The histone H3-lysine 4-methyltransferase Mll4 regulates the development of growth hormone-releasing hormone-producing neurons in the mouse hypothalamus

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In humans, inactivating mutations in MLL4, which encodes a histone H3-lysine 4-methyltransferase, lead to Kabuki syndrome (KS). While dwarfism is a cardinal feature of KS, the underlying etiology remains unclear. Here we report that Mll4 regulates the development of growth hormone-releasing hormone (GHRH)-producing neurons in the mouse hypothalamus. Our two Mll4 mutant mouse models exhibit dwarfism phenotype and impairment of the developmental programs for GHRH-neurons. Our ChIP-seq analysis reveals that, in the developing mouse hypothalamus, Mll4 interacts with the transcription factor Nrf1 to trigger the expression of GHRH-neuronal genes. Interestingly, the deficiency of Mll4 results in a marked reduction of histone marks of active transcription, while treatment with the histone deacetylase inhibitor AR-42 rescues the histone mark signature and restores GHRH-neuronal production in Mll4 mutant mice. Our results suggest that the developmental dysregulation of Mll4-directed epigenetic control of transcription plays a role in the development of GHRH-neurons and dwarfism phenotype in mice.
abuki syndrome (KS), caused by haploinsufficiency of MLL4 or UTX, is a human developmental disorder that affects multiple tissues. One of the consistent cardinal features of KS is stunted growth and postnatal short stature. For instance, a report on growth data in 39 KS patients revealed that postnatal growth retardation is a clinical feature in all cases. To date, however, the molecular etiology underlying stunted growth and short stature in KS patients remains ambiguous. The postnatal growth is controlled by the hypothalamus–pituitary gland–liver axis. First, growth hormone-releasing hormone (GHRH)-neurons in the hypothalamus release GHRH, which then stimulates secretion of the growth hormone (GH) from the pituitary gland. GH, in turn, induces the expression of insulin-like growth factor 1 (IGF1) in the liver, which controls bone epiphyseal growth plates development, and glucose homeostasis.

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KISS1-neurons are one of tyrosine hydroxylase (Th) neurons that include GHRH neurons, but only in ~22% of Npy-GFP mice that express GFP in AgRP-neurons. Together, our study revealed that the deficiency of Mll4-directed epigenetic control of GHRH-neurons results in a severe reduction of GHRH neurons and dwarfism in the mouse, providing crucial insights into the molecular etiology underlying dwarfism in human KS patients.

Results

Mll4+/− mice exhibit dwarfism and the reduction of GHRH neurons. To test if Mll4 haploinsufficiency leads to dwarfism in mice as it does in human, we monitored the growth of Mll4+/− mice, in which a single Mll4 allele is inactivated. Mll4+/− mice showed a significant reduction in body weight, linear length, and DNA levels for Ifg1 in the liver (Fig. 1a, b, Supplementary Fig. 1a), indicating that Mll4+/− mice have a deficit in GH signaling and faithfully recapitulate one of the cardinal features of KS, postnatal growth deficiency.

To test the role of Mll4 in hypothalamic GHRH neurons, we monitored the expression pattern of Mll4 in the ARC. GHRH neurons are one of tyrosine hydroxylase (Th) neurons in the ARC. Interestingly, Mll4 was expressed in ~88% of Th+ neurons that include GHRH neurons, but only in ~22% of Npy+ AgRP-neurons at postnatal day 33 (P33) in Npy-Gfp reporter mice that express Gfp in AgRP-neurons (Fig. 1c). In contrast, Mll4 was expressed in ~59% of Pomp+ cells in P65 Pomc-Gfp reporter mice. These results indicate that most GHRH-neurons express Mll4, whereas only a subset of AgRP- and Pomc-neurons express Mll4.

Next, we tested if the number of GHRH-, AgRP-, and Pomc-neurons changes in Mll4+/− mice using a panel of markers. GHRH-neurons significantly reduced, as determined by GHRH-neuronal markers, Ghrh, Th, and Dlx1, in Mll4+/− mice relative to littermate WT control mice (Fig. 1e). In contrast, the number of Pomc-neurons did not change and AgRP-neurons mildly increased in Mll4+/− mice (Fig. 1e). Our data indicate that the generation or survival of GHRH-neurons is impaired in Mll4+/− mice, and also suggest that the deficiency of GHRH-neurons is
likely a strong contributing factor to the dwarfism in Mll4 haploinsufficiency.

