Transcriptional and Post-transcriptional Control of Nhlh2 with Differing Energy Status

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ABSTRACT

Nescient Helix Loop Helix 2 (Nhlh2) is a member of the basic helix-loop-helix transcription factor family. Mice with a targeted deletion of Nhlh2, called N2KO mice, show adult onset obesity in both males and females. Nhlh2 regulates other genes by binding to the E-box in the promoter region of these genes. This transcription factor regulates many other transcription factors including MC4R and PC1/3 which are associated with human obesity. The Nhlh2 promoter has been analyzed for putative transcription factors binding sites. These putative binding sites have been tested to be the regulators of Nhlh2 by transactivation assays with mutant promoters, Electrophoretic Shift Assay (EMSA), and Chromatin Immunoprecipitation Assay (ChIP) as methods to investigate the DNA-protein binding.

The results of these experiments showed that the Nhlh2 promoter has five Signal Transducer and Activator of Transcription 3 (Stat3) binding site motifs at -47, -65, -80, -281, -294 and two Nuclear Factor Kappa-Light-Chain-Enhancer of Activated B Cells (NFκB) binding site motifs at -67 and -135. While NFκB acts as a negative regulator of Nhlh2, this research showed that Stat3 acts as a regulator for the Nhlh2 basal

expression and leptin stimulation. The ChIP assay using chromatin from mouse hypothalamus and antibodies against Stat3 and the NFκB subunits P50, P65, and c-Rel demonstrated that all of these antibodies were able to pull down the part of the Nhlh2 promoter containing the binding sites of Stat3 and NFκB. The EMSA results not only demonstrated that NFκB and Stat3 binding site motifs are real binding sites, but also exists the possibility of a relationship between these transcription factors to regulate Nhlh2 expression with leptin stimulation.

An effort in analyzing the human NHLH2 3'UTR showed that one of the SNPs located at position 1568 in the NHLH2 mRNA ($NHLH2^{A1568G}$) which converts adenosine to guanine might have the potential to decrease the mRNA stability. For more investigation about this SNP, the mouse Nhlh2 tail was cloned into 2 different vectors and these vectors were subjected to site directed mutagenesis to create the 3'UTR SNP that convert A to G. One of these vectors used luciferase as a reporter gene for expression while the other one was used to measure Nhlh2 mRNA stability. These vectors were transfected into hypothalamic cell line N29/2 to test the effect of this SNP on Nhlh2 expression. This study demonstrated that this SNP down regulated luciferase expression and also decreased Nhlh2 mRNA stability.

Taken together, this study demonstrated that Nhlh2 could be regulated transcriptionally by both NFκB and Stat3 transcription factors and post-transcripitionally by the 3'UTR SNP that converts adenosine to guanine.

This dissertation is dedicated to:

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helped, supported and encouraged me a lot during my PhD life, I do not know what my life would have been without her support and help.

List of abbreviations

ARC	Arcuate Nucleus
bHLH	Basic Helix-Loop-Helix
bp	Base pair
α-MSH	α-Melanocyte Stimulating Hormone
ChIP	Chromatin Immunoprecipitation
CNS	Central Nervous System (CNS)
EMSA	Electrophoretic Shift Assay
EtBr	Ethidium Bromide
Ικκ	IκB Kinase
LepR	Leptin Receptor
LH	Lateral Hypothalamus
MC4R	Melanocortin 4 Receptor (Human)
Mc4r	Melanocortin 4 Receptor (mouse)
MC4RKO	Melanocortin 4 Receptor Knockout Mouse
NFκB	Nuclear Factor Kappa-Light-Chain-Enhancer of Activated B Cells
NHLH2	Nescient Helix-Loop-Helix 2 (Human)
Nhlh2	Nescient Helix-Loop-Helix 2 (Mouse)
NHPP	National Hormone and Peptide Program
N2KO	Nhlh2 Knockout Mouse
PVN	Paraventricular Nucleus
PC1/3	Prohormone Convertase 1/3

POMC	Pro-opiomelanocortin
qPCR	Quantitative Polymerase Chain Reaction
RISC	RNA-Induced Silencing Complex
SNP	Single Nucleotide Polymorphism
STAT-3	Signal Transducer and Activator of Transcription 3
TRH	Thyroid Releasing Hormone
UTR	Untranslated Region
VMH	Ventromedial Hypothalamic Nucleus
WHO	World Health Organization
WT	Wild Type

Attribution

Dr. Donald W. Bowden is a professor in Department of Biochemistry, Wake Forest University School of Medicine and Dr. Nichole D. Palmer is a post-doctorate in Department of Biochemistry, Wake Forest University School of Medicine. Both of them worked on genomic analysis that found an association between a SNP in the NHLH2 gene and hip-waist ratio in a group of African Americans.

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Chapter 1

Introduction:

Obesity is a worldwide problem. The World Health Organization (WHO) reported that more than one billion are considered as overweight adults and 300 million as clinically obese. This problem affects both the developed and the developing countries including all age groups and socioeconomic classes (*1*). More than 155 million school age children and 22 million children under age 5 are considered severely overweight (*2*). Obesity is usually associated with diseases that threaten life such as insulin resistance, type 2 diabetes, cardio-vascular disease and different types of cancers. Also it is associated with many other health problems such as, skin problems, respiratory difficulties and infertility (*1*). These diseases and many other side effects of obesity cost the US between 5% and 7% of the total annual medical expenditures (*3*).

Body weight stability can be affected by different factors with genetic, physiological and behavioral factors being the most important (*4*). Although changes in life style and diets are liklely to be a significant factor in increasing the existence of obesity in childhood and adults during the last few decades (*5, 6*), it is clear that obesity has a large fundamental genetic component and is important to understand the effect of genetic variation in controlling energy homeostasis and its influence on obesity (*7, 8*). The heritability of body weight is estimated to be 0.78 which has increased to 0.81 over the first 25 years (*9, 10*).

Moreover, the heritability of body mass index (BMI) of adult and children reached 0.7 (*11*). Many studies illustrate that there are about 11 mutations in human genes that comprise the known monogenetic forms of obesity, and 5% of all forms of obesity are caused by mutation in the MC4R gene (*12*). In addition to MC4R, many other genes are involved in body weight regulation such as PC1 and POMC.

Many of the genes linked to genetic forms of obesity are expressed in the brain, and specifically in an area of the brain called the hypothalamus. The brain plays a significant role in regulating energy homeostasis by modulating both energy expenditure and food intake. This process is driven by different parts of the brain (*13*). It is well known that the central nervous system, especially the hypothalamus, plays a major role in regulating body weight, independently of the effects of body weight (*4*). The paraventicular nucleus (PVN) which lies in the anterior hypothalamus regulates many neural pathways that affect energy homeostasis (*14*). The ventromedial hypothalamic nucleus (VMH) has been considered as a satiety center. Stimulation of this part inhibits feeding, whereas lesions in VMH increase food intake (*15*). Nhlh2 is expressed in the hypothalamus—particularly the arcuate (ARC), paraventricular (PVN), ventromedial (VMH) and lateral hypothalamus (LH) (*16*).

Gene transcription:

In eukaryotes, several RNA polymerases share the task of synthesizing RNA in the nucleus. RNA polymerase II is one of three enzymes which catalyze the transcription of DNA to synthesize messenger RNA. This enzyme requires transcription factors to recognize its target on the gene promoter to initiate transcription (*17*). Extracellular signaling molecules stimulate or suppress gene expression by targeting transcription factors in the nucleus, which bind to the DNA (*18*). Activation of signal transduction pathways in tissues and neurons of the brain work to control body weight and energy expenditure. Transcription factors are major players in controlling these processes. Transcription factors regulate gene expression by binding to cis-regulatory elements within the upstream noncoding sequence of genes. Moreover, many posttranscriptional regulatory elements, including miRNA, may have considerable effects on gene expression (*19*). Although much research concentrates on changes in transcriptional regulation, gene expression is affected at many levels. Studying all of these levels together may help give a complete picture and better understanding of gene expression.

Transcriptional and Post-transcriptional regulation regulation of genes related to body weight and obesity:

Nescient helix-loop-helix 2 (Nhlh2) is one of the hypothalamic genes that demonstrate a significant influence on energy balance and body weight. This

gene is a member of the basic helix-loop-helix (bHLH) transcription factor family, which bind to E-box sequences (CANNTG) and form heterodimers with other bHLH family members to regulate transcription (*20, 21*). Mice which have deletion of this gene (N2KO mice) display adult onset obesity which becomes obvious after puberty by 12 weeks of age (*22*). The expression of Nhlh2 is influenced by changing the feeding status and energy availability. Expression of Nhlh2 decreased when mice were food deprived, while feeding for 2 hours following deprivation increased its expression. In situ hybridization analysis and quantitative polymerase chain reaction (qPCR) demonstrated that leptin injection after food deprivation also increase the Nhlh2 expression in mouse hypothalamus (*23*). Exposing mice to cold temperature reduces the expression of Nhlh2 while return to room temperature increases Nhlh2 expression (*23*). Besides the importance of Nhlh2 in energy homeostasis, the protein might have an important role in the developing of nervous system during murine embryogenesis (*24*). N2KO mice showed disruptions in the hypothalamic pituitary axis by decrease gonadotropin circulation. These disruptions are associated with loss of normal sexual behavior and reduced male fertility (*25*). Mice containing mutations in both Nhlh1 and the Nhlh2 show elimination and morphological changes of GnRH-1 neurons in different parts of the mouse brain suggesting that they have an important role in the development of the GnRH-1 neurons (*26*). While Nhlh2 itself is important for controlling energy expenditure, it also controls other genes related to energy homeostasis and obesity. Mice with a

targeted deletion of the melanocortin-4-receptor (MC4RKO) showed adult-onset obesity associated with lack of physical activity, but without reducing body temperature or increased food intake (*27*).This gene is directly transactivated by the transcription factor Nhlh2 when induced by leptin (Wankhade and Good, 2011). The prohormone convertase gene, Pcsk1 (PC1/3) requires both Nhlh2 and STAT3 for the full transcriptional response after leptin stimulation (*28*). The pro-opiomelanocortin POMC is cleaved by the prohormone convertases, PC1/3 to produce α-melanocyte stimulating hormone (αMSH) that regulates the body's response to energy availability.

Studying the transcriptional regulation and post-transcriptional regulation that is mediated by microRNAs might help in interventions to treat the disorders that are caused by abnormal gene expression. The miRNA regulates gene expression by either degrading the mRNA by pairing it with the mRNA or inhibiting the synthesis of protein from the gene mRNA (*29*).This inhibition of protein translation is achieved by Watson–Crick base pairing of the miRNA to its target in the 3' untranslated region (3'UTR) of the mRNA (*30, 31*). This complement is important and critical in the seeding region, nucleotides 2 to 8 from the 5' end of the miRNA, which binds to its target to inhibit translation of mRNA to protein (*32*). Because of this mechanism of the miRNA to interact with its target, the single nucleotide polymorphisms in the 3'UTR might affect this mechanism by eliminating legitimate miRNA binding sites or create illegitimate

miRNA binding sites. This might explain why many diseases are associated with SNPs in the 3'UTR.

Nhlh2 is one of the genes that play a role in energy expenditure and obesity. This gene has Stat3 and NFκB putative binding sites in its promoter. So the Stat3 and the NFκB have the potential to be the regulator of this gene. This study has been designed to determine if these binding sites are real binding sites for Stat3 and NFκB and if so how the Stat3 and NFκB affect the Nhlh2 expression. While the Nhlh2 can be driven by transcription factors that bind to its promoter, there is a possibility that the Nhlh2 expression is affected by post-transcriptional factors. The Nhlh2 has a SNP in the 3'UTR that has the potential to create illegitimate binding site for miRNA which might affect the stability of the Nhlh2 mRNA and it has the potential to eliminate mRNA stabilizing protein called hnRNP-U protein. According to this data, the second part of this research was designed to determine the effect of this SNP on the stability of Nhlh2 mRNA.

Chapter 2

Specific Aims:

Genetics and Obesity:

Obesity or overweight is defined as an increase in body weight, usually fat tissue, which may cause adverse health consequences. The Center of Disease Control and Prevention defined it as: “Overweight and obesity are both labels for ranges of weight that are greater than what is generally considered healthy for a given height. The terms also identify ranges of weight that have been shown to increase the likelihood of certain diseases and other health problems”(*33*).

Overweight and obesity occur when the balance between energy intake and expenditure are disrupted. This disruption happens when the energy coming from food intake exceeds the energy expenditure which includes basal metabolism, exercise and thermogenesis (*34*). Nhlh2 is one of the genes that play a role in the regulation of adult body weight. Target deletion of Nhlh2 transcription factor in mice (N2KO mice) causes adult-onset obesity. The increase in body weight is not associated with increased food intake in these animals; rather it is due to failure to exhibit normal levels of physical activity when given access to a running wheel (*35*). Nhlh2 is expressed in the hypothalamus—particularly the arcuate (ARC), paraventricular (PVN), ventromedial (VMH) and lateral hypothalamus (LH) (*16*). These regions also express many of the neuropeptides and receptors that regulate body weight. Nhlh2 is also expressed

in the paraventricular nucleus (PVN), where it acts to regulate the expression of pro-thyrotrophin releasing hormone (TRH) and prohormone convertases (PC1) (*16*). PC1 affects the processing of neuropeptides in the hypothalamus that inhibits feeding, including αMSH and TRH, which could affect hyperphagia (*36*). The PC1 and PC2 enzymes are necessary to cleave the POMC to produce α-melanocyte stimulating hormone (αMSH). N2KO mice show a reduction in prohormone convertases PC1 and PC2 levels reaching to 50%, and 40 % of normal mice respectively. These reductions in the PC1 and PC2 result in lower of the α-melanocyte stimulating hormone (αMSH) compared to WT mice. While PC1 is important for αMSH production, the Nhlh2 is necessary for full transcription of PC1/3 in the hypothalamus (*28*) and it regulates the MC4R directly by binding to the E-box in the MC4R promoter(*37*). The MC4R is important for energy homeostasis because the MC4R knockout mice displayed adult-onset obesity associated with lack of physical activity, but without reducing body temperature or increased food intake (*27*).

Leptin signals and transcriptional regulation of Nhlh2:

Works from our lab show that leptin is required to induce each of PC1 and Mc4r promoters' activity. Leptin is one of the most important regulators of food intake and energy homeostasis. This cytokine is secreted by adipocytes and targets neurons within the hypothalamic arcuate nucleus (ARC) (*38*). There are many signal transduction pathways activated by LepR. Restoration of the leptin

receptor in the ARC of lepR mutant mice normalized the physical activity levels without significant changes in food intake (*39*). Also, leptin signals cause phosphorylation of insulin-receptor substrates which lead to activation of PI-3 kinase and downstream regulation of gene expression through NFκB transcription (*40*). Thus, NFκB may be upstream of the Nhlh2 transcription factor and be responsible for regulation of its expression.

Food deprivation decreased Nhlh2 expression in the mice and injection of leptin after food deprivation caused an increase of Nhlh2 expression in both the arcuate (ARC) and paraventricular (PVN) nucleus of the hypothalamus (*23*). Stat3 is one of the important transcription factors that play a role in mediating growth factors and cytokines (*41*). It is also important for regulating mouse hypothalamus gene expressions through the leptin pathway (*42*). Leptin treatment causes rapid phosphorylation of Stat3 in the hypothalamus of mice (*43*). Preliminary results in our laboratory show an interaction of Stat3 with one of its binding motif on the Nhlh2 promoter using EMSA. About 1.2 kb of murine Nhlh2 promoter has been analyzed to identify the promoter elements controlling Nhlh2 expression. The proximal promoter region of human and mouse Nhlh2 contains five conserved putative Stat3 binding motifs, and two conserved NFκB sites, each of which could play a role in modulating Nhlh2 expression during changes in energy availability. Both Stat3 and NFκB can be affected by peripheral leptin injection (*44, 45*).

Together these results support a hypothesis that the peripheral signals of excess energy stimulate Stat3 and/ or NFκB to bind to the Nhlh2 promoter, and increase the level of Nhlh2 mRNA. Nhlh2 itself is a target of leptin signaling and the N2KO mice become obese due to lack of physical exercise. Based on this data, it is hypothesized that an increase in available energy stimulates Stat3 or/and NFκB-dependent transcriptional activation of the Nhlh2 gene in the arcuate nucleus or paraventricular nucleus of the hypothalamus**.**

Post-transcriptional regulation of Nhlh2:

Many posttranscriptional regulatory elements, including miRNA, may have considerable effects on gene expression (*19*). MicroRNAs (miRNAs) are noncoding RNAs which play a role in regulating gene expression and it start to take significant importance in molecular biology. Because of the base-pairing requirements for miRNA/mRNA interaction the single nucleotide polymorphisms (SNPs) that can reside in the miRNA binding sites may affect the miRNA function by eliminating existing binding sites or creating illegitimate ones. Therefore the SNPs in the miRNA gene or in the miRNA binding sites may change the gene expression (*46*). The NHLH2 gene has 23 known SNPs, one of them located in the 3'UTR at the position 1568 in the NHLH2 mRNA ($NHLH2^{A1568G}$). It was found that this SNP has an association with the hip-waist ratio in humans (Bowden and Good, unpublished). This SNP within the 3'UTR of NHLH2 may affect the stability of mRNA and the expression of this gene. While Nhlh2 is a transcription factor

and regulates up to 7000 different genes (*47*), it is also a target of regulation during changes in energy availability. Based on these data, it is hypothesized that the existence of mouse Nhlh2 SNP in the 3'UTR of the Nhlh2 gene may decrease the stability of Nhlh2 expression.

