Identification of the molecular mechanisms underlying restless legs syndrome

Faezeh Sarayloo

Department of Human Genetics

Faculty of Medicine

McGill University

Montreal, Quebec, Canada

December 2019

A thesis submitted to McGill University in partial fulfillment of the requirements of the degree of Doctor of Philosophy

Abstract

Restless legs syndrome (RLS) is a sleep-related sensory-motor disorder. It is characterized by uncomfortable sensations in the legs, mostly during the evening or at night. These symptoms can partially be relieved by movement, so the patients have to stand up or walk in the middle of night at their rest time to alleviate the sensory symptoms. RLS consequences are decreased quality of life and the mental health. This disorder is highly frequent in the elderly in the European descent populations. Currently, RLS can be considered as one of the most common and probably least known neurological conditions. This is a complex disorder with both genetic and environmental factors contributing to its development. Genome-wide association studies (GWAS) have identified 19 loci associated with RLS, among which common variants within *MEIS1* (Myeloid ecotropic viral insertion site 1) and *SKOR1* (SKI family transcriptional corepressor 1) showed significant associations. Despite the high prevalence of this disease, and the several genetic studies performed on it, very little is known about the neurobiological mechanisms that lead to development of RLS. The work done in this thesis investigates the functions of two RLS-associated genes, i.e. *MEIS1* and *SKOR1.* We showed that there are direct links between MEIS1 and *SKOR1* where MEIS1 has a regulatory role over *SKOR1* expression. Furthermore, we studied the transcriptome-wide regulatory role of MEIS1 in RLS using *in vitro* cell models and identified novel genes and pathways that are regulated by MEIS1, and possibly involved in RLS biology. Additionally, postmortem brain tissues of RLS individuals were implemented to validate the cell line findings. We observed that mineral absorption pathway was significantly enriched in both *in vitro* cell models and the brain tissues. Due to the crucial role of iron in RLS pathogenesis, enrichment of mineral absorption pathway is a meaningful finding in RLS research. Moreover, we also performed investigations on the regulatory role of SKOR1 in RLS and found pathways related to iron

metabolism and also neurodevelopment. To our knowledge, the regulatory role of SKOR1 over the genes in these pathways is reported for first time in this work.

Résumé

Le syndrome d'impatiences des membres à l'éveil (SIME) ou syndrome des jambes sans repos (SJSR) est un trouble sensori-moteur lié au sommeil. Il se caractérise par des sensations inconfortables dans les jambes, principalement le soir ou la nuit. Les symptômes peuvent en partie être soulagés par le mouvement, de sorte que les patients doivent se lever ou marcher au milieu de la nuit ou pendant leurs périodes de repos, pour atténuer les symptômes sensoriels. Les conséquences du SIME sont une diminution de la qualité de vie et des problèmes de santé mentale. Ce trouble est très fréquent chez les personnes âgées des populations d'origine européenne. Actuellement, le SIME peut être considéré comme l'une des affections neurologiques les plus courantes et probablement les moins connues. Il s'agit d'un trouble complexe dont des facteurs génétiques et environnementaux contribuent à son développement. Des études d'association pangénomique (GWAS) ont identifié 19 loci associés au SIME, parmi lesquels des variants communs dans MEIS1 et SKOR1 ont montré des associations significatives. Malgré la forte prévalence de cette maladie et les nombreuses études génétiques réalisées, on en sait très peu sur les mécanismes neurobiologiques qui conduisent au développement du syndrome. Le travail effectué dans cette thèse a permis d'étudier les fonctions de ces deux gènes. Nous avons ainsi montré qu'il existe des liens directs entre MEIS1 et SKOR1, MEIS1 jouant un rôle de régulation sur l'expression de SKOR1. De plus, nous avons étudié le rôle de régulateur transcriptionnel de MEIS1 à l'échelle du génome dans des systèmes cellulaires *in vitro* et identifié de nouveaux gènes et voies régulés par MEIS1, et possiblement impliqués dans la biologie de ce trouble. En outre, des tissus cérébraux postmortem de patients atteints du SIME ont été utilisés pour valider les résultats obtenus au niveau cellulaire. Nous avons ainsi observé que la voie d'absorption des minéraux était considérablement enrichie à la fois dans les modèles cellulaires *in vitro* et dans les tissus cérébraux. En raison du rôle crucial du fer dans la pathogénèse du SIME, l'enrichissement

de la voie d'absorption des minéraux est une découverte significative. De plus, nous avons également mené des recherches sur le rôle régulateur de SKOR1 dans le SIME et découvert des voies liées au métabolisme du fer et au neuro-développement. À notre connaissance, le rôle régulateur de SKOR1 dans ces voies particulières est décrit pour la première fois dans ce travail.

Table of Contents

List of Abbreviations

BBB	Blood-brain barrier
C. elegans	*Caenorhabditis elegans*
cDNA	Complementary DNA
ChIP-Seq	Chromatin immunoprecipitation followed by high-throughput sequencing
CI	Confidence interval
CML	Chronic myelogenous leukemia
CNS	Central nervous system
CRE	Cis-regulatory elements
CRI	Chronic renal insufficiency
CSF	Cerebrospinal fluid
Ct	Threshold cycle
DEG	Differentially expressed gene
df	Degrees of freedom
DGE	Differential gene expression
DMEM	Dulbecco's Modified Eagle Medium
EMSA	Electrophoretic mobility shift assay
ESRD	End-stage renal disease
FCM	Ferric carboxymaltose
FTN	Ferritin
GO	Gene Ontology
GWAS	Genome-wide association study
GWL	Genome-wide linkage

HCNR	Highly conserved noncoding regions
HTS	High-throughput sequencing
IDA	Iron deficiency anemia
IGB	Integrated genome browser
IMDM	Iscove's Modifed Dulbecco's Medium
iRLS	Idiopatic restless legs syndrome
IRLSSG	International restless legs syndrome study group
IV	Intravenous
IVTT	*In vitro* transcription/translation
KEGG	Kyoto encyclopedia of genes and genomes
KO	knockout
LCL	Lymphoblastoid cell lines
LD	Linkage disequilibrium
LGE	Lateral ganglionic eminences
LOD	Logarithm of odds ratio
logFC	Log fold change
MD	Mean-difference
MDS	Multidimensional scale
MEIS1	Myeloid ecotropic viral insertion site 1
MRI	Magnetic resonance imaging
mRNA	Messenger RNA
MS	Multiple sclerosis
MT	Metallothionein
MW	Molecular weight

NIH	National institute of health
OR	Odds ratio
ORF	Open reading frame
PD	Parkinson's disease
PLMS	Periodic limb movements during sleep
PLMW	Periodic limb movements during wakefulness
PMI	Postmortem interval
q-RT-PCR	Quantitative reverse transcriptase polymerase chain reaction
RIN	RNA integrity number
RLS	Restless legs syndrome
RNA-Seq	RNA sequencing
RNAi	RNA interference
ROS	Reactive oxygen species
rRNA	Ribosomal RNA
siRNA	Small interference RNA
SKOR1	SKI family transcriptional corepressor 1
SNP	Single nucleotide polymorphism
TF	Transcription factor
UTR	Untranslated regions
WED	Willis-Ekbom disease
WT	Wild-type

List of Figures

List of Tables

Acknowledgments

I would like to start with expressing my sincere gratitude to my supervisor Dr. Guy Rouleau. It has been an honor to be a part of your team. Working with you helped me develop my skills as an independent researcher. My special thanks to my co-supervisor Dr. Patrick Dion. You were not only our supervisor in the lab, but also a friend. I am always thankful for all your understanding, support, kindness and mentorship during my graduate studies. I would like to thank Ross MacKay and Rimi Joshi at the Human Genetics department for making all the administrative procedures fast and smooth. I would also like to extend my appreciation to my supervisory committee members, Dr. Gustavo Turecki and Dr. Wayne Sossin.

At Rouleau lab, I had the amazing opportunity to work with bright, supportive and helpful people. I would like to thank Helene for always being helpful in coordinating the lab and also assisting with the expression studies and also the brain dissections. I am grateful to Alexandre and Dan for always willing to answer my bioinformatic questions and helping with my data analyses. I am grateful to Daniel for his help in molecular cloning procedures. I am thankful to Aida for her kind support and guidance since the day I joined the lab.

I had wonderful friends at the Rouleau lab that my experience could not have been the same without them. Our travels together to different conferences and all the amazing time and hard laughter that we had made my PhD life more joyful. Gabrielle was my friend since my very first months in Canada and was always there for me to help me adapt to my new city and new lab. Zoe and Fulya were wonderful friends that we could share our happiness and sadness. I am grateful to Paria, Rachel, Maria, Parizad and Kavin for all the fun and laughter in the short but high-quality time that we had together. Thanks Jay, for revising some parts of my thesis and for being very nice, supportive and motivating in any scientific discussion. I am thankful to Cal for being so

supportive, positive and hard-working that motivates people around you. I am also grateful to the former lab members who are my good friends now, Sadaf, Amelie and Amirtha.

I am grateful to my sister, Fatemeh and my brother-in-law, Majid, for always being there for me. For supporting me since my first day in Montreal in January 2014. For always listening to my complaints in my hard and challenging days and always pushing me forward, encouraging me and believing in me. Special thanks to my mom, Masoomeh, for teaching me to live with my heart and care about people around me. For believing in educating oneself and educating others and for being extra patient, supportive and encouraging during my challenging days. Without you, I could not have finished this journey. To my dad, Mahmoud for making our education a priority, for planting big dreams in our mind since we were so little and for teaching us to do valuable and meaningful work.

Lastly but most importantly, I would like to thank my best friend, my husband, Arman. For being a constant source of support and kindness throughout this journey. Thanks for making everything easier and more fun in my life. For giving me the courage to listen to my heart and choose what I really want in my career and life, even if it scares me most. For ALWAYS being super kind and making me laugh in my hard and challenging days. Having you alongside me made this journey amazing.

Contribution to original knowledge

The work presented in this thesis represents an original contribution to the biological knowledge of restless legs syndrome. The main approaches used in this work are protein-DNA binding assays, reporter assays, human cell line manipulations and high-throughput transcriptome sequencing. The results are presented in Chapters 3, 4, and 5. Chapter 3 introduces a novel finding of a direct binding between two significant RLS genetic contributors (i.e. *MEIS1* and *SKOR1*). Chapter 4 reveals a regulatory role for MEIS1 in RLS with introducing new genes (e.g. *HMOX1, VDR* and members of metallothionein family) and enrichment of a new pathway (i.e. mineral absorption). Chapter 5 shows a transcriptional regulatory role for SKOR1 in RLS with enrichment of RLS relevant pathways (e.g. ferroptosis and neurodevelopment). Chapters 3 and 4 have been shared with the scientific community throughout publications in peer reviewed journals. A version of Chapter 5 is drafted for publication as well.

Format of the Thesis

The work described in this thesis was performed under the co-supervision of Dr. Guy Rouleau and Dr. Patrick Dion. It is traditionally formatted and follows the Thesis Preparation Guidelines by the Department of Graduate and Postdoctoral Studies. This thesis contains 7 chapters. Chapter 1 is an introduction to the thesis with the overall rationales, objectives and hypotheses. It also contains modified sections of a review article that was published by the thesis author at *Frontiers in Neurology.* Chapter 3 is published at *Scientific Reports.* Chapter 4 is published at *PLoS ONE.* Version of Chapter 5 is prepared for publication.

Contribution of Authors

Chapter 1. *MEIS1* and restless legs syndrome; a comprehensive review [1].

Faezeh Sarayloo contributed to the original concept of the article, did the literature search, wrote the original version of the manuscript and reviewed it as it progressed. Patrick Dion and Guy Rouleau contributed to the original concept of the manuscript and reviewed the manuscript.

Chapter 3. A direct interaction between two Restless Legs Syndrome predisposing genes: *MEIS1* and *SKOR1* [2].

Helene Catoire contributed to the original concept of the project and performed expression experiments. Wrote the original version of the manuscript and reviewed it as it progressed. Faezeh Sarayloo contributed to the interpretation of results and performed expression experiments. Assisted with the redaction of the manuscript. Karim Mourabit Amari performed electrophoretic mobility shift assays. Sergio Apuzzo designed and performed electrophoretic mobility shift assays. Alanna Grant assembled luciferase assays expression vectors and measured luciferase expression. Daniel Rochefort designed and assembled luciferase assays expression vectors. Lan Xiong contributed to the concept of the project and its interpretation. Jacques Montplaisir contributed blood samples, clinical information of RLS patients. Christopher Earley contributed brain samples from RLS patients. Gustavo Turecki contributed brain samples from control individuals. Patrick Dion designed studies and contributed to the original concept of testing the interactions. Wrote and reviewed the manuscript. Guy Rouleau contributed to the original concept of the project and oversaw its progression. Wrote and reviewed the manuscript.

Chapter 4. Mineral absorption is an enriched pathway in a brain region of restless legs syndrome patients with reduced *MEIS1* expression [3].

Faezeh Sarayloo contributed to the original concept of the project and performed cell culture and molecular experiments, analyzed the data, wrote the original version of the manuscript and reviewed it as it progressed. Alexandre Dionne-Laporte performed bioinformatic analysis of RNA-Seq data. Helene Catoire contributed to brain RNA extraction and q-RT-PCR analysis. Daniel Rochefort contributed to CRISPR-Cas9 experiment. Gabrielle Houle contributed to the interpretation of results. Jay Ross contributed to the interpretation of results. Fulya Akçimen contributed to the interpretation of results. Rachel De Barros Oliveira contributed to the interpretation of results. Gustavo Turecki contributed to the interpretation of results. Patrick Dion contributed to the original concept of the project, reviewed the manuscript. Guy Rouleau contributed to the original concept of the project, oversaw its progression, reviewed the manuscript.

Chapter 5. SKOR1 has a transcriptional regulatory role on genes involved in pathways related to restless legs syndrome.

Faezeh Sarayloo contributed to the original concept of the project and performed cell culture and molecular experiments, analyzed the data, wrote the original version of the manuscript and reviewed it as it progressed. Dan Spiegelman assisted with the bioinformatic analysis of RNA-Seq data. Daniel Rochefort designed the anti-SKOR1 peptide sequence. Fulya Akçimen contributed to the interpretation of results. Rachel De Barros Oliveira contributed to the interpretation of results. Patrick Dion contributed to the original concept of the project, reviewed the manuscript. Guy Rouleau contributed to the original concept of the project, oversaw its progression, reviewed the manuscript.

Chapter 1 Introduction

"*Wherefore to some, when being abed they betake themselves to sleep, presently in the arms and legs, leapings and contractions of the tendons, and so great a restlessness and tossing of their members ensue, that the diseased are no more able to sleep, than if they were in a place of the greatest torture*".

First definition of restless legs syndrome, Sir Thomas Willis, 1672

Restless legs syndrome (RLS) is a sleep-related sensory-motor disorder. The first description of RLS was provided by Sir Thomas Willis in 1672 when he reported a condition where patients had difficulty in sleeping because of discomfort in their limbs. It was only after 1945 that a more detailed description of this disease was made by the Swedish neurologist Karl Ekbom, hence RLS is also referred to as Willis-Ekbom disease (WED) [4]. Currently in 2019, RLS can be considered as one of the most wide-spread and probably least known conditions.

Diagnostic criteria

The diagnostic criteria of RLS were first established by International Restless Legs Syndrome Study Group (IRLSSG) during a workshop held at the National Institute of Health (NIH) in 1995. The original diagnostic criteria were subsequently updated in 2003 and more recently in 2014. This last update suggested that it is essential for an RLS diagnosis to meet all the following five criteria [5]:

1. An urge to move the legs usually but not always accompanied by, or felt to be caused by, uncomfortable and unpleasant sensations in the legs.

2. The urge to move the legs and any accompanying unpleasant sensations begin or worsen during periods of rest or inactivity such as lying down or sitting.
3. The urge to move the legs and any accompanying unpleasant sensations are partially or totally relieved by movement, such as walking or stretching, at least as long as the activity continues.
4. The urge to move the legs and any accompanying unpleasant sensations during rest or inactivity only occur or are worse in the evening or night than during the day.
5. The occurrence of the above features is not solely accounted for as symptoms primary to another medical or a behavioral condition (e.g. myalgia, venous stasis, leg edema, arthritis, leg cramps, positional discomfort, habitual foot tapping) [5].

Overall, the frequency of the symptoms can range from less than once a year to daily, and also their severity can range from mild annoyances to severe disruptions of sleep that warrant medical treatment. Moreover, the symptoms may be absent for different periods of time. There are also additional features that can support the diagnosis of RLS, for instance signs of periodic limb movements (PLMs), either while awake (PLMW) or during sleep (PLMS), effectiveness of dopaminergic treatments, and a family history of the disease [5].

Epidemiology

According to a review made by Koo [4] in 2015, over 50 epidemiological studies were conducted for RLS. These studies were performed across the populations of five of the six inhabited continents (not Australia); though most of them studied individuals from North America and Europe. Comparisons of the different epidemiological studies have been complicated since each one took different ascertainment criteria into account when estimated the prevalence of RLS.

Some studies only considered one question, though most took three or four diagnostic criteria into consideration. However, regardless of the methodology used, most studies reported RLS to be a prevalent condition. It is also noteworthy that many of the cases included in these epidemiological studies only showed mild symptoms and did not received medical attention.

A breakdown of the reported RLS prevalence rates in different continents has been provided in a review by Koo [4]. In Africa, a prevalence rate ranging between 0.03% and 0.47% was reported based on two studies performed in Tanzania [6, 7]. In Asia, 13 studies have been performed, mostly in South Korea, and these reported a prevalence ranging between 0.9% and 12.1% [8-12]. Overall, it is also worth pointing-out that distinctly higher prevalence was observed in the studies that required a positive answer to only one of the questions, as such these studies included in a larger number of individuals being included in the study. By comparisons, studies that required four affirmative criteria reported estimates as low as 1.0%.

Most RLS epidemiological studies were conducted across populations from European countries. The prevalence was diverse and ranged between 3.2% and 24.2% (lowest in Turkey and highest in France) [13-24]. North American studies showed the prevalence of RLS to be between 0.4% and 18.3% [25-31]. In South America, the prevalence ranged from 2.0% to 20.2% (lowest in Ecuador and highest in Argentina) [32-35].

Having a more detailed look at the prevalence rates, there is an overall higher trend of RLS in northern European countries, as such it appears that among other factors, latitude could influence the onset and/or progression of RLS (Figure 1). This higher prevalence in northern European countries suggests that sun exposure, vitamin D, and daylight time might have an effect

on RLS development. RLS is more common in women than men and its prevalence increases with age in European and North American populations [36]. The condition is also more prevalent in individuals with iron deficiency or kidney disease [37].

Figure 1. Higher RLS prevalence in countries with a higher latitude in the Europe. This figure shows RLS incidence per 1,000 individuals in each city or country composed of 500 men and 500 women. Aug, Augsburg, Germany; Bur, Bursa, Turkey; Dor, Dortmung, Germany; Kan, Kandira, Turkey; Lar, Larissa, Greece; Mer, Mersin, Turkey; Pom, Pomerania, Germany; Rey, Reykjavok, Iceland; S Ty, South Tyrolia, Italy; Som, Sombor, Serbia; StE, Saint Etienne, France; Upp, Uppsala, Sweden [4]. Adapted from Koo, *Sleep Med Clin,* 2005.