The inactivation of Mll4 in the developing hypothalamus leads to a drastic reduction of GHRH neurons and dwarfism. To test if Mll4 plays a cell-autonomous role in GHRH-neuronal development, we generated Mll4 conditional knockout (Mll4-cKO) mice, in which Mll4 was deleted in the embryonic ARC, by crossing Mll4<sup>f/f</sup> and Nkx2-1-Cre mice<sup>21,31</sup>. In Mll4-cKO mice, Mll4 expression was largely eliminated in the developing ARC, but not in surrounding tissues, by embryonic day (E) 12.5
and their littermate control mice (Fig. 2b, c), indicating that the development of GHRH-neurons was impaired in the absence of Mll4. Mll4-cKO mice also showed a striking reduction of GHRH-neurons at P65 (Fig. 2b, c), indicating that the developmental defects of GHRH-neurons in Mll4-cKO embryos were not recovered at adult stage. In contrast to GHRH-neurons, the number of AgRP-neurons did not significantly change in Mll4-cKO mice (Fig. 2d).

We also tested the marker for other hypothalamic neurons: Trh, a marker for the dorsomedial hypothalamus that does not express Nkx2-1, and Sf1, a marker for a subregion of the ventromedial hypothalamus that expresses Nkx2-1. Neither Sf1+ nor Trh+ cells were significantly altered in their numbers in Mll4-cKO mice (Supplementary Fig. 2), highlighting a relatively selective loss of GHRH-neurons in the Mll4-deficient hypothalamus.

To determine if the mere expression of Ghrh gene alone or the developmental program for GHRH-neurons was disrupted in Mll4-cKO mice, we examined the expression pattern of transcription factors that are crucial to drive GHRH-neuronal development. We have previously reported that Dlx1 and its transcription factors that are crucial to drive GHRH-neuronal developmental program for GHRH-neurons was disrupted in Mll4-cKO mice, which include GHRH-neurons (Fig. 2e), but the levels of Isl1 and Mash1 did not significantly change in Mll4-cKO mice (Fig. 2f).

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Next, we examined if Mll4-cKO mice exhibit any growth phenotypes, reflecting the developmental deficiency of GHRH-neurons. Mll4-cKO mice showed a marked reduction of both body weight and linear length (Fig. 3a–c). Furthermore, serum glucose levels and hepatic IGF1 mRNA levels drastically decreased in Mll4-cKO mice (Fig. 3d, e), indicating deficits in the signaling of GH, downstream of GHRH. Milder but similar results were also obtained with Mll4-cHET (for conditional heterozygous knockout) mice (Supplementary Fig. 1b).

Together, our results establish that Mll4 is required for the proper generation of GHRH-neurons in the developing hypothalamus. Our data also clearly link the stunted growth of Mll4 mutant mice to the impaired development of GHRH-neurons.

Nrf1 is a major partner transcription factor of Mll4 in developing hypothalamus. To determine the molecular mechanism by which Mll4 directs the development of GHRH-neurons, we defined genome-wide Mll4-binding loci in the developing hypothalamus by performing ChIP-seq in the mouse hypothalamus at E15, a time point when GHRH-neurons are being actively specified, with the ChIP-seq quality Mll4 antibody that we developed. Our ChIP-seq analyses identified 2541 Mll4-bound ChIP-seq peaks (p < 0.001, FDR < 10%). Interestingly, ~85.5% of Mll4 ChIP-seq peaks were located in the promoters and gene bodies and only ~14.5% of Mll4-bound peaks were found in the intragenic regions in the developing hypothalamus (Fig. 4a). This Mll4-occupancy pattern differs from the previous finding that Mll4 is mainly enriched in the intragenic enhancer regions in the adipocytes.