The main objective of this study is to understand the regulation of the Nhlh2 gene during different energy availability with concentration on the transcriptional regulation of Nhlh2. It is important to understand the regulation of this gene because the mice which have deletion of this transcription factor (N2KO mice) display adult onset obesity. Also, it seems that the expression of this gene is affected by feeding status and energy availability.

Specific aim 1: To determine which transcription factor(s) regulate the expression of Nhlh2 in different energy availability states: The proximal promoter region of Nhlh2 contains five putative Stat3 binding motifs and two putative NFκB sites. The preliminary data available from some experiments in our laboratory support and indicate that the Stat3 and the NFκB transcriptional factors are strong candidates to be regulators of this gene.

Specific aim 2: Study the effect of mutation in 3'UTR of Nhlh2 on mRNA stability. There is a SNP location at the position 1568 in the human NHLH2 mRNA ($NHLH2^{A1568G}$) within the 3'UTR of the NHLH2 gene, with the potential to affect the NHLH2 mRNA stability-binding proteins. This SNP was found to have

an association with hip-waist ratio in humans. Therefore, it is important to investigate if this SNP affects the stability of mRNA and the expression of Nhlh2.

Chapter 3

Nescient helix-loop-helix 2 (Nhlh2) is regulated by Stat3 and NFκB transcription factors.

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Introduction:

The incidence of overweight and obesity is considered an important public health issue in the United States. Since it is associated with many disease including cardiovascular disease, type 2 diabetes, hypertension and different kind of cancers, Healthy People 2010 considered overweight and obesity as one of the most important health indicators. While many factors play a role in maintaining body weight stability, genetic, physiological and behavioral factors are the most important at the biological level. It is now obvious that the central nervous system, especially the hypothalamus, plays a major role in regulating body weight, independently of the effects of body weight (*4*). Nescient helix-loop-helix 2 (Nhlh2), a member of the basic helix-loop-helix (bHLH) transcription factor family, is expressed in the adult hypothalamus. Mice which have deletion of this transcription factor (N2KO mice) display adult onset obesity. Obesity in the Nhlh2 knockout mice (N2KO) becomes obvious by 12 weeks of age (*22*). Food deprivation causes a decrease in Nhlh2 expression when compared to ad lib fed-mice while feeding for two hours after deprivation causes an increase in Nhlh2 expression. In situ hybridization analysis and quantitative polymerase chain reaction (qPCR) showed increase in Nhlh2 expression in both the arcuate and paraventricular nucleus of the hypothalamus when mice were injected by leptin (*23*). These results suggest that the expression of Nhlh2 is affected and changed by feeding status and energy availability.

Activation of signal transduction pathways in tissues and neurons of the brain by peripheral signals of energy availability eventually lead to controlled body weight. Transcription factors are major players in controlling these processes by binding to cis-regulatory elements within the upstream non-coding sequence of genes. Transcription factors and the gene regulated by these transcription factors are the targets of these signal transduction pathways. Transcription factors are grouped into different families by the motifs they use to regulate gene expression. The Nhlh2 transcription factor is a member of the basic helix-loop-helix transcription (bHLH) family, which bind to DNA through their basic domain at a motif in promoters termed the E-box motif, CANNTG(*48*) Nhlh2 is expressed in the hypothalamus—particularly the arcuate (ARC), paraventricular (PVN), ventromedial (VMH) and lateral hypothalamus (LH) (*16*). While Nhlh2 itself is a transcription factor and regulates more than 7000 different genes (*47*), it is also the target of regulation following changes in energy availability.

There are additionally eight transcription factors which are known to play key roles in hypothalamic gene regulation and have the potential to be affected by changes in energy balance (*44*). Peripheral leptin injection can increase the expression of Stat3 transcription factor via JAK-kinase dependent phosphorylation, homodimerization and translocation to the nucleus (*44*). Stat3 also plays an important role in mediating the responses to growth factors and cytokines (*41*), and in stimulating hypothalamic gene expression following leptin

(*42*). There are many signal transduction pathways that are activated by LepR. Leptin is secreted by adipocytes and targets leptin receptor (LepR) expressed neurons within the hypothalamus (*38*), resulting in stimulation of JAK2 kinase , and then rapid phosphorylation and nuclear translocation of Stat3 (*43, 49-54*).

The inflammatory pathway transcription factor, Nuclear Factor Kappa B (NFκB) can also be affected by leptin-dependent signaling (*45*). The nuclear factor kappa B (NFκB) is one of the transcription factors that expressed in central nervous system neurons as a response for inflammation (*55*). NFκB is found as a dimer in the cytoplasm which bound to the inhibitory protein IκB. The IκB inhibitory protein is degraded when subjected to Phosphorylation by IκB kinase (Iκκ) which induce the transfer of NFκB from cytosol to the nucleus where it controls the transcription of many genes including genes related to metabolism and obesity (*56-58*). Restoration of leptin receptor in the ARC of lepR mutant mice normalized the physical activity levels without significant changes in food intake (*39*). Also, it was found that leptin signals cause phosphorylation of insulin-receptor substrates which leads to the activation of PI-3 kinase and downstream regulation of gene expression through NFκB transcription (*40*). So, it is possible that leptin receptor signaling may impact Nhlh2 expression in the ARC leading to those downstream effects on physical activity.

It is well known that the Nhlh2 plays a significant role in physical activity control and it controls the expression of different genes that regulate the body

weight, which in turn affect weight gain, such as MC4R and PC1/3. But the transcriptional regulation of Nhlh2 still needs to be studied. In this study, we are trying to determine the transcription factors that modulate the Nhlh2 expression under different state of energy availability. As mentioned before, both Stat3 and NFκB are involved in leptin stimulation pathway. Moreover, leptin treatment increase the Nhlh2 mRNA levels (*23*). According to these results, we hypothesized that the leptin modulate the expression of Nhlh2 through transcriptional regulation of Stat3 and/ or NFκB on the Nhlh2 promoter.

Results

Putative binding sites for Stat3 and NFκB are present on the Nhlh2 promoter.

In order to understand the transcriptional regulation of mouse Nhlh2 gene, the proximal promoter was analyzed. After analyzing the mouse Nhlh2 promoter (NC_000069.5), five different binding site motifs for Stat3 were found at -47, -65, -80, -281, -294 and two binding site motifs of NFκB also were found at -67 and -135 (Figure 1). The Blast analysis also revealed a unique site, conserved between mouse (NC_000069.5) and human (NM_005599.3) Nhlh2/NHLH2 promoters with overlap between two sites of Stat3 and one of the NFκB binding site motifs. Interestingly, the analysis shows that both of the NFκB binding sites at -67 and -135 are identical between human and mouse. Whereas, only the 3rd Stat3 site at -80 and the 4th Stat3 site at -65, which overlaps with the 2nd NFκB, are identical between human and mouse.

Leptin induced expression of Nhlh2

To investigate if the Nhlh2 proximal promoter responds to leptin *in vitro*, the hypothalamic cell line N29/2 was transfected with either empty vector or vectors containing various lengths of Nhlh2 proximal promoter up to -950 bp. As shown in Figure 2A, and as a separate experiment in Figure 2B, a minimal promoter of 163 base pairs (bp) was sufficient to confer leptin stimulation of the

Nhlh2 promoter under these conditions ($P < 0.05$). In all cases, the empty vector showed no effect of leptin stimulation. Interestingly longer promoter constructs up to -950 bp indicated the presence of a possible negative control region between -819 bp and -950 bp, but investigation of these was outside of the scope of this project.

For more understanding of the Nhlh2 response to leptin signal, the N29/2 cells were transfected with either empty vector or vector which has the minimal Nhlh2 proximal promoter region (163 bp). While leptin stimulation for 2 hrs after 12 hrs of serum deprivation did not affect the cells transfected with empty pGL3-basic vector, it significantly increased luciferase transcription in N29/2 cells transfected with the 163 bp of Nhlh2 promoter construct ($P < 0.05$) (Figure 2B).

NFκB acts as a negative regulator of Nhlh2.

To test whether the NFκB binding site motifs affect leptin-induced transcriptional regulation of Nhlh2, various constructs with substitution mutations in each of the NFκB binding sites were created for use in transactivation assay (Figure 3A). These mutations were designed to eliminate NFκB binding and transactivation, as shown for similar NFκB sites (*59*).

The WT promoter showed the expected increase with leptin stimulation. However, mutation in the 1st NFκB binding site at -135 did not significantly affect basal expression of Nhlh2, but did significantly increase the expression of Nhlh2

following leptin treatment when compared to the WT promoter. Mutation of the 2nd NFκB binding site at -67 was similar in not affecting basal expression and while slightly increased, did not significantly affect transactivation of the Nhlh2 promoter construct following leptin stimulation. The construct with the NFκB -67 mutation no longer shows significant induction with leptin. Substitution mutations in both NFκB binding sites still results in significant induction of the Nhlh2 promoter construct by leptin, but to an even greater extent than the WT construct alone (Figure 3B).

Stat3 is responsible for the basal and leptin treatment activity of the Nhlh2 promoter.

To test whether the Stat3 sites were functional for the Nhlh2 promoter, substitution mutations were created in each of the Stat3 binding site motifs (Figure 4A). Again, mutations were generated that would completely eliminate Stat3 binding, as demonstrated by previous work (*60*). When compared with expression levels of the WT promoter (WT), mutation of the 3rd site of Stat3 at -80 results in a complete loss of both basal promoter activity and the promoter response to leptin ($P < 0.05$). Mutation of the 4th site at -65 increased the basal expression of Nhlh2 ($P < 0.05$), but also eliminated leptin stimulation ($P < 0.05$) (Figure 4B). Moreover, the mutation of the 5th site of Stat3 at -47 increased the basal expression of Nhlh2, and like the 4th site mutation, resulted in a loss of leptin stimulation ($P < 0.05$) (Figure 4B). Combination of the 3rd site mutation

with either the 4th or 5th site mutations or 4^{th} and 5^{th} sites showed the dominant effect of the 3rd site mutation with elimination of both basal and leptin-induced expression. These results together suggest that the 3rd site of Stat3 is required for both basal and leptin stimulation expression levels of Nhlh2. A construct containing both the 4th and 5th site mutations were expressed at a level significantly higher than the WT construct, but like the single 4th and 5th site mutations, with a loss of leptin induction. These results suggest that the 4th and 5th Stat3 binding sites may exert a slightly negative effect on basal expression of the Nhlh2 promoter, but are required induction of transcription following leptin stimulation.

Chromatin Immunoprecipitation (ChIP) and EMSA reveal that both the Stat3 and NFκB bind to the Nhlh2 Promoter

The transfection experiments demonstrated that Stat3 is the key factor for Nhlh2 expression, but they do not confirm that Stat3 itself acts directly on the Nhlh2 promoter and can bind to the endogenous promoter. For further support to these results and to determine whether Stat3 and/or NFκB can bind to the Nhlh2 promoter in hypothalamic tissues, a ChIP assay using antibodies against Stat3 or the NFκB subunits, P50, P65, and c-Rel was performed on mice. Chromatins were prepared from hypothalami of mice injected with leptin. The bound chromatins were immunoprecipitated using a Stat3, P50, P65, and c-Rel antibodies to pull down all regions bound by these transcription factors. Primers

were designed to amplify the Nhlh2 promoter region containing the putative Stat3 and NFκB binding sites motifs (Table 1). As shown in Figure 5A, an antibody to Stat3 was able to pull down the minimal Nhlh2 promoter containing the putative Stat3 and NFκB motifs. This same region was pulled down by the NFκB subunit-specific antibodies for P50, P65, and c-Rel NFκB subunits. These results demonstrate that following leptin stimulation of mice, Stat3, and the NFκB subunits (P50, P65, and c-Rel) could be found on the endogenous Nhlh2 promoter in hypothalamic extracts from mice. PCR for Nhlh2 promoter performed as input for a positive control (lane 1, Figure 5A and b), and beads alone (without antibody, lane 3, Figure 5A; lane 2, Figure 5B) as a negative control were as expected. The ChIP assays demonstrated specific transcription factor binding of the endogenous Nhlh2 promoter in hypothalamic extract from leptin-stimulated mice with Stat3, and the NFκB p65, c-Rel and p50 subunits (Figure 5B). Together with the results from the transfection and mutation analysis experiments, these results support the role for both Stat3 and NFκB transcription factors in transcriptional regulation of Nhlh2 following leptin stimulation.

For a better understanding of the mechanism, including the specific NFκB and Stat3 motifs involved gel mobility shift experiments were carried out. Oligonucleotides, encoding the different sites of Stat3 and NFκB located between -1 and -163 of the Nhlh2 promoter (Table 2), were incubated with nuclear extract prepared from N29/2 cells treated with leptin. As shown in Figure 5C, a mobility shift band indicated by the arrow NFκB was observed when nuclear extracts

were incubated with oligonucleotides containing the 1st NFκB binding site motif but not with the 2nd NFκB binding site motifs. Incubation of the extracts with oligonucleotide containing a mutation in the 1st NFκB binding site decreased the band density (figure 5C, lane 3), while a competition reaction with the cold oligonucleotide containing the 1st NFκB site eliminated the binding (Figure 5D, lane2). When an oligonucleotide containing the 2nd NFκB binding site at -67, as well as both of the 3rd and 4th Stat3 binding sites was used, two major bands were detected when incubated with nuclear extract (Figure 4B). In Figure 5E, nuclear extract:oligonucleotide complexes were co-incubated with antibodies against either Stat3 or each of the NFκB subunits. Two major bands were detected with supershifting when treated with Stat3, p50, p65, and c-Rel antibodies (Figure 5E, lanes 1, 2, 3 and 4). Previous published reports have seen a similar pattern (*61*), and suggest to us that both the upper and the lower bands represent the Stat3 as either homodimer or as a heterodimer with one of the NFκB subunit. In previous reports, and based on the size of the proteins involved in the complex, the upper band most likely includes either the p65 homodimer bound to Stat3 or a p50-c-Rel heterodimer bound to Stat3. The lower band represents oligonucleotides bound to the p50-p65 heterodimer--Stat3 complex. These interpretations are confirmed with the supershift experiments as both the upper and lower bands were supershifted with Stat3, p50, and p65. Conversely, the lower band, which contains only the p50 and p65 subunits did not supershift with the anti c-Rel antibody. The lane numbers 5 shows the two major bands

without supershift. Interestingly, the incubation of the cold oligonucleotide containing only the 3rd Stat3 binding site reduced the density of both bands (Figure 5E, lane 6). These results support the conclusion that the 3rd site of Stat3 plays a key role in regulating Nhlh2 but also suggest the existence of a relationship between the Stat3 and NFκB that coordinates leptin-induced transactivation through this complex binding motif found on the Nhlh2 promoter.

Discussion

The study presented here extends previous work in mice, to now identify the mechanism and promoter motifs controlling leptin-induction of Nhlh2. We show that the proximal promoter region of Nhlh2 contains five putative Stat3 binding motifs. As well as two NFκB binding site motifs, the 2nd of which overlaps two of the Stat3 motifs.

The shorter Nhlh2 promoter of 163 bp, which has 3 Stat3 and two NFκB binding site motifs was sufficient to confer leptin-induced expression, and was higher than expression levels of a longer 950 bp construct. These results suggested that this part of the Nhlh2 promoter might contain the binding sites of the transcripton factor/factors controlling the stimulation of Nhlh2 by leptin. Therefore, the short construct was used to study the tranactivation of Nhlh2. The transfection of short Nhlh2 promoter demonstrated that the treatment with leptin after serum deprivation caused a 2-fold increase in the expression when compared to untreated promoter. Since the treatment with leptin increased the reporter expression, this indicates that Nhlh2 promoter itself is target of leptin signal. This has implications on both the expression of Nhlh2 and many other potential Nhlh2 target genes downstream this transcription factor. This led us to do more investigation to determine the transcription factor/factors that control the stimulation of leptin for Nhlh2 expression.

The transactivation experiment of the mutants NFκB reveal that the 1st NFκB binding site acts as a negative regulator of Nhlh2, while the 2nd one did not affect the basal Nhlh2 expression but eliminated the effect of leptin. The effect of the 2nd NFκB binding site cannot be discussed away from the effect of Stat3 binding site since there is an overlap between the 2nd NFκB binding site and two other Stat3 binding sites. Thus, the effect of mutation in this site might be indirect by affecting the binding of Stat3 to their binding domain.

