in Clinvar. Particularly this variant is too common in the general population (Frequency of 2/1,000 in GnomAD) for the prevalence of IHH, which is 1/10–100.000 (Bianco and Kaiser 2009), suggesting it is also seen in ostensibly healthy individuals. Furthermore, our patient’s sense of smell is intact, which goes against Kallmann syndrome. Altogether his IHH phenotype is likely to result from the combinatorial effect of the variants in these three genes. Oligogenic etiology in IHH is well established (Sykiotis et al. 2010). His lack of obesity may be explained by a milder character of his heterozygous NHLH2 variant as suggested in the transactivation studies.

Previously, in a study exploring genetic underpinnings of extreme obesity, a heterozygous NHLH2 variant (p.A83P) was found in a single patient from a cohort of 382 morbidly obese (BMI > 36) adult individuals (Ahituv et al. 2007). No data regarding the reproductive phenotype of this patient was provided in that article. This variant was previously demonstrated by our lab to be defective in both DNA binding and transactivation (Al Rayyan et al. 2013). p.R79C in Case 1 is located remarkably close to the p.A83P. These two variants (p.R79C and p.A83P) occur in residues that are preserved in almost all species in a region known as the Myc-type, basic helix-loop-helix (bHLH) domain, which is highly likely to be functionally critical (Fig. 1).

Nlh2 pR79C variant is defective in DNA binding

Since NHLH2 is a transcription factor, we further studied one of the variants (p.R79C), which is situated within the functionally critical basic DNA-binding domain. In silico 1D structural prediction for the human WT and p.R79C variant revealed an increased disorder in the protein chain past the cysteine in position 79 (Fig. 2A). This resulted in a slight variation in the folding of the basic DNA-binding domain as seen in Fig. 2B (circles). Additional analysis of the DNA-binding domain, using the FunFold server predicted that both the WT and p.R79C NHLH2 proteins would interact with DNA. However, there was a shift in the predicted DNA-binding domain for the variant protein such that the neighboring tyrosine residue at position 78 was predicted to substitute for the missing arginine at position 79 (Fig. 2C). This shift also changed the predicted downstream DNA interaction domain at the arginine in position 85 in WT NHLH2 to a glycine at position 86 in the p.R79C variant, even though R85 is present in that protein. Chromatin
immunoprecipitation was used to test whether the in-silico changes resulted in alterations in DNA binding in vitro. As the positions of the normal amino acids in human variants are 100% conserved to those found in the mouse Nhlh2 protein (Fig. 1), a mouse Nhlh2 protein construct tagged with myc-tag was mutagenized to produce mouse p.R79C Nhlh2 variant. We have previously shown that the WT Nhlh2-myc protein can bind to Mc4R promoters using ChIP (Wankhade and Good 2011). As shown in Fig. 2D, the WT and p.R79C variant Nhlh2 proteins are produced in N29/2 hypothalamic cells at a similar level with no visible protein migration alteration or increased degradation. However, ChIP demonstrates that the p.R79C variant has little to no binding of the Mc4R promoter region, compared to WT (Fig. 2E). Together these findings confirm that the Nhlh2R79C variant confers reduced DNA binding when compared to WT Nhlh2. As aforementioned, we previously demonstrated in vitro evidence that another variant (p.A83P) nearby in the same domain from a patient with extreme obesity to be defective in both DNA binding and transactivation (Al Rayyan et al. 2013).

**NHLH2 variants show reduced transactivation of a target promoter**

Recently, the NHLH2 binding to and transactivation of the rat Kiss1 promoter was demonstrated (Leon et al. 2021). Kiss1 protein expression is essential for reproduction as kisspeptin is the final product of the GnRH pulse generator to stimulate GnRH hormone release from the GnRH neuron axons (Lehman et al. 2010). To translate our results back to the human patients, human KISS1 promoter constructs
were used to measure transactivation by WT NHLH2 and NHLH2 variants. HEK293 cells were transfected with empty vector (n:8), WT NHLH2 (n:8), or mutant NHLH2 R79C (n:7), A9L (n:5), V31M (n:8) along with the human KISS1 promoter reporter plasmids. As shown in Fig. 3, one way ANOVA followed by Tukey test demonstrated that expression of the KISS1 promoter is induced by both the WT and NHLH2 plasmids. Notably, however, there is a marked decrease in luciferase activity with the mutant variants compared to WT NHLH2, albeit the difference did not reach the statistical significance (set at < 0.05) for p.V31M.