Treatment options

Dopaminergic treatments

Over the past 10 years, the use of dopaminergic precursors (e.g. levodopa) has been the first line of treatments for RLS [38]. Levodopa can be taken up by the blood-brain barrier (BBB) and subsequently stimulate the production of dopamine. Despite the efficacy of levodopa treatments to reduce the severity of RLS symptoms, there are reports highlighting critical complications such as augmentation and tolerance. Augmentation of symptoms can emerge in the case of a sustained use of dopamine treatments and it refers to an earlier advent of the symptoms in the course of the day. Augmentation can also be accompanied by symptoms in other organs in addition to the legs. On the other hand, tolerance refers to the process of lower effectiveness of the treatment over time and the need to increase the medication dose [39-41].

Over time, the prescription of dopamine precursors for RLS has declined. Instead, longer-acting dopaminergic agents have come to be used. Dopamine agonists including pramipexole, ropinirole (binds to D3 receptors), and rotigotine (binds more efficiently to D1 and D5) have become more widely used to more effectively treat RLS [42-44]. By comparison to the use of dopamine precursors, the use of dopamine agonists is notably less associated with the development of augmentation side effect.

Gabapentinoids

Gabapentinoid drugs are also used to treat RLS [45]. These drugs are ligands of the alpha-2-delta subunit of a subset of voltage-gated calcium channels and block these channels. Gabapentinoids are also frequently used in the treatment of neuropathic pain. Studies on North

American and Japanese populations have shown the efficacy of this treatment in multiple reports. Other countries do not currently permit prescription of gabapentinoids for RLS treatment [46, 47].

Opioids

Opioids have also been used as an option for RLS treatment and have been shown to reduce RLS severity [48]. However, considering the risks of addiction and abuse, opioid formulations are only prescribed for patients with severe RLS and in cases for which treatments with dopamine agonists and gabapentinoids have already failed.

Iron supplements

Iron plays a crucial role in a number of biological processes (e.g. dopamine synthesis). Epidemiologic studies suggest that the prevalence of RLS is higher in patients suffering from iron deficiency anemia compared to the general population; this prevalence can reach 23.9% [49]. Furthermore, magnetic resonance imaging (MRI) of RLS patients' brain regions showed that the most consistent biological abnormality observed in RLS patients is brain iron deficiency within the substantia nigra, red nucleus, putamen, caudate nucleus, and thalamus [50, 51]. These observations led to the hypothesis that iron plays a major role in the RLS pathophysiology [52]. One hypothesis is that iron transport from blood circulation through the BBB into the central nervous system (CNS) could be impaired in RLS, leading to low iron levels in some brain regions [53]. It is also hypothesized that low iron in RLS brain can activate the hypoxic response pathways in the CNS [54].

A variety of iron formulations either in the form of oral intake or intravenous (IV) delivery have been implemented as RLS treatments. Oral iron treatment by ferrous sulfate did not show

significant improvements of RLS symptoms [55]. This low effectiveness can be due to the low dietary iron uptake. The emergence of IV treatment by iron sucrose solved this issue to some extent and showed more efficiency, especially in patients with low ferritin levels [56]. Another form of iron formulation, which is a high molecular weight iron dextran has been studied as a treatment for RLS. However, this medication is not considered safe enough for treatment of RLS because it can cause anaphylactic shock in some cases. An advanced iron formulation, ferric carboxymaltose (FCM), was recently designed; this iron formulation delivers iron in a more controlled manner and does not release extensive ionic iron into the serum, resulting in a reduced RLS severity [57, 58]. The current treatment options are summarized in detail in a review article by Salminen et al. [45].

RLS risk factors and comorbidities

Pregnancy

The prevalence of RLS increases in pregnant women specifically in their last trimester and approximately 11% to 27% of pregnant women have been reported to meet RLS criteria [59, 60]. Multiple pregnancies is a risk factor that increases the likelihood of developing transient RLS [61]. Low folate, low iron, and hormonal changes have been suggested to play roles in increasing RLS in pregnant women [59]. However, it is challenging to assess iron deficiency in pregnant women because they mostly take folate supplements which increases ferritin levels. There are likely links and correlations between higher levels of estradiol and RLS [62, 63].

Kidney disease

The epidemiological studies investigating the prevalence of RLS in individuals with end-stage renal disease (ESRD), patients on hemodialysis, or with chronic renal insufficiency (CRI) resulted in a range from 15% to 68% (in Hungarians and European Americans) [64, 65]. Renal

failure is considered as one of the most common associations with RLS. RLS symptoms do not ameliorate with dialysis; however, a successful kidney transplant can improve RLS symptoms. The presence of RLS in patients with renal failure and long-term dialysis can increase the mortality risk [66].

Iron deficiency

Iron deficiency in RLS has been demonstrated in three types of studies: pathophysiological, iron treatment, and epidemiological:

- Pathophysiological studies report that a reduced level of iron is observed in the brain or cerebrospinal fluid (CSF) of RLS patients in comparison to control individuals [53].
- Treatment studies suggest that either oral or IV iron treatments can ameliorate RLS symptoms. The effectiveness of iron treatment was more effective in the patients with compromised iron availabilities (either in ESRD or the blood donors). IV iron treatments resulted in long lasting improvement of the symptoms in about 20% of the treated cases [67, 68].
- Epidemiological studies report that the prevalence of RLS increases in the populations with iron deficiency or in individuals with iron deficiency anemia (IDA), compared to non-iron deficient cases [49]. Overall, there is no consistent relation between RLS and serum iron or ferritin status [52].

Neuropathy

Sensory symptoms of polyneuropathy can seem similar to RLS symptoms. However, neuropathy symptoms are usually located in the feet and not the legs, and movement cannot

alleviate the symptoms [69]. The evidence supporting an association between neuropathy and RLS is limited. For example, Gemignani et al. [70] reported that one-third of their studied neuropathy cohort had RLS, and this subset of neuropathy and RLS cases had higher chance of having small fiber sensory neuropathy than neuropathy cases without RLS. However, a subsequent and independent study reported that there was not a higher chance of developing neuropathy across patients who were first observed to have RLS [71].

Other comorbidities

A systematic review by Trenkwalder et al. reported potential associations between RLS and some cardiovascular diseases in women, diabetes (and neuropathy), migraine, and dopaminergic treatment in Parkinson's disease (PD) patients [68]. However, the evidence for existing associations between RLS and other phenotypes such as anemia without iron deficiency, hypertension, multiple sclerosis (MS), headache other than migraine, strokes, and ataxias are insufficient [68]. Overall, familial RLS with more genetic determinants (a topic that will be discussed later in this chapter) are accompanied by fewer environmental factors and comorbidities and vice versa.

Family history and genetic studies of RLS

Based on the current literature, up to 60% of idiopathic RLS (iRLS) patients have a positive family history [16, 72-75]. iRLS refers to the condition that other comorbidities are not present in the patient and genetic plays an important role in it. In 2010, a family study on Canadian RLS cases identified familial aggregation with a rate of 77.1% [76]. Some other investigations reported that iRLS cases with a positive family history have a younger age at onset as compared to the RLS cases without family history [75, 76].

Furthermore, a study by Allen et al. in 2002 [77] suggested that the prevalence of RLS increases in the first- or second-degree relatives of RLS patients. In this study, RLS patients were compared to controls, and a significantly higher frequency of RLS in the first- and second-degree relatives was observed. Genetic anticipation studies have also been performed on RLS cases to further prove the role of genetic in the disease. Genetic anticipation is described as an earlier age at onset (or more severe symptoms) from one generation to the next. In a German multiplex family, earlier onset of the symptoms was observed in the third and fourth generations compared to generations one and two [78]. Genetic anticipation has also been reported in families in Turkey and Brazil [79-81].

Twin studies have also been performed to investigate the rate of heritability and role of genetic in RLS. These investigations reported a significantly higher concordance rate of RLS in monozygotic twins compared to dizygotic twins [82-84]. The high rate of familial RLS, genetic anticipation, and high concordance rate of RLS in monozygotic twins are all in support of a genetic role in RLS. Nevertheless, RLS is a complex condition and environmental factors also contribute to its development.

Genome-wide linkage studies

Between 2001 and 2008, RLS genetic studies were focused on familial cases with the assumption that RLS is a monogenic disorder. Genome-wide linkage (GWL) approaches in large multiplex families, mostly with an autosomal dominant mode of inheritance and one with autosomal recessive inheritance pattern were conducted. These investigations did not identify a single gene causing RLS. To date, a total of eight loci have been found to segregate in the studied

families. These loci had slightly significant linkage scores (logarithm of odds ratio, LOD), in large genomic regions [85-92]. These large genomic regions underwent sequencing efforts but did not result in identification of a causally related sequence [93]. Currently, it is well understood that RLS is not a classic monogenic disorder and a single variation is likely not causing the disease; rather, it is a complex condition. Complex traits are affected by a combination of multiple genetic factors with small to moderate effect sizes in different genomic regions and also by environmental factors [94].

Genome-wide association studies

The "one-mutation, one-gene, one-outcome" model is not proper for studying complex traits. These conditions can have contributions from several genes, some of which might interact with one another in additive (epistatic) or non-additive ways, and can additionally interact with the effect of environmental factors [95]. Identification of these contributors can play an important role in obtaining effective treatments for the disease. Considering the complex nature of RLS, its genetic investigations progressed toward association studies aimed at identifying common variants with small to moderate effect sizes.

The first genome-wide association study (GWAS) for a common sleep disorder was performed on RLS in 2007 [96]. 401 patients with familial RLS and 1,644 control individuals of German and French-Canadian origin were studied in this report and common variants in three noncoding genomic regions were identified [96]. The strongest association signal was found in a 32 kb linkage disequilibrium (LD) block in intron 8 of *MEIS1* gene [96]. This association has been replicated in several follow-up studies in both familial and sporadic RLS cases (odds ratio (OR) = 1.92, 95% confidence interval (CI) 1.85 – 1.99, p-value = 2.00E-280, from a recent meta-

analysis in 2017) [97-100]. Furthermore, common variants in an intergenic region between *MAP2K5*/*SKOR1* were also found to be associated with RLS and were replicated in different populations. Overall, common genetic variants with low effect size in 19 loci were identified for RLS, each conferring a small risk for the disease [100].

Post-GWAS era

Since the emergence of GWAS in 2005, a wide range of genetic variations were found to confer risk of complex traits or phenotypes. A considerable number of these loci were also replicated in different studies, suggesting that they are true associations [101]. However, making connections between these findings and deciphering the underlying biology of the condition based on these results is usually challenging. This challenge is mainly present on two levels:

1) The identified genetic loci do not specify which variant is disease-causing [95]. This problem arises from the fact that many variants are in strong LD with the disease-associated variant and make a haplotype [102]. When there is strong LD between the genetic variants in a haplotype, distinct associations with the disease cannot be specified; this prevents researchers from identifying a single causal variant [103, 104].

2) It is not specified which gene is altered by the associated variant. This is due to the fact that a large number of candidate variants are within non-protein-coding regions and maybe located within long distances from their closest genes [105]. It is noteworthy that these disease-associated variants are in regions that are enriched with cis-regulatory elements (CREs). Changing the transcriptional regulation of these CREs over other genes can be considered as a potential role for these variants [103-105].

A look into the publications in the period of 2005 to 2016 shows a drastic increase in the number of studies reporting disease-associated variants. However, there is a large gap between these findings and follow-up functional investigations that address the possible implications of these findings. This gap is one of the main issues of the post-GWAS era (Figure 2) [95].

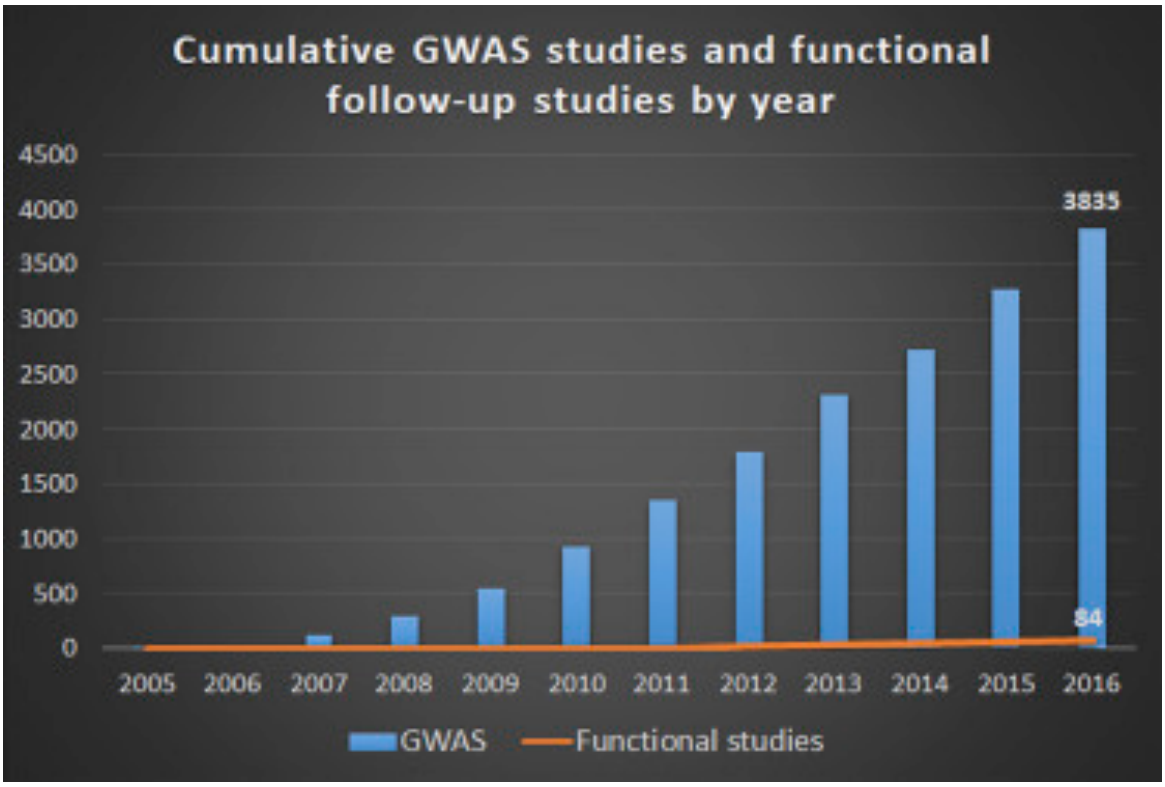


Figure 2. Functional follow-up studies of disease risk loci identified by GWAS lags behind the discoveries of new disease-associated variants [95]. Adapted from Gallagher et al., *American Journal of Human Genetics,* 2018.

MEIS1

MEIS1 contribution to RLS genetic

Both common and rare variants in *MEIS1* region have been reported to be associated with RLS. Identification of common variants in *MEIS1* was discussed in the previous section [96-100]. Contribution of rare coding variants in *MEIS1* region was also proposed to be a cause of RLS. In one of 71 familial RLS probands, an Arg272His (p.R272H) variation was found. However, this finding failed to be replicated in a case-control genotyping study across a North American cohort [106]. Another study performed a burden test on a German population and reported an excess in the rare null alleles specific to *MEIS1* isoform 1 in RLS cases compared to controls [107]. Furthermore, in a study performed by Xiong et al., 285 familial RLS probands who had received a clinical RLS diagnosis were studied. The 13 *MEIS1* exons (and their corresponding splice junctions) were sequenced, however no variants were identified [108]. Overall, these studies suggest that the contribution of the coding variants is at most a very rare cause of RLS. This observation could be expected because MEIS1 is a homeobox transcription factor and is involved in many different stages of developmental processes. Presence of functional coding variants in this gene would likely have many additional consequences, other than RLS.

MEIS1 protein functions

MEIS1 is a homeobox transcription factor (TF); this protein belongs to the three amino acid loop extension (TALE) family of homeodomain proteins. In collaboration with PBX or HOX, MEIS1 forms heterodimeric or heterotrimeric complexes and this results in higher DNA binding specificity and affinity [109]. MEIS1 has been suggested to have roles in hematopoiesis, vascular patterning [110, 111] and neurodevelopment [112-115]. MEIS1 regulates the proximodistal limb axis development [112]. This gene is highly expressed in dopaminergic neurons of the substantia

nigra and red nucleus, however its function in these cells remains unknown [113-115]. MEIS1 establishes motor neuron pool identity and their target-muscle connectivity [116]. The numerous roles for MEIS1 during development make the study of its specific role in RLS biology more challenging.

Reduced MEIS1 expression may contribute to the RLS development in a subset of patients

A follow-up functional study after the first GWAS on RLS was performed by Xiong et al. in 2009. Human lymphoblastoid cell lines (LCL) and two different brain regions (thalamus and pons) from RLS patients were implemented in an expression study. A quantitative reverse transcriptase polymerase chain reaction (q-RT-PCR) followed by western blot analysis revealed that the patients who carry the *MEIS1* RLS risk haplotype (GG/GG, rs12469063–rs2300478) express reduced levels of *MEIS1* mRNA and protein in LCL and thalamus. The authors suggest that in a subset of RLS individuals, reduced *MEIS1* expression can contribute to development of the symptoms [108].

The lower *MEIS1* expression in a subset of RLS patients was followed up in *in vivo* studies in mouse models. Young male and female mice with heterozygous *Meis1* knockout showed hyperactivity; however, no effect was observed on their anxiety-related behaviors [114, 117]. Subsequently, middle aged mice were used in another study to also consider the age-related manifestation of RLS symptoms in human [118]. In these animal models, *Meis1* haploinsufficiency in middle aged mice was associated with an increase in the activity more specific to the beginning of the rest phase of animal in a sex-dependent manner. This observation was similar to the circadian rhythm of RLS symptoms in human patients [118].

Allele-dependent cis-regulatory function of MEIS1 in the telencephalon in two animal models (mice and zebrafish)

MEIS1 contains a cluster of highly conserved noncoding regions (HCNRs), suggesting the presence of CREs [114]. Considering that most RLS-associated variants found by GWAS are within non-protein-coding regions including CREs, Spieler et al. conducted a study to explore the cis-regulatory role of the common intronic variants in *MEIS1* HCNRs [114]. RLS-associated single nucleotide polymorphism (SNP, rs12469063) is in the HCNR 617 of *MEIS1;* in this study, a reporter assay was conducted to assess the effect of this RLS risk variant on the enhancer function of HCNR 617 in transgenic mice and zebrafish:

- In mice, rs12469063 lies within a region of high interspecies conservation, has a neural enhancer activity, and has an allele-specific functional impact on the enhancer functionality of this region. The authors reported that the risk allele of rs12469063 decreases the enhancer activity of this region in LGE and MGE (lateral and medial ganglionic eminences, respectively). This effect in LGE/MGE suggests that RLS may involve the basal ganglia because these regions give rise to the basal ganglia [119]. This HCNR harboring the RLS-associated rs12469063 has an enhancer function during development, suggesting that this effect and the predisposition to RLS probably occurs during embryonic development. CREB1 has higher binding affinity to the RLS risk allele compared to the protective allele of rs12469063 (observed in an affinity chromatography).
- A reporter assay in zebrafish also confirmed the observed enhancer activity of HCNRs in mice. Two more transcriptionally active enhancers were also introduced

in these zebrafish models in addition to HCNR 617. In this report, HCNR 617 harboring rs12469063 is the only RLS-SNP dependent enhancer region [114, 120].

MEIS1 has links with iron metabolism

Unc-62 is the *MEIS1* orthologue in *Caenorhabditis elegans* (*C. elegans*) with 25% identity with *MEIS1* and almost 80% identity with its homeodomain. *Unc-62* is one of the 64 genes by which post-developmental inactivation increases the worm's life span. Changes in the iron content of the worm's media can alter the effect of *Unc-62* on the worm's life span, suggesting that *Unc-62* is involved in iron metabolism. Catoire et al. measured the effects of *Unc-62* on the expression of ferritin. It was found that post-developmental inactivation of *Unc-62* using RNA interference (RNAi) was associated with higher ferritin levels. Ferritin protein has a key role in iron metabolism, allowing excess iron storage and releasing it into a soluble and nontoxic form [121].