The mutation of the 3rd Stat3 site eliminated the reporter expression. Not only did it eliminate the expression, but it also eliminates the response to leptin treatment. In addition, the Nhlh2 promoter luciferase reporter gene assays, demonstrated that all the mutations of the Stat3 binding site motifs reduced leptin stimulation of Nhlh2 promoter activity and it was significant in the 3rd and 5th sites. These results demonstrated that the 3rd site of Stat3 is essential for basal expression of Nhlh2. While the 4th and 5th site are required for leptin stimulation. The mutations in the Stat3 binding sites and the 2nd NFκB binding site showed decrease or elimination of response to leptin, but it seems that the NFκB indirectly affect the leptin stimulation through Stat3 because the 2nd NFκB binding site overlaps with the 3rd and 4th Stat3 binding sites. While the mutation in the 2nd NFκB binding site reduced the effect of leptin, but it still kept the expression with leptin treatment higher than the expression without leptin treatment. The mutations in all of the Stat3 binding sites decreased the expression of leptin treatment to be lower than the expression without leptin and

these differences were significant in both of the 3rd and 5th sites. These results suggest that Stat3 is the key factor in regulating Nhlh2 and it is required for both basal Nhlh2 expression and leptin induction of transcription following leptin stimulation in the hypothalamic cells. It was previously demonstrated that Stat3 regulates the expression of many genes in the hypothalamus, such as PC1 and POMC (*62, 63*). Leptin stimulates phosphorylation of Stat3 in the hypothalamus (*43*). This stimulation that is achieved by the leptin receptor mediates intracellular signals via an associated Jak2 tyrosine kinase (*49*). The stimulation of JAK2 kinase causes JAK2 and Stat3 phosphorylation and then the Stat3 dimerizes and translocates to the nucleus to regulate gene transcription (*50-54*). Our results agree with and supported these results and previous work from our laboratory which show a strong link between leptin signaling and activation of the Nhlh2 gene, this link comes from the high correlation between the Nhlh2 expression and the circulating of leptin in serum(*23*). While Vella et al. (*23*) demonstrated that leptin injection and ad lib feeding after starvation increased the expression of Nhlh2 significantly, this study shows that leptin can regulate Nhlh2 expression through Stat3 transcription factor. Taken together, these data indicate that Stat3 is the key regulator of Nhlh2 and it regulates both basal expression and leptin stimulation

For further investigation and to confirm a role for Stat3 transcription factor in regulating Nhlh2 and to prove that Stat3 acts directly on the Nhlh2 promoter and binds to it, EMSA and ChIP experiments , using both of the Stat3 antibody

and the antibodies of different subunits of NFκB, have been implemented. The results of ChIP demonstrated that treating mice with leptin resulted in a marked translocation of Stat3, p65, p50, and c-Rel from cytosol to nucleus. This indicates that Stat3 regulates the Nhlh2 activity by directly interacting with its promoter. The ChIP assay showed that Stat3, P50, P65, and c-Rel are able to bind to the Nhlh2 promoter, but this assay does not determine the exact binding sites for these transcription factors to the Nhlh2 promoter which has different binding sites for NFκB and Stat3. The results of EMSA showed that the NFκB bound to the 1st NFκB binding site. The 2nd NFκB alone did not show binding, but when an oligonucleotide containing the 2nd NFκB binding site, as well as both of the 3rd and 4th Stat3 binding sites was used; two major bands were detected when incubated with nuclear extract. These two bands represent tow different complexes each of which contain Stat3 and different subunit of NFκB. This suggests the need of interaction between the Stat3 and the NFκB to control the expression on Nhlh2. Many studies support our results by identifying a relationship between Stat3 and different subunits of NFκB. These studies demonstrated that Stat3 interact with NFκB to drive the expression of other genes. In vivo studies showed that the Stat3 interacts with P65 homodimer (*64*). Stimulation of cells with inflammatory cytokines induced the formation of a complex of Stat3 and P65 to promote the serum amyloid A (SAA) gene (*65*). Stat3 also can form a complex with P50 subunit (*64*). Unphosphorylated Stat3 can interact directly with NFκB along with competition with IκB and it can bind to

both phosphorylated and unphosphorylated P65 in response to TNFα and Phosphorylated Stat3 has the ability to bind with P50 and P65 in response to IL-6 treatment (*61*).

According to the results of this study, Both of Stat3 and NFκB play a role in Nhlh2 regulation. While Stat3 regulate the Nhlh2 expression and mediate leptin stimulation of Nhlh2, NFκB acts as a negative regulator of Nhlh2. Both Stat3 and NFκB are major mediators in response to inflammation and stress. These transcription factors have been implicated in many of human diseases, such as cardiac diseases, different kinds of human cancer, liver, lung, and digestive system diseases. Since the Stat3 and the NFκB have been implicated in these diseases, it is possible that these diseases might modulate the Nhlh2 expression and in turn affect the energy expenditure.

Materials and Methods

Plasmid reporter constructs

Plasmids with different sizes of the Nhlh2 promoter were used for transfection into N29/2 cells (Cellutions Biosystems, Toronto, Ontario) to determine the minimal promoter that stimulated by leptin, the sizes of these plasmids are 163, 373, 819 and 950 bp. These fragments were cloned into Luciferase reporter plasmid pGL3-basic (Promega Corp., Madison). The minimal promoter contains the flanking sequence between -1 bp and -163 bp of the mouse Nhlh2 promoter (NC_000069.5), which contains three binding sites motifs of Stat3 and the two binding site motifs of NFκB, was used to study the transcriptional regulation of Nhlh2. Large scale plasmid preparation was performed using a Qiagen Midi Plasmid kit (Qiagen Ltd., Valencia, CA). The plasmid construct containing Stat3 was a generous gift from Dr. James Darnell, The Rockefeller University, New York, NY, and was modified to have an improved translational start site (*28*). The leptin receptor (ObRB) plasmid was a generous gift from, Christian Bjørbæk, Harvard Medical School, Boston, MA.

Site directed mutagenesis

The pGL3-basic plasmid with 163 bp of Nhlh2 proximal promoter inserted in front of luciferase have been used as a substrate for mutagenesis of the NFκB and Stat3 binding sites by using PCR site-directed mutagenesis method. PCR reactions were carried out by using iProof High fidelity DNA polymerase (BioRad)

along with WT Nhlh2 promoter construct as a template with a 5 minutes elongation time. The WT construct was used to create each of the different constructs with substitution mutations in the NFκB binding sites (1st and 2nd sites). Other constructs with substitution mutations in the Stat3 binding site were also created using the Nhlh2 promoter construct as a template. The oligonucleotides used for creating these mutations have been listed in Table 1.

The PCR was carried out in 50 µl using 5 ng of template. The first cycle was performed at 98°C for 3 min, and then followed by 35 cycles at 98°C for 10 s, 55°C for 30 s, and 72°C for 5 minutes. The reaction was terminated at 72°C for 10 min. The PCR product was stained with Ethidium Bromide (EtBr) and run on 1% agarose gel to detect the success of amplification. The PCR product was digested with DpnI enzyme to cut the template DNA and keep only the PCR product. The DpnI-digested PCR product was transformed into E-coli and cultured to extract plasmids. Plasmids were sequenced at the Virginia Bioinformatics Institute to ensure that the correct mutations were introduced and that there were no PCR errors.

Cell culture and transfections

The N29/2 cells, hypothalamic cell line, were grown in DMEM-high glucose (4.5 g/liter) medium and 4.5 Sodium pyruvate containing 10% fetal bovine serum, 100 units/ml penicillin, and 10µg/ml streptomycin (HyClone, Logan, UT). Cells were incubated at 37°C in 5% CO_2. The WT and each one of

the mutant plasmids was transfected into N29/2 cells using Effectene transfection reagent (Qiagen, Valencia, CA) following the manufacturer protocol. Cells were trypsinized, collected, and plated in 12-well plates 24 hours prior to the transfection day. Each well had 1-2 X 10^5 cells to get confluency between 40-60 % on the next day. Cells were transfected with 435 ng of DNA per well (200 ng reporter Nhlh2 plasmid or empty vector (pGL3-basic) as a control, 35 ng of CMV-β-gal plasmid, and 100 ng each of Stat3 and Leptin receptor constructs). The β gal plasmid was used as the internal control for the efficiency of transfection.

Luciferase and β -galactosidase Assays

N29/2 Cells were incubated at 37°C for 24 hrs after transfection. Cells were then subjected to serum deprivation for 12 hours followed by treatment with either 100 mM leptin (National Hormone & Peptide Program (NHPP), NIDDK, and Dr. Parlow) or vehicle (PBS) for 2 hrs in serum-free media. Cells were lysed 2 hours after leptin stimulation, in 400 µl 1X Reporter Lysis Buffer (Promega). Luciferase assay was performed using 5 µl of cell lysis (luciferase assay system Promega, Madison, WI). As an internal control for the efficiency of transfection, the β-galactosidase (β-gal) activities were measured using 25 µl of lysate (Promega, Madison, WI). In order to obtain the standardized luciferase activity, the luciferase activity of each sample was divided by the corresponding relative β-gal activity. For each assay, the basal WT promoter total luciferase activity was

taken to be 1, and the results were expressed as fold activation over the control value.

Chromatin Immunoprecipitation Assay (ChIP)

Preparation of chromatin-DNA and ChIP assays were performed using Upstate Biotechnology Chromatin Immunoprecipitation kit (Upstate, Charlottesville, VA) according to the protocol supplied by the manufacturer. Mice were starved for 24 hours followed by leptin (3 mg/kg body weight in PBS) injection. Mice were euthanized two hours after leptin treatment and dissected to isolate the hypothalamus. The hypothalamus was minced and subjected to cross-linking reactions using formaldehyde at a final concentration of 1% for 15 minutes at room temperature. The hypothalamus was washed twice with phosphate buffered saline containing protease inhibitors. Hypothalamus tissues were lysed, and chromatin was sonicated to share the DNA to get a 500-1000 bp DNA length. Following centrifugation, the chromatin was extracted and diluted 10-fold. Chromatin was precleared with Salmon Sperm DNA/Protein A Agarose-50% Slurry. Chromatin was incubated overnight at 4 ^{0}C using 2 mg mouse Stat3 (K-15: sc-483), P-50 (NLS: sc-114), P65 (F6: sc-8008), or c-Rel (N-466: sc-272) from (Santa Cruz Biotechnology) or with no antibody as a negative control. Samples were subjected to immunoprecipitation with Salmon Sperm DNA/Protein A Agarose-50% Slurry. Immunoprecipitated complexes were washed with different buffers according to the manufacturer protocol followed by

an elution twice with 250 µl of elution buffer. Protein–DNA cross links were reversed by adding 20 µl of 5 M NaCl and incubating at 65 ^{0}C overnight. DNA was recovered using Wizard clean up PCR system (Promega). Five µl of the recovered DNA was used for PCR amplification (20 cycles) with a primer set 5' ATCATGGGCTGCTTTCAACT 3' and 5' TAGCCTGGAGATGGTGGAAG 3' to amplify the Nhlh2 promoter region between -1 and -226 where two NFκB and three of the Stat3 binding sites were located. Another PCR amplification reaction was performed using 2 µl of the previous PCR product (20 cycles). The samples were electrophoresed using a 1.4% agarose gel, and visualized by ethidium bromide under UV light.

Electromobility Shift Assay (EMSA)

Double stranded oligonucleotides were annealed and labeled using T4 polynucleotide kinase (Promega, Madison, WI) and [γ_^{32}p] deoxy (d)-ATP (PerkinElmer, Waltham, MA; 3000 Ci/mmol). Labeled oligonucleotides were purified using MicroSpin G-25 columns (GE Healthcare, UK). Five micrograms of nuclear extract were incubated at room temperature in a 20 µl reaction volume for 20 minutes in binding buffer from (Promega). Labeled oligonucleotides, about 35 fmol of [γ_^{32}p] dATP-labeled probe, were added and incubated for another 20 minutes at room temperature. The DNA sequences of the double-stranded oligonucleotides used for this study are listed in table 2. For competition experiments, a 10-fold molar excess of unlabeled oligonucleotides were added to

the binding reaction. Samples were subjected to electrophoresis at a 6% non-denaturing polyacrylamide gel at 250 V for 2-3 hours to separate the DNA-complex from the free oligonucleotides. The gel was pre-run in 5X Tris-borate-EDTA buffer for 30 minutes before loading the samples. Gels were dried by heated vacuum and exposed to film (Eastman Kodak, Rochester, NY) for 4-7 days at −80°C. For supershift assays, polyclonal antibodies against Stat3 (K-15: sc-483), p50 (NLS: sc-114), p65 (F6: sc-8008), and c-Rel (N-466: sc-272) from (Santa Cruz Biotechnology) were added to the reaction mixture and incubated for 30 min at room temperature prior to the addition of the radio labeled oligonucleotides.

Experimental Animals

Mice were housed in a room with automatically controlled temperature, humidity and lighting 12 h: 12 h light:dark cycle with *ad libitum* access to food and water. Only male mice were used for ChIP experiments. Food deprivation was implemented for 24 h and then mice were injected with leptin (3 mg/kg body weight in PBS). All mice were euthanized by CO_2 two hours following leptin treatment. Brains were isolated by dissection, and a hypothalamic tissue block was made, minced by razor blade and transferred to PBS and used directly according to the experiment procedure. All animal experiments were approved by the Institutional Animal Care and Use Committees at Virginia Tech.

Figures:

Figure 1: The Nhlh2 proximal promoter region

Mouse (NC_000069.5) and Human (NM_005599.3) Nhlh2 proximal promoter region alignment. Numbering is from -1 position on both promoters. The gray shading represents putative Stat3 binding site motifs while the underlined sequences represent the NFκB binding site motifs.

Figure 1

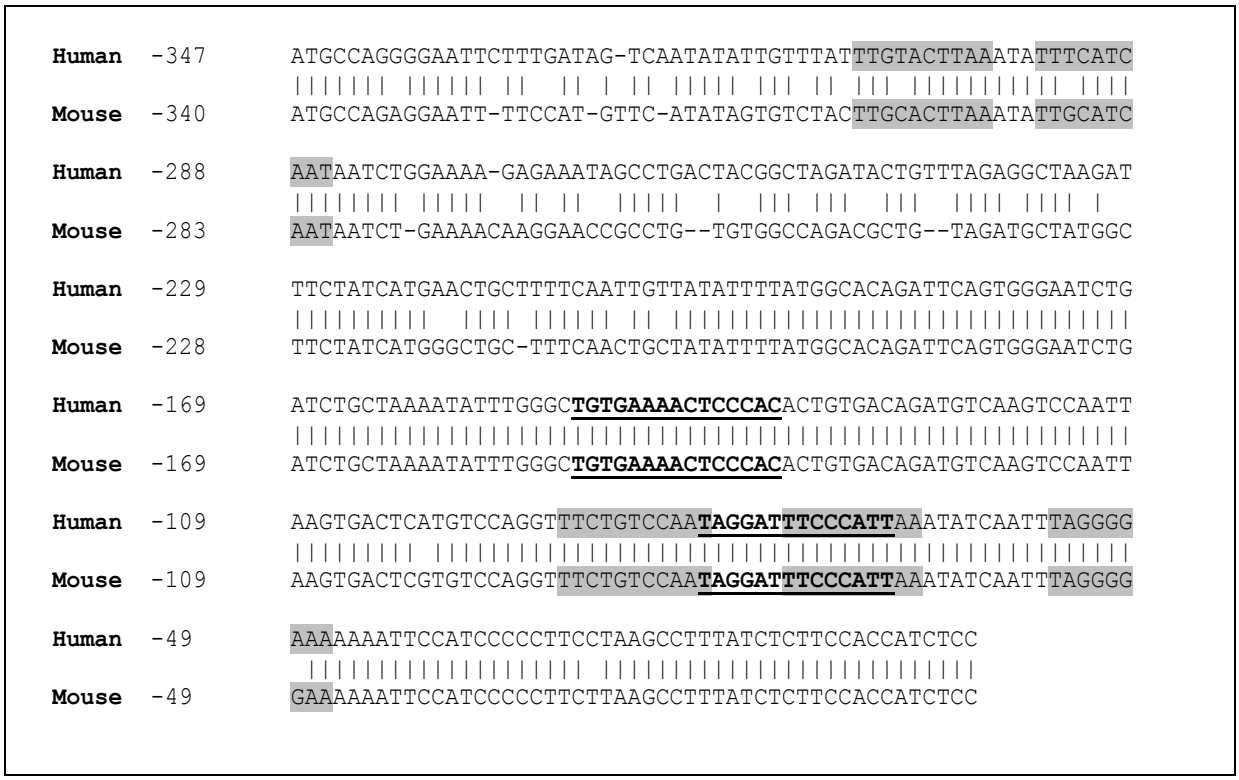

Human -347 ATGCCAGGGGAATTCTTTGATAG-TCAATATATTGTTTATTTGTACTTAAATATTTCATC
Mouse -340 ATGCCAGAGGAATT-TTCCAT-GTTC-ATATAGTGTCTACTTGCACTTAAATATTGCATC
Human -288 AATAATCTGGAAAA-GAGAAATAGCCTGACTACGGCTAGATACTGTTTAGAGGCTAAGAT
Mouse -283 AATAATCT-GAAAACAAGGAACCGCCTG--TGTGGCCAGACGCTG--TAGATGCTATGGC
Human -229 TTCTATCATGAACTGCTTTTCAATTGTTATATTTTATGGCACAGATTCAGTGGGAATCTG
Mouse -228 TTCTATCATGGGCTGC-TTTCAACTGCTATATTTTATGGCACAGATTCAGTGGGAATCTG
Human -169 ATCTGCTAAAATATTTGGGCTGTGAAAACTCCCACACTGTGACAGATGTCAAGTCCAATT
Mouse -169 ATCTGCTAAAATATTTGGGCTGTGAAAACTCCCACACTGTGACAGATGTCAAGTCCAATT
Human -109 AAGTGACTCATGTCCAGGTTTCTGTCCAATAGGATTTCCCATTAAATATCAATTTAGGGG
Mouse -109 AAGTGACTCGTGTCCAGGTTTCTGTCCAATAGGATTTCCCATTAAATATCAATTTAGGGG
Human -49 AAAAAAATTCCATCCCCCTTCCTAAGCCTTTATCTCTTCCACCATCTCC
Mouse -49 GAAAAAATTCCATCCCCCTTCTTAAGCCTTTATCTCTTCCACCATCTCC