Overall, the causal role of the variants p.A9L and p.V31M in the phenotype needs further confirmation. Particularly, p.A9L in Case 2 is inherited from a healthy mother. Additionally, the mother and sister started puberty in time, and they are not obese. There are no known specific functional attributions to the protein regions of the variants p.A9L and p.V31M, they still demonstrated decreased KISS1 transactivation. We wondered about the mechanism of these effects. GPS-MSP methyl-group specific prediction algorithm (GPS-MSP: http://msp.biocuckoo.org/index.php) identified newly created methylation sites on NHLH2 at R79 with each of the p.A9L and p.V31M variants (Supplementary Fig. 1). The acquired methylation at R79 by each of A9L and V31M would predict impaired availability of R79 to bind to target promoter regions (Chen et al. 2006). More work needs to be done to understand the role of additional methylation on R79 for Nhlh2 transactivation function, including determination of if and when WT Nhlh2 is methylated, and whether increased methylation of the transcription factor affects its ability to bind DNA as a mechanism for reducing transactivation.

**Discussion**

The hypothalamic arcuate nucleus is the hub where homeostasis of two essential systems i.e., nutrition/metabolism and reproduction, are maintained. Neither of these systems is well understood yet. Therefore, rare human cases of IHH may provide unique opportunities to gain insight into the inner workings of these two closely interacting systems. As supported by the data in this study, NHLH2, may represent a functional link between the hypothalamic metabolic circuitry and the GnRH pulse generator.

It is well recognized that nutritional or metabolic inputs are essential determinants of the initiation of puberty. Under-nutrition decreases arcuate Kiss1 expression in rodents (Castellano et al. 2005), and leptin-null mice show decreased ARC Kiss1 expression and pubertal failure (Smith et al. 2006). Moreover, leptin administration restores ARC Kiss1 expression and fertility in the leptin-null mice (Chehab et al. 1996). We have previously shown that Nhlh2 expression is significantly reduced in response to 24-h food deprivation and increased with food intake or leptin injection in the ARC POMC (Proopiomelanocortin) neurons (Vella et al. 2007). These results indicate that NHLH2 may function as a hypothalamic sensor of peripheral leptin through its role in transcriptional control of the POMC-melanocortin pathway.

Anatomically, close appositions have been observed between POMC fibers and KNDy neurons (Manfredi-Lozano et al. 2016). Recent brain-wide tracing studies determined that the largest source of synaptic input to the KNDy neurons originate from the POMC cells (Moore et al. 2019). Moreover, a developmental link between nutrient-sensing and reproductive neuropeptide-synthesizing neuronal populations was demonstrated by Sanz and colleagues (Sanz et al. 2015). These investigators have shown that Pomc-expressing progenitor cells not only give rise to POMC cells but also Kisspeptin (KNDy) cells in the ARC (Sanz et al. 2015). Notably, high levels of Nhlh2 expression were detected in the adult mouse in neurons scattered throughout the ARC (Jing et al. 2004). Consistent with these foregoing findings, a recent study suggested a leptin signaling pathway from POMC neurons to KNDy neurons in controlling reproduction. This study showed that the stimulation of central α-MSH signaling robustly activated the reproductive axis in pubertal rats, whereas chronic inhibition of MC3/4R delayed puberty (Manfredi-Lozano et al. 2016). Despite all this evidence, the relationship between metabolic circuitry and KNDy cells is not well established. In this study, we present clinical and human genetic evidence for the involvement of a potentially critical gene (NHLH2) to represent the

![Fig. 3 Transactivation of Human KISS1 promoter by WT NHLH2 and Mutant NHLH2. HEK293 cells were transfected with empty vector (n:8), WT NHLH2 (n:8), or mutant NHLH2 R79C (n:7), A9L (n:5), V31M (n:8) along with the human KISS1 promoter reporter plasmids. Bars represent mean ± SEM. Statistically significantly differences are shown with different letters in the figure: Empty vector vs WT: (p = 0.0003), WT vs R79C: (p = 0.04), WT vs A9L: (p = 0.01), WT vs V31M: (p = 0.06). One-Way ANOVA followed by Tukey test was used for statistical analysis.](image-url)
connection between metabolic circuitry and pubertal development. We identified inactivating rare sequence variants in *NHLH2* in patients who have both IHH and late-onset obesity. These findings remarkably complement those of the *Nhlh2* knock-out mice (N2KO), which we have created and characterized over the past two decades (Good et al. 1997). The N2KO mouse, similar to its human counterpart, features both hypogonadotropic hypogonadism (IH) and adult-onset obesity (Good et al. 1997). Notably, two independent research groups confirmed the HH plus late-onset obesity phenotype in the N2KO mice (Cigliati et al. 2007; Krüger et al. 2004). Two recent mouse models have provided support to our original whole-body knock-out model. Leon et al. have reported that in *vivo* ablation of *Nhlh2* from Kiss1 neurons using *Kiss1*-cre;*Nhlh2*fl/fl mice induced a male-specific delay in puberty onset and subfertility along with milder reproductive changes in the female mice. That model also showed impaired response to leptin, among other metabolic changes (Leon et al. 2021). In the other study, the investigators created an Nhlh2 overexpression model by the lentivirus injection into the hypothalami of mice. NHLH2 overexpression resulted in the prevention of the development of obesity on a high-fat diet (Carraro et al. 2021).