To test further the role of Nrf1 in Mll4-directed gene regulation in the developing hypothalamus, we performed de novo motif analyses on the top 300 Mll4 ChIP-seq peaks using the algorithms MEME, DREME, and TOMTOM. Intriguingly, a single motif, which resembles the consensus binding site for the transcription factor Nrf1, was found in ~92% of the top 300 Mll4 ChIP-seq peaks (Fig. 4b). The Nrf1 motif was enriched in the summit of Mll4 ChIP-seq peaks (Fig. 4c). This genome-wide analyses indicate that Nrf1 is primarily responsible for recruiting Mll4 to most of Mll4-target loci in E15 developing hypothalamus.

Notably, Mll4 and Nrf1 co-occupied the promoter of the Nrf1 gene itself (Fig. 4i), raising the possibility that Nrf1 auto-regulates its own transcription by recruiting Mll4 as a transcriptional coactivator. Indeed, the ectopic expression of Nrf1 enhanced the transcriptional activity of the Nrf1/Mll4-binding region in the Nrf1 gene in HEK293 cells, as monitored using luciferase reporter assays (Fig. 4j). The expression of Nrf1 was severely reduced in the ARC region of Mll4-cKO mice relative to their littermate controls at E12.5 and P0 (Fig. 4k), supporting the notion that Mll4 induces the expression of Nrf1 in the developing hypothalamus (Fig. 4g). Further supporting this notion, Mll4 associated with Nrf1 in HEK293 cells, as determined by co-immunoprecipitation (coIP) assays (Fig. 4h).

Nrf1 mediates the recruitment of Mll4 to a majority of Mll4-target genes in the developing hypothalamus (Fig. 4g). Further supporting this notion, Mll4 associated with Nrf1 in HEK293 cells, as determined by co-immunoprecipitation (coIP) assays (Fig. 4h).

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and Nrf1 ChIP-seq datasets with our scRNA-seq analyses of E15 ARC neurons, which revealed 83 genes that are most specifically enriched in developing GHRH-neurons relative to other ARC neuronal types. Among the 83 genes, 7 genes recruit both Mll4 and Nrf1 to the same loci (Fig. 5a, b, Supplementary Fig. 3), suggesting that Nrf1 mobilizes Mll4 to these 7 genes thereby activating their expression (Fig. 4l). These Nrf1-Mll4-co-occupancy genes include two transcription factor genes Prox1 and Pbx3 and five non-transcription factor genes, Vat1, Flywch2, Plekhi1, Tmem200a, and Them6 (Fig. 5a, b, Supplementary Fig. 3). Among the 83 genes, 24 genes including the transcription factor gene Egr1, are associated with the genomic regions that
recruit only Nrf1, not Mll4, thus representing potential ‘indirect’ Mll4-target genes (Fig. 4l). Interestingly, Mll4 binds to the promoter/5′UTR of the Dlx1 gene, but Nrf1 does not (Fig. 5a, b), suggesting that Mll4 induces Dlx1 expression in the developing hypothalamus by collaborating with other transcription factor.

Overall, our systematic bioinformatic analyses revealed several ‘direct’ and ‘indirect’ target genes of Mll4 in the developing GHRH-neurons. Notably, these Mll4-target genes include Dlx1, Prox1, Egr1, and Pbx3, which have been shown or proposed to play a role in the development of GHRH-neurons10,34.

To validate the ChIP-seq data, we performed independent ChIP assays in E15 hypothalamus using antibodies against Mll4 and Nrf1. Consistent with ChIP-seq data, both Mll4 and Nrf1 were recruited to the common ChIP-seq peak regions for Mll4 and Nrf1 regions annotated to Prox1, Vat1, Flywch2, and Pbx3, but not to the negative control genomic region Untr6 (for Untranslated region on chromosome 6)42 (Fig. 5c). Also, Nrf1 bound to ChIP-seq peak regions in Resp18 and Egr1, and Mll4 bound to Dlx1 (Fig. 5c).

To test if Nrf1/Mll4-bound genomic genes act as enhancers responding to Nrf1 and Mll4, we generated the luciferase reporters linked to Nrf1-Mll4-co-occupancy regions annotated to Vat1 and Flywch2, named Vat1::Luc and Flywch2::Luc. Both luciferase reporters were activated by Nrf1 in a dose-dependent manner and suppressed by Mll4 knockdown with shRNA against Mll4 in HEK293 cells (Fig. 5d), suggesting that...
Nrf1 and Mll4 enhance the transcription activity of their binding regions in cells.

