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Melanocortin 4 Receptor is a Transcriptional Target of Nescient Helix-Loop-Helix-2

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Abstract

Melanocortin 4 receptor (*Mc4r/MC4R*) is a G-Protein coupled receptor that is expressed in the hypothalamus and implicated in body weight control. Mutations in *MC4R* are the most frequent cause of monogenetic forms of human obesity. Despite its importance, the *MC4R* signaling pathways and transcriptional regulation underlying the melanocortin pathway are far from being fully understood. The transcription factor Nescient Helix-Loop-Helix 2 (*Nhlh2*) influences the melanocortin pathway through transcriptional regulation of prohormone convertase I, which influences the production of melanocortin peptides. In the present study, *Nhlh2*'s role as a transcriptional regulator of *Mc4r* has been demonstrated. *Nhlh2* knockout mice have reduced hypothalamic expression of *Mc4r* mRNA, suggesting that it could be a direct or indirect transcriptional regulator of the *Mc4r* promoter. To demonstrate direct transcriptional regulation, chromatin immunoprecipitation and electrophoretic gel shift assays show that *Nhlh2* binds to the E-Boxes located at -551, -366 and +54 on the *Mc4r* promoter. Leptin-induced transactivation of the *Mc4r* promoter is significantly higher in the presence of exogenously added *Nhlh2*. SiRNA knockdown of *Nhlh2* leads to significantly reduced endogenous *Mc4r* mRNA expression levels in N29/2 cell line. Transactivation using promoters with mutations in each of the E-Boxes results in significantly reduced transactivation efficiency compared to the WT *Mc4r* promoter, suggesting that *Nhlh2* regulates *Mc4r* transcription through these sites. Findings from these studies, combined with previous work implicating *Nhlh2* as a transcriptional regulator of both the *Mc4r* gene and the melanocortin pathway, suggest that *Nhlh2*'s transcriptional activity directly influences the human and rodent body weight control pathways.

Keywords

Nhlh2; *Mc4r*; hypothalamus; E-box; obesity; leptin

1. INTRODUCTION

The prevalence of obesity is 32.2% among adult men and 35.5% among adult women in the United States, which is significantly higher than just one decade ago (Flegal et al., 2010).

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While changes in food choices and reduced physical activity are the two important factors leading to today's increasing rate of obesity (Kopelman, 2000), it is crucial to consider the role of genetics in controlling body weight and energy homeostasis, especially in regard to the influence genetics can have on obesity. Obesity-related traits such as Body Mass Index (BMI), fat mass and leptin levels are 40–70% heritable (Allison et al., 1994, Comuzzie et al., 1995, Comuzzie et al., 1996). Monogenic forms of obesity caused by polymorphism(s)/ mutation(s) in genes such as carboxypeptidase E, Leptin, Leptin receptor, Tubby, melanocortin 4 receptor (*MC4R*) and Proopiomelanocortin (POMC) leads to impaired energy homeostasis, which dramatically impair body weight regulation (Geffroy et al., 1995, Hall et al., 1993, Huszar et al., 1997, Kleyn et al., 1996, Mountjoy and Wong, 1997, Prochazka and Leiter, 1991, Tartaglia et al., 1995). Considering the importance of genes in transmitting traits to the next generation, detailed studies of the biological consequences of genetic polymorphisms are necessary to better understand the molecular physiology of obesity.

Mutations in the *MC4R* gene constitute one of the most common causes of human monogenic obesity (Farooqi et al., 2000, Roth et al., 2009). In addition to mutations affecting the amino acid sequence of *MC4R*, several groups have identified polymorphisms in non-coding regions of the gene. Of those, four are known to be in the 5' promoter region (Lubrano-Berthelie et al., 2003), with two of these polymorphisms potentially affecting the binding site for basic Helix-Loop Helix (bHLH) transcription factors. The first is a Single Nucleotide Polymorphism (SNP) at in the *MC4R* promoter located at -178bp, which is found in both obese and non-obese controls at a frequency of 5% and changes E-Box #1 from CATCTG to AATCTG, effectively removing the E-box motif (Lubrano-Berthelie et al., 2003). The second mutation is a deletion, -20delGC (*MC4R*^{ΔGC}) which changes E-Box #2 from CAGCTG to CA(del)TG, again effectively removing the E-box motif (Valli-Jaakola et al., 2006). This deletion was found in two extremely obese yet unrelated children and their relatives (Valli-Jaakola et al., 2006). When first identified, the deletion was found to be within a perfect consensus site for *NHLH2*. It was then shown that indeed, human *NHLH2* could bind to that E-Box (Valli-Jaakola et al., 2006). However, transactivation differences between the normal and the promoter containing the 2bp deletion could not be demonstrated (Valli-Jaakola et al., 2006). Few studies have analyzed transcriptional activity of the normal human *MC4R* promoter. The only published study on mouse *Mc4r* regulation shows that the minimal promoter required for activity in neuronal and non-neuronal cells lines lies between -130 and +10 (Daniel et al., 2005) of the mouse gene.