The lower *MEIS1* expression observed in thalamus samples obtained from RLS patients carrying the *MEIS1* risk haplotype was associated with an increased mRNA level of light and heavy chain ferritin (*FTN*), as well as *DMT1*. *DMT1* encodes a proton-coupled metal transporter that carries iron from the extracellular domain to the cytoplasm and is expressed in the endosomes of brain capillary endothelial cells denoting the BBB [122-124]. This study suggested a regulatory role for MEIS1 on iron management proteins. Detailed links between *MEIS1* and RLS are discussed in a review article by thesis author [1] and are summarized in Figure 3.

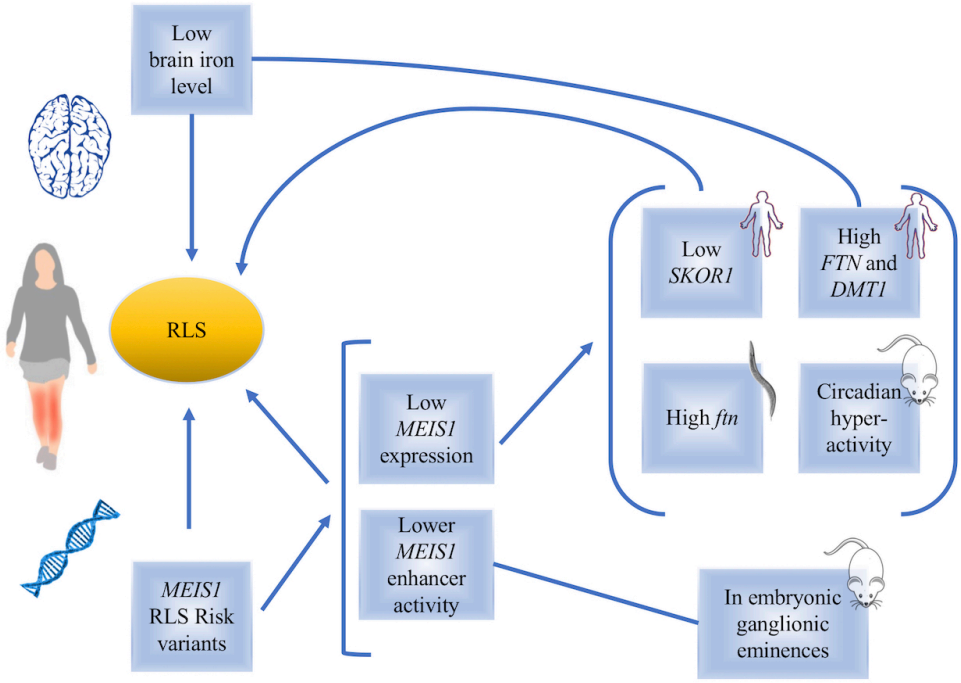


Figure 3. An overview of the links between *MEIS1* and RLS mechanisms [1]. Adapted from Sarayloo et al, *Frontiers in Neurology,* 2019.

As mentioned in the previous sections, some RLS patients respond well to iron treatment. This led some studies to interrogate the underlying pathways differing between responders versus non-responders. Some studies investigated the iron uptake at the blood-brain barrier and the possible roles of MEIS1 in this regard [125]. Unpublished data by Connor et al. 2017 presented in a review article reported that treatment of a cell culture model of BBB with iron chelators is associated with an increase in *MEIS1* expression. Conversely, iron loading decreased *MEIS1* expression (the small sample size in this report indicates that more investigations remain to be done to further validate this observation) [54, 126]. These observations suggest a novel role for MEIS1 in the BBB which warrants further examination.

Hypoxia has also been reported to be an activated mechanism in a number of cell types in RLS patients [127-130]. Hypoxia is related to cellular iron deficiency and functional studies on the role of MEIS1 in conditions other than RLS suggest roles for MEIS1 in hypoxic pathways. For example, studies on the possible roles of MEIS1 in leukemia reported that loss of MEIS1 led to oxidative stress, and hypoxia reversed this oxidative stress and prevented the progression of leukemia in MEIS1-deficient cells [131].

The links between MEIS1 and other sleep related disorders

RLS is a common and complex sleep-related disorder and the pleiotropic effects of its predisposing genes on other common sleep-related disorders is plausible. Insomnia is characterized by problems in falling asleep or maintaining sleep. The heritability of insomnia is estimated to be 38% and 59% in men and women, respectively; this suggests that genetic factors play an important role in insomnia. A UK Biobank GWAS on insomnia showed that *MEIS1* had the strongest association signal, suggesting *MEIS1* may be a shared genetic risk factor for RLS

and insomnia [132-134]. However, the pleiotropic effect of *MEIS1* in RLS and insomnia is controversial; some studies suggest that the association of *MEIS1* with insomnia only comes from the inclusion of RLS cases in the insomnia studies [135]. Others argue that the overlap in the phenotype of RLS and insomnia could drive only some, and not all of the *MEIS1*'s association with insomnia, thus *MEIS1* may have a pleiotropic effect on RLS and insomnia [132]. A part of such inconsistencies arises from the heterogeneous phenotypic definition of insomnia itself which can lead to the inclusion of a number of RLS cases.

Periodic leg movement during sleep (PLMS) are present in approximately 80% of RLS cases and several GWAS on PLMS also showed associations with *MEIS1* [136-140]. This pleiotropic effect can arise from *MEIS1*'s wide expression pattern during the development [141]. During development, *MEIS1* is expressed in granule cell lineage cells and astrocytes in the cerebellum. In post-embryonic neuronal tissues, it is expressed in the adult mouse brain in cerebellar granule cells, the forebrain and importantly, in dopaminergic neurons of the substantia nigra (as shown in the Allen Brain Atlas of the mouse brain, http://mouse.brain-map.org). Current literature supports no genetic links between RBD (REM sleep behavior disorder) and *MEIS1*.

SKOR1

Variants in the promoter region of *SKOR1* were found to be associated with RLS and were replicated in different populations [2, 96, 99, 100]. Despite the importance of *SKOR1* in RLS genetic, little is known regarding the actual function of this gene in RLS underlying pathways. Also, little is known about the actual functional properties of SKOR1 protein in general. The Genotype-Tissue Expression (GTEx) database [142] shows that *SKOR1* has its highest expression in cerebellum and cerebellar hemisphere. In the embryonic CNS of mice, *Skor1* is expressed

posterior to the midbrain-hindbrain border in a certain subset of post-mitotic neurons. In the developing spinal cord in mice, *Skor1* is selectively expressed in dorsal horn interneurons; in this region, a homeodomain transcription factor, Lbx1, is required for proper specification [143]. Skor1 interacts with Lbx1 and they cooperatively repress transcription, suggesting that Skor1 acts as a transcriptional corepressor for Lbx1 in regulating cell fate determination in the dorsal spinal cord (*SKOR1* was previously known as *LBXCOR1*) [143].

Whole transcriptome sequencing

Understanding the transcriptome is necessary for the identification of the functional features of the genome as well as understanding the underlying molecular mechanisms contributing to the development of the diseases. Since the advent of RNA Sequencing (RNA-Seq) a decade ago, it has become a ubiquitous tool for interpretation of the genomic functions. Microarrays were used in the past for identification of differential gene expression (DGE), however the hybridization nature of microarrays limits the abilities of this method. RNA-Seq is a genome-wide transcriptome analysis method used to quantify RNA levels by using high-throughput sequencing (HTS) approaches via sequencing complementary DNA (cDNA) [144].

The standard procedure of RNA-Seq starts with RNA extraction from tissues or cell lines. The majority of the RNA content in cells is comprised of ribosomal RNA (rRNA); messenger RNA (mRNA) enrichment (poly(A) selection) or rRNA depletion (Ribo-Zero) are the main methods used to circumvent this issue [145]. Library preparation is performed on RNA samples by converting them to cDNA followed by ligating adaptors to the cDNA sequences. These short fragments will be sequenced on sequencing platforms (usually Illumina) with different read depths depending on the objectives and the available funds of the study. Basically, higher read depths can

result in the identification of more differentially expressed genes (DEGs). Subsequently, the reads will be aligned to a reference genome or are assembled *de novo*, after which their overlaps with the transcripts will be counted and normalized in different replicates. Based on the study objectives, statistical models will be fit to the data and the DEGs between different conditions will be discovered.

Hypotheses and objectives

Functional or expression studies on the association signals found by RLS genetic studies are still in an early stage and there is still a high demand for more in-depth investigations. Our group has done a number of studies investigating the functions of MEIS1 and to a lesser extent SKOR1 in RLS. Based on our previous work and also the literature:

1) We hypothesized that in addition to *MEIS1,* the other known RLS-associated noncoding variants might also have a regulatory effect on the genes harboring them (similar to the effect of *MEIS1* risk haplotype on its mRNA and protein expression). The objective for Chapter 3 was to test these effects on material obtained from RLS patients (lymphoblastoid cell line, pons, and thalamus) harboring different RLS-associated variants (by the time of this study, noncoding regions of *MEIS1*, *BTBD9, SKOR1,* and *MAP2K5* were found to harbor RLS-associated variants). Based on the results from this experiment, we found that there are links between two of these genes (*MEIS1* and *SKOR1*, published at *Scientific Reports*, 2018 [2]).

2) The data obtained in Chapter 3 showed a transcriptional regulatory role for MEIS1 in RLS. Moreover, MEIS1 is a homeobox-containing transcription factor with regulatory roles on iron management proteins in *C. elegans* [124]. Despite its contribution to RLS

genetic, no direct role is found for this gene in the known RLS pathways. We hypothesized that the reduced expression of *MEIS1* in a subset of RLS patients might lead to changes in the expression levels of its downstream genes that might play crucial roles more directly related to RLS. The objective of Chapter 4 was to generate human cell models with dysregulated *MEIS1* expression, a condition similar to a subset of RLS patients, followed by whole transcriptome sequencing. Subsequently, RLS patients' brain material with reduced *MEIS1* expression also underwent RNA sequencing and their results were cross-validated with the cell line data (published at *PLoS ONE*, 2019 [3]).

3) Based on the significant contribution of *SKOR1* in RLS genetic, its direct links with *MEIS1*, and its reduced expression in a subset of the patients (Chapter 3), we investigated the role of *SKOR1* in RLS pathways in Chapter 5. Currently, there is a limited understanding of the actual function of SKOR1. The studies so far suggest that SKOR1 performs as a transcriptional corepressor in regulating cell fate determination in the dorsal spinal cord [143]. We hypothesized that SKOR1 might also have a transcriptional regulatory function in RLS (an event similar to what was observed for MEIS1 in Chapter 4). The objective of Chapter 5 was to use human cell lines to manipulate *SKOR1* expression followed by RNA sequencing to find downstream genes and pathways for SKOR1, possibly related to RLS (manuscript in preparation).

Chapter 2 Material and methods

LCL culture

We selected LCL samples from RLS patients that were previously established by transformation with EB virus using standard protocols. These LCL samples were grown at 37°C and 5% CO_2 in RPMI 1640 medium (Invitrogen) supplemented with 15% (v/v) heat-inactivated fetal calf serum (Sigma-Aldrich), 0.29 mg/ml of l-glutamine, 100 U/ml of penicillin and 100 µg/ml of streptomycin (Invitrogen). At an approximate density of 1×10^6 cells/ml, the cells were harvested for the follow-up experiments [2, 108].

Postmortem RLS brain tissues

Brain tissues (thalamus and upper pons) were autopsied from 31 physician diagnosed RLS patients (Caucasians of European ancestry) from the Harvard Brain Tissue Resource Center. Final diagnosis was made by an RLS expert based on available questionnaires and medical records but blinded to the genotype information; same samples were previously used in our earlier RLS reports [108, 124].

RNA isolation and quantitative reverse transcriptase polymerase chain reaction (q-RT-PCR)

0.2 g of frozen brain tissues (thalamus and pons) were used for RNA extraction using the RNeasy® Lipid Tissue kit (Qiagen). For the case of LCL samples or human cell lines, RNA was extracted from 5 million cells using the RNeasy® mini kit (Qiagen). Reverse transcription of RNA to cDNA was performed from 1µg of total RNA using either a mix of oligo-dT and random primers and the Quantitect® Reverse Transcription kit (Qiagen) or the SuperScript® VILO™ cDNA Synthesis Kit (Invitrogen). q-RT-PCR was performed using TaqMan method (Applied

Biosystems) with probes and primers designed by Applied Biosystems specific for *BTBD9* (Hs00537653_m1), *MAP2K5* (Hs00177134_m1), *MEIS1* (Hs00180020-m1), *MT2A* (Hs02379661_g1), *CNTFR* (Hs00181798_m1), *CNTNAP4* (Hs00369159_m1), *VDR* (Hs01045843_m1), *HMOX1* (Hs01110250_m1), *SLC30A1 (Hs00253602_m1), MT1X* (Hs00745167_sH), *ATP1A1* (Hs00167556_m1), *SIX4* (Hs00213614_m1), *COL9A3* (Hs00951243_m1), *BAG3* (Hs00188713_m1), *EVC2* (Hs00377633_m1) and a custom probe designed at the junction of two exons of *SKOR1* (AJ1RULU). PCR conditions were as follows: 50°C for 2 min, 95°C for 10 min, followed by 40 cycles at 95°C for 15 sec (denaturation) and 60°C for 1 min (annealing and extension). Fluorescent signals were captured using the ABI PRISM® 7900HT Sequence Detection System (Applied Biosystems) or QuantStudio™ 7 Flex Real-Time PCR System and Software (v1.0, Applied Biosystems). Expression levels were determined by converting the threshold cycle (Ct) values using the $2^{-\Delta\Delta Ct}$ method and were normalized using the human 18S rRNA gene with commercial primer-probe mix (Applied Biosystems) or the human *POLR2A* control (Hs00172187_m1). All experiments were run in triplicate and independent cDNA synthesis was performed twice.

Small interference RNA (siRNA) assays

100 pmol of *MEIS1* specific siRNA (*Qiagen, Hs_MEIS1_10, #SI04321331*) was used for transfection of HeLa cells using the jetPRIME® (*Polyplus*) transfection reagent; 48 hours post-transfection, *MEIS1* expression was decreased with 70% efficiency. The negative control cells were transfected with low GC duplex control siRNA. This experiment was performed in triplicate.

Case-control RLS association study

TaqMan assays C__11771023_10 was used for the genotyping of rs4776976 (C/T) following the manufacturer's instructions and the genotypes were determined using the Applied Biosystems 7900 Fast Real-Time Polymerase Chain Reaction (PCR) System and Safety Data Sheet (SDS) software (v. 2.2.2). Genotype distribution deviation from the Hardy–Weinberg equilibrium was checked by the goodness-of-fit with one degree of freedom (df). Differences in sex and age were tested by $\chi 2$ (Chi-squared) or Student's t-tests, respectively. rs4776976 association with RLS was performed using the PLINK program [2, 146].

Electrophoretic mobility shift assay (EMSA)

We tested the binding affinity of MEIS1 and Pbx1 to *SKOR1* promoter at the three potential sites with an electrophoretic mobility shift assay. PCS2 vectors were designed to express either wild-type or mutated MEIS1 (N51S, since 51 is a crucial position of homeobox domains) or Pbx1 (*provided by Dr. Featherstone*). *In vitro* transcription/translation (IVTT) (*TNT Coupled Reticulocyte Lysate Systems part #TB126, Promega*) method was used to purify the proteins. On the other hand, the following oligonucleotides were used for the *SKOR1* promoter DNA probes: (SKOR1_$^{+}$2.6_top: tca aac ttg ggc cgg aca gta tga tta att aca gtt taa tt; SKOR1_$^{+}$2.6_bottom: taa tta aac tgt aat taa tca tac tgt ccg gcc caa gtt tg; SKOR1_$^{-}$1,76_top: tgc act cca gcc tgg gtg aca gag tga gtg aga ttc cat ttc; SKOR1_$^{-}$1,76_bottom: tga aat gga atc tca ctc act ctg tca ccc agg ctg gag tgc; SKOR1_$^{-}$8,7_top: tgg ccc cag ttc aca atg aca ggc agg tgc ctc ctc tgc ttc; SKOR1_$^{-}$8,7_bottom: tga agc aga gga ggc acc tgc ctg tca ttg tga act ggg gcc; Consensus_top: tac tgc tgc gat gat tga cag ccg cct cg; Consensus_bottom: tcg agg cgg ctg tca atc atc gca gca gt) in SSC buffer, marked with dATP 32P using the Large (Klenow) Fragment DNA polymerase (*Invitrogen*) and purified using Microspin™ G50 columns (*GE Healthcare*). The purified proteins and the DNA probes were

incubated for 20 minutes at room temperature with Poly DiDc 0,1 U/μL (*Novagen*) and BindBuffer (Glycerol 24%, KCl 200 mM, TRIS pH 7,5 20mM, MgCl2 6 mM, EDTA pH 8,5 0,2 mM, Dithiothreitol 2 mM) for identification of protein-DNA interactions and the mix was then loaded on a 30% acrylamide:bisacrylamide gel. Electrophoresis was performed at 125 Volts for 45 minutes. The gel was then dried under vacuum at 80°C for 45 minutes and autoradiography was performed with 48 hours exposure time.

Luciferase reporter assay constructs

The luciferase reporter assays were performed by insertion of each *SKOR1* promoter with positive EMSA results in a single PGL3 vector expressing the firefly luciferase gene. Each vector contains one of the potential MEIS1/Pbx1 binding sites. P1 segment contains the MEIS1/Pbx1 binding site ~8.7kb upstream *SKOR1* ATG start site. This fragment (5,287 bp) was amplified with Phusion DNA polymerase (*New England Biolabs*) from BAC clone RP11-207J8, using the following primers: fwd_5'-gag ctc tta cgc gtg cta gcc cgg gct cga gag ggt gcc tgt ggt gtg gga cgg tag g-3', rev_5'-ctg act aat tga gat gca gat cgc aga tct taa att gtc ttg acc cct tgc tgg ttt tt-3'.

rs4776976 SNP is located 17 bp upstream P1 fragment, so this region was generated with two alternatives (with the C risk allele or with the T protective allele) using QuikChange® Site-Directed Mutagenesis (*Agilent*). Furthermore, P2 segment with the ~1.76kb MEIS1/Pbx1 binding site upstream *SKOR1* ATG start site is the closest segment to the ATG start codon and contains the ~1.76kb MEIS1/Pbx1 binding site (1,535 bp). This region was also amplified from same BAC clone using the following primers: fwd_5'-gct ctt acg cgt gct agc ccg ggg tcg acg cca aaa aga ggg aag aac c-3', rev_5'-cta att gag atg cag atc gca gat ctc gag acc agg tcc cac ttg act tg-3'. The three fragments were cloned with the sequence and ligation independent cloning (SLIC) method [147] at XhoI site of pGL3 vector (Promega) which contains SV40 promoter upstream the firefly

luciferase gene. Proper clones were selected based on Sanger sequencing (*Genome Quebec Innovation Centre*) [2].

Luciferase reporter assay

100,000 HeLa cells were plated in a 24-well plate and after 24 hours they were transfected with 100 or 200 ng of one of the following constructs: empty PGL3-Promoter vector, P1 with risk variant, P1 with non-risk variant or P2 in PGL3 vectors. Each of these vectors were co-transfected with 100 ng of Renilla luciferase vector and 25 pmol of siRNA (*MEIS1* or negative control) using the attractene transfection reagent (*Qiagen*). 48 hours post-transfection, cells were lysed with 100 ul of passive lysis buffer from the Dual-Luciferase® reporter assay (*Promega*). Firefly and Renilla luciferase activity were then read using the same kit and a Synergy 4 microplate reader (*Biotek*). A ratio between firefly and Renilla expression was used to assess the luciferase expression [2].