We predicted that the expression of the direct and indirect Mll4-target genes in mouse developing GHRH-neurons would be downregulated in the absence of Mll4. To test this idea, we examined the expression levels of potential Mll4-target genes in the ARC of Mll4-cKO mice using in situ hybridization (ISH) and immunohistochemistry (IHC) analyses. Mll4-cKO mice showed significant downregulation of all tested target genes, such as Pbx3 and PlekhG1, which are annotated to Nrf1-Mll4-co-occupancy regions (i.e., direct Mll4-target genes), and Pik3r1 and Resp18, which are associated with Nrf1 peak but not Mll4 peak.
(i.e., indirect Mll4-target genes) (Fig. 6a). While Dlx1, Prox1, and Egr1 are highly and significantly enriched in developing GHRH-neurons, they are also expressed in other ARC neuronal types, such as the expression of Dlx1 in developing n8/n9 TH-neurons\(^1\). Thus, to monitor the expression of Dlx1, Prox1, and Egr1 in developing GHRH-neurons, we performed the double immunofluorescence staining with a combination of antibodies against Dlx1 and Prox1 or Egr1. Dlx1/Prox1- and Dlx1/Egr1 double-positive cells in the ARC were markedly reduced in the ARC of Mll4-cKO mice relative to their littermate controls (Fig. 6b), indicating the downregulation of these transcription factors in GHRH-neuronal lineage and the impaired transcription program directing the development of GHRH-neurons.

Together, these studies strongly suggest that Mll4 governs GHRH-neuronal development by inducing transcription program crucial for GHRH-neuronal differentiation and also by upregulating non-transcription factor genes that are relatively specifically enriched in developing GHRH-neurons.

**Mll4 directs GHRH-neuronal development via its epigenetic regulatory activity.** Next, to ask if Mll4 triggers the transcriptional activation of its target genes in the GHRH-neuronal lineage via its epigenetic regulatory activity that induces transcriptionally active chromatin landscape, we sought for the method to trigger transcriptionally active chromatin, marked by H3K27ac, in Mll4-deficient mice. Given that the HDAC inhibitor AR-42 restored the active chromatin in the hippocampus and also rescued the hippocampal memory defects in Mll4\(^{-/-}\) mice, we employed AR-42. We performed daily intraperitoneal injection of 50 mg/kg of AR-42 into pregnant dams from 9 days after a vaginal plug detection until harvesting the embryos (Fig. 7a). We first monitored the active chromatin marks at E12.5. H3K4me1 and H3K27ac levels in Mll4-cKO embryos (Fig. 7b, Supplementary Fig. 4b), indicating that deficiency of GHRH-neuronal developmental program in Mll4-null hypothalamus was at least partially repaired by AR-42 treatment. Collectively, these data suggest that the restored active chromatin landscapes by AR-42 led to the partial rescue of GHRH-neuronal development in Mll4-cKO mice.

**Discussion**

Mutations in *MLLA* result in human developmental disorder KS, whose hallmarks include dwarfism\(^1\). Providing important insights into the etiology for the dwarfism in KS, our studies uncovered that Mll4 governs the development of mouse GHRH-neurons during hypothalamic development by establishing transcriptionally active chromatin landscapes in collaboration with Nrf1 and other partner transcription factors (Fig. 8). First, the two distinct mouse models, *Mll4*\(^{+/-}\) mice, which mimic the haploinsufficiency for *Mll4* in KS, and *Mll4*-cKO mice, in which *Mll4* was inactivated in the developing hypothalamus, showed impaired GHRH-neuronal development, reduced hepatic *Igf1* levels, and stunted growth. Second, our comprehensive genome-wide ChIP-seq studies for Mll4 and Nrf1 uncovered that Mll4 partners mainly with Nrf1 to activate the expression of GHRH-neuronal genes in the developing hypothalamus. Notably, Mll4, and Nrf1 collaborate to trigger the expression of the *Nrf1* gene itself, suggesting that Mll4 indirectly controls Nrf1 alone-occupied genes via increasing Nrf1 levels (Fig. 8). Our studies also suggest that Mll4 may cooperate with other transcription factors. For instance, Mll4 may be recruited to the promoter/5' UTR region of *Dlx1* by an unknown partner transcription factor. Last, the HDAC inhibitor AR-42 restored the active chromatin marks, followed by a partially rescued GHRH-neuronal developmental program, in the ARC of Mll4-cKO mice.