Knockout mice provide a model for understanding the role that a single gene may play in the etiology of more complex human diseases. Deletion of Nescient helix-loop-helix 2 (*Nhlh2*) in mice (N2KO mice) results in adult-onset obesity with reduced exercise-associated energy expenditure (Coyle et al., 2002, Good et al., 1997). *Nhlh2* is a basic helix-loop-helix transcription factor that binds to the canonical E-Box motifs, CANNTG (Atchley and Fitch, 1997). *Nhlh2* is predominantly expressed in the paraventricular (PVN) and arcuate nucleus of the hypothalamus (Jing et al., 2004), where its expression is modulated by energy availability (Vella et al., 2007, Vella and Good, 2010). One of the gene regulatory targets of *Nhlh2* is the neuropeptide processing enzyme, prohormone convertase 1/3 (*PCKS1/Pcsk1*), a gene also implicated in the etiology of human obesity (Jackson et al., 1997). In response to leptin stimulation, *Nhlh2* forms a heterodimer with the signal transducer and activator of transcription 3 (*Stat3*) transcription factor, leading to increased *Pcsk1* expression in hypothalamic neurons (Fox and Good, 2008). N2KO mice have reduced expression of *Pcsk1* mRNA and the *POMC* neuropeptides that are normally processed by *Pcsk1*, including alpha melanocyte stimulating hormone (α -MSH) (Jing et al., 2004). α -MSH normally binds to the *Mc4r* receptor, thus, animals with a targeted deletion of *Nhlh2* have an overall defect in melanocortin signaling through transcriptional regulation of *Pcsk1* and reduced processing

of *POMC* neuropeptides. Recent work from our laboratory supports *Nhlh2*'s role in peripheral pathways, which are controlled through the melanocortin pathway (Wankhade et al., 2010).

We have been working to identify other possible targets of *Nhlh2*. The recently established role of *Nhlh2* in transcriptional regulation of *Pcsk1* (Fox and Good, 2008), earlier work from our laboratory explaining *Nhlh2*'s fluctuating expression level with energy availability (Vella et al., 2007), as well as the putative polymorphic *Nhlh2* binding site in the human *MC4R* promoter (Valli-Jaakola et al., 2006), substantiates the hypothesis that *Mc4r* may also be a direct transcriptional target of *Nhlh2*. While Valli-Jaakola and colleagues were not able to show transcriptional regulation in their system using HeLa cells, we believed that use of the *Nhlh2* knockout mice, and hypothalamic cell lines would verify the polymorphic site as an *Nhlh2* transactivation site. Our studies asked if *Nhlh2* is required for normal *Mc4r* regulation in response to leptin, and further, we whether *Nhlh2* regulates the *Mc4r* promoter. In extending the work of Valli-Jaakola, we examined all three putative E-box motifs for *Nhlh2*-mediated regulation of *Mc4r*.

2. MATERIAL AND METHODS

2.1. Experimental animals

The Institutional Animal Care and Use Committee (IACUC) at Virginia Polytechnic Institute and State University approved all animal protocols. Animal colony maintenance, breeding and genotyping have been described previously (Jing et al., 2004). N2KO and normal mice were maintained in 12 h light/12 h dark conditions with *ad libitum* access to food (4.5% crude fat) and water. Only male mice were used for all experiments in order to eliminate the need for estrous cycle analysis in female mice (WT N=12, N2KO N=10). All mice were euthanized by CO₂ asphyxiation at 13.00 h to standardize hormone and steroid levels that fluctuate hourly.

2.2. Quantitative real-time PCR (qRT-PCR) from whole hypothalamus

After euthanization, brains were isolated by dissection. A hypothalamic block was isolated using the Mouse Brain Matrix (Zivic Laboratories Inc., Pittsburgh, PA, USA) to properly dissect the hypothalamus. The brain segment was put into 4 M guanidine isothiocyanate buffer and homogenized. Samples were layered over 5.7 M cesium chloride buffer and spun for 18 h at 120,000 g at 20 °C. The supernatant was discarded and RNA was resuspended in DEPC water. RNA was then DNase treated. cDNA was created using reverse transcriptase in a magnesium buffer (Promega Corporation, Madison, WI, USA) for 1 h at 42 °C by using 1 µg of total RNA. Quantitative real-time PCR (qRT-PCR) for *Mc4r* expression in the hypothalamus was performed using Mouse *Mc4r* primers (TABLE 1). Mouse β -actin was used as a housekeeping control gene. QPCR was performed by using the 7900HT PCR system and the Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA). mRNA levels of *Mc4r* were normalized against β -actin by using Δ CT method. Normalized levels of mRNA were measured in triplicate per individual mouse from which sample means were calculated for each mouse. No template control wells were run for all reactions to confirm the absence of contamination and primer-dimer formation. All primer sets are analyzed for primer-dimer formation, specificity of target (sequencing of the amplicon), and melting curve analysis. Data are reported as the fold-difference from the WT *ad lib* fed experimental group. Student's *t* test between pairs was used with an alpha level of 0.05 to determine significance for all analyses. The data presented are means $\pm$ SE with **p*<0.05, ***p*<0.01.

2.3. Block of *Nhlh2* expression using Small Inhibitory RNA

The N29/2 cell line possesses endogenous *Nhlh2* expression. Silencing of *Nhlh2* expression was accomplished using *Silencer*[®] select predesigned siRNA (Ambion Austin TX) targeted to the coding region. *Silencer*[®] Select Negative Control siRNA, a scrambled siRNA that should not affect any genes (Ambion, Austin, TX) was used as a negative control. β -actin levels were measured in all cells, as its levels should not be affected by either of the siRNAs. Cells were transfected using HiPerfect (Qiagen, Valencia, CA) siRNA transfection reagent and were collected after 24 hrs for RNA isolation using Trizol method. Quantitative real-time PCR was used to access relative expression levels for β -actin, *Nhlh2* and *Mc4r* in the presence of either the scrambled or *Nhlh2* siRNA.