Generation and characterization of the human neuroblastoma cell lines overexpressing MEIS1 gene

MEIS1 open reading frame (ORF, NM_002398.2, Open Biosystems, clone ID: 5531644) was sub-cloned into pcDNA3.1+ (at BamH1 and EcoR1 restriction cloning sites). pcDNA3.1+ is a mammalian expression vector with CMV promoter and contains a neomycin marker for the selection of stable cell lines. We performed Sanger sequencing (Genome Quebec Innovation Centre) to validate the sequence of the cloned cDNA. jetPRIME® (Polyplus) was used to transfect human neuroblastoma cell lines (SK-N-SH cells [148]). Stable cell lines overexpressing *MEIS1* were selected using G418 antibiotic (the optimum G418 concentration for SK-N-SH cells was 500 ug/ml, based on a standard curve). 48 hours post-transfection, the cells were maintained in DMEM

(Dulbecco's Modified Eagle Medium) containing 500 ug/ml G418 and the media was changed every other day for a period of four weeks to obtain stable cell lines overexpressing *MEIS1*. The case and the control conditions were done in at least triplicates. We performed western blot analysis using polyclonal anti-MEIS1 from Abnova (Catalog number: H00004211-A01, http://www.abnova.com) to characterize the MEIS1 overexpression. The western blot condition was optimized as follows: 1:500 primary antibody dilution in PBS-T buffer with skim milk, primary incubation at room temperature for one hour and secondary incubation using goat anti-mouse IgG overnight at 4°C.

Human cell lines with SKOR1 overexpression or knockout

SKOR1 ORF (ENST00000341418.5, Open biosystem, Clone ID: 100068272) has a high expression level in Brain-Cerebellum and Brain-Cerebellar Hemisphere based on GTEx. This ORF was also sub-cloned in pcDNA3.1+ mammalian expression vector with the strong CMV promotor. The cDNA of the plasmid was validated by Sanger sequencing (Genome Quebec Innovation Centre). We used jetPRIME® (Polyplus) to transfect HAP1 and HEK293 cells with this vector. 48 hours post-transfection, the cells were transferred to culture media which contained 500 ug/ml G418 and the media were changed every other day for a period of four weeks (in at least triplicates) to obtain stable cell lines overexpressing *SKOR1*. On the other hand, *SKOR1* knockout HAP1 cells were also used as a complementary to the *SKOR1* overexpressing cells. HAP1 cells are partially-haploid human cell line. This cell line is derived from the male chronic myelogenous leukemia (CML) cell line KBM-7. HAP1 cells contain a single copy of each chromosome, except for a heterozygous 30-megabase fragment of chromosome 15 [149]. These cells are widely used for the purpose of knockout (KO) cell line generation, since they are partially haploid and more easily

and efficiently knocked out [150]. The HAP1 wild-type and HAP1 *SKOR1*-KO cells (Catalog number: HZGHC006614c012) were commercially obtained from Horizon Discovery. Additional information about the cell line and the experimental approaches to generate a particular KO strain can be found at the following website: https://www.horizondiscovery.com. HAP1 cells were cultivated in IMDM (Iscove's Modifed Dulbecco's Medium) (Gibco) and HEK293 cells in DMEM (Dulbecco's Modified Eagle Medium) (Gibco); both supplemented with 10% fetal bovine serum (FCS, Biochrom) and 1% penicillin/streptomycin (Pen/Strep, Sigma) at 37 °C and 5% CO2.

Polyclonal anti-SKOR1 generation and characterization

Two different peptide sequences were used for SKOR1 polyclonal antibody generation (peptide 1: MELRKKLEREFQSLKDN and peptide 2: SNRFPDDEDAQEETE). The design of Peptide 1 was done by our group: SKOR1_HUMAN protein sequence (UniProt identifier: P84550-1) was used as reference sequence for epitope analysis in the peptide sequence design procedure. We implemented the Jameson-Wolfe Antigenic index profile prediction track from the software Protean (DNASTAR v5.0.5) to identify the immune-reactive regions of the SKOR1 protein. The design was based on the following criteria: lack of N-terminal glutamine or asparagine and C-terminal proline or glycine, no internal cysteine and no multiple serine, proline or glutamine residues in the sequence. This peptide sequence is common to all *SKOR1* isoforms. Peptide 2 sequence was suggested and used in a western blot analysis in a study by Arndt et al. [151]. For each of the peptide sequences, two New Zealand white rabbits were immunized at Capralogics Inc.; detailed protocol can be found at https://www.capralogics.com/rabbit-antibodies. After 13 weeks, final bleeds were used for antibody characterization by western blot analysis using HEK293 cells overexpressing *SKOR1* ORF. The western blot conditions were optimized as

follows: 1:100 final bleed dilution in PBS-T buffer with skim milk, primary incubation at room temperature for one hour and secondary incubation using goat anti-rabbit IgG overnight at 4°C. Endogenous levels of the SKOR1 protein cannot be detected on western blots due to the low abundance of the protein.

High-throughput RNA sequencing of human cell lines

We used RNeasy® mini kit (Qiagen) to extract the total RNA content from the human cell lines. During the RNA extraction, a randomization process was used to ensure that no batch effects were generated. Synergy H4 Hybrid Multi-Mode Microplate reader from BioTek was used for RNA quantification. 250 ng total RNA obtained from human cell lines was provided to the McGill University and Génome Québec Innovation Centre (*MEIS1* manipulated cells) or to Macrogen Inc (*SKOR1* manipulated cells). RNA Integrity Number (RIN) was assessed using the 2100 Bioanalyzer instrument, together with the 2100 Expert software and Bioanalyzer assays (RIN was over 9 for all the cell line RNA samples). Illumina Ribo-Zero rRNA Removal Kit (Human/Mouse/Rat) was used to target eukaryotic long noncoding and coding transcripts (rRNA-depleted libraries).

RNA sequencing [152] of *MEIS1* manipulated cells was performed on the Illumina HiSeq 2500 platform at 100bp paired-end reads with a total of 80 million reads per sample at McGill University and Génome Québec Innovation Centre. The RNA-Seq of *SKOR1* manipulated cells was performed on the Illumina HiSeq 4000 platform at 150bp paired-end reads with a total of 100 million reads per sample (at Macrogen Inc).

High-throughput RNA sequencing of RLS brain tissues

Based on our previous study where we reported a reduced *MEIS1* expression level in cells and thalamus tissues obtained from a subset of RLS patients carrying the *MEIS1* risk haplotype (GG/GG) [108], we used the same brain tissues for RNA-Seq study. Ten thalamus samples showing highly contrasting levels of *MEIS1* expression were used for RNA sequencing; five samples with substantially lower levels of *MEIS1* expression (*MEIS1* risk haplotype carriers, GG/GG) by comparison to five samples with higher expression levels (non-risk, AA/TT or heterozygous carriers, AG/TG). We further validated their differential *MEIS1* expression using a TaqMan gene expression assay (p-value < 0.001). Pons tissues were also used in that previous study, however *MEIS1* expression was not significantly reduced as a result of *MEIS1* haplotype in this region. Nevertheless, we used pons samples only based on their *MEIS1* expression level, regardless of their genotype, to assess whether the previous observations will replicate. Moreover, no group differences regarding the postmortem interval (PMI), age, sex or RIN were present in the thalamus or pons groups Table 1.

Table 1. Demographics for RNA-Seq Study. Thalamus and pons brain sample gender ratios and group demographics means (presented as mean ± SEM). Non-parametric Wilcoxon tests showed that there are no significant differences between groups for any of these variables [3]. Modified from Sarayloo et al. *PLoS ONE,* 2019.

Status	**Sample size**	**Gender**	**Age (year)**	**Postmortem Interval (hour)**	**RIN**
Thalamus High *MEIS1*	n = 5	4F / 1M	84.4 ± 5.938	13.08 ± 3.099	2.82 ± 0.124
Thalamus Low *MEIS1*	n = 5	4F / 1M	84.2 ± 2.354	17.63 ± 3.159	2.54 ± 0.306
Pons High *MEIS1*	n = 5	5F / 0 M	83.2 ± 5.978	14.5 ± 3.541	2.9 ± 0.138
Pons Low *MEIS1*	n = 5	5F / 0 M	85.4 ± 2.249	17.12 ± 3.253	2.5 ± 0.176

0.2 g of frozen brain tissues (thalamus and pons) were used for RNA extraction using the RNeasy® Lipid Tissue kit (Qiagen). Similar to human cell line RNA extraction, a randomization process was used to ensure that no batch effects were generated. The concentration of RNA was measured by the Synergy H4 Hybrid Multi-Mode Microplate reader from BioTek and 2.5ug RNA was provided to Macrogenlab Inc. The RIN was assessed by the 2100 Bioanalyzer instrument, together with the 2100 Expert software and Bioanalyzer assays, however all the RINs were below 7. This means these samples contain degraded RNA and they might be removed during the Ribo-Zero process; this can introduce biases to the results. On the other hand, poly (A) selection method can also introduce a strong 3′ bias [153]. Therefore, we alternatively used RNA Access method for these highly precious but degraded RNA samples [154]. RNA sequencing [152] was performed on the Illumina HiSeq 2500 platform at 100bp paired-end reads with a total of 80 million reads per sample at Macrogenlab Inc.

Bioinformatic analyses of RNA-Seq data

STAR v2.5.1b was used to align the FASTQ files of the paired-end reads to the human genome reference (GRCh37/hg19 assembly) [155]. Picard v1.123 was used to mark duplicates and calculate exonic/intronic/intergenic rates (https://broadinstitute.github.io/picard/). HTSeq-count was used for gene-level quantification and all the genes with less than 1 read per sample were removed (http://www-huber.embl.de/users/anders/HTSeq/doc/overview.html). The library size for each sample was estimated using the number of mapped reads in the BAM file (ttp://samtools.sourceforge.net/). Rnaseq.py v3.0.0 pipeline from GenPipes was used for all the steps mentioned above [156].

Differential gene expression was determined by EdgeR v3.5.2 [157]. An exact test with no covariate was used for the RNA-Seq data analysis of the cell line data with *MEIS1* dysregulation. For the case of human cell lines with *SKOR1* dysregulation, Quasi-likelihood F-tests was implemented with batches as covariates in an additive model. Likelihood ratio test including age, postmortem interval (PMI), RNA integrity number (RIN) and batches as covariates was performed for the brain tissues in an additive model. Including RIN in the additive model of brain DGE analysis can retrieve the biologically meaningful results from these low RIN but precious RNA samples [158]. BiomaRt v2.14.0 R package (http://www.bioconductor.org/packages/release/bioc/html/biomaRt.html) was incorporated for gene annotations. The list of significantly DEGs was defined at FDR < 0.05 and were used in the pathway analysis [159, 160].

Pathway analysis

Enrichment analysis for predicted target genes was conducted using EnrichR database (http://amp.pharm.mssm.edu/Enrichr/), which is a comprehensive gene set enrichment analysis web server (in Chapter 4, we also validated the results obtained from EnrichR by the Database for Annotation, Visualization and Integrated Discovery (DAVID, v6.8) [159-162]. KEGG pathway analysis and three categories of GO (http://geneontology.org) functional annotation, including biological process (BP), cellular component (CC) and molecular function (MF), were included in this study. The significantly enriched pathways with FDR < 0.05 were selected. In Chapter 5, we also visualized the interactions between the pathways using ClueGO and CluePedia (Cytoscape plug-in) [163].

Ethics statement

Lymphoblastoid cell lines were obtained from RLS patients who signed an informed consent form before the study. The LCL biobanking was reviewed and approved by the institutional review board of McGill University (project # 2015-164, MP-CUSM-14-051). RLS brain tissues were provided by the Harvard Brain Tissue Resource Center. This is supported in part by a Public Health Service Grant (R24MH068855), with permission from the RLS Brain Bank Tissue Review Committee through the RLS Foundation. In the case-control association study of rs4776976 with RLS, all participants signed an informed consent at enrollment. The study protocols were approved by the institutional ethics review boards. This study was approved by Comité d'éthique de la recherche du Centre hospitalier de l'Université de Montréal and McGill University ethics. All methods were performed in accordance with the relevant guidelines and regulations of McGill University (REB NEU-14-051).

Chapter 3 A direct interaction between two restless legs syndrome predisposing genes: *MEIS1* and *SKOR1*

Preface

The first large scale genome-wide association study performed for RLS in 2007 reported three noncoding genomic regions within the following genes: *MEIS1*, *BTBD9* and *MAP2K5*/*SKOR1* [96]. All three of these association signals were subsequently replicated in independent studies in different populations. While our understanding of RLS genetics benefited from the advancements made by these association studies, limited explanations remained about their functions in RLS pathogenic pathways. In 2009, a link was found between the risk haplotype (rs12469063/rs2300478: GG/GG) within *MEIS1* noncoding regions and its mRNA and protein expression. In that study, an examination of patient-derived material obtained from RLS cases revealed that a subset of patients that carried *MEIS1* risk haplotype, had a reduced mRNA and protein expression of this gene [108]. This observation raised the possibility of a regulatory effect from these noncoding variants toward the expression of the RLS candidate genes. Based on these findings, Chapter 3 of this thesis investigates the effects of the well-established (at the time this study was initiated) RLS-associated risk variants on their gene expression levels. This investigation involved genotyping and q-RT-PCR analysis of lymphoblastoid cell lines and autopsied brain tissues (pons and thalamus) obtained from individuals who received RLS diagnosis from physicians specialized in sleep disorders. The results with positive regulatory effects were further investigated and characterized using protein-DNA binding and promoter reporter assays.

Results:

BTBD9, MAP2K5 and SKOR1 expression based on their corresponding RLS-associated risk variants

Our previous study highlighted a decreased level of *MEIS1* mRNA and protein expression in lymphoblastoid cell line and thalamus samples of RLS patients who carried the *MEIS1* risk haplotype [108]. Here, we examined if other RLS-associated risk variants might also affect the mRNA expression levels of the genes harboring them. For this aim, we performed q-RT-PCR on biological material of RLS cases (LCL and brain tissues: thalamus and pons) to test for changes in the expression levels of RLS-associated genes based on their genotype (by the time this study was initiated, variants within noncoding regions of *MEIS1, BTBD9, MAP2K5 and SKOR1* were identified) [96].

rs3923809 is located in intron 5 of *BTBD9* (A is the RLS risk allele). *BTBD9* mRNA expression was measured in LCL samples obtained from RLS patients and was compared between individuals with G/G, A/G and A/A alleles, but no significant differences were detected. However, we only had access to four LCL from individuals homozygous for the non-risk allele (G/G) and this small sample size could decrease the power to detect a true difference. Next, we measured *BTBD9* mRNA levels in the two brain regions to further explore the possible regulatory effects of this SNP. On average, in the thalamus and pons of RLS patients who were heterozygous (A/G) carriers of rs3923809, there seemed to be an increased level of *BTBD9* mRNA, however the distribution of the samples was too broad to conclude any significant differences between individuals carrying either the A/A or A/G alleles. Moreover, the brain regions homozygous for the G/G allele were not available for this study and this prevented us from reaching a conclusion (Figure 4).

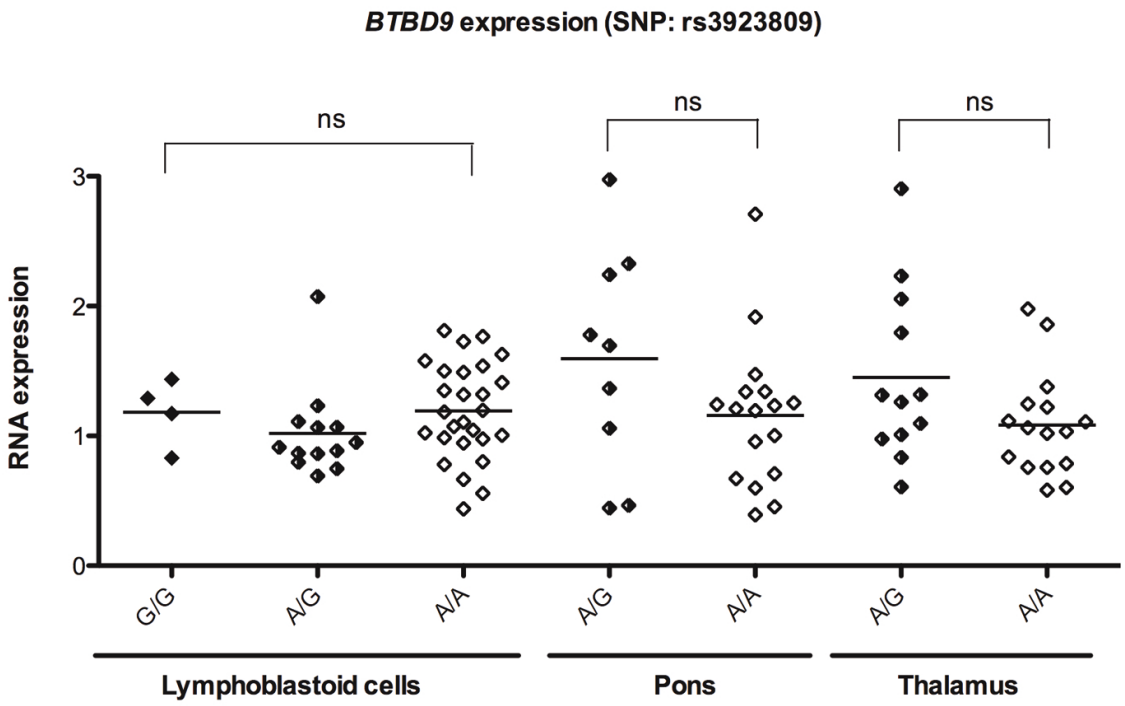


Figure 4. q-RT-PCR measurement of BTBD9 expression as a function of rs3923809 genotype (A is the RLS risk allele). No significant difference was observed in LCL, pons or thalamus samples from RLS patients. Adapted from Catoire et al. *Scientific Reports,* 2018.

rs3784709 is located in the intergenic region between *MAP2K5* and *SKOR1* (C is the RLS risk allele). We measured the mRNA expression of both *SKOR1* and *MAP2K5* and compared their expression between RLS individuals with T/T, C/T and T/T alleles, however we did not observe any effect as a result of this risk allele (Figure 5). This is also noteworthy that the small number of RLS brain samples that were homozygous for the non-risk allele limited the power of this test. Nonetheless, the q-RT-PCR assays performed on LCL suggested that the mRNA levels of the two genes were not affected by the presence of the risk allele.