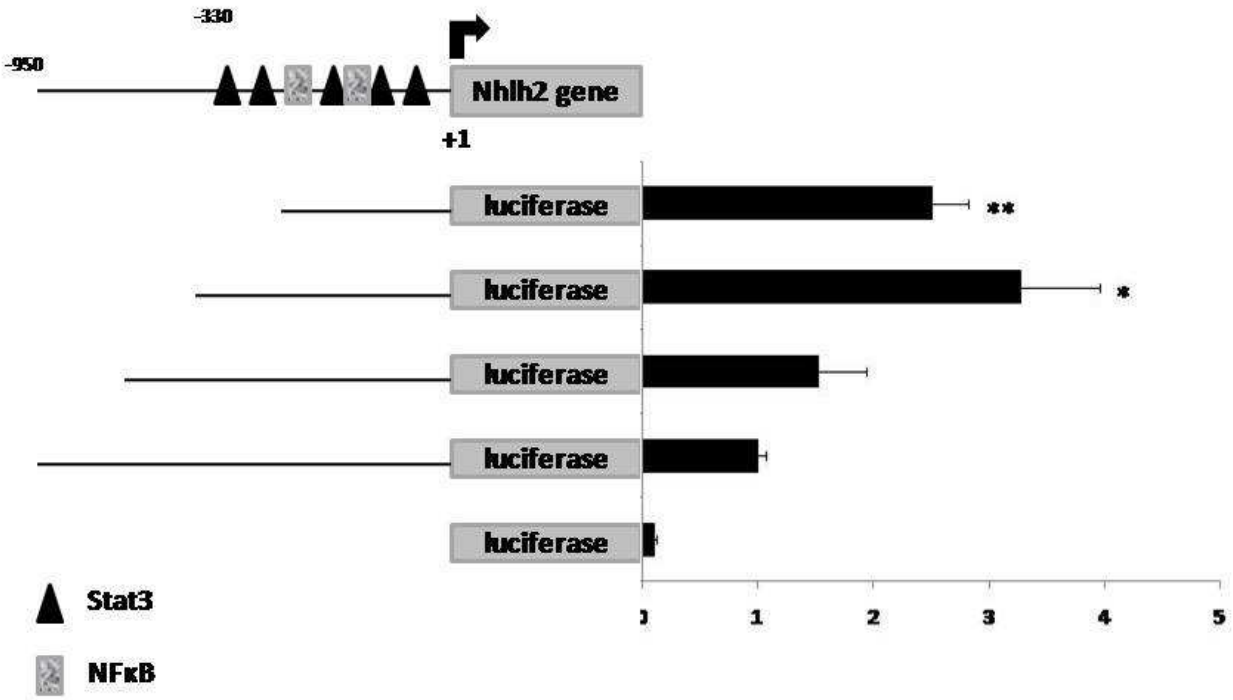
Figure 2: The short proximal promoter of Nhlh2 enhances the Nhlh2 expression and Leptin induce Nhlh2 expression after 12 hours deprivation.

A. Activity of the WT Nhlh2-luc reporter transfected into N29/2 cells and stimulated with leptin after 12 hours of serum deprivation (black bars). The gray bars with lines represent the reporter vectors with different length of the Nhlh2 promoter which used for transfection. The sizes of the promoter are 163, 373, 819 and 950 bp. Luciferase activity was measured and normalized to the expression of β-gal-encoding protein. The differences between the long Nhlh2 promoter and the shorter ones are presented by means ± SE *P < 0.05, ** P < 0.01.

B. The control is plasmid PGL3-Basic without the Nhlh2 promoter. Activity of Nhlh2 promoter reporter transfected into N29/2 cells and serum deprived for 12 hours (*gray bars*) or serum deprived and followed by leptin treatment (100 mM leptin) for 2 hours (*black bars*). The Nhlh2 luciferase activity was measured and normalized to the expression of β-gal-encoding protein. Luciferase activity is presented relative to the values obtained in cells transfected with WT Nlhlh2-luc plasmid and subjected to serum deprivation for 12 hours. The data presented are means ± SE *P < 0.05 compared to WT subjected to serum deprivation.

Figure 2

A.


-330
-950
Nhlh2 gene
+1
luciferase
luciferase
luciferase
luciferase
luciferase
**
*
Stat3
NFκB
0
1
2
3
4
5

B.

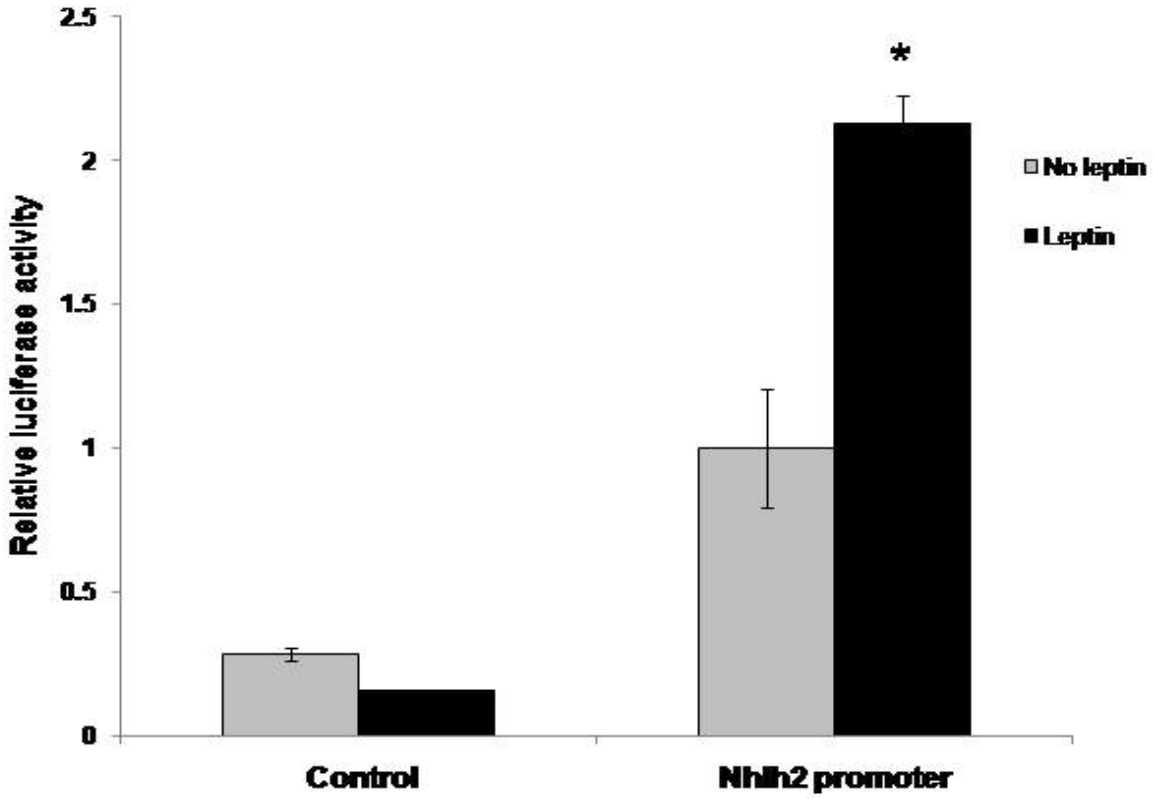

2.5
2
1.5
1
0.5
0
Relative luciferase activity
*
No leptin
Leptin
Control
Nhlh2 promoter

Figure 3: The 1st site of NFκB motifs on Nhlh2 promoter plays as a negative regulator of Nhlh2 when treated with leptin.

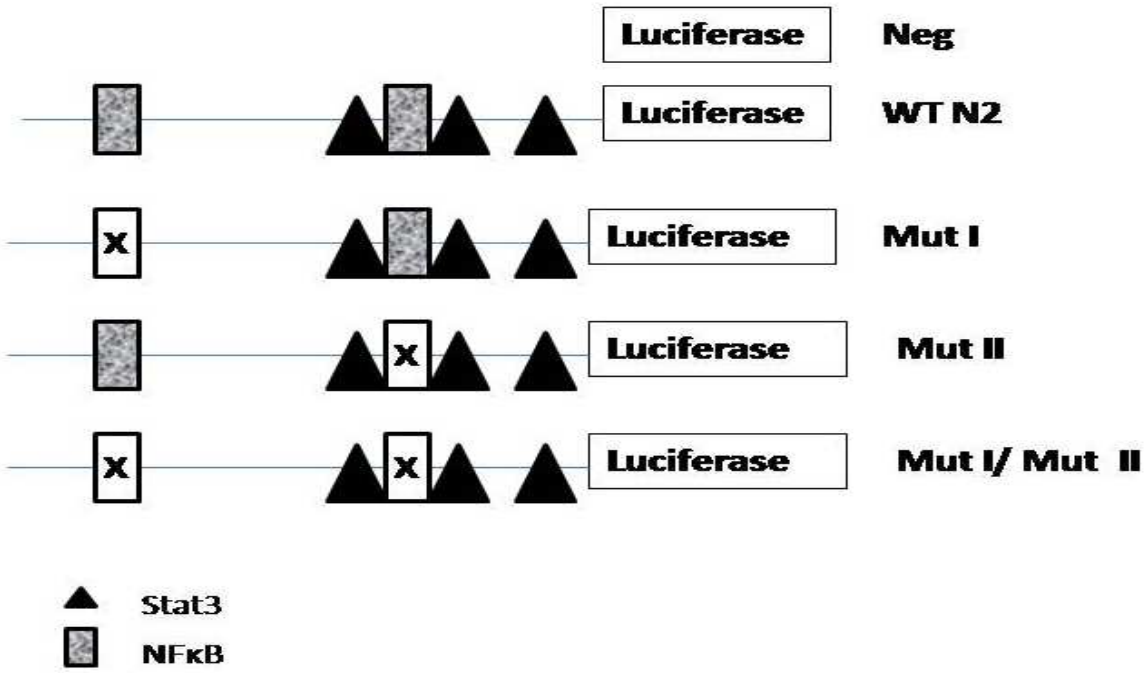
A. Substitution mutations in all NFκB sites were created. WT Nhlh2-luc plasmid contains the promoter region with three Stat3, and two NFκB putative binding sites. Plasmids containing the promoter with mutations at -135, -67 are stated as Mut I and Mut II respectively. A fourth plasmid with mutations in both NFκB binding sites is labeled as Mut I/ Mut II. The table in the bottom shows the sequence of the WT and the mutations in each site.

B. The control is plasmid PGL3-Basic without the Nhlh2 promoter. The difference in luciferase activity between the control and the Nhlh2-luc was significant ($P < 0.01$). N29/2 cells were transfected with WT Nhlh2-luc plasmid (WT), Nhlh2-luc plasmid contains mutation in the 1st NFκB motif (MutI), Nhlh2-luc plasmid contains mutation in the 2nd NFκB motifs (Mut II), or Nhlh2-luc plasmid contains mutations in both 1st and 2nd NFκB motifs (Mut I/ Mut II). All of them were subjected to serum deprivation for 12 hours (No leptin), or serum deprivation for 12 hours followed by leptin treatment (100 mM leptin) for 2 h (Leptin). Activity of the WT Nhlh2-luc reporter (WT), Mut I , Mut II, and Mut I/ Mut II transfected into N29/2 cells were subjected to serum deprivation for 12 h (*grey bars*) or serum deprivation for 12 h followed by 2 hours leptin treatment (*black bars*).

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 Nhlh2-luc reporter (WT). The data presented are means ± SE and the significant differences stated as a = ($P < 0.05$) compared to WT without leptin, a* = ($P < 0.01$) compared to WT without leptin, b = ($P < 0.05$) compared to WT with leptin, c = ($P < 0.05$) within pairs.

Figure 3

A.



Putative NFκB sites	WT sequence	Mutant sequence
-144 WT/Mut I	TGTGAAAACTCCCACA	TGTGAAACT**TAA**ACA
-77 WT/ Mut II	TAGGATTTCCCATT	TA**AT**ATTTCCCATT

General consensus sequence of NFkB: gggRNNYYcc

R= Purine, Y= pyrmidine

B.

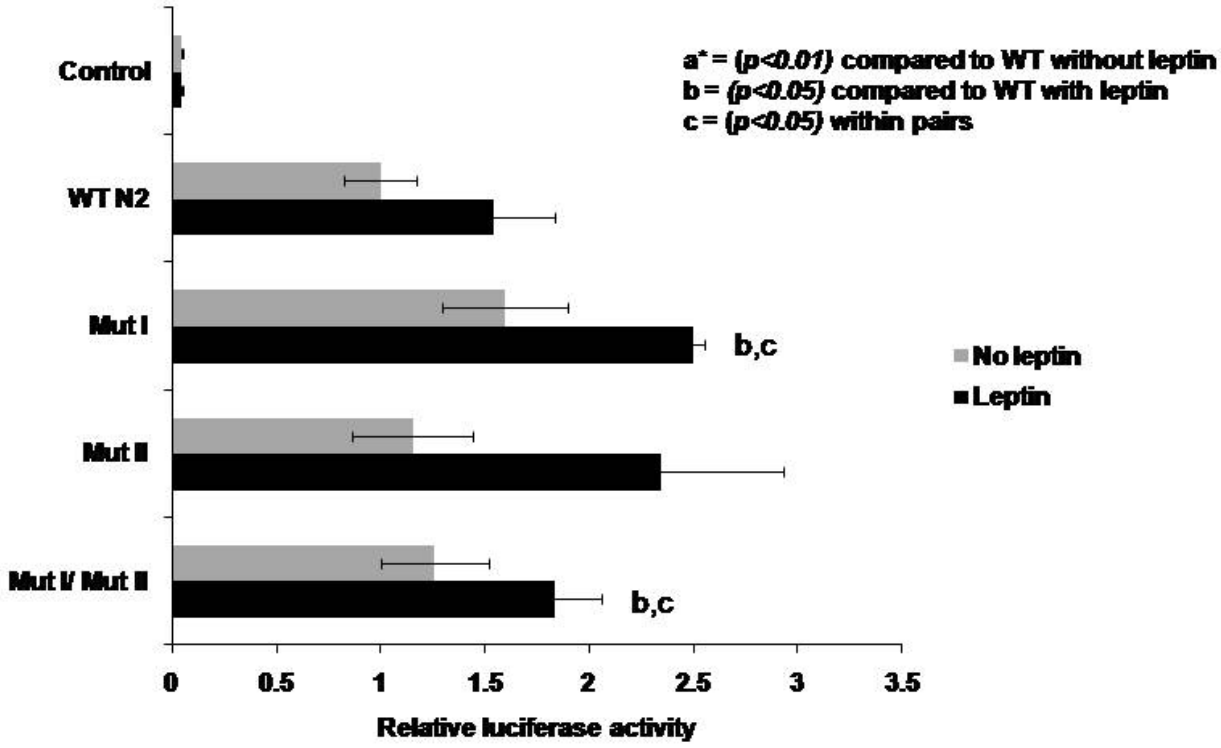

a* = (p<0.01) compared to WT without leptin
b = (p<0.05) compared to WT with leptin
c = (p<0.05) within pairs
Control
WT N2
Mut I
Mut II
Mut I/ Mut II
b,c
b,c
No leptin
Leptin
0
0.5
1
1.5
2
2.5
3
3.5
Relative luciferase activity

Figure 4: The 3rd site of Stat3 motifs on Nhlh2 promoter plays as a positive regulator of Nhlh2 when treated with leptin.