2.4. Plasmid expression constructs and *Mc4r* promoter reporter constructs

The plasmid reporter construct containing the *Mc4r* promoter was a gift of Dr. Kathleen Mountjoy, University of Auckland, New Zealand. The original vector was prepared by subcloning a region containing 1098 bp of mouse *Mc4r* promoter sequence and 426 bp of 5' untranslated region (UTR) inserted into pGL3 basic (Promega, Madison, WI) (Daniel et al., 2005). Using the above plasmid, a shorter reporter construct was prepared by amplifying an 800 bp region, which contained all three E-Box sites of the *Mc4r* promoter. Primers used are listed in Table 1. HindIII sites in the forward and reverse primer were used to insert the PCR-generated 800bp *Mc4r* fragment into the pGL3 basic vector by using a site between the luciferase coding sequence and the SV40 polyadenylation sequence. The resulting plasmid was prepared using a Qiagen Maxi Plasmid kit (Qiagen Ltd., Valencia, CA) and confirmed using DNA sequencing through the Virginia Tech Core Laboratory facility. Plasmid constructs containing *STAT3* and leptin receptor (ObRB) were a generous gift from Dr. James Darnel of Rockefeller University, New York, NY, and Christian Bjørnbæk of Harvard Medical School, Boston, MA.

2.5. Site directed mutagenesis

The *Mc4r* E-Box mutant constructs were generated by using PCR site-directed mutagenesis. PCR reaction was performed by using Platinum *Pfx* DNA Polymerase (Invitrogen) along with WT *Mc4r* promoter construct as a template with a 5 minute elongation time. Primer sequences (Table 1) with mutations designed for each individual E-Box site were used. The PCR cycle used was; denaturation step-15 seconds at 94⁰ C, annealing- 55⁰ C for 30 seconds and elongation time of 1 minute per kb. PCR amplification was confirmed by running 5 ul of PCR product stained with Ethidium Bromide (EtBr) run on a 1% agarose gel and visualized under UV light. Subsequently, the PCR product was digested with *DpnI* to remove any template DNA. After visualizing DNA on the 1% agarose gel containing EtBr, the *DpnI*-digested PCR product was transformed, prepared and then sequenced at the Virginia Tech Core Laboratory Facility.

2.6. Cell culture and transfections

The hypothalamic N29/2 cell line (Cellutions Biosystems, Toronto, Ontario) were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 10ug/ml streptomycin (HyClone, Logan, UT) at 37°C in 5% CO₂. Cells were transfected using Effectene transfection reagent (Qiagen, Valencia, CA) according to the manufacturer's recommendations. Briefly, cells were plated into 12-well plates at a concentration of 2×10⁵ cells/ml 24 hours before transfection and were transfected with 535ng of DNA per well (200ng reporter *Mc4r* plasmid; 35ng of CMV- β -gal plasmid; and 100 ng each of the *Nhlh2*, *Stat3*, leptin receptor (*ObRb*) and/or empty vector [pcDNA-zeo]). Addition of *Stat3* and *ObRb* optimized leptin signaling in N29/2 cells. Furthermore, while we show that *Nhlh2* is expressed by the N29/2 cell line, addition of

exogenous *Nhlh2* can further increase *Mc4r* levels in these cells. Thus, all three expression constructs were routinely added in transfection experiments. The CMV- β -gal plasmid was used as the internal control to check the transfection efficiency. Transfections were reproduced in triplicate, and total input DNA was kept constant and controlled by using the empty vector where appropriate.

2.7. Luciferase and β -galactosidase Assays

Transfected cells were serum-starved overnight and treated with leptin (1.2 μ g/ml of media) (rmLEPT, National Hormone and Peptide Program [NHPP], NIDDK) for 2 hr the following day. Cells were lysed and collected in reporter lysis buffer (Promega, Madison, WI). Two hours after stimulation cells were lysed in 300 μ l Reporter Lysis Buffer (Promega) according to the manufacturer's recommendations. A 5 μ l aliquot was used for the luciferase (Luciferase assay system, Promega, Madison, WI) and a 20 μ l aliquot was used for β -galactosidase assays (Promega, Madison, WI). For each assay, the basal WT promoter total luciferase activity normalized against β -galactosidase activity was taken to be 1, and results were expressed as fold activation over the control value. The data presented are means $\pm$ SE of three of more independent experiments. An alpha level of 0.05 was used to determine significance for all analyses.

2.8. Chromatin Immunoprecipitation Assay (ChIP)

ChIP assays were performed using the Chromatin Immunoprecipitation (ChIP) kit (Upstate Biotechnology Corp/Millipore, Billerica, MA) according to the manufacturer's recommendations. Cells were transfected with a vector containing myc-tagged *Nhlh2* protein (a gift from Dr. Thomas Braun, University of Halle-Wittenberg, Germany) and an expression vector for *Stat3* and treated with leptin for 15 minutes. Proteins bound to DNA were cross-linked using formaldehyde at a final concentration of 1% for 15 minutes at 37°C. Protein-DNA complexes were immuno-precipitated using myc-tag mouse monoclonal primary antibody (Cell Signaling, Danvers, MA). *Nhlh2*-myc promoter complexes were measured by PCR. Primers (TABLE 1) flanking the region of the *Mc4r* promoter where all three E-Boxes are located were used for PCR amplification. The samples were electrophoresed using a 1% agarose gel, and visualized by ethidium bromide staining.