*Mll4*-cKO mice showed not only a reduced number of *Ghrh* expressing cells but also a downregulation of many genes highly enriched in developing GHRH-neurons\(^1\), indicating that Mll4 inactivation led to impaired GHRH-neuronal development, rather than just downregulation of the *Ghrh* gene. These GHRH-neuronal genes include transcription factor genes *Dlx1*, *Egr1*, *Pbx3*, and *Prox1*, which are directly bound and controlled by Mll4 according to our ChIP-seq and subsequent analyses. Given that Dlx1 is crucial for GHRH-neuron generation and other transcription factors are candidates for key regulators of GHRH-neuronal development\(^1,34\), we propose that Mll4 activates the central gene regulatory network for GHRH-neuronal development, thus serving as a primary epigenetic regulator for GHRH-neuronal production (Fig. 8).

The findings that KS patients respond well to recombinant human GH in catch-up growth\(^5\) indicate that KS patients have an intact GH signaling pathway. These studies imply that the
**Fig. 5 Target genes of MII4 in developing mouse GHRH neurons.**

**a** List of genes in developing mouse GHRH-neurons, which are associated with either MII4 or Nrf1 ChIP-seq peaks or both MII4 and Nrf1 ChIP-seq peaks. Genes encoding transcription factors are highlighted in blue.

**b** MII4 and Nrf1 ChIP-seq peaks for representative target genes of MII4 in developing GHRH-neurons. The location of each peak with regard to its associated gene is marked by an arrow for the direction of the gene and a square for the 5' UTR (both in blue).

**c** Independent validation ChIP experiments with E15 hypothalamus and IgG (controls) and antibodies against MII4 and Nrf1 reveal recruitment of MII4 and Nrf1 to representative target genes of MII4. Column bars represent mean of three independent experiments, error bars indicate the SD.

**d** Luciferase reporter assays reveal that the common ChIP-seq peak areas for MII4 and Nrf1 in Vat1 and Flywch2 are responsive to ectopic expression of Nrf1 and shRNA against MII4. Column bars represent mean of three independent experiments, error bars indicate the SEM. Statistical differences were determined by two-sided Student’s t-test; not significant (ns), *p < 0.05, **p < 0.01, ***p < 0.001, and ****p < 0.0001.
Fig. 6 Mll4-cKO show reduced expression of Mll4-target genes in developing GHRH-neurons. a ISH analyses of expression of Mll4-target genes in Mll4-cKO and their littermate controls at indicated stages. b IHC analyses of coexpression of transcription factors whose genes are targeted by Mll4; the number of Dlx1 only, Prox1 only, and Dlx1/Prox1 double-positive cells at E17.5, and the number of Dlx1 only, Egr1 only, and Dlx1/Egr1 double-positive cells at P0 in Mll4-cKO and controls. The number of mice used is as indicated below each genotype in parenthesis (a, b). The location of ISH/IHC images is schematically shown (a), which applies to all images in (a, b). Statistical differences were determined by two-sided Student’s t-test; *p < 0.05, **p < 0.01, and ***p < 0.001. Column bars represent mean, error bars indicate the SD. Scale bars, 100 µm.
The hypothalamus–pituitary axis producing GH is defective in KS. While our studies demonstrate an essential role of Mll4 for the generation of GHRH neurons, it is possible that Mll4 plays an additional role in GH production in the pituitary. In this regard, it is noteworthy that Nkx2-1 regulates GH and prolactin transcription in the rat pituitary. Notably, Nkx2-1 is also expressed in other cell types of the hypothalamus as well as forebrain and lung. Therefore, we do not exclude the possibility that the dwarfism observed with our Mll4-cKO mice may also involve deletion of Mll4 in Nkx2-1+ non-GHRH-neuronal populations in the hypothalamus or Nkx2-1+ cell types in the pituitary, forebrain or lung, either directly or indirectly. In particular, further studies are needed to investigate the role of Mll4 in the development and function of the pituitary, particularly regarding the pituitary production of GH.