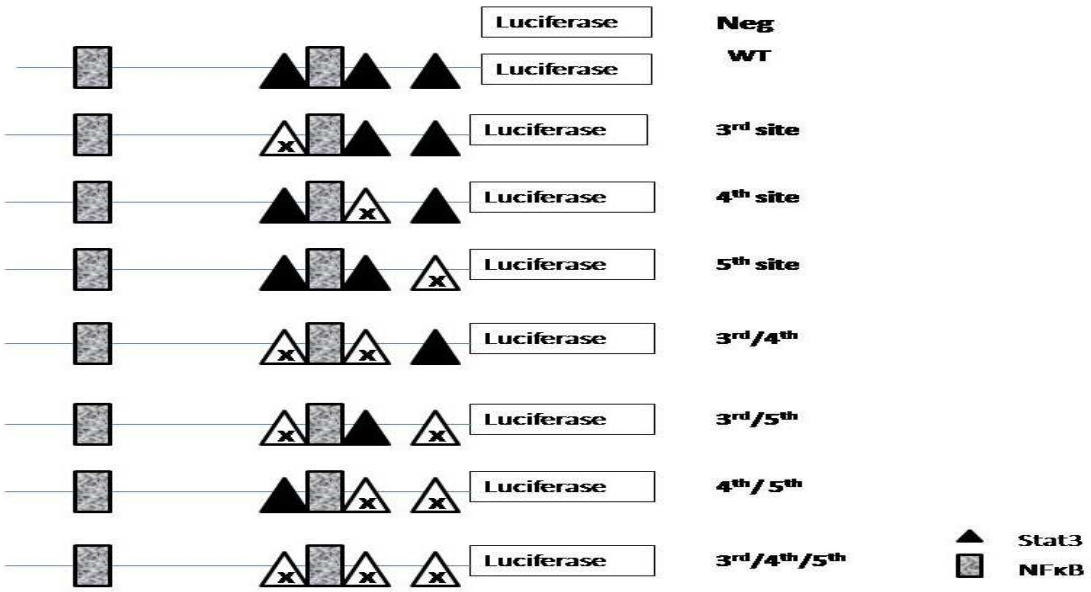
A. Substitution mutations in all Stat3 sites have been created. WT Nhlh2-luc plasmid has a promoter region with three Stat3, and two NFκB putative binding sites. Plasmids containing the promoter with mutations -80, -65, and -47 are stated as a 3rd site, 4th site and 5th site respectively. 3rd/4th has mutations in both 3rd and 4th sites. 3rd/5th has mutations in both 3rd and 5th sites. 4th /5th has mutations in both 4th and 5th sites. 3rd/ 4th/ 5th has mutations in 3rd, 4th, and 5th sites. The table in the bottom showing the sequence of the WT and the mutations in each site.

B. The control is plasmid PGL3-Basic without the Nhlh2 promoter. The difference in luciferase activity between the control and the Nhlh2-luc was significant ($P < 0.05$). N29/2 cells were transfected with WT Nhlh2-luc plasmid (WT), Nhlh2-luc plasmid contains mutation in the 3rd Stat3 motifs (3rd site), Nhlh2-luc plasmid contains mutation in the 4th Stat3 motif (4th Site), Nhlh2-luc plasmid contains mutation in the 5th Stat3 motif (5th Site), Nhlh2-luc plasmid contains mutations in both 3rd and 4th Stat3 sites (3rd/4th). Nhlh2-luc plasmid contains mutations in both 3rd and 5th Stat3 sites (3rd/5th). Nhlh2-luc plasmid contains mutations in both 4th and 5th Stat3 sites (4th/5th). Nhlh2-luc plasmid contains mutations in all of 3rd, 4th and 5th Stat3 sites (3rd4th/5th). All of them were subjected to

serum deprivation for 12 hours (No leptin), or serum deprivation for 12 hours followed by leptin treatment (100 mM leptin) for 2 hours (Leptin). Activity of the WT Nhlh2-luc reporter (WT), 3rd site, 4th site, 5th site,3rd/4th, 3rd/5th, 4th/5th, or 3rd/4th/5th transfected into N29/2 cells were serum deprived for 12 hours (grey bars) or serum deprived for 12 hours followed by 2 hours leptin treatment (black bars). 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 Nhlh2-luc reporter (WT). The data presented are means ± SE and the significant differences stated a = ($P < 0.05$) compared to WT without leptin, a* = ($P < 0.01$) compared to WT without leptin, b = ($P < 0.05$) compared to WT with leptin, b* = ($p < 0.01$) compared to WT with leptin, c = ($P < 0.05$) within pairs.

Figure 4

A.



Putative Stat3 sites	WT sequence	Mutant sequence
-90 WT/ 3rd Site Mut	TTCTGTCCAAT	**GCG**TGTCCAAT
-74 WT/ 4th Site Mut	TTCCCATTAA	TTCCCATT**GG**
-56 WT/ 5t Site Mut	TTAGGGGGAA	**GC**AGGGGGAA

General consensus sequence of Stat3: $(TT(N)_{4\text{-}6}\ AA)$

B.

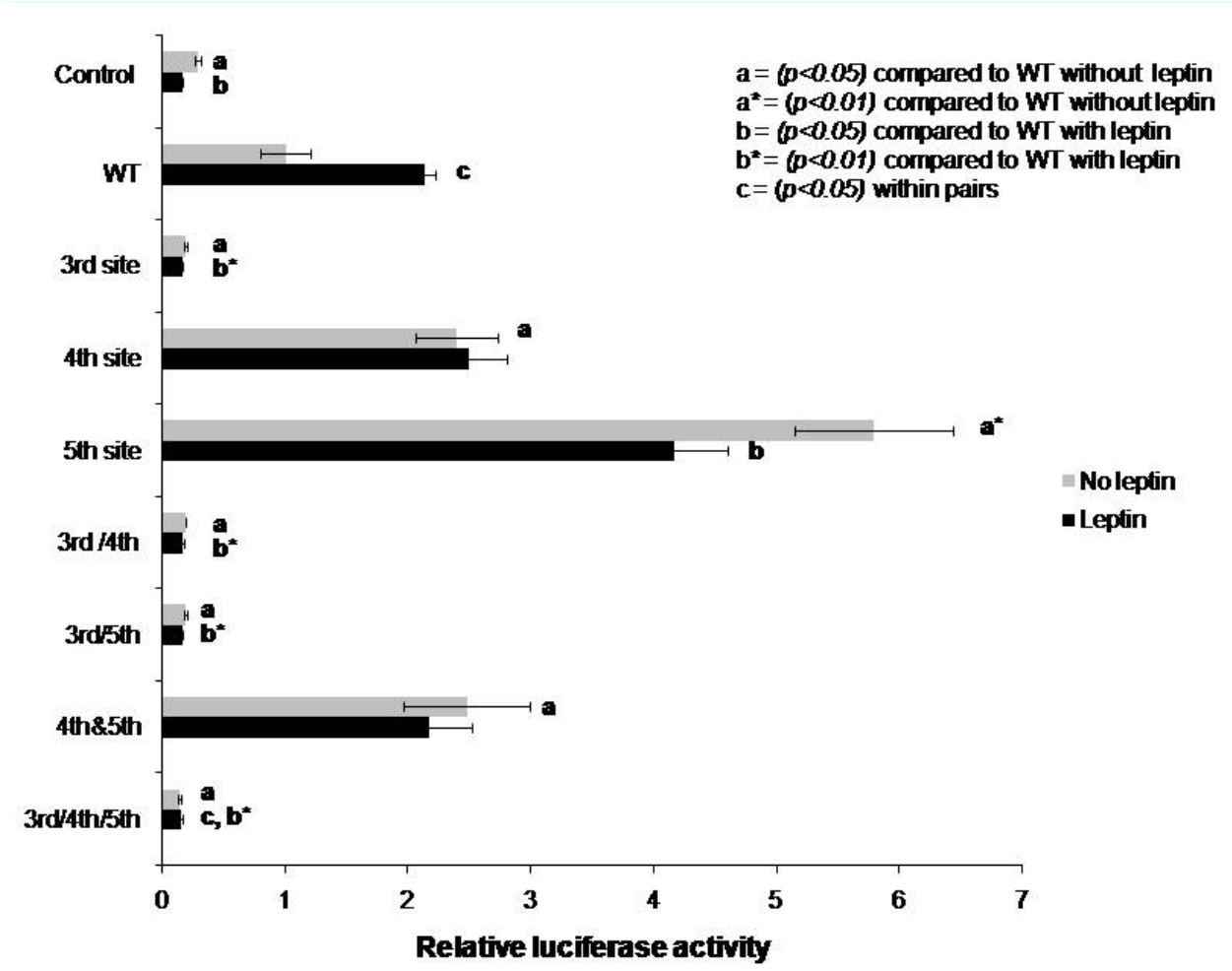

a = (p<0.05) compared to WT without leptin
a* = (p<0.01) compared to WT without leptin
b = (p<0.05) compared to WT with leptin
b* = (p<0.01) compared to WT with leptin
c = (p<0.05) within pairs
Control
WT
3rd site
4th site
5th site
3rd /4th
3rd/5th
4th&5th
3rd/4th/5th
a
b
c
b*
a*
c, b*
No leptin
Leptin
0
1
2
3
4
5
6
7
Relative luciferase activity

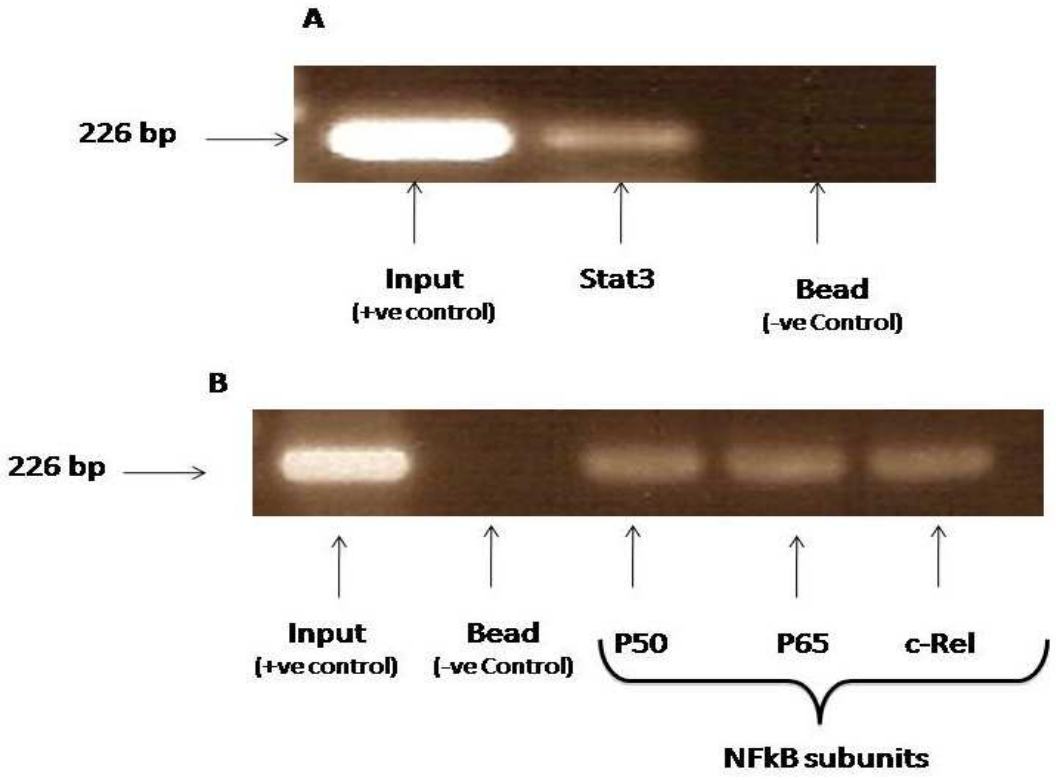
Figure 5: ChIP and EMSA demonstrate that Stat3 and NFκB bind their binding motifs on the Nhlh2 promoter.

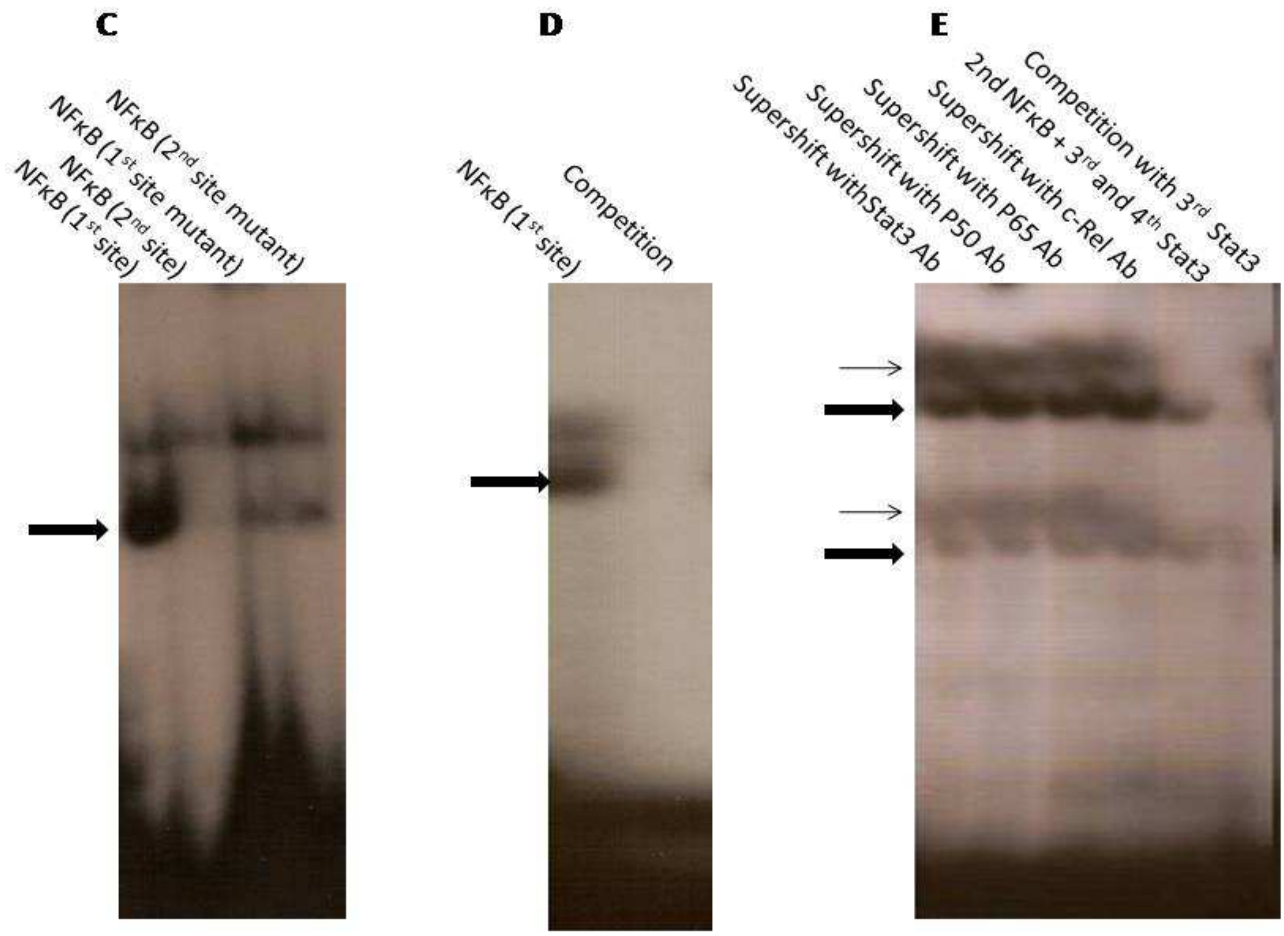
A ChIP assay demonstrates binding of Stat3, P50, P65, and c-Rel to the Nhlh2 promoter. Cross-linked chromatin from mice hypothalamus stimulated with leptin for 2 hours after 24 hours starvation were incubated with antibodies against Stat3, p50, p65, and c-Rel . Immunoprecipitates were decrosslinked and analyzed by PCR using primers amplifying the Nhlh2 promoter region containing the Stat3 and NFκB binding sites. The input (Figure 5 A and B, lane 1) included in the PCR represents 20% of the total chromatin without immunoprecipitation. A ChIP was performed for beads only (no antibody) negative control (Figure 5A, lane 3 and B, lane 2). Figure 5A, Lane 2 showing the Nhlh2 promoter immunoprecipitated with the Stat3 chromatin complex. While figure 5B, Lanes 3, 4 and 5 represent the Nhlh2 promoter immunoprecipitated with the p50, p65, and c-Rel chromatin complexes respectively.

Nuclear extract from N29/2 cells treated with leptin after serum deprivation for 12 hours were incubated with labeled oligonucleotide and ran in polyacrylamide gel. EMSA experiments reveal binding of NFκB to the 1st NFκB binding motifs at -135 but not to the 2nd one at -67 (Figure 5 C). Oligonucleotides include the 3rd Stat3, 2nd NFκB, and 4th stat3 binding sites motifs were used to investigate the ability of Stat3 and NFκB to bind to these motifs and determine the relationship between the Stat3

and the NFκB in these binding sites. (Figure 5E, Lanes 1,2,3,and 4) represents the Oligonucleotides contains the 3rd Stat3, 2nd NFκB, and 4th stat3 binding sites motifs incubated with Stat3, p50, p65, or c-Rel antibodies for supershift while the 5th lane does not include any antibody. The 6th lane represent the hot oligonucleotides include the 3rd Stat3, 2nd NFκB, and 4th stat3 binding sites motifs with cold oligos represent the 3rd Stat3 binding site motifs. The thick arrow points to the shifted complex, while the thin arrow points to the supershifted complex.