2.9. Electrophoretic Mobility Shift Assay (EMSA)

Oligonucleotides were annealed and labeled with T4 polynucleotide kinase (Promega, Madison, WI) and [γ -³²P] deoxy (d)-ATP (PerkinElmer, Waltham, MA; 3000 Ci/mmol). Oligonucleotides used for EMSA analysis are listed in TABLE 1. Labeled oligonucleotides were used as probes or remained unlabeled as competitors. A total of 5 μ g protein was incubated with 35 fmol of [γ -³²P] dATP-labeled probe in binding buffer (Promega) for 10 minutes at room temperature. For competition experiments, a 10-fold molar excess of unlabeled oligonucleotide was added to the binding reaction. DNA-protein complexes were separated from free DNA by electrophoresis on a 6% non-denaturing polyacrylamide gel. All gels were pre-run in 0.5X Tris-borate-EDTA buffer for 30 minutes prior to electrophoresis at 25V for 1–2 hours. Gels were dried under vacuum and exposed to film (Eastman Kodak, Rochester, NY).

2.10. Statistical analysis

All values are expressed as mean $\pm$ SEM unless indicated otherwise. Comparison of means between two groups was made using unpaired two-tailed Student's T-test. *P* values were calculated using statistical analysis function in Microsoft Excel® (2007 version). Significance is expressed at **p*≤0.05; ***p*≤0.01.

3. RESULTS

3.1. *Mc4r* mRNA levels are reduced in the absence of *Nhlh2*

To investigate the effect of *Nhlh2* deletion on *Mc4r* mRNA levels, expression was measured using quantitative RT-PCR (qRT-PCR) of hypothalamic RNA isolated from WT and N2KO mice. Hypothalamic *Mc4r* mRNA levels were significantly reduced by 80% in N2KO mice compared to WT mice (Fig. 1; * $p \leq 0.05$).

The N29/2 cell line is an immortalized cell line used as a model of hypothalamic neurons (Belsham et al., 2004) and previously used by our laboratory to study *Pcsk1* regulation by *Nhlh2* (Fox and Good, 2008). RT-PCR was used to determine whether both *Nhlh2* and *Mc4r* are expressed in the N29/2 cell line. As shown in Fig. 2A, both *Nhlh2* and *Mc4r* are expressed in two different RNA isolates of the N29/2 cell line. siRNA targeting of *Nhlh2* mRNA was used to reduce *Nhlh2* expression in N29/2 cells. Cells transfected with siRNA targeted at *Nhlh2* showed reduced expression (83% compared to scrambled control) of the endogenous *Nhlh2* levels. This level of reduced *Nhlh2* in N29/2 cells led to a significant reduction (97% compared to scrambled control) in endogenous *Mc4r* mRNA expression. Neither of the siRNAs affected β -actin levels. Likewise, scrambled siRNA did not affect expression of any of the tested genes (Fig. 2B).

3.2. *Nhlh2* positively regulates *Mc4r* promoter activity *in vitro*

Polymorphisms in the *MC4R* promoter that potentially affect the binding sites for *Nhlh2* have been reported (Valli-Jaakola et al., 2006). The sequence of the mouse *Mc4r* promoter was analyzed for possible E-box motifs that could be putative binding sites for *Nhlh2*. As shown in Fig. 3A, the mouse *Mc4r* promoter has three of these putative binding motifs at -551, -366 and +54, relative to the transcription start site. One of these (the 1st E-Box, at -553) is identical to the site shown to bind *Nhlh2* on the murine *Pcsk1* promoter (Fox and Good, 2008), although the sequence at this first site differs between mouse and human (Fig. 3B). The 2nd E-Box, which is conserved between the mouse and human genes, is identical to the E-Box shown to interact with Bex2 and LMO2 protein in a DNA binding complex (Han et al., 2005) (Fig. 3B). The 3rd E-Box is not similar between human and mouse, nor does it match any known *Nhlh2* binding site.

Reduced *Mc4r* mRNA levels in N2KO mice, and in *Nhlh2*-depleted hypothalamic cell lines, as well as the presence of *Nhlh2* binding motifs in the *Mc4r* promoter region made it likely that *Nhlh2* may directly transactivate the *Mc4r* promoter. To test whether *Nhlh2* transactivates the *Mc4r* promoter, an 803-bp fragment (-639 to +139, Fig. 3A) of the murine *Mc4r* promoter containing all three putative E-Box motifs was subcloned into the pGL3 luciferase reporter plasmid. Exogenous overexpression of *Nhlh2* in N29/2 cells lead to a significant 1.2 fold increase in *Mc4r* promoter transactivation activity compared to cells not transfected with *Nhlh2* (Fig. 3C). Together with reduced *Mc4r* expression in N2KO mice, the reduction in *Mc4r* RNA following siRNA downregulation of endogenous *Nhlh2* in a hypothalamic cell line, these results from overexpression of *Nhlh2* in N29/2 cells suggest that *Nhlh2* is required for leptin-induced *Mc4r* promoter activity.