The hippocampal memory defects of Mll4+/− mice can be rescued by the HDAC inhibitor AR-42 or a ketogenic diet that increases the endogenous HDAC inhibitor β-hydroxybutyrate. Both methods
robustly increased H3K27ac levels\(^ {43,47}\). Here, we discovered that AR-42 also rescued the expression of Ghrh and other GHRH-neuronal genes impaired in the ARC of Mll4-cKO embryos. Notably, we wished to further test if the restored expression of Ghrh by AR-42 also reverses the postnatal stunted growth of Mll4-cKO mice, but AR-42-treated pups showed perinatal lethality to severe developmental defects, consistent with the reported embryonic lethality of knockout mouse models for several Hdac genes\(^ {48}\). Interestingly, we found that both H3K27ac and H3K4me1 increased upon AR-42 treatment in Mll4-cKO mice, raising the question of which enzyme contributed to the induction of H3K4me1 in the absence of Mll4. The compensatory increase in H3, the paralog of Mll4, in the ARC region of Mll4-cKO mice (Supplementary Fig. 4c) may have been responsible for the increased H3K4me1 levels following AR-42 treatment in Mll4-cKO mice, which warrants future studies. Given that Mll3 mutation has not been linked to KS or dwarfishism in human, Mll3 is unlikely to function redundantly with Mll4 in the hypothalamic at least under normal condition.

Our results also highlight the intimate link between the two enhancer marks H3K27ac and H3K4me1/2. Mll4 has been shown to play a role to recruit H3K27-acyltransferases p300 and CBP to its target genes\(^ {24-26}\). Combined with these previous findings, our study suggests that Mll4 orchestrates the establishment of transcriptionally active chromatin landscape. It remains to be addressed whether Mll4-directed H3K4me1/2 modification plays an active role in gene induction or simply serves as a co-occurring mark with H3K27ac. In this regard, it will be interesting to test whether the rescue of GHRH-neuronal gene expression requires a restoration of levels of H3K27ac alone or both H3K27ac and H3K4me1/2. To this end, the chemical inhibitors of the H3K4-methyltransferase activity of Mll4 and the H3K27-acetyltransferase activity of p300 can be explored. Such inhibitors exist for p300 (ref. \(^{19}\)), but not for Mll4 yet. Overall, our results suggest that the missing epigenetic regulatory activity of Mll4 is the main driver for the loss of GHRH-neurons in Mll4-cKO mice, and further provide the proof-of-concept that Mll4 can be a feasible target to develop epigenetic therapeutics for KS.

In summary, by combining mouse genetics, genome-wide studies, and pharmacological approaches, we demonstrated that Mll4 directs the development of mouse GHRH-neurons via its ability to modulate H3K4me1/2 and H3K27ac levels on its target genes during hypothalamus development. We also found Nrf1 as the major partner transcription factor of Mll4 in directing the development of mouse GHRH-neurons. These results strongly suggest that the dysregulation of Mll4-directed epigenetic regulation of GHRH-neuronal genes is likely a molecular etiology underlying the dwarfishism in human KS patients.

Methods

Mouse work. All mice were maintained on a normal 12 h light, 12 h dark cycle with ad libitum access to chow and water, unless otherwise noted. Mll4\(^ {+/−}\), Mll4\(^ {−/−}\), Pomp-Gfp, Npy-Gfp, and Nkx2-1-Cre mice have been described previously\(^ {11,27,29-31}\). Mll4\(^ {+/−}\) mice were crossed with Mll4\(^ {+/−}\):Nkx2-1-Cre mice to generate Mll4\(^ {−/−}\):Nkx2-1Cre mice (Mll4-cKO mice). Pregnant mice were intraperitoneally injected with AR-42 (30 mg/kg, purchased from Selleck Chemicals) or vehicle (0.5% methylcellulose, 0.1% Tween-80, water) from 9 days after detecting a vaginal plug till E12.5 or E17 for analyses. All studies were approved by the Institutional Animal Care & Use Committee of University at Buffalo and Oregon Health & Science University.