Case 1 and 2 in this study suffer from congenital cryptorchidism, suggesting severe congenital hypogonadism. As the fetal testicular descent and phallus growth are androgen-dependent, consequently, male infants with severe IHH present with cryptorchidism and/or microphallus. These observations suggest that with NHLH2 deficiency, the hypophysis of the HPG axis starts in utero. Microphallus and reduced anal-genital length as the first notable phenotypes in the N2KO mice are also consistent with this observation (Good et al. 1997). Both males and female N2KO mice fail to exhibit normal sex behavior, and they are severely subfertile. Female N2KO mice have abnormally long estrous cycles with an extension of proestrus and reduction of time in estrous (Good and Braun 2013). Overall, in the reproductive sense, males are notably more severely affected in the N2KO mice. Consistent with this observation, the patients presented here and the one (p.A83P) partially described in the literature are all males despite NHLH2 being on an autosome, chromosome 1. As noted above, the recent Kiss1 neuron-only ablation of Nhlh2 featured male-specific delay in puberty onset (Leon et al. 2021). This sex predilection with NHLH2 has been repeatedly observed in a phenotypically similar condition: Prader Willi Syndrome (PWS), which is also characterized by IH and obesity. In PWS, IHH is present in most males (Noordam et al. 2021). Whereas this condition is less apparent in girls since breast development occurs in almost all cases, although menarche is usually unattained. Furthermore, up to date, there are no reports of fertility by PWS men, while several pregnancies in women with PWS were noted (Noordam et al. 2021). Nhlh2 is intimately implicated in the pathogenesis of PWS. NHLH2 and PC1 are the two most severely reduced proteins in the stem cells from patients with PWS due to a minimal deletion encompassing SNORD116, a non-coding RNA gene within the PWS deleted/causative region (Burnett et al. 2017). Like the cases with *NHLH2* variants, in PWS too, males are reproductively more severely affected. Furthermore, we have very recently reported that Nhlh2 is post-transcriptionally regulated by Snord116, the minimal critical gene for PWS (Kocher et al. 2021). We have shown that higher expression of Snord116 leads to more stable Nhlh2 mRNA and subsequently a higher level of translated Nhlh2, which is expected to prevent PWS phenotype (Kocher et al. 2021). NHLH2 has already been suggested to control transcription of many genes, thus a broader role in the energy balance (Polex-Wolf et al. 2017). Taken together, these results may solidify the role of Nhlh2 in the pathogenesis of PWS.

As elaborated above, the absence of both IHH and obesity in the heterozygous mother and sister from Family 2 can be explained by male favoritism in NHLH2 phenotypes. Alternatively, reduced penetrance, variable expression, oligogenic etiology (Sykiotis et al. 2010), and clinical reversibility (Sidhoum et al. 2014) are well-recognized features to complicate pedigrees in IHH. The segregation of the phenotype with the NHLH2 variant in the pedigree of Case 1 indicates an autosomal recessive inheritance. In all other three cases, an autosomal dominant inheritance is likely. Therefore, this syndrome seminally described here in humans may be either an autosomal recessive or a dominant disorder.