3.3. *Nhlh2* binds to all the three E-Boxes on the *Mc4r* promoter

To determine whether *Nhlh2* can bind to the native *Mc4r* promoter, a Chromatin Immunoprecipitation (ChIP) assay was performed on cells expressing myc-tagged *Nhlh2*. Chromatin from N29/2 cells was immunoprecipitated using an anti-myc antibody to pull down all regions bound to the N2-myc fusion protein. Primers to the *Mc4r* promoter region that amplify a region of DNA containing all the three putative E-Box motifs were then used to detect the immunoprecipitated chromatin. PCR on a *Mc4r* promoter plasmid (data not

shown) and the ChIP input material (genomic DNA), confirmed the primers used were specific for the endogenous *Mc4r* promoter containing all three putative E-Box motifs. The antibody to the myc-tagged *Nhlh2* protein pulled down the region of the *Mc4r* promoter containing all three putative E-box motifs. In cells not transfected with *Nhlh2*-myc as well as in a no-antibody control, the *Mc4r* promoter, was not pulled down (Fig. 4A). These data indicated that *Nhlh2* can interact with the endogenous *Mc4r* promoter.

To specifically identify which of the three E-Box motifs *Nhlh2* interacts with, Electrophoretic Gel Mobility Shift Assays (EMSA) were performed. Oligonucleotide sets (Table 1) representing each of the three E-Box sites in *Mc4r* promoter were used for EMSA. An oligonucleotide set designed to the E-Box motif present on the *neclin* promoter were used as a positive control. Binding of *Nhlh2* to the *neclin* promoter has been documented elsewhere (Kruger et al., 2004). Nuclear extract prepared from N29/2 cells transfected with *Nhlh2*-myc show that *Nhlh2* can bind to all the three E-Boxes on the *Mc4r* promoter (1st 'CAAATG' -551, 2nd 'CAGCTG' -366, and 3rd 'CAGATG' +54), with a similar intensity to its known binding site on the *neclin* promoter ('CACATG') (Fig. 4B, lane a). Oligonucleotide sets with mutations that eliminate the E-Box motif did not bind, or showed reduced binding to *Nhlh2* (Fig. 4B, lane b). Nuclear extract not transfected with *Nhlh2*-myc showed no binding to the oligonucleotide set with the *Neclin* promoter E-Box, or to any of the *Mc4r* promoter E-box oligonucleotide sets (Fig. 4B, lane c and d). Competition analysis with excess cold oligonucleotide specific to each E-Box confirmed that this binding was specific with a reduction in total binding for all motifs over extract alone (Fig. 4B, lane e). Supershift with N2-Myc antibody (Fig. 4B, lane f) showed disturbed or reduced binding in all the four oligonucleotides as compared to the WT.

3.4. *Nhlh2* requires all three E-Box motifs to induce promoter transactivation of *Mc4r* in N29/2 cell line

ChIP and EMSA confirmed *Nhlh2* binding to all three of the E-Boxes located on *Mc4r* promoter. To determine which of these motifs were responsible for directing transcription of *Mc4r* by *Nhlh2*, mutations in each of the motifs were created using site directed mutagenesis (Fig. 5A). As shown previously, addition of exogenous *Nhlh2* further increases expression of the *Mc4r*-luciferase reporter construct over the N29/2 cells, which do contain low levels of *Nhlh2* (Fig. 5B). However, mutation of each E-Box in turn led to significant reductions in *Mc4r* transactivation in cells both with exogenously-added and endogenously-expressed *Nhlh2* (Fig. 5B). Mutation of first E-Box (*Mc4r*-MUT-1) reduced *Mc4r* expression by 22-fold in the absence and 29-fold in the presence of exogenous *Nhlh2* (**p<0.01), with the *Mc4r*-MUT-1 promoter showing almost no transactivation compared to the WT *Mc4r* promoter (Fig. 5B). Mutations in the second (*Mc4r*-MUT-2) or third (*Mc4r*-MUT-3) also led to significant reductions of promoter activity (**p<0.01) as compared to WT (Fig. 5B), affecting *Mc4r* promoter transactivation both in the presence and absence of exogenously-added *Nhlh2*.

4. DISCUSSION

Polymorphisms in the protein coding region of the *MC4R* gene are strongly linked to monogenetic forms of obesity in humans (Farooqi and O'Rahilly, 2008). Although there is abundant literature available supporting *Mc4r*'s role in peripheral body weight and food intake regulations, there has been very few efforts to characterize the transcriptional regulation of *Mc4r*. The results presented here identify a new regulatory target for the basic helix-loop-helix transcription factor *Nhlh2*, placing it as a critical upstream regulator of melanocortin signaling through direct transcriptional regulation of the *Mc4r* gene, and linking it to a promoter mutation in the human *MC4R* gene that is associated with obesity in two human cohorts.