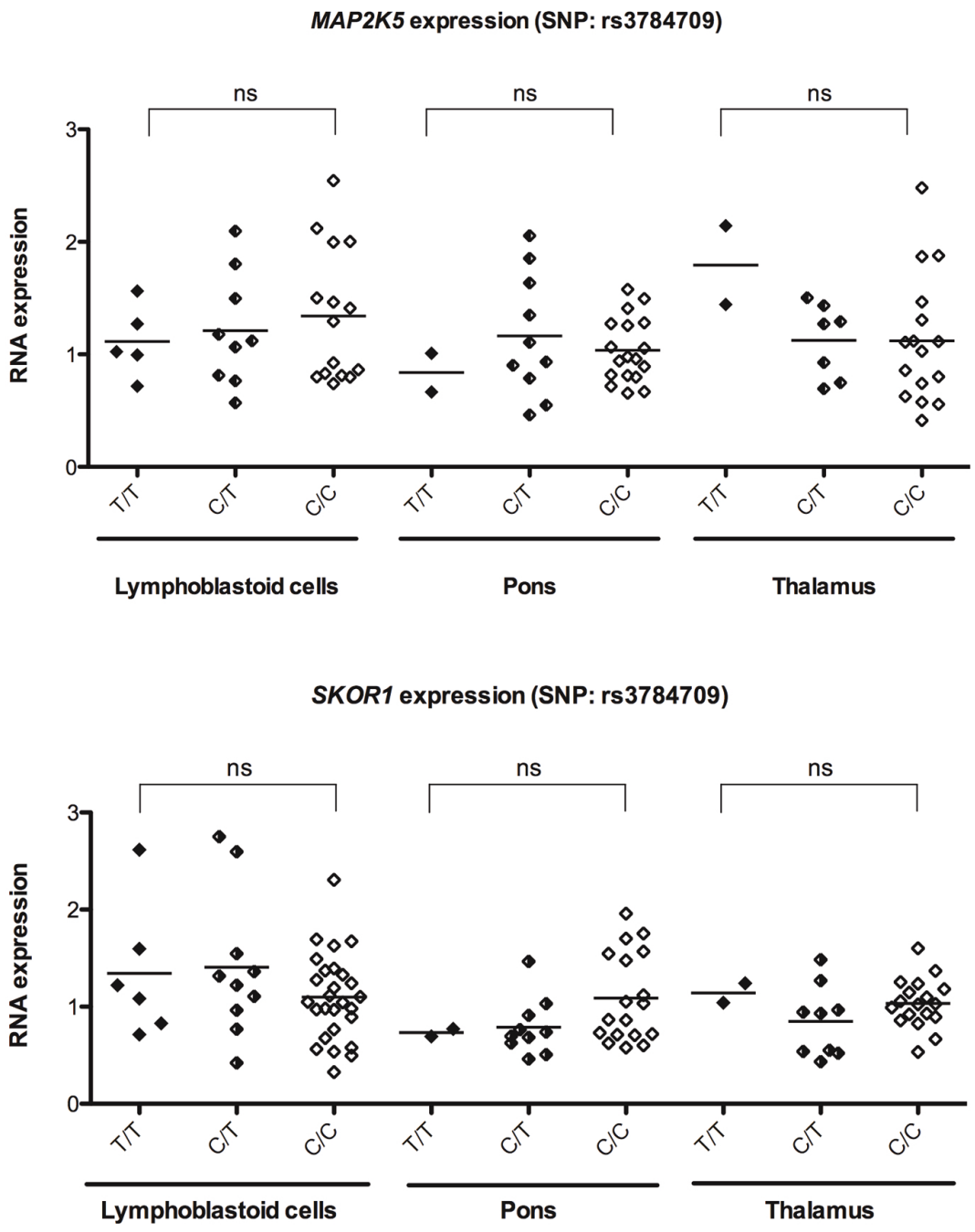


Figure 5. q-RT-PCR measurement of MAP2K5 (top) and SKOR1 (bottom) expression as a function of rs3784709 genotype (C is the risk allele). No significant difference was observed in LCL, pons or thalamus samples from RLS patients. Adapted from Catoire et al. *Scientific Reports,* 2018.

A link between SKOR1 expression and MEIS1 risk haplotype

After the observation where no links were present between the RLS risk variants and the expression levels of the genes harbouring them, we investigated the links between each risk variant and the other RLS-associated genes. We did not observe any significant effect, except for the case of *SKOR1* expression. We observed changes in *SKOR1* mRNA expression according to the *MEIS1* risk haplotype. *SKOR1* mRNA expression in LCL was significantly decreased (p-value = 0.0202) in RLS cases that were homozygous carriers of the *MEIS1* risk haplotype (rs12469063/rs2300478: GG/GG) by comparison to the non-risk carriers (AA/TT). Similar q-RT-PCR measurements were performed on *SKOR1* mRNA expression on thalamus samples obtained from RLS individuals carrying the same *MEIS1* haplotypes and a decreased *SKOR1* mRNA level was observed again (p-value = 0.0174). Pons samples did not show any significant differences in the *SKOR1* mRNA level (p-value = 0.1519, Figure 6). The decreased level of *SKOR1* mRNA expression in LCL and thalamus had a similar pattern to the *MEIS1* decreased expression as a result of its RLS risk haplotype [108]. This raises the possibility of a transcriptional regulatory effect from MEIS1 protein as a transcription factor on *SKOR1*.

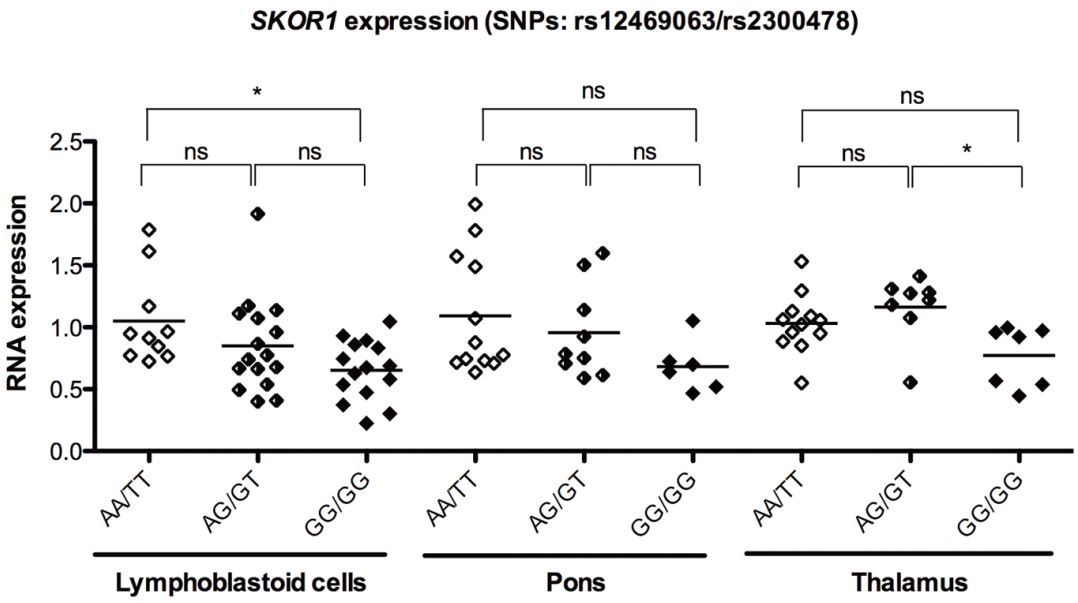


Figure 6. q-RT-PCR measurement of SKOR1 expression as a function of MEIS1 risk haplotype. A significant decrease of expression in LCL and thalamus samples with the MEIS1 risk haplotype (GG/GG) was observed. Adapted from Catoire et al. *Scientific Reports,* 2018.

Reduced MEIS1 expression is associated with reduced SKOR1 expression

Due to a similar trend in the reduced expression of *MEIS1* and *SKOR1* in LCL and thalamus samples obtained from individuals harbouring the *MEIS1* risk haplotype (GG/GG), we started to investigate the links between these two genes in more detail. We used human cell lines (HeLa cells) and silenced *MEIS1* using a specific siRNA targeting *MEIS1* (~70% efficiency in mRNA reduction). This reduction was associated with a 64% decrease of *SKOR1* mRNA level (p-value < 0.0001, Figure 7). This observation further supports a link between *MEIS1* and *SKOR1* and suggests that MEIS1 might act as an upstream activator for *SKOR1*.

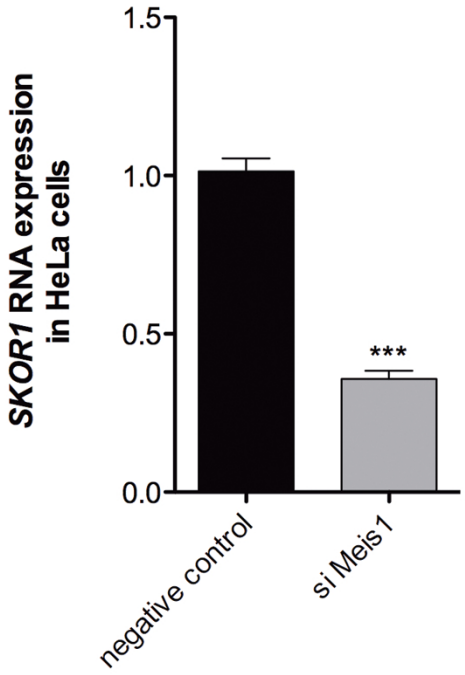


Figure 7. Decreased SKOR1 mRNA expression in human HeLa cells with silenced MEIS1 (by siRNA). Adapted from Catoire et al. *Scientific Reports,* 2018.

MEIS1 binding sites within SKOR1 promoter region

MEIS1 is a homeobox containing transcription factor with direct DNA binding properties. Based on the links found between *MEIS1* and *SKOR1,* we hypothesized that MEIS1 transcription factor might regulate the expression of *SKOR1* by binding to its promoter region. A literature search showed that MEIS1 forms *in vivo* heterodimers with a member of another family of homeobox genes, PBXs (pre-B cell leukemia transcription factors like Pbx1; reviewed by Longobardi et al. [164]). The DNA binding affinity of these proteins is higher when they form heterodimers [165, 166]. We investigated the presence of MEIS1/PBX1 consensus DNA binding sites [165-167] in the upstream and downstream untranslated regions (UTRs) of *SKOR1* and all of *SKOR1* intronic sequences. Three potential MEIS1/PBX1 binding sites were found (Figure 8):

1. At ~8.7kb upstream ATG start site (TGACAGgcAGgT).
2. At ~1.76kb upstream ATG start site (TGACAGagTGAg).
3. At ~2.6kb downstream ATG start site, in intron 2 of the canonical isoform of *SKOR1* (GGACAGtaTGAT).

Our search was focused on the hexameric consensus DNA binding sites; a search for MEIS1's octameric consensus binding (TGATTG/TAT), as reported by Penkov et al., did not result in finding any sites in *SKOR1* promoter regions [168].

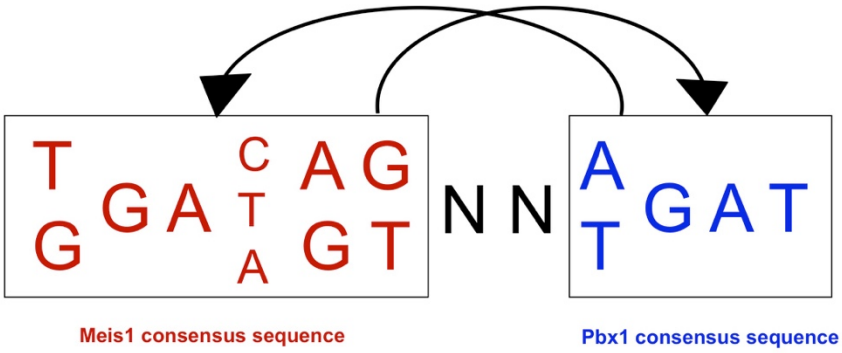


Figure 8. The consensus MEIS1/PBX1 DNA binding sequences. Adapted from Catoire et al. *Scientific Reports,* 2018.

Electrophoretic mobility shift assay shows a direct DNA binding role for MEIS1 in two SKOR1 promoter regions

pBluescript vectors with either the wild-type or mutant (to serve as negative control) forms of the coding sequences of the proteins (Meis1 or Pbx1) were used. We used *TNT Coupled Reticulocyte Lysate Systems* (Promega) to purify the proteins. The purified proteins were used in electrophoretic mobility shift assay to examine their binding to the three consensus DNA binding regions: ~8.7kb and ~1.76kb sites upstream and ~2.6kb site downstream *SKOR1*'s ATG, Figure 9). We observed strong direct DNA bindings in both upstream ATG start sites (~8.7kb and ~1.76kb), however MEIS1 did not bind to the ~2.6kb downstream ATG site, and therefore is unlikely to directly affect *SKOR1* expression via that region. Overall, these *in vitro* EMSA results indicate a physical interaction between MEIS1 protein and two regions in *SKOR1* promoter. In light of the decreased *SKOR1* expression in RLS cases with homozygous *MEIS1* risk haplotype (Figure 6), these direct bindings of MEIS1 to *SKOR1* promoter regions strongly suggests a regulatory role for MEIS1 over *SKOR1* in RLS.

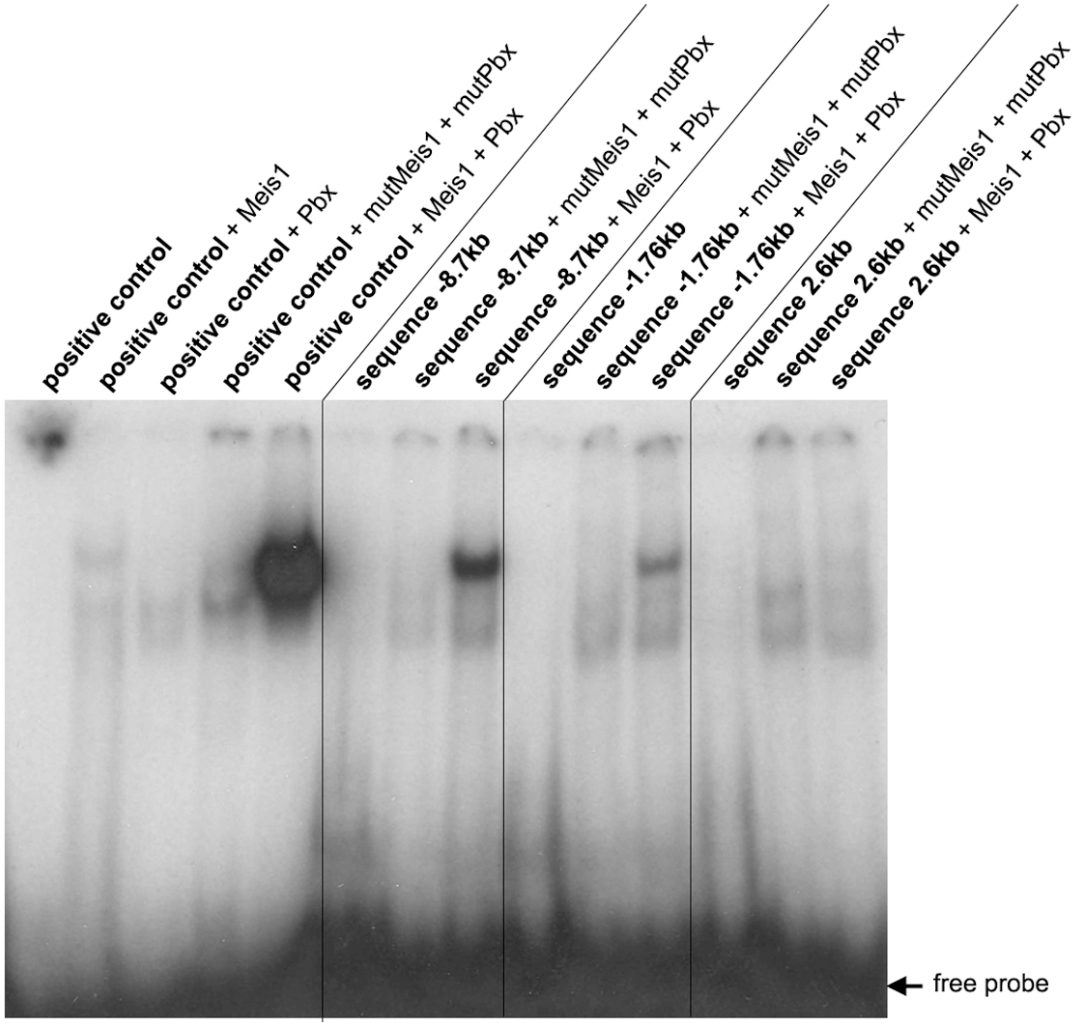


Figure 9. EMSA showing a direct binding for MEIS1/PBX1 to two SKOR1 promoter regions. Adapted from Catoire et al. *Scientific Reports,* 2018.

Furthermore, to assess the conservation of these MEIS1 binding sites in *SKOR1* promoter, we used the publicly available Meis1 ChIP-Seq (Chromatin immunoprecipitation followed by high-throughput sequencing) data in mice (GEO database #GSM2188919) [169]. This analysis resulted in the identification of two Meis1 binding sites upstream *Skor1* gene (mm8 genome assembly). This suggests that these regions are likely real elements involved in the activation of *SKOR1*. Integrated genome browser (IGB) was used to visualize these binding regions (Figure 10) [170].

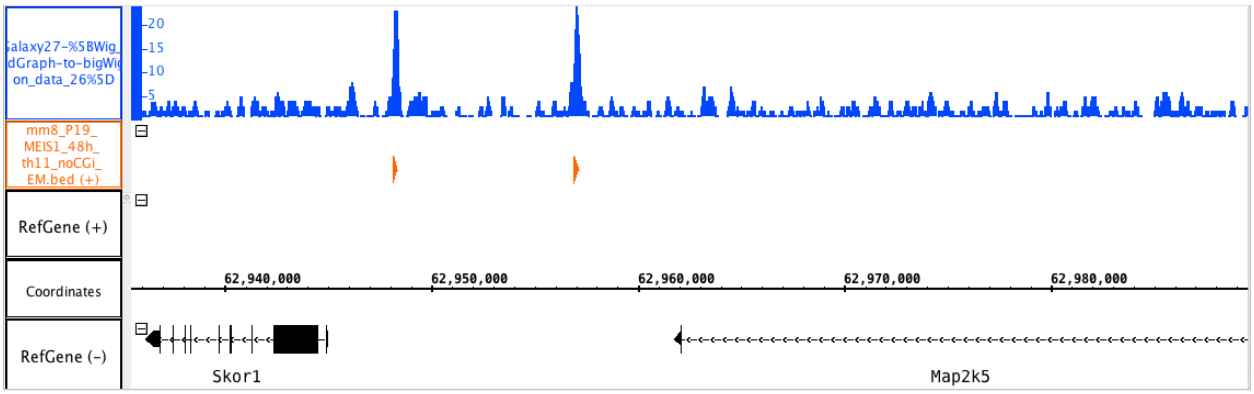


Figure 10. IGB view of the Meis1 ChIP-Seq data available from GEO database (GSE82314) from Mahe et al. [169]. Two peaks upstream Skor1 gene (in orange) show MEIS1 direct DNA binding sites. Adapted from Catoire et al. *Scientific Reports,* 2018.

A case-control association study of an additional SNP close to the ~8.7kb MEIS1 binding site in SKOR1 promoter

In parallel with the functional studies of RLS, our group had also performed an association study on 401 RLS cases and 588 controls of unrelated individuals with European ancestry (recruited as previously described) [171]. The average age at enrollment of RLS patients was 52.5 ± 14.9 years, with 38.5% men and they were diagnosed based on the IRLSSG criteria [5]. The average age at enrollment of the control population was 53.0 ± 16.1 years, with 38.2% men and there was no significant difference in sex and age between the cases and controls. The risk allele (C) frequency of rs4776976 is 0.848 in RLS cases and 0.776 in controls, respectively (the genotype distribution fits with the Hardy-Weinberg equilibrium). The p-value for the association test of the C risk allele is 0.0001267. Based on gnom Aggregation Database, the rs4776976 RLS risk allele (C) is the most common allele in the European population (0.8) compared to its non-risk T allele (0.2) [172]. rs4776976 is located 17 bp upstream the MEIS1 binding site in *SKOR1* promoter region (the ~8.7kb site). With the identification of this additional SNP, we also considered its effects on *SKOR1* gene expression and on the binding affinity of MEIS1 to this promoter region of *SKOR1,* in the following two sections.

There is no link between rs4776976 and SKOR1 mRNA expression level

We revised our mRNA expression data based on the two possible genotypes (C and T) to examine if the rs4776976 SNP influences *SKOR1* expression (Figure 11). No significant effect was observed from the risk allele on *SKOR1* expression in the LCL, pons or thalamus of patient samples.