Figure 5


A
226 bp
Input
(+ve control)
Stat3
Bead
(-ve Control)
B
226 bp
Input
(+ve control)
Bead
(-ve Control)
P50
P65
c-Rel
NFkB subunits


C
NFκB (1st site)
NFκB (2nd site)
NFκB (1st site mutant)
NFκB (2nd site mutant)
D
NFκB (1st site)
Competition
E
Supershift withStat3 Ab
Supershift with P50 Ab
Supershift with P65 Ab
Supershift with c-Rel Ab
2nd NFκB + 3rd and 4th Stat3
Competition with 3rd Stat3

Tables

Table 1: Oligonucleotides used for site directed mutagenesis

PRIMER	SEQUENCE 5'—3'
NFκB 1st site (Mutant), sense	5' TGGGCTGTGAAAACT**TAA**ACACTGTGACAGATG 3'
NFκB 1st site (Mutant), antisense	5 'CATCTGTCACAGTGTTTAAGTTTTCACAGCCCA 3'
NFκB 2nd site (Mutant), sense	5 'AGGTTTCTGTCCAATAATATTTCCCATTAAATAT 3'
NFκB 2nd site (Mutant), antisense	5 'ATATTTAATGGGAAATATTATTGGACAGAAACCT 3'
3rd Stat3 (Mutant), sense	5' TAAGTGACTCGTGTCCAGGTGCGTGTCCAATAGGA TTTCCCAT 3'
3rd Stat3 (Mutant),antisense	5' ATGGGAAATCCTATTGGACACGCACCTGGACACG AGTCACTTA 3'
4th Stat3 (Mutant), sense	5' TAGGATTTCCCATTGGATATCAATTTAGGG 3'
4th Stat3 (Mutant),antisense	5' CCCTAAATTGATATCCAATGGGAAATCCTA 3'
5th Stat3 site (Mutant), sense	5' ATTAAATATCAATGCAGGGGGAAAAAAT 3'
5th Stat3 site (Mutant), antisense	5' ATTTTTTCCCCCTGCATTGATATTTAAT 3'
4th and 5th Stat3 mutants, sense	5' ATTGGATATCAATGCAGGGGGAAAAAAT-3'
4th and 5th Stat3 mutants, antisense	5' ATTTTTTCCCCCTGCATTGATATCCAAT 3'

Table 2: Oligonucleotides used for EMSA

PRIMER	SEQUENCE 5'—3'
NFκB 1st site, sense	5'GGCTGTGAAAACTCCCACACTG 3'
NFκB 1st site, antisense	5'CAGTGTGGGAGTTTTCACAGCC 3'
NFκB 2nd site, sense	5' CCAATAGGATTTCCCATTAAAT 3'
NFκB 2nd site, antisense	5' ATTTAATGGGAAATCCTATTGG 3'
NFκB 1st site (Mutant), sense	5' GGCTGTGAAAACTTAAACACTG 3'
NFκB 1st site (Mutant), antisense	5' CAGTGTTTAAGTTTTCACAGCC 3'
NFκB 2nd site (Mutant), sense	5' CCAATAATATTTCCCATTAAAT 3'
NFκB 2nd site (Mutant), antisense	5' ATTTAATGGGAAATATTATTGG 3'
3rd and 4th Stat3 and 2nd NFκB sites, sense	5' AGGTTTCTGTCCAATAGGATTTCCCATTAAATAT 3'
3rd and 4th Stat3 and 2nd NFκB sites, antisense	5' ATATTTAATGGGAAATCCTATTGGACAGAAACCT 3'
3rd Stat3 (WT), sense	5' GTCCAGGTTTCTGTCCAATAGGATTT 3'
3rd Stat3 (WT), antisense	5' AAATCCTATTGGACAGAAACCTGGAC 3'

Chapter 4

A Single Nucleotide Polymorphism in Nescient Helix Loop Helix 2 Gene Linked to Body Weight Phenotypes Reduces mRNA Stability

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Running title: Nhlh2 3'UTR SNP reduces its mRNA stability

Key Words: Nhlh2, NHLH2, mRNA, miRNA, 3'Untranslated Region, SNP, Hip-Waist Ratio, African Americans, Obesity

Introduction:

In spite of the transcription being a necessary step in gene expression, this does not mean that it is the only or even the main factor affecting the final concentration of the gene product produced. Many posttranscriptional regulatory elements, including microRNA (miRNA) binding sites, may have considerable effects on gene expression (*19*). miRNAs are noncoding RNAs which play a role in regulating gene expression and it starts to take on significant importance in molecular biology. They are small single stranded RNAs, approximately 22-nucleotides (nt) long, that modulate gene expression by eliminating or reducing the target gene expression. The miRNA pass through different processes until they reach mature miRNA. The first step is the synthesis of pri-miRNA which is transcribed from the miRNA gene and formed the hairpin structure. In the nucleus, the pri-miRNA is processed by Dorsh and is transferred by exportin-5 to the cell cytoplasm where a process controled by Dicer ribonuclease result in the release of a 22 nucleotide double strand RNA (*66*). This small RNA is then incorporated with RNA-induced silencing complex (RISC) and released a single strand miRNA as mature miRNA which has the ability to inhibit translation or degrade mRNA (*67*).

Studies on the mechanism of the miRNA action are still relatively new, and therefore more investigation is needed. Until now, research in this area revealed

that the miRNA regulates gene expression by either degrading the mRNA by pairing with the mRNA or inhibiting the translation of gene mRNA to protein (*29*).The miRNA inhibits protein translation through Watson–Crick base pairing to target the 3' untranslated region (3'UTR) of the messenger RNA (*30, 31*). A specific region called the seeding region is nucleotides 2 to 8 from the 5' end of the miRNA which binds to its target by Watson-Crick complementary. This is the most important part of the miRNA (*32*). So, the effectiveness of the miRNA depends on the complementary between the 5' end of the miRNA with its target and it is more important than the complementary in the miRNA 3' end. This rule has been confirmed by Brennecke et al. (*68*) when they found that a complementary of seven or more bases of the miRNA 5' end with its target is enough to affect gene regulation and the miRNA with less than that needs compensatory pairing in the miRNA 3' end to be functional. So there is a possibility that a SNP eliminates a miRNA target or makes a new sequence that matches perfectly to the seeding region of a miRNA that is not found in the given RNA (*69*). Because of the base-pairing requirements for miRNA/mRNA interaction, the single nucleotide polymorphisms (SNPs) that can reside in the miRNA binding site may affect the miRNA function by eliminating existing binding sites or creating illegitimate ones. Also SNPs in the miRNA genes themselves may affect the miRNA function. Therefore the SNPs in the miRNA gene or in the miRNA binding site may change gene expression (*46*).

Since their discovery two decades ago, the research illustrated that the miRNA had a significant role in regulating many biological and pathological processes. More than 8000 miRNA sequences have been found in different species including mammals, plants and viruses (*70*). Research reveals that the human genome has more than 600 miRNA and it is predicted that it may have more than 1000 miRNA (*71*). Each miRNA has the ability to bind to about 100 different targets (*72*). So, it is predicted that the human miRNAs might have the ability to regulate about 30% of the protein coding genes in the human genome (*73*). In the last decade, much research has indicated the importance of miRNA in different cellular processes such as proliferation, differentiation, cell growth, survival, and death. This implicates SNP in a miRNA binding site may affect the ability of miRNA to interact with its binding site results in change of gene expression which can cause risk of diseases. Much of this research reveals important evidence that the miRNAs involved in the carcinogenesis. The first study demonstrated the role of the miRNA in tumor growth identified incidence of chronic lymphocytic leukemia in B-cell caused by loss of miR-15a and miR-16-1 at chromosome 13q14.3 (*74*). miRNAs play a role in regulating gene expression and influence many biological processes including adipocyte differentiation, metabolic integration, insulin resistance and appetite. The miRNAs have been investigated to determine their targets which lead to better understanding of pathways of many metabolic diseases. This will improve the ability to invent different ways for obesity and many other diseases treatment (*75*). Research

reveals that the miRNAs play a significant role in obesity and many pathways that related to obesity such as glucose homeostasis, diabetes and adipogenesis. Krek et al. (*76*) found that miR–375 has an important effect on beta cells regulation function which affect the glucose homeostasis and they illustrated that both miR-124a and let-7b were highly expressed in the pancreatic islet cells and they important for blood glucose regulation. The *MiR-9* plays a role in insulin regulation by down regulating the Onecut-2 transcription factor which plays as a negative regulator of secretin (*77*). Other researchers demonstrated that miR-122 has a significant effect on controlling the triglyceride metabolism and cholesterol synthesis in mouse liver (*78*) and miR-*143* is important in adipocyte differentiation of human (*79*). The hypothalamic brain-derived neurotrophic factor (BDNF) is one of factors that play an important role in regulation energy balance and it has an important effect on obesity (*80*). It was found that the pyramidal neurons which express the BDNF have high levels of Dicer and Risc which are essential components of miRNA biosynthesis and effectiveness (*81, 82*). Many other studies found a relationship between SNPs in miRNA binding sites and risk of diseases. A SNP in the 3'UTR of the human SLITRK1 gene created illegitimate miRNA binding site for miR-189, which has been found to cause Tourette syndrome by inhibiting the expression of this gene (*83*). By analyzing SNPs that change the sequence of miRNA binding sites and alter their ability to bind to their targets, an association has been found between one of these SNPs located at the insulin receptor gene with cancer risk (*84*). A SNP in the Mir717,

which targets about 91 gene associated with obesity, affects its seeding region and has been found in the lean mouse strain 129/Sv (*85*). The SNP in the 3'UTR of the hAT_1R gene which located at position 1166 and convert A to C , at the cis regulatory element where the miR-155 interact with its target, is associated with hypertension and many other disorder related to cardiovascular disease and its existence in the 3'UTR decreased the expression of reporter gene(*86*).

The NHLH2 gene has 23 known SNPs as of the dbSNP Build 133 of the SNP database, one of them located at the position 1568 in the NHLH2 mRNA ($NHLH2^{A1568G}$ rs11805084). This SNP, which converts Adenosine to Guanine, is located in the 3'UTR of the NHLH2 mRNA. Based on the location of the SNP in the 3'UTR in proximity to AUUUA elements that can control mRNA stability (*87, 88*), we asked whether this SNP affected mRNA Stability of Nhlh2. Further, we examined the relationship of this SNP to body weight phenotypes in an African American cohort.

Data analysis showed that a polymorphism in the 3'-tail rs11805084 ($NHLH2^{A1568G}$) has an association with hip waist ratio of a group of 509 African Americans suffering from Insulin Resistance Atherosclerosis. This data showed a significant association between this SNP and the BMI adjusted waist measurements ($p = 0.043$) and waist-hip ratios ($p = 0.035$) (Bowden, D., and Good, D., unpublished). The miRNAs have the ability to affect mRNA stability

and translation (*89*), so it is possible that the NHLH2^{A1568G} SNP affects posttranscriptional regulation of NHLH2 and the expression of this gene by disrupting a putative miRNA binding site or creating illegitimate miRNA binding sites. Therefore, this SNP may have a clinical importance issues. In this study, the biological consequences of the SNP on Nhlh2 mRNA stability and protein levels have been analyzed. The objective of this study is to determine if the mutations in the 3'UTR of Nhlh2 affect protein translation or mRNA stability or if it has no effect on Nhlh2 mRNA levels. According to this information, we hypothesize that the hunman NHLH2 3'UTR SNP which changes a conserved A residue to G affects the stability of its mRNA and thus the expression of the Nhlh2. The Nhlh2 mouse with engineered mutation was used to test the hypothesis.

Results:

Nhlh2 3'UTR SNP destabilizes the Nhlh2 mRNA.

The effect of Nhlh2 3'UTR SNP has been assayed to measure the differences in mRNA stability between construct containing the Nhlh2 coding region with the 3'UTR and another one that has the same sequence but contains the Nhlh2 3'UTR SNP. Nucleotide substitutions corresponding to the Nhlh2 3'UTR SNP have been tested to determine whether this SNP has an effect on Nhlh2 mRNA stability. The Nhlh2 mRNA decay assays were implemented using hypothalamic cell line N29/2 transfected with plasmid fused with Nhlh2 sequence, attached to myc tag to distinguish it from the endogenous Nhlh2, with or without Nhlh2 3'UTR SNP. Transcription was inhibited using actinomycin D, allowing for RNA harvesting at 30, 60, 120 and 240 minutes after actinomycin D treatment to determine half-life of the mRNA species. The Nhlh2 mRNA at each time point was measured by real time quantitative PCR (Q-PCR) using a primer set to detect only the myc-tagged mRNA (Figure 1B). The presence of SNP in the Nhlh2 3'UTR decreased the mRNA levels significantly ($P < 0.01$). The analysis Nhlh2 3'UTR sequence shows that induced mutation in the mouse Nhlh2 has the potential to create illegitimate binding site for mmu-miR1905 miRNA.

Presence of the Nhlh2 3'UTR SNP decreases reporter protein levels.

To study the importance of this SNP in regulating Nhlh2, a vector with the 3'UTR SNP cloned downstream luciferase as a reporter gene was used. This vector contained the CMV promoter, which regulates the expression of the reporter luciferase gene fused to 3'UTRs.This assay has been used to detect the difference in mRNA stability in a similar previous studies (*90*). Hypothalamic cell line N29/2 has been used for luciferase assay. The luciferase activity of the Luciferase-pcDNA3 plasmid which has the CMV promoter and the SV40 poly (A) signal gave relatively similar expression compared to the plasmid which had Nhlh2 3'UTR instead of SV40 poly A. These results suggested that substitution of SV40 with the Nhlh2 3'UTR did not affect the Luciferase-pcDNA3 plasmid activity. The luciferase activities of the constructs containing the Nhlh2 3'UTR containing the SNP were 38.5% lower than the construct without SNP ($P < 0.01$) (Figure 1). These results suggest that the Nhlh2 3'UTR SNP down regulates the Nhlh2 expression.

Discussion

The NHLH2 gene has been analyzed for SNPs. There are a total of 10 promoter SNPs, 8 SNPs in intronic sequences, and 7 SNPs in either the 3' or 5' UTR of the mRNA (data not shown, but available on NCBI SNP database). One of the SNPs in the 3'UTR is of interest because of its location with a putative miRNA binding site, and AU-rich sequence elements (ARE) binding protein site (for mRNA stability). So this SNP has the potential to alter the NHLH2 mRNA and protein level. SNPs could influence gene expression through different mechanisms. SNPs in the 5' flanking region might modulate gene transcription by eliminating binding sites of transcription factor to the promoter or by creating binding site for transcription factor that do not exist normally. SNPs in the coding regions might affect the structure of the protein produced from that gene and it might affect its properties. The SNP in the Nhlh2 3'UTR might affect the miRNA binding site or the mRNA stabilizing protein binding site. Our analysis supports this and shows that induced mutation in the mouse Nhlh2 has the potential to create illegitimate binding site for mmu-miR1905 miRNA.

In this study we were able to prove that the SNP that engineered in mouse Nhlh2 3'UTR reduces the Nhlh2 expression and we demonstrated that by using two different methods. First, Nhlh2 mRNA quantity of transcripts either containing the SNP or WT was measured by real time PCR. Second, a luciferase reporter gene construct was used to demonstrate that the effect of this SNP was

transferable to a non-related gene, luciferase. Both of the luciferase method and the real time PCR gave the same results, demonstrating that the Nhlh2 3'UTR SNP decreases the stability of Nhlh2 mRNA transcript. It is possible that the SNP in the 3'UTR created a new sequence match the binding site of a miRNA that is not found in the given RNA (*69*). In this study, the effect of mouse induced mutation in the Nhlh2 3'UTR has been investigated to determine the effect of this SNP on mRNA stability. While the SNP in the 3'UTR of the Nhlh2 has the potential to create illegitimate miRNA bind site and delete mRNA stability-binding proteins site, the molecular effect of this SNP on the Nhlh2 expression remains to be defined.

The effect of the mouse Nhlh2 3'UTR SNP has a clear effect on the Nhlh2 mRNA stability. Since this SNP decreased the mRNA stability, it might create illegitimate miRNA binding sites which are responsible for this destabilization. An investigation on the ability of the Nhlh2 3'UTR to create a sequence match to miRNA that did not originally exist without this SNP shows that this SNP created a sequence has the potential to bind with the mmu-miR1905 miRNA. It is possible that this SNP may change the secondary structure of the 3'UTR which is an important factor to determine the protein binding site. Also the secondary structure is important to affect the rate of mRNA cleavage by endonucleotases. This SNP could affect mRNA abundance by influencing the stability of gene mRNA by affecting the polyadenylation site selection. The Nhlh2 has 8 AU rich regions, these regions might be a target of different proteins which bind to these

regulatory elements and destabilize or stabilize the mRNA. Yugami et al. (*91*) have shown an interaction of hnRNP-U protein with the 3'UTR of the human NHLH2, and increased stability during interaction and they found that the hnRNP-U mRNA stability targets are also greater with increased energy availability (*91*). The NHLH2 3'UTR SNP is in one of the 8 AU rich regions and it is possible that converting the A to G at this position results in eliminating the binding site of stabilizing protein hnRNP-U.