Knockdown of *Nhlh2* in cells using siRNA or in mice using the N2KO mouse corroborates the role for *Nhlh2* in *Mc4r* regulation. In mice, absence of *Nhlh2* leads to development of adult onset obesity with reduced physical activity (Coyle et al., 2002). Reduced physical activity and obesity are two characteristics of *Mc4r* knockout mice and humans with polymorphisms/mutations in *Mc4r* (Farooqi et al., 2000, Huszar et al., 1997, Yeo et al., 1998). Recent work from our group demonstrated a reduction in peripheral β -adrenergic receptors in white and brown adipose tissues, suggesting a central melanocortin defect and consistent with the results presented here (Wankhade et al., 2010). In previous work, we also showed that *Nhlh2* forms a heterodimeric complex with STAT3 to coordinate the regulation of the *Pcsk1* gene at an overlapping Stat:E-Box motif on the *Pcsk1* promoter (TTATATTCAAATG) (Fox and Good, 2008). Reduced expression of *Pcsk1* in N2KO mice leads to reduced synthesis of one of the fully processed *POMC* peptides, α -MSH, and the Thyrotropin-Releasing Hormone (*TRH*) peptide. Our new results suggest that deletion of *Nhlh2* has a double effect on melanocortin signaling, reducing both levels of the receptor and of the peptide binding to that receptor. Together with the finding of reduced *Mc4r* mRNA levels in N2KO mice shown herein, it can be concluded that animals with a deletion of *Nhlh2* have an overall defect in hypothalamic melanocortin signaling.

Promoter analysis revealed three E-Box binding motifs for *Nhlh2*. In addition, results also identified both new and confirmed E-Box motifs that interact with *Nhlh2*. E-Box #1 at -553 (CAAATG) on the mouse *Mc4r* promoter is identical to the E-Box identified for the PC1/3 promoter (Fox and Good, 2008). The E-Box on the *Pcsk1* promoter overlaps with a Stat3 motif on that promoter, which is not found on the *Mc4r* promoter. While we previously showed an *Nhlh2*/Stat3 interaction on the *Pcsk1* promoter, given the lack of a *Stat3* motif, we do not believe this interaction would exist or be necessary for *Mc4r* transactivation. While Stat3 protein activity is transactivated by leptin to regulate target genes, *Nhlh2* mRNA levels are increased up to 100-fold following either leptin injection or food intake (Vella et al., 2007), suggesting that *Mc4r* levels would be up-regulated following leptin stimulation. E-Box #2 (CAGCTG) has previously been found to bind *Nhlh2* in a complex with Bex2 and LMO2, although a specific gene that is regulated by this complex was not identified by the study, which used all synthetic promoters (Han et al., 2005). The sequence of E-Box #2 at -361 is identical to that E-Box in the human *MC4R* promoter, which when mutated by a 2-bp deletion is linked to obesity in two different human families (Valli-Jaakola et al., 2006). E-Box #3 (CAGATG) at +47 in 5'UTR of the *Mc4r* is a novel *Nhlh2* binding motif and is interesting in that it is located past the transcriptional start site of *Mc4r*. A 2003 paper identified two mutations in E-Box #1 in both obese children and adults (Lubrano-Bertheliet et al., 2003), although the most prevalent one (A/C in CATCTG at -178bp according to their gene map), present in up to 5% of their sample, is also present in some non-obese controls (Jacobson et al., 2002).

The present study reveals that the hypothalamic cell line N29/2 endogenously expresses both *Mc4r* and *Nhlh2*, making it a good model system for studying hypothalamic gene expression. We have previously shown that N29/2 cells also respond to leptin stimulation with activation of Stat3 and PC1/3 (Fox and Good, 2008). The *Mc4r* promoter reporter construct used in these studies was expressed in the native N29/2 cells, and its expression could be increased when a construct overexpressing exogenous *Nhlh2* was added. Likewise, use of a *Mc4r* promoter luciferase construct with a mutation affecting any of the individual E-Boxes led to significantly reduced promoter induction, either in the native N29/2 cells or in cells overexpressing *Nhlh2*. The first E-Box (CAAATG) mutation led to no promoter activation in the presence of exogenous *Nhlh2*. This E-Box may be the major contributor to *Mc4r* transactivation, and are consistent with earlier reports showing the CAAATG E-Box sequence has one of the highest affinities for *Nhlh2*, among other E-Boxes (Han et al., 2005). Mutations in the second and third E-Boxes (CAGCTG and CAGATG, respectively)

also led to significantly reduced promoter transactivation. As these motifs were mutated individually and still have an active 1st E-Box site, they may be involved in fine-tuning the overall response of the promoter to energy availability signals such as *Nhlh2*. Overall, these results suggest that all three E-Boxes are critical for *Mc4r* transcription. Relating this back to the human *MC4R* promoter mutation suggests that a polymorphism that eliminates even one of these E-Box motifs could have deleterious effects on hypothalamic *MC4R* expression. Future studies to identify more *MC4R* promoter mutations and evaluate their impact on *MC4R* expression are warranted.