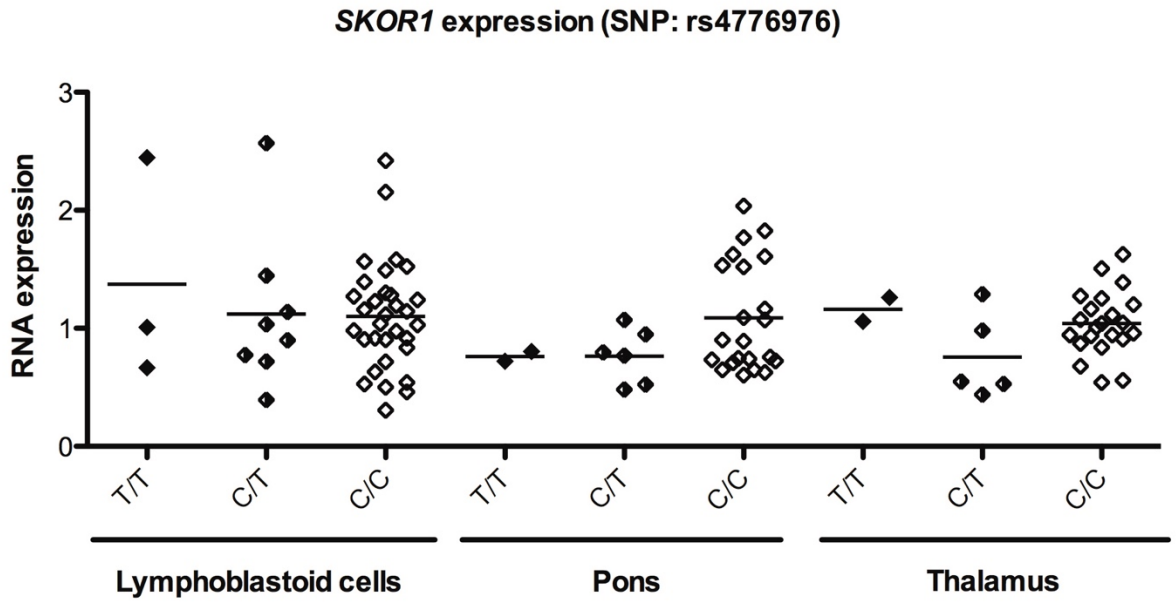


Figure 11. q-RT-PCR measurement of SKOR1 expression as a function of rs4776976 (C is the risk allele). No significant difference was observed in LCL, pons or thalamus samples from RLS patients. Adapted from Catoire et al. *Scientific Reports,* 2018.

The direct binding of MEIS1 to SKOR1 promoter region has a positive regulatory effect on SKOR1 expression

To examine whether MEIS1 binding to *SKOR1* also influences its expression and measure if the C or T alleles of rs4776976 affect the regulatory role of MEIS1 at the ~8.7kb site, we used dual-luciferase reporter assay and obtained quantitative measures of expression. We cloned the two *SKOR1* promoter regions that showed positive bindings to MEIS1 (based on EMSA results) into two separate fragments upstream a firefly luciferase reporter (PGL3, Promega): P1 contained the ~8.7kb site with either C or T alleles of rs4776976 SNP, and P2 contained the ~1.76kb site (Figure 12). These PGL3 vectors in addition to an empty PGL3 vector were separately co-transfected into HeLa cells, as well as a vector expressing a *Renilla* luciferase.

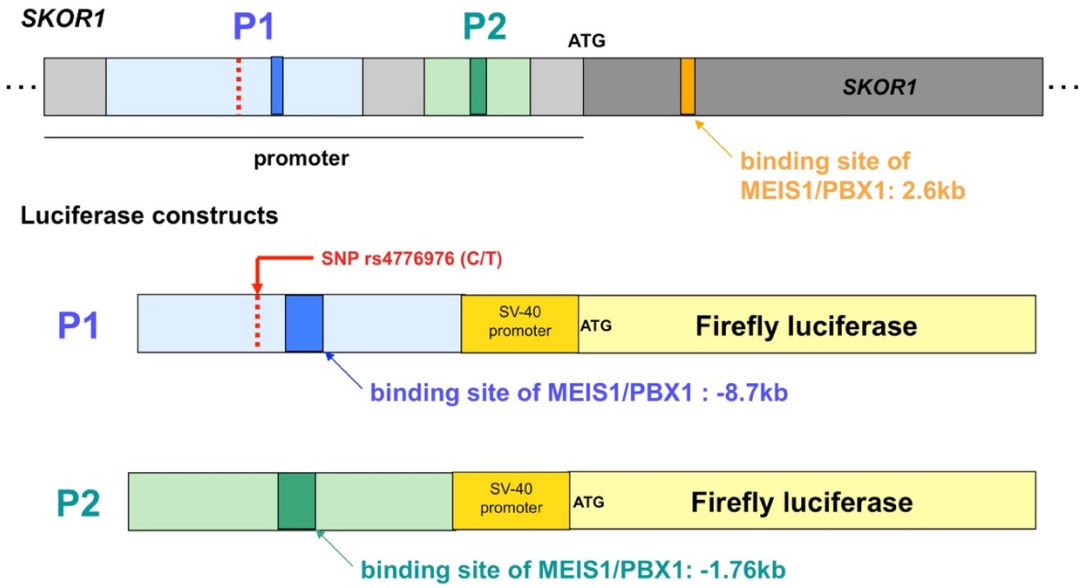


Figure 12. Representation of luciferase expression vectors. The SKOR1 promoter regions with positive binding to MEIS1 were cloned as two separate segments (P1 or P2). The rs4776976 SNP close to the P1 fragment was constructed with the two possible alleles C or T to detect the effect of these alleles on MEIS1 regulatory role over SKOR1. Adapted from Catoire et al. *Scientific Reports,* 2018.

Each pGL3 luciferase vector was co-transfected with either siRNA targeting *MEIS1* or control low GC duplex siRNA in HeLa cells. The reason for down-regulating *MEIS1* in these cells was to mimic the decreased expression of *MEIS1* known to be associated with its risk haplotype in a subset of RLS patients. The luciferase expression in each of these conditions was calculated as a percentage between cells co-transfected with the *MEIS1* siRNA and the negative siRNA control (these values were normalized from the firefly and Renilla signals, Figure 13).

In the condition where *MEIS1* was down-regulated by siRNA, we observed a significant decrease (~40%) of luciferase expression in P1 fragment with the rs4776976 C risk allele (p-value < 0.001). However, P1 fragment with the rs4776976 T non-risk allele did not alter the luciferase expression. This suggests that the RLS-associated risk allele (C) results in binding of MEIS1/PBX1 to the P1 segment of *SKOR1* promoter region. Moreover, we observed a significant decrease of expression (p-value < 0.01) in the P2 *SKOR1* promoter region. These results further approve the data obtained from previous sections of this chapter, demonstrating that MEIS1 physically binds to *SKOR1* promoter regions and acts as a direct activator of *SKOR1* expression. We also showed that the rs4776976 SNP plays an important role for MEIS1 binding to *SKOR1* promoter, where the presence of C allele results in *SKOR1* gene being regulated by MEIS1.

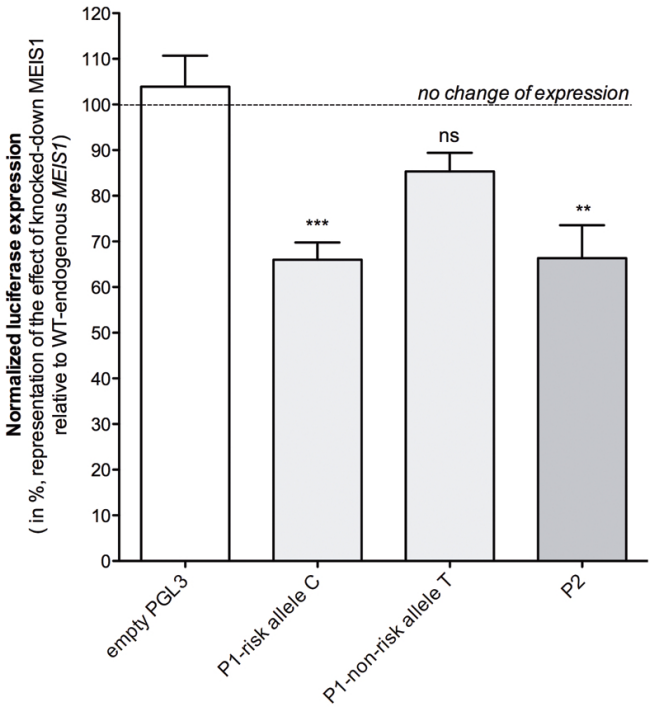


Figure 13. Luciferase reporter assay. Y-axis represents the ratio of luciferase expression (ratio firefly / Renilla luciferase expression) as percentage of samples with MEIS1 downregulated (targeted with MEIS1 siRNA) in comparison to samples with wild-type endogenous MEIS1 expression (negative control siRNA). As indicated in the figure, P1 segment (harbouring the C risk allele) and P2 segment show decreased luciferase expression as a result of MEIS1 downregulation using siRNA. $^{**}p < 0.01$ and $^{***}p < 0.001$ compared as indicated. Adapted from Catoire et al. *Scientific Reports,* 2018.

Chapter 4 Mineral absorption is an enriched pathway in a brain region of restless legs syndrome patients with reduced *MEIS1* expression

Preface

MEIS1 significantly contributes to RLS genetic, however we know little about the functions this gene plays in RLS pathogenic pathways. MEIS1 is a member of homeobox containing transcription factors and based on the current literature (also the results presented in Chapter 3), it appears to have a transcriptional regulatory role over other genes that might be involved in RLS in a more direct manner [2, 100]. Chapter 4 of this thesis is focused on the identification of MEIS1 downstream targets in a genome-wide context (by RNA sequencing), and pathway analysis to see if the dysregulated genes would be related to what is known about RLS. The first set of transcriptomic data was derived from SK-N-SH cells (a neuronal-like human cell line [148]) overexpressing *MEIS1* as well as wild-type SK-N-SH cells. We hypothesized that the differentially expressed genes between these conditions could be downstream MEIS1 regulatory function. In order to establish any links between these DEGs and RLS pathways, we postulated that their expression should also be altered in the brains of RLS cases with the *MEIS1* risk haplotype (who also had reduced levels of *MEIS1* expression) [108]. Therefore, we also generated RNA-Seq data from postmortem brain tissues obtained from RLS cases. The analysis of the transcriptomic data and cross validation of the results across the different conditions shed light on new genes and pathways that might contribute to the mechanisms underlying RLS.

Results

Characterization of MEIS1 overexpression in SK-N-SH cells

SK-N-SH cells were stably transfected by pcDNA3.1+ vector with the *MEIS1* ORF sub-cloned downstream the CMV promoter. *MEIS1* overexpression (*MEIS1*-OE) was first characterized by western blot immunodetection (Figure 14). MEIS1 protein molecular weight (MW) is 43 kDa and SK-N-SH cells overexpressing it show a notably higher level of MEIS1 by comparison to their control wild-type SK-N-SH cells.

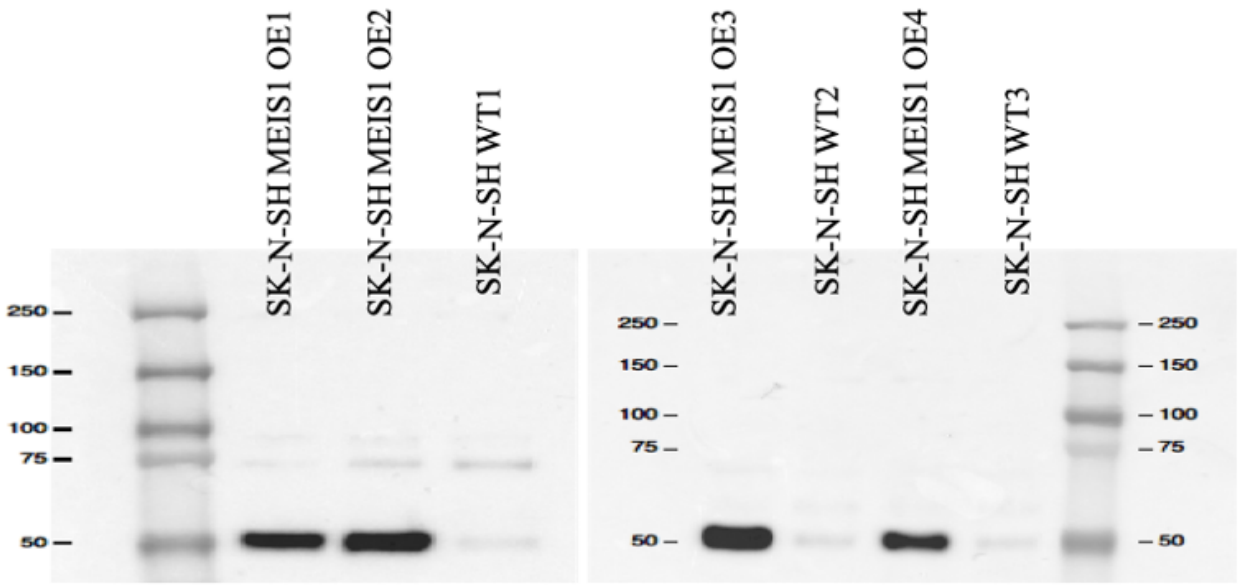


Figure 14. Western blot immunodetection of MEIS1-OE cells. MEIS1 is highly overexpressed in SK-N-SH cells (Four replicates were generated, only clones 1 to 3 were used for RNA-Seq, MEIS1 MW is 43 kDa). Adapted from Sarayloo et al. *PLoS ONE,* 2019.

Subsequently, we confirmed the overexpression of *MEIS1* at the RNA level after performing RNA-Seq and analyzing the data. Volcano plot of the RNA-Seq data is presented in Figure 15 and *MEIS1* is the most differentially expressed gene (log fold change (logFC) = 5.15, FDR = 2.60E-16).

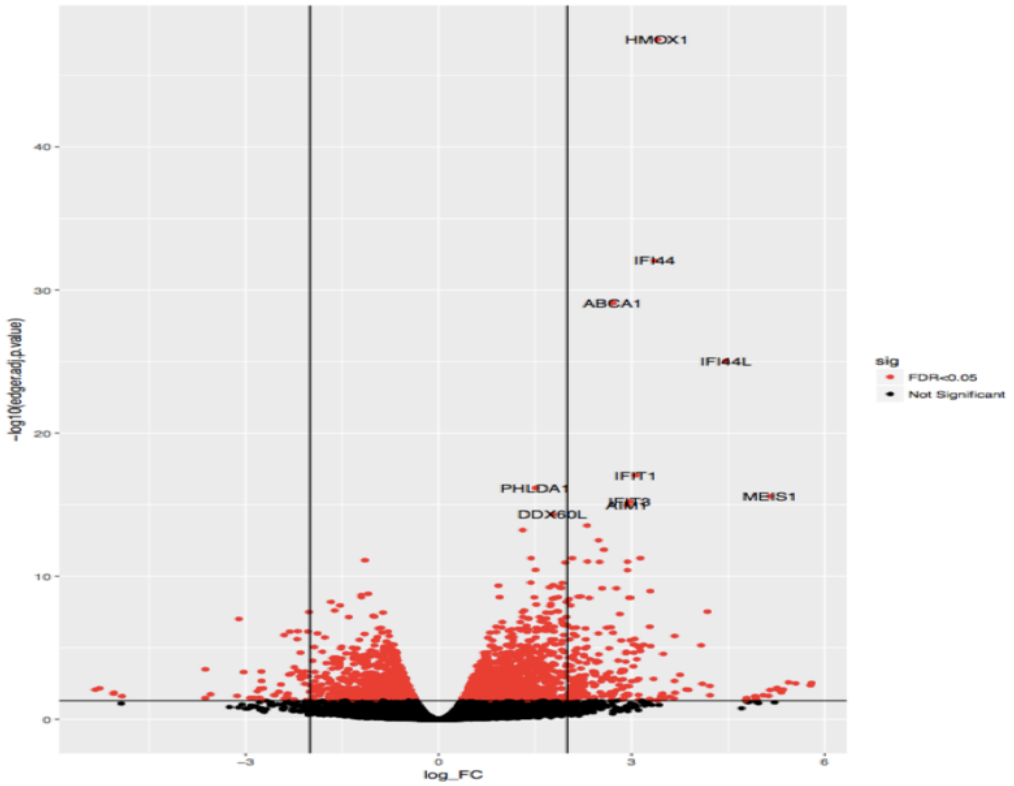


Figure 15. Volcano plot of DEGs in SK-N-SH cells overexpressing MEIS1. MEIS1 is significantly differentially expressed (logFC=5.15, FDR=2.60E-16). Adapted from Sarayloo et al. *PLoS ONE,* 2019.

Differential gene expression analysis of SK-N-SH cells overexpressing MEIS1

The RNA-Seq data obtained from the *MEIS1*-OE SK-N-SH cells were analyzed and compared with the wild-type cells to identify the differentially expressed genes. We implemented edgeR algorithm [157] for this aim; 1,374 and 937 genes were significantly up- or down-regulated, respectively (FDR < 0.05). We also generated another set of human HEK293 cell line with *MEIS1* gene knocked out by CRISPR-Cas9 to further validate the DEGs and refine the data to a smaller list for the follow-up analyses [3]. Only the DEGs from SK-N-SH *MEIS1*-OE cells that were also replicated in the *MEIS1*-KO cells were selected for further analysis. As a result, 128 genes were possibly activated, and 239 genes were possibly repressed by MEIS1 regulatory function (Figure 16).

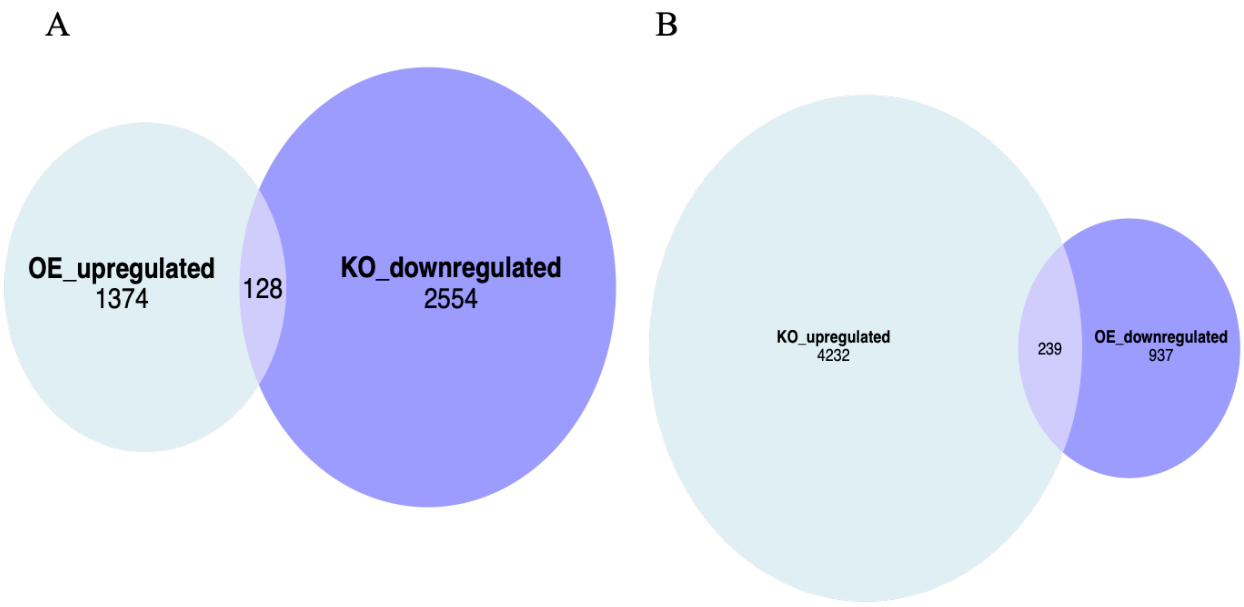


Figure 16. Venn diagram of the overlapping genes in the DEGs in the *MEIS1*-OE and *MEIS1*-KO datasets. **A.** 128 genes are possibly activated by MEIS1. **B.** 239 genes are possibly repressed by MEIS1. Adapted from Sarayloo et al. *PLoS ONE,* 2019.