The molecular effects of this SNP using an in vitro assay were used to detect differences in mRNA stability. This SNP was engineered into mouse Nhlh2 and was analyzed in hypothalamic cell line (N29/2) that expresses endogenous Nhlh2. While using luciferase as a reporter gene is a way to avoid problems to discriminate between the endogenous mRNA and the mRNA under investigation, myc tag has been used in the QPCR experiment by attaching to the Nhlh2 coding region to differentiate it from the endogenous Nhlh2 by designing primers detecting both the myc tag and the Nhlh2. While the QPCR experiment demonstrated that the Nhlh2 3'UTR SNP affected Nhlh2 expression level, using the luciferase as a reporter gene instead of the Nhlh2 coding region in the second experiment demonstrated that the effect of Nhlh2 3'UTR on mRNA stability is protein independent. Regardless of whether the SNP with Nhlh2 tail is linked to the native protein coding region or to luciferase, it affects mRNA stability. So, the SNP in the tail can independently affect any protein level that attached to the mutant Nhlh2 tail.

The results of this study demonstrated that the induced SNP in the mouse Nhlh2 3'UTR SNP which change the conserved A residue to G has the potential to affect human NHLH2 mRNA level. This result supports the hypothesis that the SNP which convert A to G in the NHLH2 tail ($NHLH2^{A1568G}$ SNP) affect the stability of mRNA and so the expression of N. This study represents the first evidence of a direct effect of Nhlh2 3'UTR SNP on Nhlh2 gene expression. These findings will assist in the interpretation of factors affecting the NHLH2 expression.

MATERIALS AND METHODS:

Generation of constructs

In order to test the hypothesis that the Nhlh2 3'UTR SNP which converts A to G affects the stability of the Nhlh2 mRNA, two constructs have been created. One has the mouse Nhlh2 sequence with myc tag while the other has luciferase as a reporter gene attached to the Nhlh2 3'UTR. Each one of these construct has been used as a template to create Nhlh2 3'UTR SNP to convert A to G.

Myc-tag Nhlh2 sequence construct.

It is expected that the Nhlh2 3'UTR SNP will result in create of a miRNA binding site; this may result in destabilization of the Nhlh2 mRNA or a lack of specific regulation of Nhlh2 mRNA under certain physiological conditions. To analyze the mRNA stability, Actinomycin assay was used to measure mRNA stability. N29/2 cells were transfected with either WT Nhlh2 expression vector or an expression vector in which the Nhlh2 3'UTR SNP has been created by site-directed mutagenesis. Twenty-four hours following transfection, cells were treated with Actinomycin D (5 μg/ml) for 30, 60, 120 and 240 min. RNA was prepared and used in real-time Q-PCR analysis for Nhlh2 mRNA. The 18s ribosomal gene was used as a control.

A 1.2 kb fragment of Nhlh2 3'UTR was excised from a vector containing mouse Nhlh2 sequence using SacI and NotI enzymes. The 3'UTR of Nhlh2 was

cloned into PCS2-MT vector which already contained the myc tag attached to mouse Nhlh2 coding sequence. This plasmid was cut with SacI and NotI enzymes to open an insertion sites for the 3'UTR. The 3'UTR was inserted directly after the Nhlh2 coding sequence. The feature myc tag attached to Nhlh2 was used to differentiate between the indigenous Nhlh2 and the Nhlh2 expressed from this vector. To create another vector which has Nhlh2 3'UTR SNP, this construct was used as a template for site directed mutagenesis reaction (Figure 1A).

Luciferase-3'UTR reporter constructs

Chimeric mRNA between Nhlh2 and firefly luciferase were constructed as a second method to study mRNA stability. The full-length of Nhlh2 3'UTR were cloned downstream of the firefly luciferase gene in Luciferase-pcDNA3. Site directed mutagenesis of the WT construct was used to produce the 3'UTR SNP-containing construct (Figure 3). Luciferase is a long-lived protein and it has been used to study ARE- and miRNA mediated inhibition of translation (*90*).

A 1338 bp fragment of Nhlh2 3'UTR was amplified from mouse Nhlh2 sequence using Hot Master Taq DNA Polymerase (5-Prime) and cloned into pBluescript. The 3'UTR then digested by ApaI and SmaI enzymes and cloned into pcDNA3 vector features is driven by the CMV promoter and it has luciferase as a reporter gene. The pcDNA3 was cut with ApaI and SmaI to get rid of SV-40 and insert the Nhlh2 3'UTR directly downstream of the luciferase coding

sequence. The plasmid was subjected to restriction enzyme digestion followed by agarose gel electrophoresis to assess the incidence of insertion followed by sequencing to verify the insertion (Figure 2A). This construct has been used as a template to create another plasmid with mutation in the 3; UTR at site 1568 which convert adenosine to guanine.

Site Directed mutagenesis:

The mutation has been created by PCR site-directed mutagenesis method. PCR reactions have been performed using iProof High fidelity DNA polymerase (BioRad) along with WT Nhlh2 3'UTR constructs as a template with an 8 or 5 minutes elongation time for the 1st plasmid and 2nd plasmid respectively. Two primers which have the mutation in the middle of them have been used for this purpose; the forward primer was 5' GCAGGATTTGAGCTTGGTGGGACTTTAACCCCAAGATAG 3' and the reverse was 5' CTATCTTGGGGTTAAAGTCCCACCAAGCTCAAATCCTGC 3'. The PCRs were carried out using 5 ng of template in 50 µl reaction. The first cycle was performed at 98°C for 3 min, and then followed by 35 cycles at 98°C for 10 s, 55°C for 30 s, and 72°C for 5 minutes. The reaction was terminated at 72°C for 10 min. The PCR product was subjected to agarose gel electrophoresis (1% agarose gel) and stained with Ethidium Bromide (EtBr) to detect the successful of amplification. The PCR product was digested with DpnI to break the template DNA and keep only the PCR product. The DpnI-digested PCR product was

transformed into E-coli and cultured to amplify the plasmid and extract it. The presence of the appropriate mutations was verified by DNA sequence analysis at the Virginia Bioinformatics Institute to ensure that the correct mutations were introduced and that there were no PCR-errors generated. Reporter plasmid DNA was purified using QIAGEN Midi-Prep kits (Valencia, CA).

Transfection for luciferase assay:

The hypothalamic cell line N29/2 (Cellutions Biosystems, Toronto, Ontario), were grown in DMEM-high glucose (4.5 g/liter) medium and 4.5 Sodium pyruvate containing 10% fetal bovine serum, 100 units/ml penicillin, and 10ug/ml streptomycin (HyClone, Logan, UT). Cells were incubated in 37°C under a 95% air and 5% CO2 atmosphere. Transient transfection of the cells was performed in 12-well tissue culture plates using Effectene transfection reagent (Qiagen, Valencia, CA) following the manufacturer instructions. Cells were seeded into the wells about 24 hours prior to transfection. Each well was seeded with 1.2 ml of media with 1-2 x 10^5 cells per ml in order to get 40-60 % confluency after 24 hours. Twenty four hours following cells seeding, each well received 400 ng of luciferase reporter plasmid (WT) or the mutant plasmid. All wells received 35 ng of β-gal plasmid as an internal control plasmid. Cells were washed with PBS 24 hours after transfection and lysate were collected for luciferase and β-gal assay. To assay luciferase activity, 5 µl of lysis was placed in the wells of a white 96-well plate and 100 µl luciferase assay buffer (Promega, Madison, WI) was injected

sequentially; relative light units were measured by (GLOMAX Multi Detection System) from (Promega, Madison, WI). The β-galactosidase activity was taken to be the rate of increase of absorbance at 450 nm. In order to measure the relative luciferase activity, the luciferase activity of each sample was divided by the corresponding relative β-gal activity. Three independent transfection experiments were performed to confirm the results. In each experiment, triplicate wells were transfected, and luciferase and β-galactosidase activities for each well were determined in triplicates. The effect of Nhlh2 3'UTR SNP on the luciferase activity was compared with that of the wild-type construct using paired t tests.

mRNA stability assays

The assay was performed on the hypothalamic N29/2 cell line. The cells were maintained in DMEM-high glucose (4.5 g/liter) medium and 4.5 Sodium pyruvate containing 10% fetal bovine serum, 100 units/ml penicillin, and 10ug/ml streptomycin (HyClone, Logan, UT) at 37°C in 5% CO2. Cells were transfected with a total amount of 400 ng of DNA at 40-60 % confluence in 12-well plates using Effectene transfection reagent (Qiagen, Valencia, CA) following the manufacturers protocol. To analyze the mRNA stability, Actinomycin assay was used to measure mRNA stability. Twenty-four hours following transfection, cells were treated with media having 5 µg/ ml Actinomycin D (Sigma, Saint Louis, Missouri) for 30, 60, 120 and 240 min. Total RNA was extracted using the Trizol extraction protocol. The RNA used to synthesize cDNA. The Nhlh2 mRNA levels

were measured using the SYBRGreen PCR master mix (Applied Biosystems, Foster city, California), using forward primer 5' ATGGAGAGCTTGGGCGACCTCA 3' and reverse primer 5' TTGGTCCGGACTCAGCATCATCGAAT 3' which target the sequence flanking both of myc tag and the Nhlh2 coding region to differentiate between the endogenous Nhlh2 which was already expressed in N29/2 cell line and the Nhlh2 expressed from the transfected DNA. The Nhlh2 mRNA expression levels were normalized to the level of 18s rRNA housekeeping gene using the forward primer 5' CGCCGCTAGAGGTGAAATTC 3' and the reverse primer 5' TTGGCAAATGCTTTCGCTC 3'. Real-Time PCR experiments were performed on a 7900 HT Fast Real-Time PCR System (Applied Biosystems, Foster city, California).

Figures:

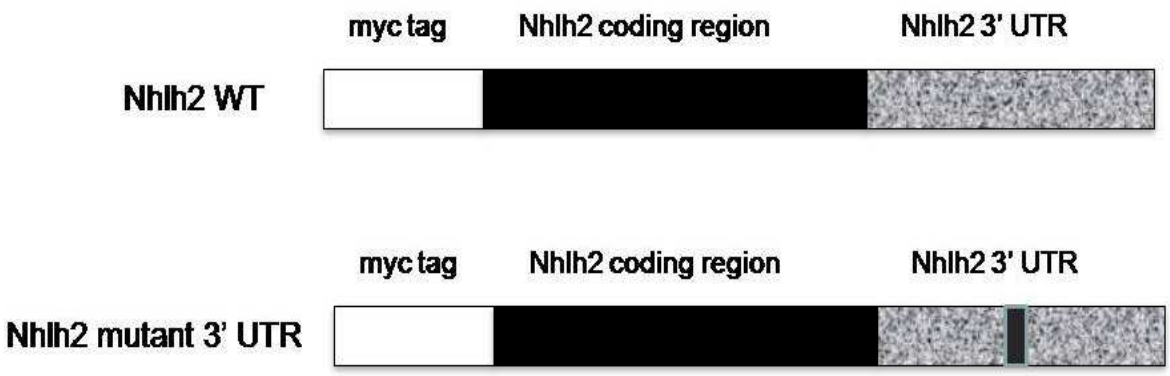
Figure 1: Effect of Nhlh2 3'UTR SNP on mRNA stability.

A. Mouse Nhlh2 sequence has been fused into PCS2-MT vector. The Nhlh2 3'UTR region was introduced using restriction enzymes SacI and NotI enzymes were used to insert the Nhlh2 3'UTR sequences. This vector transfected into N29/2 cell (Nhlh2 WT) or the same construct but contain mutation in the Nhlh2 3'UTR (Nhlh2 mutant 3'UTR).

B. N29/2 cells transfected with the construct containing the WT Nhlh2 myc tail (black diamond) or with the same plasmid containing the mutant Nhlh2 myc tail construct (grey squares). Cells were treated with actinomycin D and RNA was harvested at different time points after the Actinomycin D treatment. The 18s rRNA housekeeping was used as a control. The data shown are the means ± SEM ** $P < 0.01$.

Figure 1

A.



WT GCAGGATTTGAGCTTGGTGAGACTTTAACCCCAAGATAGG

Mutant GCAGGATTTGAGCTTGGTGGGACTTTAACCCCAAGATAGG

B.

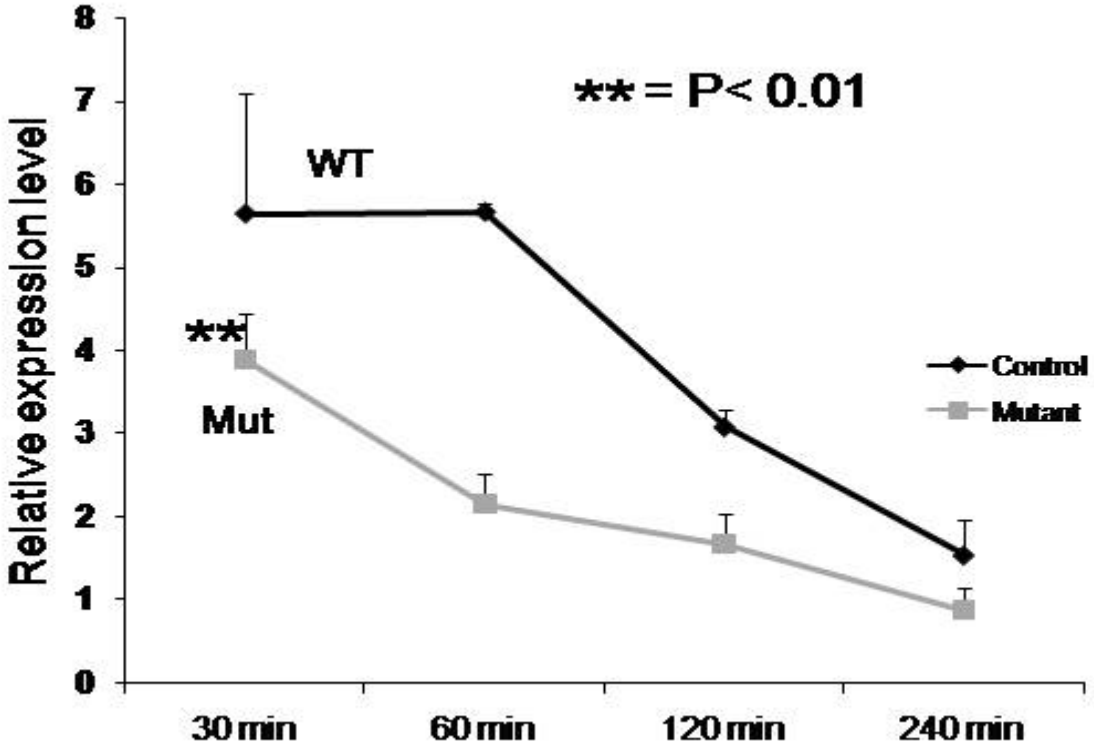

Relative expression level
8
7
6
5
4
3
2
1
0
** = P< 0.01
WT
**
Mut
Control
Mutant
30 min
60 min
120 min
240 min

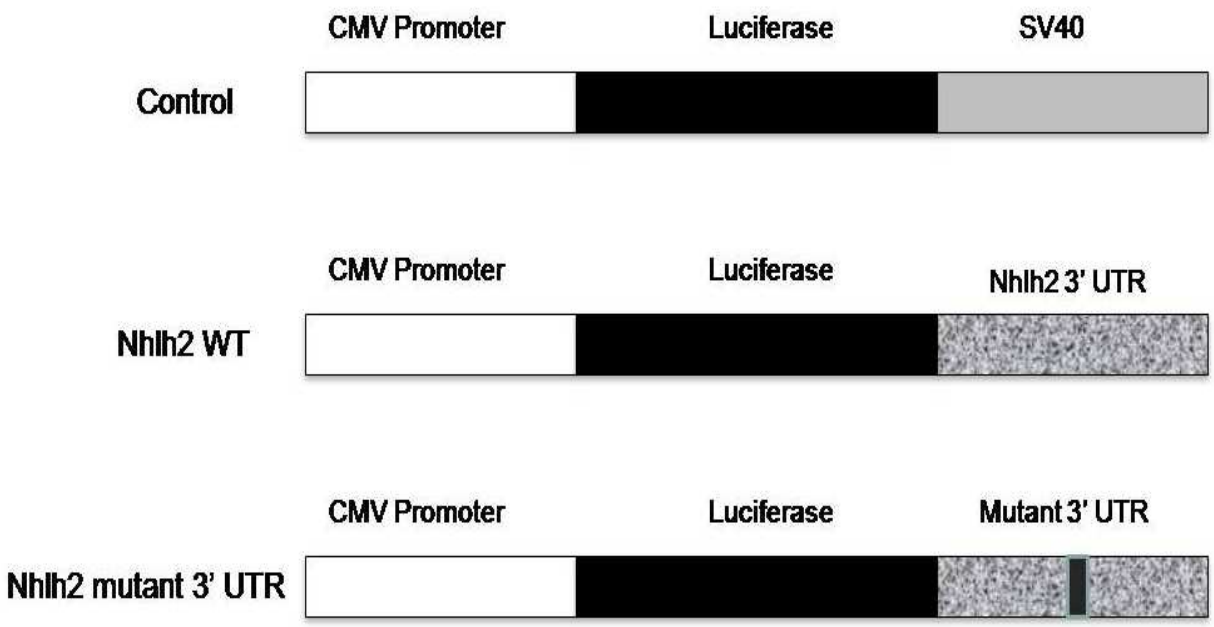
Figure 2: Effect of Nhlh2 3'UTR SNP on luciferase activity.