In studying hypothalamic gene regulation, we feel it is necessary to use longer promoter constructs and attempt to use hypothalamic cell lines, rather than cell lines such as HeLa and COS, which may not have all of the neuronal signaling molecules and thus may not behave like a neuronal cell. Previous studies of the mouse *Mc4R* promoter showed that the minimal promoter required for activity in both neuronal and non-neuronal cells lines was between -130 and +10 (Daniel et al., 2005). Valli-Jaakola and colleagues used just this minimal promoter, which does not contain E-box 1 or 3 in their studies (Valli-Jaakola et al., 2006). As shown by mutation analysis, elimination of E-box 1 or 3 in neuronal cells results in complete abrogation of transcriptional activity by the reporter construct. Expression is still present in non-neuronal cells (Valli-Jaakola et al., 2006). In addition, it is likely that leptin stimulation is necessary for *Nhlh2* transcriptional activity towards both *Mc4r* and *Pcsk1* genes (Wankhade and Good, unpublished observations)(Fox and Good, 2008)

In summary, we used mice and a neuronal cell line model to confirm that the obesity-linked deletion (CA(del)TG⁻²⁰) in the human *MC4R* promoter (Valli-Jaakola et al., 2006) has the potential to affect expression of the *MC4R/Mc4r* gene. We also identified two other E-Box motifs that affect *Mc4r* transcription and are conserved in the human promoter. The studies in this paper also identify a new binding site for the bHLH transcription factor *Nhlh2*, which can help in future studies to characterize other hypothalamic promoter targets for this transcription factor. Together with already published studies, these new data help solidify *Nhlh2*'s role in hypothalamic energy balance regulation by demonstrating its role in transcriptional expression of the receptor and controlling levels of the ligand necessary for full hypothalamic melanocortin signaling in response to leptin or food intake.

Highlights

- Mutations in *MC4R* gene are the most frequent cause of monogenetic human obesity
- *Nhlh2* knockout mice are obese with reduced hypothalamic expression of *Mc4r* mRNA
- *Nhlh2* binds to all three E-boxes on the *Mc4r* promoter
- A human deletion on the *MC4R* promoter prevents *Nhlh2* binding and is linked to obesity
- *Nhlh2* is required for leptin-induced transcription of the *Mc4r* gene

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Fig. 1. Hypothalamic *Mc4r* mRNA levels in WT and N2KO mice

Whole hypothalamic RNA was used to determine the relative expression level of *Mc4r* RNA in the two genotypes. *Mc4r* mRNA levels were measured using qRT-PCR to quantify relative expression levels of *Mc4r* mRNA. All results were normalized to β -actin levels, which have previously been shown not to differ between WT and N2KO mice (Vella et al., 2007). The results are expressed as mean $\pm$ SE; * p < 0.05; (WT N= 12 and N2KO N=10 animals).

Fig. 2. SiRNA knockdown of *Nhlh2* in N29/2 cells reduces *Mc4r* expression

A. PCR was used to amplify *Nhlh2* and *Mc4r* from two different isolates of cDNA obtained from N29/2 cells. Previous work with both primer sets confirms specificity of the primers (see methods for further details). **B.** SiRNA to mouse *Nhlh2* (N2siRNA) was used to knockdown endogenous *Nhlh2* expression in the N29/2 cell line. Scrambled siRNA (Scrambled) was used as a negative control. After transfection, cells were collected and used to isolate total RNA. mRNA expression levels of β -actin, *Nhlh2* and *Mc4r* in each condition was determined using qRT-PCR. The results are expressed as mean $\pm$ SE; * p < 0.05;

Fig. 3. The *Mc4r* promoter contains E-box motifs and its activity is enhanced in presence of *Nhlh2* in transactivation assays

A. An 803-bp fragment (−648 to +117) of the murine *Mc4r* promoter is shown. The three putative *Nhlh2* binding sites (squared in gray) are numbered serially from the transcription start site.

B. Comparison of human and mouse *Mc4r* promoter using accession numbers AY236539.1 and AF201662.1 respectively human and mouse. Base pair numbers given correspond to the numbering in the NCBI database for those accession numbers, respectively, at the “C” residue in each E-Box motif.

C. Activity of the WT *Mc4r*-luc reporter transfected into N29/2 cells in the absence (*gray bars*) or presence (*black bars*) of *Nhlh2*. Luciferase activity was measured and normalized to the expression of β -gal-encoding protein. Normalized activity is presented relative to the values obtained in cells transfected with empty vector PGL-3-luc alone $\pm$ SE; ** $p < 0.01$.

Fig. 4. ChIP and EMSA reveal *Nhlh2* binds all three E-Box motifs on the *Mc4r* promoter

A. ChIP assay demonstrating binding of *Nhlh2*-myc to the *Mc4r* promoter. Lane a, PCR positive control using *Mc4r* plasmid; Lane b, Total input chromatin, representing 10% of the total chromatin. Lane c, Negative control using cells not transfected with *Nhlh2*-myc. Lane d, Amplification of the *Mc4r* promoter following immunoprecipitation with the *Nhlh2*-myc antibody.

B. EMSA experiments demonstrating binding of *Nhlh2* to all three E-Box binding sites and positive control Necdin. Binding to the positive control motif from the Necdin promoter, the E-Box motif at -551 (CAAATG), the E-Box motif at -366 (CAGCTG) and the E-Box motif at +54 (CAGATG) is shown. Extract transfected with *Nhlh2* mixed with WT oligonucleotides for each site (Lane a); Extract transfected with *Nhlh2* with mutant oligonucleotides for each site (Lane b); WT oligonucleotide with nuclear extract from mock-transfected cells (Lane c); Mutant oligonucleotides with nuclear extract from mock-transfected cells (Lane d); 100X competition with cold oligonucleotide, and nuclear extract containing *Nhlh2* (Lane e); Supershift using an antibody to c-myc, which will recognize the myc-tagged *Nhlh2* protein (Lane f).