MYT1 as an RLS-associated gene is differentially expressed in the human cell lines with MEIS1 dysregulation

Considering that our group has previously reported a positive regulatory role for MEIS1 on the expression of *SKOR1* (Chapter 3) [2], we analyzed the data in search for the possibility of MEIS1 regulating the expression of other RLS-associated genes. The DGE analysis of our data revealed the expression of myelin transcription factor 1 (*MYT1* gene) to be negatively regulated by MEIS1 in the cross-validated RNA-Seq data of human cell lines. *MYT1* is expressed in the neuronal progenitor cells and is involved in neuronal differentiation [173]. The association of variants within *MYT1* with RLS was reported in the most recent meta-analysis of RLS GWAS on cases with European ancestry (p-value = 3.36E-14, OR = 1.13 95%CI 1.08-1.17) [100].

Tissue enrichment analysis of the DEGs activated in the cell lines with MEIS1 dysregulation

The 128 DEGs activated and the 239 DEGs repressed in the *MEIS1* dysregulated cells were analyzed for tissue enrichment based on the GNF SymAtlas gene expression [174]. Detailed information regarding the tissues with enrichment of the DEGs that are activated in the cell line models are listed in Table 2. The most significant result after correction for multiple testing was caudate nucleus (a component of basal ganglia, FDR = 7.40E-09); 61 out of the 128 DEGs are expressed in caudate nucleus.

This tissue enrichment is interesting in regard to RLS biology; brain iron deficiency is the most consistent biological abnormality observed in RLS patients. This reduced iron level is observed within the substantia nigra, red nucleus, putamen, caudate nucleus and thalamus [50, 51]. The enrichment of the genes that are expressed in this brain region in this dataset is in co-occurrence with the well-established iron deficiency that was observed in this brain region of RLS

patients examined by brain MRI. This further emphasizes on the links that were previously made between MEIS1 and iron metabolism in RLS [124].

Table 2. Tissues with significant enrichment of the DEGs activated in *MEIS1* dysregulated human cell lines. Caudate nucleus is the tissue with the most significant enrichment of the DEGs. Adapted from Sarayloo et al. *PLoS ONE,* 2019.

Category	Term	p-value	FDR
GNF_U133A_QUARTILE	Caudatenucleus_3rd	9.40E-11	7.40E-09
GNF_U133A_QUARTILE	Heart_3rd	1.80E-07	6.90E-06
GNF_U133A_QUARTILE	Ovary_3rd	1.00E-04	1.90E-03
GNF_U133A_QUARTILE	Adrenal Cortex_3rd	9.10E-05	2.40E-03
GNF_U133A_QUARTILE	Atrioventricular node_3rd	5.10E-04	7.90E-03
GNF_U133A_QUARTILE	Pancreas_3rd	1.20E-03	1.30E-02
GNF_U133A_QUARTILE	Trachea_3rd	1.20E-03	1.50E-02
GNF_U133A_QUARTILE	Medulla Oblongata_3rd	2.20E-03	2.10E-02

The only enriched pathway in the DEGs in the human cell lines with MEIS1 dysregulation is mineral absorption

Pathway enrichment analysis of the DEGs obtained from our transcriptomic studies was performed by Enrichr and we subsequently confirmed the results using the Database for Annotation, Visualization and Integrated Discovery (DAVID) [159-162]. Mineral absorption was the only pathway that was significantly enriched in the DEGs lists after correction for multiple testing, with MEIS1 having an activator function (FDR = 0.0001, Table 3).

Table 3. Mineral absorption as the only enriched pathway in the DEGs activated in *MEIS1* dysregulated human cell lines. Adapted from Sarayloo et al. *PLoS ONE,* 2019.

Term	**p-value**	**FDR**	**Genes**
Mineral absorption	8.69E-07	1.09E-04	*MT2A, VDR, HMOX1, SLC30A1, MT1X, ATP1A1*

The genes present in this enriched pathway are: *HMOX1*, *VDR*, *MT2A*, *MT1X*, *ATP1A1* and *SLC30A1*. *HMOX1* (heme oxygenase 1) is required for the degradation of heme to biliverdin, free iron, and carbon monoxide; it shows interesting links to RLS pathways because of its involvement in iron metabolism. A previous report showed a small association signal for *HMOX1* with RLS in a cohort of Spanish cases [175]. *VDR* (Vitamin D receptor), the other gene present in this enriched pathway, also showed an association with RLS in Spanish cases [176]. Furthermore, several investigations have reported possible links between vitamin D and RLS [177-179]. Even though, association signals between *HMOX1* and *VDR* have been previously reported for RLS, to our knowledge this is the first study that reports a possible regulatory role for MEIS1 over these genes. This substantiates how the transcriptional regulatory role of MEIS1 might contribute to RLS. Members of metallothionein (MT) family of proteins were also present in this enriched pathway (i.e. *MT2A* and *MT1X*). MTs bind intracellular metals and also have a protective role against free radicals generated by oxidative stress [180].

Investigation of the publicly available ChIP-Seq data for MEIS1 DNA binding sites

MEIS1 is a homeobox containing transcription factor with direct DNA binding abilities. To predict whether the regulatory role of MEIS1 over the genes in the mineral absorption pathway (listed in Table 3) is by direct binding of this transcription factor to the promoter regions of these genes, we investigated the Meis1 ChIP-Seq data. This data was generated in mice and is publicly available from GEO database (GSE82314, by Mahe et al.) [169]. Promoter regions of *VDR* and *ATP1A1* orthologs in mice showed peaks in this ChIP-Seq data; this suggests that MEIS1 possibly regulates these genes by directly binding to their promoter sequences. The results are visualized in Figure 17 using IGB [170].

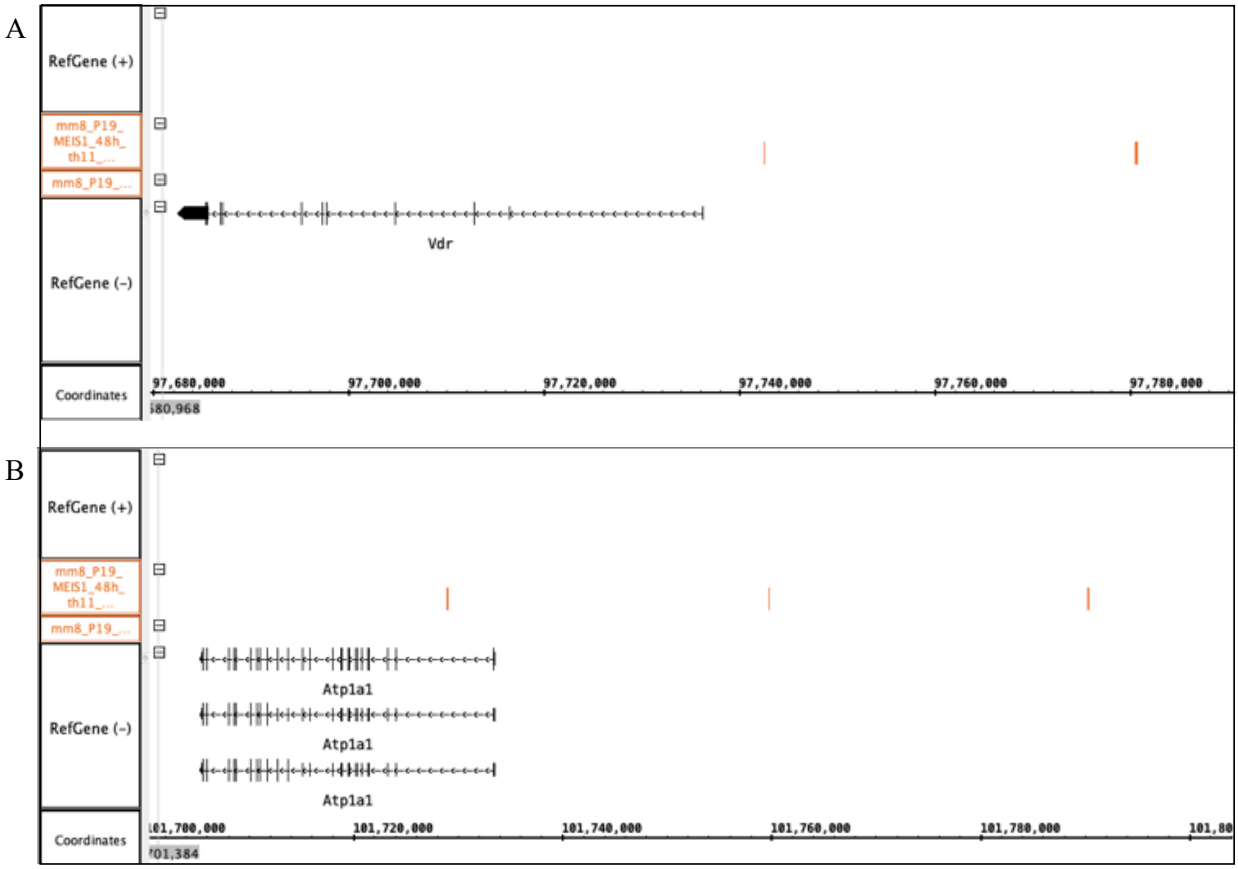


Figure 17. IGB view of the Meis1 ChIP-Seq data of genes present in the cell line mineral absorption pathway. **A.** Two peaks upstream Vdr (in orange). **B.** Two peaks upstream Atp1a1 and one peak in its first intron (in orange). Adapted from Sarayloo et al. *PLoS ONE,* 2019.

RNA sequencing of postmortem brain tissues of RLS cases

Our group previously reported a reduced *MEIS1* mRNA and protein expression in a subset of RLS patients that were harbouring the *MEIS1* risk haplotype (GG/GG) [108]. To build on this report, we performed an RNA-Seq experiment using the same brain tissues that were used in our previous study, i.e. thalamus and pons. The RNA-Seq data was generated from ten thalamus samples that showed highly contrasting levels of *MEIS1* expression. In this set, five samples had substantially lower levels of *MEIS1* expression (carriers of *MEIS1* risk haplotype, GG/GG) compared to five samples with higher expression levels (non-risk, AA/TT or heterozygous carriers, AG/TG). This reduced expression of *MEIS1* in these brain samples was verified using a TaqMan gene expression assay (p-value < 0.001). Our previous report though, did not reveal a reduced *MEIS1* expression as a result of *MEIS1* risk haplotype in the pons brain region. Nevertheless, we also used pons samples, but only based on the different levels of *MEIS1* expression. Therefore, we also assessed whether the previous observations in pons would replicate by RNA-Seq. 434 and 15 genes were differentially expressed in thalamus and pons, respectively (FDR < 0.05). These results flag candidate genes that may be downstream to the transcription factor activity of MEIS1 and can be used to further validate the observations made in the human cell lines.

Mineral absorption is also an enriched pathway in the RLS brain samples with reduced MEIS1 expression levels

Similar to our cell line enrichment analysis, we used Enrichr in conjunction with the Kyoto encyclopedia of genes and genomes (KEGG) database to analyze the DEGs possibly regulated by MEIS1 in RLS brain tissues. Each tissue was separately analyzed to obtain the enrichment of the genes that were either potentially repressed or activated in brain samples with *MEIS1* dysregulation. This analysis also resulted in the identification of mineral absorption as an enriched

pathway in DEGs that were repressed in samples of contrasting *MEIS1* expression in thalamus (FDR = 0.046, Table 4). However, this pathway was not enriched in the pons samples with contrasting *MEIS1* expression. This observation is in line with our previous publication where only thalamus samples showed reduced *MEIS1* expression in the RLS patients with *MEIS1* risk haplotype [108].

Mineral absorption pathway was the only shared pathway that was enriched in both RNA-Seq datasets obtained from human cell lines and RLS brains. Furthermore, a closer look at the results shows that MTs are a prominent family of proteins in this pathway across these different datasets. The transcriptional regulatory role of MEIS1 over MTs seems to be positive in cell lines but negative in the brains. This opposite direction may be explained by various mediatory pathways or genes that are present in *in vitro* cell lines by comparison to the more complex and heterogenous nature of the brain tissue. The overall procedure and the results of the cross-validated transcriptomic analyzed performed in Chapter 4 are summarized in Figure 18.

Table 4. Enriched pathways in the genes with repressed expression in thalamus samples with contrasting *MEIS1* expression. Adapted from Sarayloo et al. *PLoS ONE,* 2019.

Term	Overlap	p-value	FDR	Genes
Protein processing in endoplasmic reticulum	10/165	5.95E-06	8.45E-04	*DNAJA1, DNAJB1, HSP90AA1, HSPH1, HSPA1L, HSPA4L, HSPA6, TRAF2, HSPA1B, HSPA1A*
Antigen processing and presentation	6/77	1.15E-04	8.19E-03	*HSP90AA1, HSPA1L, HSPA4, HSPA6, HSPA1B, HSPA1A*
Legionellosis	5/55	2.10E-04	9.96E-03	*HSPA1L, HSPA6, HSPA1B, HSPD1, HSPA1A*

Estrogen signaling pathway	7/137	4.36E-04	1.55E-02	*HSP90AA1, HSPA1L, HSPA6, FKBP4, EGFR, HSPA1B, HSPA1A*
Mineral absorption	4/51	1.62E-03	4.60E-02	*MT2A, MT1M, MT1F, MT1G*

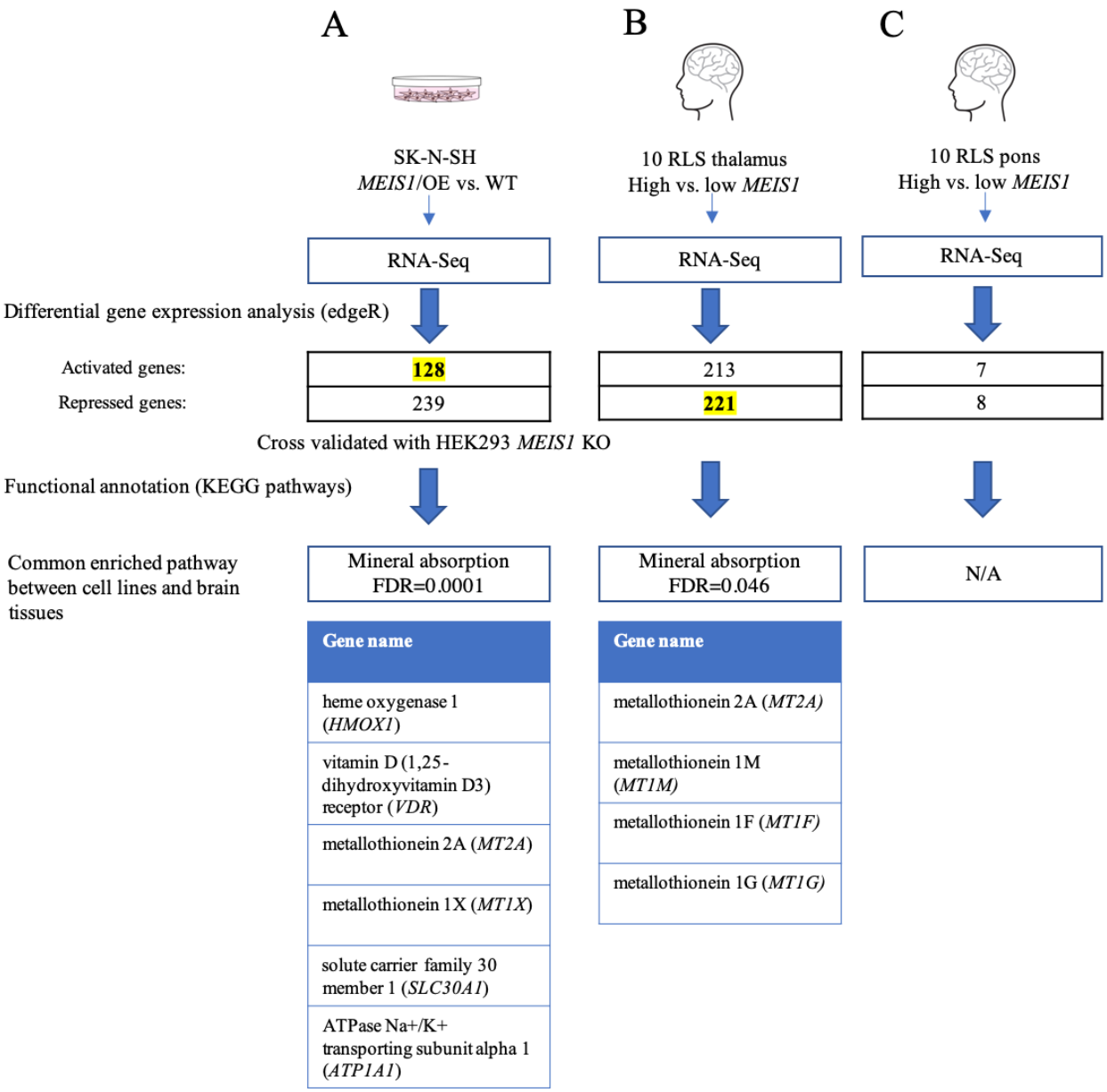


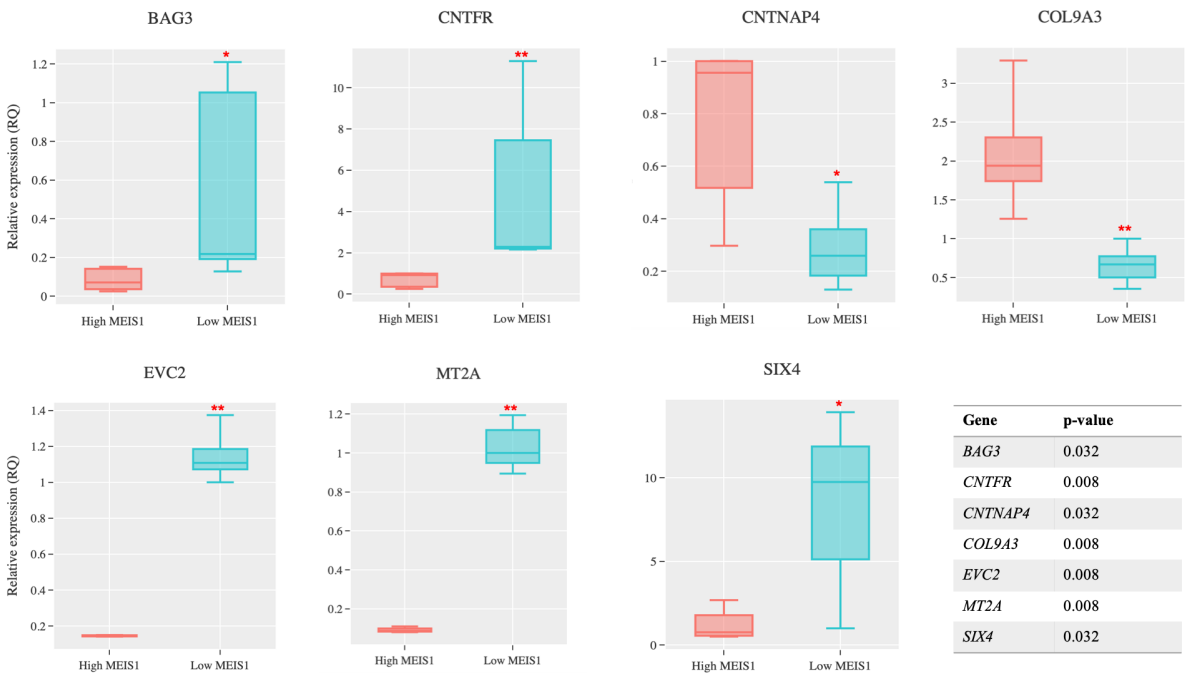
Figure 18. Representation of the procedures and the results of the transcriptomic analyses of human cell lines and brain tissue. Adapted from Sarayloo et al. *PLoS ONE,* 2019.