Cell culture and luciferase assay. HEK293 cells were obtained from ATCC and cultured in DMEM supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Thermo Scientific), penicillin/streptomycin, and 1-glutamine (both Lonza). For luciferase assays, cells were seeded into 48-well plates and transfected with Nrf1-, Vat1-, or Flywch2-luciferase reporters and expression vectors for Nrf1 or Dlx1 or control shRNA or sh-Mll4 (ref. \(^{17}\)) using SuperFect (Qiagen) according to the manufacturer’s instructions. Two days after transfection, luciferase activity was measured. The actin-β-galactosidase plasmid was cotransfected for normalization of the luciferase activities to transfection efficiency. Data were shown in relative luciferase units (mean ± SEM).

ColIP. For colIP, HEK293 cells were transfected with expression vector for Flag-Nrf1 using calcium phosphate transfection method. Transfected HEK293 cells were subsequently dissolved in RIPA buffer and rotated for 2 h at 4 °C. After centrifugation, the lysate was subjected to immunoprecipitation using 1 mg of Mll4 antibody o/n, followed by 2 h incubation with Protein A/agarose beads (Thermo Fisher Scientific). Beads were then dissolved in SDS loading buffer and separated on SDS-PAGE and transferred to nitrocellulose membranes. The membranes were blocked in Odyssey blocking buffer, and incubated with primary antibodies against Flag tag and incubated o/n. The blots were stripped with secondary antibodies, and fluorescence was detected with the Odyssey System (LI-COR), as shown in Fig. 4h (the un cropped original image in Supplementary Fig. 5).

RNA isolation and quantitative RT-PCR. Total RNA was isolated with Trizol and converted to cDNA with a RevertAid kit (Thermo Scientific). Twenty nanograms of cDNA was used for qPCR with the ViaA7 platform and primers for amplification of Ifg1 and Gcupdh. Ifg1 mRNA expression values were normalized to Gcupdh using the ΔΔCt method. Relative or fold expression levels were calculated from three individual experimental replicates.

ChIP and ChIP-seq. ChIP on E15.5 hypothalamus was performed using our homemade Mll4 and Nrf1 antibodies (ref. \(^{37}\) and Supplementary Fig. 6). Hypothalamus were dissected out, crosslinked for 10 min with PFA, and then quenched by 2× Tris-EDTA. Chromatin was then sonicated in 200–400 bp fragments. Chromatin was then 10× diluted in Chep dilution buffer, and, for immunoclearing, incubated with IgG and protein A agarose beads for 1 h. ChIP antibodies were added for 6 h incubation followed by 1 h of incubation with Protein A agarose beads. Then, the beads were washed with RIPA buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, pH 8.0, 150 mM NaCl), high salt buffer (same components as in TSE, except 500 mM NaCl).
ISH and IHC. Embryonic and P0 brains were removed and fixed in 4% PFA, cryoprotected with sucrose gradients, and frozen in OCT blocks, followed by sectioning using a microtome with a thickness of 12 μm per section. P33 and P65 mice were intraperitoneally injected with Avertin before performing standard perfusion with PBS and 4% PFA, followed by fixation in 4% PFA for 6h. DNA was subsequently isolated and subject to ChIP-seq.3,21

Sections 84

Quantification and statistical analyses. For quantification of ISH/IHC images, serial sectioning was performed on embryos/mouse brain with the distance between sections 84–216 μm. One slide from each mouse that contains matched sections was used to compare controls and mutants. Zeiss Axioplan 2 imaging 2 with apotome was used to image ISH and IHC results. Integrated density measurement in image J software was used to analyze densitometry. For cell counting, specifically immunostained cells in the arcuate nucleus were counted. Quantifications were done by analyzing three rostral to caudal sections for each embryo/mouse and at least three embryos/mice per each experimental group. Statistical differences were determined by two-tailed Student’s t-test. Statistical significance is displayed as follows: ns for not significant, *p < 0.05, **p < 0.01, ***p < 0.001, and ****p < 0.0001.

Data availability

All data supporting the findings of this study are provided within the paper and its supplementary information. A source data file is provided with this paper. The ChIP-seq dataset has been deposited in GEO (GSE149439). All additional information will be made available upon reasonable request to the authors. Source data are provided with this paper.

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