A. Luciferase reporter constructs used in transfection assays were created from a luciferase-pcDNA3 vector plasmid. The Nhlh2 3'UTR were cloned downstream of the firefly luciferase gene in uciferase-pcDNA3 vector. The SV40 were replaced with Nhlh2 3'UTR sequences. The luciferase-pcDNA3 used as a control treatment (control), the plasmid which has the luciferase with 3'UTR named (Nhlh2 WT), while the one which has mutation in 3'UTR named (Nhlh2 mutant 3'UTR).

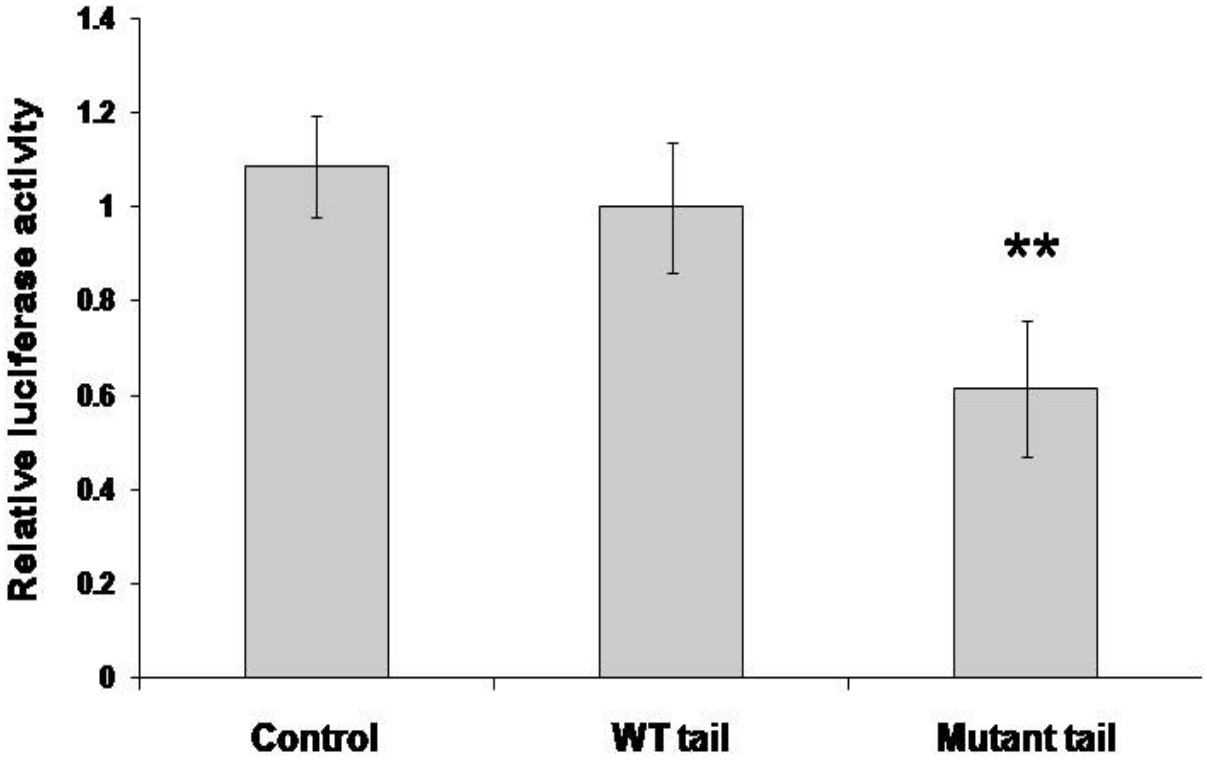
B. Luciferase reporter plasmid with 3'UTR fused downstream luciferase was labeled as (WT tail), while the plasmid containing substitution of adenosine with guanine labeled as (mutant tail). The luciferase-pcDNA3 used as a control treatment (control). Cells were harvested 24 hours after transfection and assessed for luciferase activity. Relative β-galactocidase was used as a control for the transfection efficiency. The data shown are the means ± SEM *P < 0.05, ** P < 0.01.

Figure 2

A.


CMV Promoter
Luciferase
SV40
Control
CMV Promoter
Luciferase
Nhlh2 3' UTR
Nhlh2 WT
CMV Promoter
Luciferase
Mutant 3' UTR
Nhlh2 mutant 3' UTR

B.


Relative luciferase activity
1.4
1.2
1
0.8
0.6
0.4
0.2
0
**
Control
WT tail
Mutant tail

Chapter 5

Implications and future directions

Mice containing deletion of Nhlh2 (N2KO mice) is the only obese mouse model which has deletion of the neuronal bHLH transcription factor causing adult onset obesity. The weight gain in the N2KO mice is due to reduced physical activity rather than increased food intake (*35*). This gives a good opportunity to study the rule of exercise in controlling body weight. It is clear that the Nhlh2 is one of the essential genes that play a significant role in maintaining body weight, but there is a need to understand the mechanisms and determine the factors that modulate the expression of this gene. So, understanding the mechanisms controlling the Nhlh2 might give a better understanding of how the other genes downstream from the Nhlh2 maintain body weight.

While Nhlh2 is one of the transcription factors which are already known as an important gene that plays a role in regulating body weight. It also regulates many other genes involved in maintaining body weight and obesity. This study was designed to understand the mechanisms that regulate the expression of Nhlh2 under different states of energy availability. In particular, were trying to determine the transcription factors that control the Nhlh2 expression and the posttranscription factors that modulate the Nhlh2 mRNA stability. The first part of this study concentrated on determination of the transcription factors that regulate the Nhlh2. Determination of these transcription factors not only help to

understand how this gene is regulated, but also might guide us to understand how the other genes downstream from Nhlh2 is regulated which can lead to a better understanding of the physiology of maintaining body weight.

The analysis of the Nhlh2 promoter showed that it has five putative binding sites for Stat3 and two for NFκB. We were able to demonstrate that both Stat3 and NFκB can bind to their binding sites on the Nhlh2 promoter. We also were able to show that both the Stat3 and the NFκB participate in Nhlh2 regulation. While the NFκB acts as a negative regulator, the Stat3 is responsible for the basal expression of Nhlh2 and leptin stimulation (Figure 1). This finding helps to better understand how leptin affects the transcriptional regulation of Nhlh2 and in turn how it regulates body weight.

It is well known that Stat family transcription factors are normally exist as inactive forms in the cytoplasm. In response to growth hormones and cytokines such as leptin, IL6, IL5, or interferon, these transcription factors are phosphorylated and translocated to the nucleus, where they bind to DNA cis elements in target promoters to regulate gene expression. Thus, changes in Stat3 nuclear translocation in response to these cytokines might affect the expression of Nhlh2 and in turn the response to energy expenditure. On the other hand, NFκB is one of the transcription factors that respond to inflammatory cytokines such as IL-6. The NFκB proteins are inactive in the cytoplasm attached to the IκB family inhibitors. In response to inflammatory cytokines, or stress, the IkBs are phosphorylated and triggers free NFκB to translocate to the nucleus and

activate transcription of genes related to immunity. According to our results, The NFκB plays as a negative regulator of Nhlh2; so when the body responds to inflammation or stress, it might decrease the Nhlh2 expression which in turn affects the energy expenditure and body weight. Both of Stat3 and NFκB, which play a role in Nhlh2 regulation, are major mediators in response to inflammation. These transcription factors have been implicated in many of human diseases, including different kinds of human cancer, cardiac, liver, lung, and digestive system diseases. Since the Stat3 and the NFκB have been implicated in these diseases, it is possible that these diseases might modulate the Nhlh2 expression.

The results of the Nhlh2 transcriptional regulation determine the transcription factors that regulate the Nhlh2 expression under different energy states. These results will lead to a better understanding of the physiological aspects of exercise and obesity since this gene has an obvious effect on voluntary exercise and the development of obesity. These results opened up different directions for research and a better understanding of Nhlh2 expression. The transfection experiments demonstrated the importance of Stat3 and NFκB in regulating Nhlh2 with an emphasis on the effect of Stat3 on both the basal expression of Nhlh2 and the expression after leptin stimulation. The results of EMSA showed that both Stat3 and NFκB are bound as a complex to the Nhlh2 promoter in the sequence that has overlapping between their binding sites. This interesting result opens many directions of research that can be done to investigate the kind of relationship between these transcription factors to regulate

Nhlh2 expression. It is possible that the expression of Nhlh2 is controlled by co-regulation between Stat3 and NFκB especially in the site that has overlapping between the Stat3 and the NFκB binding sites, so more research is needed to determine the relationship between these two transcription factors to regulate Nhlh2. Another direction is to identify if the Stat3 in this complex is phosphorylated or not because research shows a possibility of interaction between unphosphorylated Stat3 and NFκB to drive different pathways that the phosphorylated Stat3 do.

The second part of this research concentrated on studying the effect of a SNP in the human NHLH2 tail on its expression. SNPs in the 3'UTR might influence the stability of gene mRNA by eliminating a microRNA target or make a new sequence match on the binding site of a microRNA that is not found in the given RNA (*69*). The NHLH2 gene has 23 SNPs; one of these SNPs located in the 3'UTR has the potential to create microRNA binding sites. In this part of my study, the ability of this SNP to down regulate or increase the expression of human NHLH2 was investigated by using engineered mouse Nhlh2 3'UTR SNP. By using two different methods, real time PCR to measure the quantity of Nhlh2 mRNA and luciferase as a reporter gene, we were able to demonstrate that the Nhlh2 3'UTR SNP has the ability to decrease the stability of Nhlh2 mRNA (Figure 1). Findings from this part of my research give a better understanding of how the Nhlh2 could be regulated and it will help to link human genetic mutations to changes in the translation of Nhlh2 which in turn affect the body weight and the

obesity in the human with its complication on the individuals' health. Because of its effect on reducing the expression of Nhlh2, this SNP might create illegitimate miRNA binding site or it might eliminate the binding site of the mRNA stabilizing protein hnRNP(*91*). Since this SNP reduced the expression of Nhlh2, it might modulate the energy balance of the individual carriers of this SNP by reducing energy expenditure and increase body weight. These findings open up the possibility to do more research to invent a treatment to the people who have this kind of SNP.

Data analysis showed that a polymorphism in the human NHLH2 3'-tail rs11805084 ($NHLH2^{A1568G}$) has an association with hip waist ratio of a group of 509 African Americans suffering from Insulin Resistance Atherosclerosis (Bowden and Good, unpublished). This data showed a significant association between this SNP and the BMI adjusted waist measurements. While the NHLH2 has 23 SNPs distributed between the promoter SNPs, introns, and the UTR regions, this mutation in NHLH2 3'UTR appears to contribute to differences in human body weight. These results opened different directions for study. We already know that this SNP reduced the expression of Nhlh2, it is now important to determine how this SNP affects the expression. Our analysis of this SNP revealed that it has the ability to create miRNA binding sites that did not exist without this SNP. This miRNA is (mmu-miR1905) which belongs to (MIPF0000753; mir-1905) gene family. The miRBase accession number of this miRNA is MIMAT0007866 and its mature sequence is

CACCAGUCCCACCACGCGGUAG. The gene of this miRNA locates on murine Chromosome 3. The next step is to determine if this miRNA is expressed in the hypothalamus cells. If the results showed that this miRNA is expressed in the hypothalamus cells, the effects of this miRNA on the Nhlh2 expression will be determined. If this did not show any effect of the miRNA on the Nhlh2 expression, it is possible that this SNP affects the binding site of mRNA stabilizing proteins. Yugami et al. (*91*) showed an interaction between one of the mRNA stabilizing protein ,called hnRNP-U, with the NHLH2 mRNA which increased its stability during the interaction. The $NHLH2^{A1568G}$ SNP has the potential to affect the binding site of the hnRNP protein and result in decreasing the stability of it mRNA. So, the other direction of research will be testing the possibility that the hnRNP-U binds to the ARE modified by the $NHLH2^{A1568G}$SNP, and that the variant disrupts binding.

In this study we were able to determine the transcripton factors that control the expression of Nhlh2. As a conclusion of this study, the Nhlh2 is regulated at transcriptional level by both of Stat3 and NFκB transcription factor. While the Stat3 is required for the basal Nhlh2 expression and induction of transcription following leptin stimulation, the NFκB acts as an inhibitor of Nhlh2 expression. Also we demonstrated that the Nhlh2 3'UTR SNP decrease the Nlhh2 mRNA stability significantly. These results support our hypothesis that the Nhlh2 is regulated by Stat3 and NFκB at the transcriptional level and by the

Nhlh2 3'UTR SNP at the post-transcriptional level. This study represents the first evidence of a direct effect of Nhlh2 3'UTR SNP on Nhlh2 gene expression.

In the first part of this study, we were able to show by the transactivation experiments that both Stat3 and NFκB are involved in the Nhlh2 transcriptional regulation with evidence that the Stat3 is the key player in regulating basal and leptin stimulation of Nhlh2. Moreover, the DNA_protein binding experiments demonstrated that the binding sites of the Stat3 and the NFκB on the Nhlh2 promoter are real binding sites. Our lab was able to prove that the Nhlh2 is the transcriptional regulator of genes related to energy expenditure and obesity such as MC4R and PC1/3, while this study determined the transcriptional regulator of the Nhlh2 itself. In the second part of the study, we studied the effect of the Nhlh2 3'UTR SNP on its mRNA stability and we were able to demonstrate that this SNP can decrease the stability of the Nhlh2 mRNA and down regulate it.

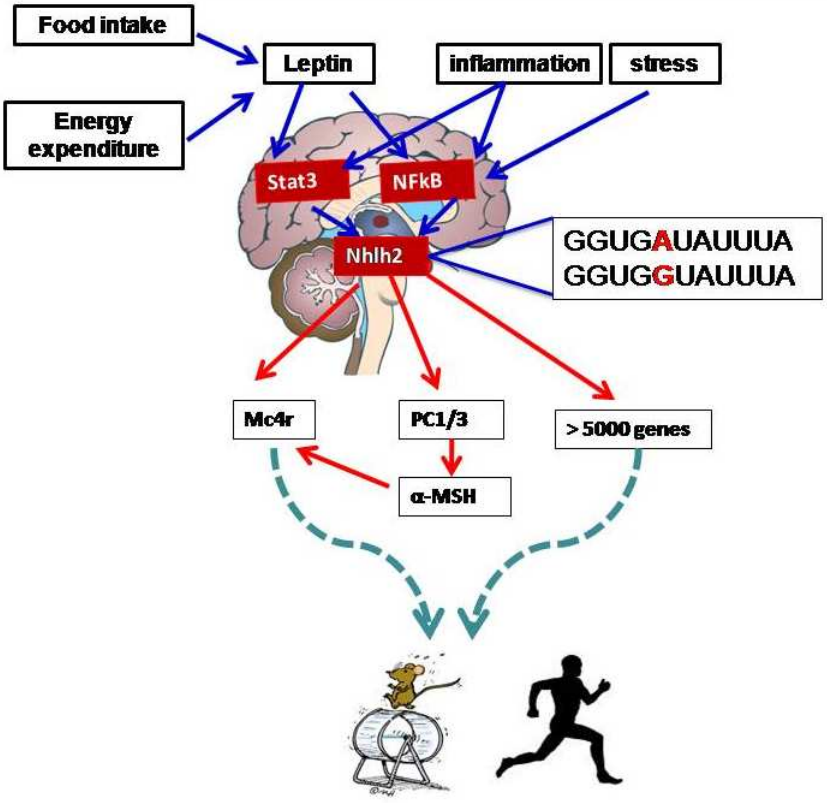
Taken together, the results of this study demonstrated that the Nhlh2 is regulated transcriptionally by Stat3 and NFκB and posttranscriptionally by factors that potentially affect its mRNA stability. These results in revealed the role of Stat3, NFκB, and the Nhlh2 3'UTR SNP in regulating the expression of Nhlh2. These findings help to better understand of the factors affecting the Nhlh2 expression at molecular level. Because of the effect of the expression of this gene on physical activity, energy expenditure and in turn on obesity, these results might provide good information to prevent or treat human obesity related to this gene. Understanding the molecular mechanisms by which this gene

regulates physical activity and body weight, could help in improving human health. This understanding is necessary for any therapeutic interventions to treat obese people and mitigate the obesity effects on human health.

Figure 1: Hypothetical model of Nhlh2 regulation by Stat3, NFκB and Nhlh2 SNP in the 3'UTR.

Nhlh2 is regulated transcriptionally by both Stat3 and NFκB in response to leptin stimulation. The changes in Stat3 and NFκB regulation in the hypothalamus might affect the expression of Nhlh2 and the other genes downstream the Nhlh2 which in turn affect the energy expenditure. The Nhlh2 could be regulated posttranscriptionally by mutation in the Nhlh2 3'UTR SNP which down regulate the expression of Nhlh2 by decreasing the Nhlh2 mRNA stability.

Figure 1


Food intake
Leptin
inflammation
stress
Energy expenditure
Stat3
NFkB
Nhlh2
GGUGAUAUUUA
GGUGGUAUUUA
Mc4r
PC1/3
> 5000 genes
α-MSH

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