A **B**

Fig. 5. All three E-Box motifs on *Mc4r* promoter are critical for its full transactivation in presence of *Nhlh2*

A. Substitution mutations in all three E-Box sites were made in the *Mc4r* promoter. Filled boxes show the sequence of the intact E-Box motifs. Open boxes show the sequence of the mutant E-Boxes for each site. Mutant nucleotides are shown as lower case letters. The negative control PGL3 plasmid contains only the luciferase gene and no *Mc4r* promoter DNA.

B. Transactivation assays performed by using WT *Mc4r* promoter and with the promoter with mutations in each individual E-Box motifs. Activity of the WT *Mc4r*-luc reporter (WT) transfected into N29/2 cells co-transfected in the presence (*black bars*) or absence (*gray*)

bars) of *Nhlh2*. The luciferase activity was measured and normalized to the expression of β -gal-encoding protein. Activity is presented relative to the values obtained in cells transfected with PGL3-luc alone $\pm$ SE, which is set to 1.** $p < 0.01$; to empty

TABLE 1

Oligonucleotides used in qRT-PCR, PCR, subcloning, mutagenesis, ChIP and EMSA experiments

Primer	Sequence 5'--3'	Purpose
<i>Mc4r</i> forward	GGAAGATGAACTCCACCCACC	qRT-PCR & ChIP
<i>Mc4r</i> reverse	AATGGGTCTGGAAACCATCGTC	
β -actin forward	GGAATCCTGTGGCATCCAT	qRT-PCR
β -actin reverse	GGAGGAGCAATGATCTTGATCT	
<i>Mc4r</i> Promoter Forward	GGCCAAGCTTGTTCACAGGCAATACGCTTTC	Plasmid reporter construct
<i>Mc4r</i> Promoter Reverse	GGCCAAGCTTTTCTCTCTCTCAGTGCGGC	
<i>Mc4r</i> E-Box Site 1 Sense	GCAGAACTGCAAATGGAGAAACAGCT	EMSA
<i>Mc4r</i> E-Box Site 1 antisense	AGCTGTTTCTCCATTTGCAGTTTCTGC	EMSA
<i>Mc4r</i> mutant E-Box Site 1 Sense	GCAGAACTGCATGGAGAAACAGCT	EMSA
<i>Mc4r</i> mutant E-Box Site 1 antisense	AGCTGTTTCTCCATGCAGTTTCTGC	EMSA
<i>Mc4r</i> E-Box Site 2 Sense	GGAGCCAGGACAGCTGCTTTTCATTTC	EMSA
<i>Mc4r</i> E-Box Site 2 antisense	GAAATGAAAAGCAGCTGTCCTGGCTCC	EMSA
<i>Mc4r</i> Mutant E-Box Site 2 Sense	GGAGCCAGGACATGCTTTTCATTTC	EMSA
<i>Mc4r</i> Mutant E-Box Site 2 antisense	GAAATGAAAAGCATGTCCTGGCTCC	EMSA
<i>Mc4r</i> E-Box Site 3 Sense	TGTGGGCGCGCAGATGCAGACGCGGCT	EMSA
<i>Mc4r</i> E-Box Site 3 antisense	AGCCGCGTCTGCATCTGCGCGCCACA	EMSA
<i>Mc4r</i> Mutant E-Box Site 3 Sense	TGTGGGCGCGCATGCAGACGCGGCT	EMSA
<i>Mc4r</i> Mutant E-Box Site 3 antisense	AGCCGCGTCTGCATGCGCGCCACA	EMSA
Necdin sense	GGGCCCTCATTTTCATGTGGGGCC	EMSA
Necdin antisense	CCCCAGGCCCCACATGAAAATGA	EMSA
Necdin mutant sense	GGATGGGTGCGTGGGGCC	EMSA
Necdin mutant antisense	GGCCCCACGCACCCATCC	EMSA
E-box site 1 mutant sense	CCGTTAGAGCAGAACTGCAACGGGAGAAACAGCTACCAGCACG	Site Directed Mutagenesis
E-box site 1 mutant anti-sense	CGTGCTGGTAGCTGTTTCTCCCGTTGCAGTTTCTGCTCTAACGG	
E-box site 2 mutant sense	GCCTGCTTCGGGAGCCAGGACAGAGGCTTTTCATTTCTTTTTTTAT	Site Directed Mutagenesis
E-box site 2 mutant anti-sense	ATAAAAAAAGAAATGAAAAGCctGTGCTCCTGGCTCCCGAAGCAGGC	
E-box site 3 mutant sense	GCGGTGTGAGTGTGGGCGCGCAGCGGCAGACGCGGCTCCAGCA	Site Directed Mutagenesis
E-box site 3 mutant anti-sense	TGCTGGGAGCCGCGTCTGCCgCTGCGCGCCACACTCACACCGC	
<i>Nhlh2</i> Forward	CAGTTGGCGTGAAGAGGTAGA	qRT-PCR
<i>Nhlh2</i> Reverse	AATGCCACGAGAAATACCA	
GAPDH Forward	AGGTCGGTGTGAACGGATTG	qRT-PCR
GAPDH Reverse	TGTAGACCATGTAGTTGAGGTCA	