Unlike with the other gene mutations in the Leptin-POMC-melanocortin pathway, i.e. LEP, LEPR, POMC, and MC4R (where obesity has an early onset by six years of age), patients and mice with deficient NHLH2 develop obesity late after childhood (juvenile) stage. As elaborated below, NHLH2 is a transcription factor, regulating many different genes, and may have feedback loops for both orexigenic and anorexigenic pathways. Mouse studies indicate that Nhlh2 also controls exercise and energy expenditure pathways. These various associations may culminate in net temporal effects at different stages of life (Good and Braun 2013). We have previously shown that for both male and female mice N2KO, weight gain occurs after 7 weeks of age, becoming significant by 7 weeks of age in females and 10–12 weeks of age in males. However, reduced motivated physical activity is evident by 4 weeks of age in the N2KO (Good and Braun 2013). The natural history of obesity in the patients with NHLH2 variants again overlaps with that of N2KO mice. In both case 1 and 2, obesity started developing in early teen years and progressed over several years to severe obesity (BMI > 120% of the BMI at 95th percentile or BMI > 35 whichever is lower). Likewise, the BMI of the previously reported patient with p.A83P was > 36 (Ahituv et al. 2007). Current knowledge and new findings in this study regarding
energy balance-reproduction connection is schematized in Fig. 4. In this model, leptin is secreted by adipose tissue in proportion to fat mass, acts on neurons in the hypothalamus by binding its receptor, activating the STAT3 signaling pathway (Good and Braun 2013). Stat3 activity on the proximal NHLH2 promoter is required for both basal Nhlh2 expression and leptin induction of Nhlh2 gene transcription (Al Rayyan et al. 2014). Stat3 knock-out model also causes obesity and infertility, recapitulating the N2KO phenotype (Gao et al. 2004). The PCSK1 gene requires both Nhlh2 and Stat3 for its leptin-induced expression (Fox and Good 2008). PCSK1, in turn, encodes for the enzyme needed to process POMC to produce neuropeptides, most notably αMSH. The N2KO mice have a 50% reduction in αMSH, which is the ligand for MC4R (Jing et al. 2004). Mutations in MC4R are the most frequent cause of monogenic forms of human obesity. MC4R itself, too, has been shown to be a transcriptional target of Nhlh2 (Wankhade and Good 2011), and a 2 base-pair deletion which removes the NHLH2 E-box motif in the MC4R promoter has been found in a proband with early-onset obesity (Valli-Jaakola et al. 2006). For the sake of clarity, these interactions are depicted in Fig. 4 in three different cells; however, they are likely to be operative in the same hypothalamic ARC cell. Even beyond the leptin-melanocortin pathway, NHLH2 has already been suggested to control transcription of many other metabolically and reproductively important genes, thus further supporting its potential coordinator role. Specifically, NHLH2 directly controls transcription of the Necdin, another NHLH2 cluster gene. NHLH2 also mediates differentiation/migration of GnRH-1 neurons (Good and Braun 2013). Likewise, hypothalamic GnRH-1 content is reduced in adult Nhlh2(−/−) mice (Good and Braun 2013). Moreover, NPY, the cardinal orexigenic neurons, were reduced in the ARC but not in the PVN in Nhlh2−/− hypothalami (Good and Braun 2013). Together, these facts support NHLH2 for a coordinating role between metabolism and reproduction.

Setmelanotide, a melanocortin 4 receptor agonist, was recently FDA-approved for patients six years and older with obesity due to mutations in POMC, PCSK1, or LEPR (Clement et al. 2020). Considering the intimate role of NHLH2 in the Leptin, POMC, MC4R pathway as documented here and elsewhere, Setmelanotide can be a treatment option for patients with inactivating NHLH2 mutations.

In conclusion, this is the first time that inactivating human variants in NHLH2 have been reported in probands with both IHH and obesity. We have provided evidence that these variants lead to a reduction in Nhlh2 binding to Mc4r promoter and KISS1 transactivation. NHLH2 may therefore constitute a functional link between the metabolic circuitry and the GnRH pulse generator in the hypothalamus, with failure of this task by inactivating mutations resulting in late-onset obesity and absence of puberty (thus infertility) in both mice and humans.

Fig. 4 The interface between metabolism and reproduction in the hypothalamic arcuate nucleus. The diagram depicts the relationships among various gene products in the leptin-melanocortin-kisspeptin pathway.

*NHLH2 promoting the MC4R transcription may also be operative in the Kisspeptin/KNDy neuron.
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Declarations

Conflict of interest The authors declare no competing interests.

Ethics approval This study was performed in line with the principles of the Declaration of Helsinki. The study was approved by the Ethics Committee of the Cukurova University Faculty of Medicine (Approval no: 82 Nov 2, 2018) and by the institutional review board of the University of Mississippi Medical Center (Approval no: 2017-0238, Jan 27, 2018).

Informed consent to participate All individuals and/or their legal guardians provided written informed consent.

Data availability The functional studies data that support the findings of this study are available on reasonable request from the corresponding author. The significant genetics variant data that support the findings of this study are available on reasonable request from the corresponding author. The raw human genetics data are not publicly available due to consent form restrictions.

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