Seven genes are shared between DEGs from human cell lines and thalamus samples with dysregulated MEIS1 expression

Among the 15 genes that were identified to be differentially expressed in the pons samples, there was no genes overlapping with the human cell line DEGs. However, we identified seven genes that were common between the thalamus and cell lines RNA-Seq data (Table 5). We also performed q-RT-PCR and their differential expression was validated by this method as well (Figure 19). Among these seven genes, *CNTNAP4* encodes a protein involved in dopaminergic synaptic transmission [181] and *CNTFR* which encodes a receptor for ciliary neurotrophic factor that is involved in neuronal survival. The functions of these two genes are related to the known RLS pathways [182]. In this list, *MT2A* is present in the mineral absorption pathway and is a member of metallothionein family of proteins.

Table 5. The genes overlapping between the DEGs in thalamus and human cell lines with *MEIS1* dysregulation. Adapted from Sarayloo et al. *PLoS ONE,* 2019.

Gene	logFC	p-value	FDR
BAG3	2.91	4.42E-13	4.02E-09
CNTFR	1.67	1.09E-06	7.99E-04
CNTNAP4	-1.45	2.92E-05	8.65E-03
COL9A3	-2.025	5.83E-06	2.835E-03
EVC2	1.60	2.29E-04	3.735E-02
MT2A	1.31	3.79E-06	2.05E-03
SIX4	2.62	2.21E-08	4.01E-05



Gene	p-value
BAG3	0.032
CNTFR	0.008
CNTNAP4	0.032
COL9A3	0.008
EVC2	0.008
MT2A	0.008
SIX4	0.032

Figure 19. q-RT-PCR validation of the DEGs common between thalamus and cell lines and their corresponding p-values. The differential expression of the genes in high vs. low MEIS1 content samples was assessed by non-parametric Wilcoxon test. *p≤0.05; **p≤0.01. Adapted from Sarayloo et al. *PLoS ONE,* 2019.

To account for the possibility of an effect from the RIN of brain RNA samples on identification of the true DEGs, we also performed q-RT-PCR on all the six genes present in the mineral absorption pathway obtain from human cell lines that were not replicated in the brain RNA-Seq (Table 3, except *MT2A*). However, none of the human cell line specific mineral absorption pathway genes showed differential expression in thalamus (this confirms the results obtained from RNA-Seq, Figure 20).

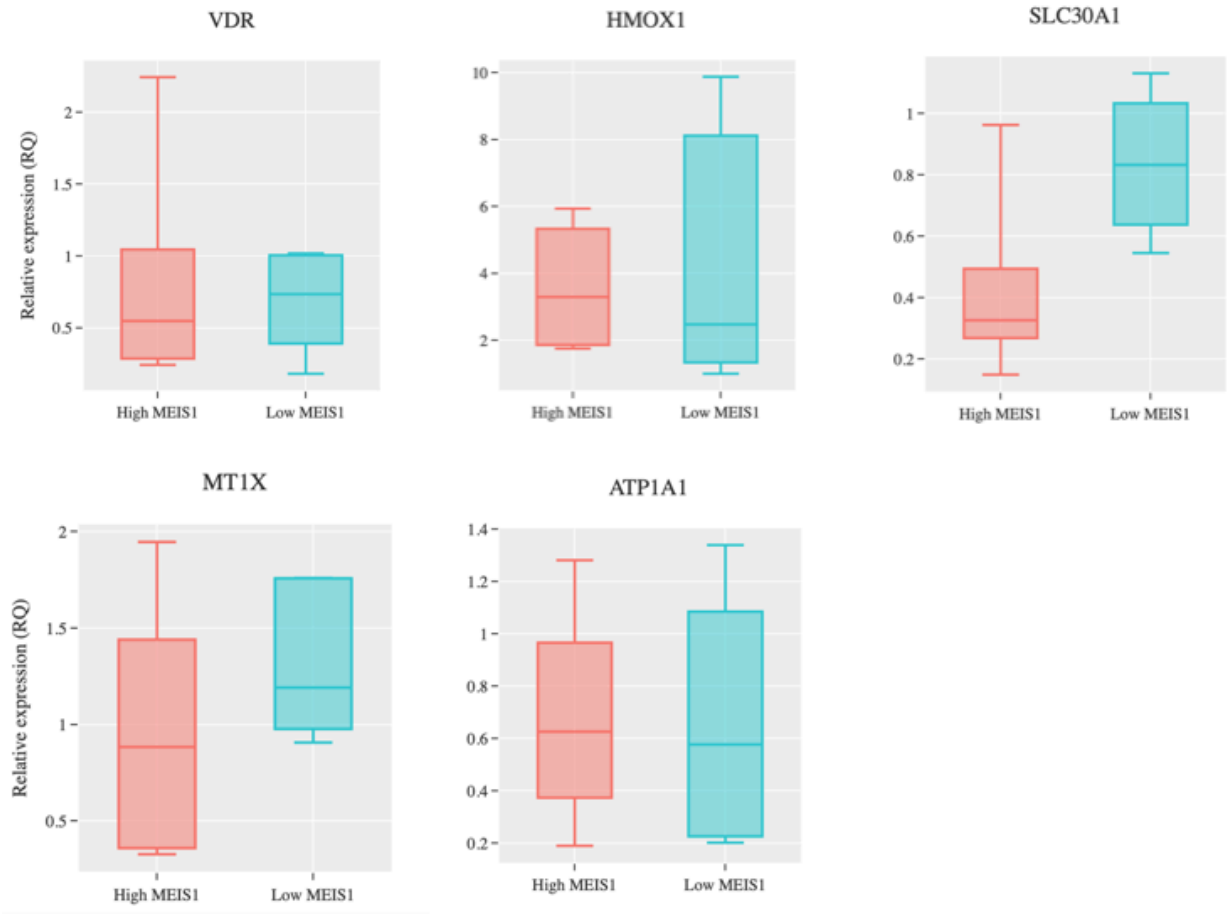


Figure 20. q-RT-PCR measurement of the genes only present in the cell line mineral absorption pathway in the thalamus samples with high vs. low MEIS1 content. No significant differential expression was observed (p-value > 0.05). Adapted from Sarayloo et al. *PLoS ONE,* 2019.

We further assessed the possibility of direct binding of MEIS1 protein to the promoter regions of these seven genes common between human cell lines and thalamus samples. Similar to the earlier section of this chapter, we used the publicly available Meis1 ChIP-Seq data in mice (GEO database, GSE82314) [169]. ChIP-Seq peaks were present in the orthologs of *BAG3, CNTNAP4* and *EVC2*; this suggests that MEIS1 has a direct regulatory role on these genes (Figure 21). These direct bindings need to be further validated in appropriate human cell lines.

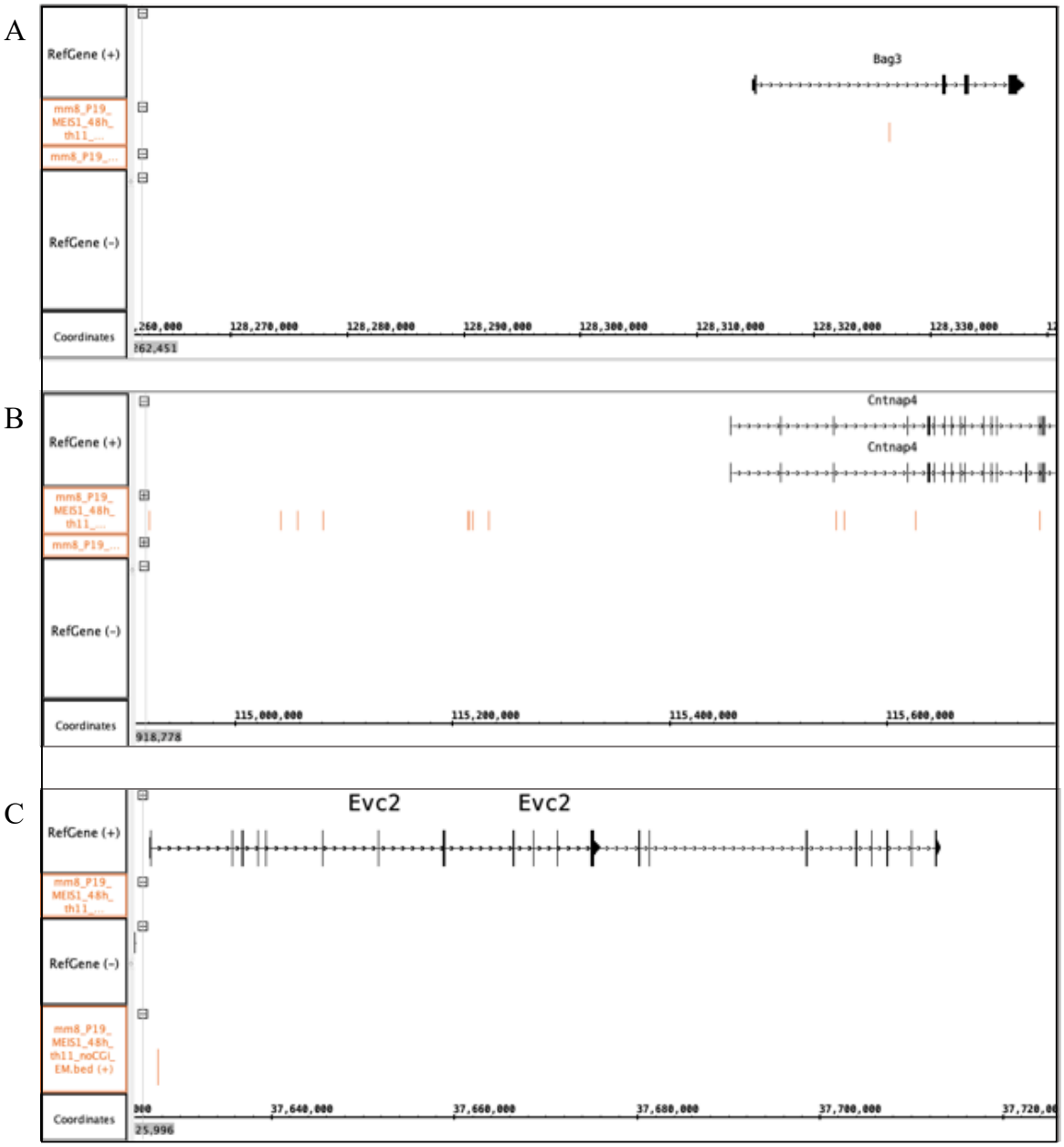


Figure 21. IGB view of the Meis1 ChIP-Seq data of genes common between human cell lines and thalamus DEGs. **A.** One peak in intron 1 of Bag3 (in orange). **B.** Six peaks upstream Cntnap4 (in orange). **C.** One peak in intron 1 of Evc2 (in orange). Adapted from Sarayloo et al. *PLoS ONE,* 2019.

Chapter 5 SKOR1 has a transcriptional regulatory role on genes involved in pathways related to restless legs syndrome

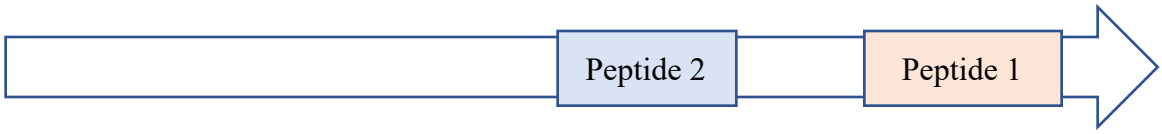
Preface

A number of independent studies have reported associations between common variants located within noncoding regions of *SKOR1* and RLS. While several genetic studies discussing *SKOR1* have emerged since the 2007 GWAS [96], the current literature still provides limited knowledge regarding SKOR1 function; a shortcoming which might partially be due to the lack of high-quality antibodies specific for the protein. Based on the human Genotype-Tissue Expression (GTEx) database [36] we know the cerebellum (more specifically the cerebellar hemisphere) is the region with the highest *SKOR1* expression. A limited number of expression and functional studies conducted in mice revealed *Skor*1 to be expressed a subset of post-mitotic neurons present in the posterior area of the midbrain-hindbrain border within the embryonic central nervous system. Moreover, in the developing spinal cord of mice, *Skor1* is selectively expressed in the dorsal horn interneurons; in this area a homeodomain transcription factor, Lbx1, is required for proper specification [37]. Skor1 interacts with Lbx1 and they cooperatively repress transcription, suggesting that Skor1 acts as a transcriptional corepressor for Lbx1 in regulating cell fate determination in the dorsal spinal cord (*SKOR1*was previously known as *LBXCOR1*) [37]. Despite the importance of *SKOR1* in RLS genetic, very little is known about the role of its product in the onset and progression of RLS. In Chapter 3, we showed that MEIS1 has a regulatory role over *SKOR1* and reduced expression of *MEIS1* in LCL and thalamus of RLS patients is accompanied with *SKOR1* reduced expression. Given the fact that *SKOR1*reduced expression can also play a role in RLS pathways and based on the transcriptional corepressor functionality of this protein in the nervous system, Chapter 5 investigates the transcriptional regulatory role of SKOR1 in two different cell lines by RNA sequencing. For this aim, we also generated a specific polyclonal anti-SKOR1, a much-needed tool for future SKOR1 studies.

Results

A new polyclonal anti-SKOR1

The generation of polyclonal anti-SKOR1 was carried out using two different peptide sequences specific for human SKOR1 to immunize duplicates of New Zealand white rabbits. Peptide1: MELRKKLEREFQSLKDN (#6033 and #6034) has 100% identity in human/mouse/rat, while peptide2: SNRFPDDEDAQEETE (#6035 and #6036) is only specific to human. The locations of the two peptides in SKOR1 protein, and the complete peptide sequence of SKOR1 are shown in Figure 22.



SKOR1 peptide sequence (965 aa)

MALLCGLGQVTLRIWVSLPSQSENGIGFLAARAFLRSGGMEALTTQLGPGREGSSSPNSKQELQ
PYSGSSALKPNQVGETSLYGVPIVSLVIDGQERLCLAQISNTLLKNYSYNEIHNRRVALGITCVQ
CTPVQLEILRRAGAMPISSRRCGMITKREAERLCKSFLGEHKPPKLPENFAFDVVHECAWGSRG
SFIPARYNSSRAKCIKCGYCSMYFSPNKFIFHSHRTPDAKYTQPDAANFNSWRRHLKLSDKSAT
DELSHAWEDVKAMFNGGTRKRTFSLQGGGGGGANGGSGGQGKGGAGGGGGGGPGCGAEM
APGPPPHKSLRCGEDEAAGPPGPPPPHPQRGLGLATGASGPAGPGGPGGGAGVRSYPVIPVPSK
GFGLLQKLPPPLFPHPYGFPTAFGLCPKKDDPVLGAGEPKGGPGTGSGGGGAGTGGGAGGPGA
SHLPPGAGAGPGGGAMFWGHQPSGAAKDAAAVAAAAAAATVYPTFPMFWPAAGSLPVPSYP
AAQSQAKAVAAAVAAAAAAAAAAAGSGAPEPLDGAEPAKESGLGAEERCPSALSRGPLDED
GTDEALPPPLAPLPPPPPPPARKGSYVSAFRPVVKDTESIAKLYGSAREAYGAGPARGPGPGAG
SGGYVSPDFLSEGSSSYNSASPDVDTADEPEVDVE**SNRFPDDEDAQEETE**PSAPSAGGGPDGE
QPTGPPSATSSGADGPANSPDGGSPRPRRRLGPPPAGRPAFGDLAAEDLVRRPERSPPSGGGGY
ELREPCGPLGGPAPAKVFAPERDEHVKSAAVALGPAASYVCTPEAHEPDKEDNHSPADDLETR
KSYPDQRSISQPSPANTDRGEDGLTLDVTGTHLVEKDIENLAREELQKLLLEQ**MELRKKLERE**
FQSLKDNFQDQMKRELAYREEMVQQLQIVRDTLCNELDQERKARYAIQQKLKEAHDALHHF
SCKMLTPRHCTGNCSFK PPLLP

Figure 22. Schematic of the locations of peptides 1 and 2 in the SKOR1 protein. Lower panel shows the complete peptide sequence of SKOR1 used as the reference for the epitope analysis (UniProt identifier: P84550-1).

Characterization of the newly produced polyclonal anti-SKOR1

13 weeks post-immunization of the rabbits, their bleeds were used in a western blot analysis, to test the affinity and specificity of the antibodies within the different antisera. This characterization was performed using HEK293 cells overexpressing *SKOR1* open reading frame; this ORF was sub-cloned in pcDNA3.1+ vector under CMV promoter. The final bleeds obtained from both replicates of the peptide sequences were tested and the western blot results are presented in Figure 23 and Figure 24. The antiserum derived from Peptide 1 (rabbit #6034) showed a strong overexpression of SKOR1 with a low background noise produced from non-specific bindings and was selected for the follow-up experiments. SKOR1 MW is ~100 kDa, however in this experiment the overexpressed protein migrated slightly higher, which can be due to post-translational modifications. Endogenous levels of SKOR1 protein could not be detected on western blots because the HEK293 cells express a very low level of the protein. The low endogenous expression level of *SKOR1* was also the reason for not using *SKOR1*-KO cells for characterizing the new antibody.

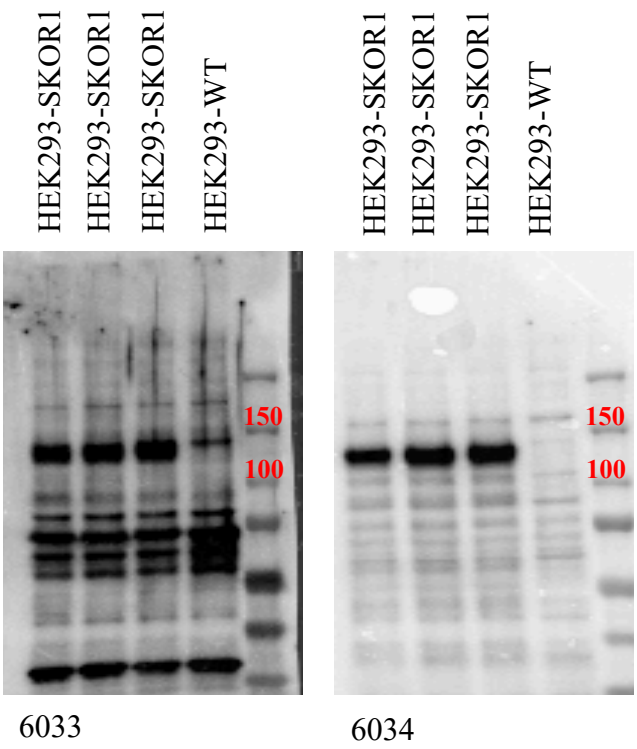


Figure 23. Western blot analyses of the polyclonal anti-SKOR1 generated in New Zealand rabbits. Rabbits #6033 and #6034 were immunized by peptide 1.

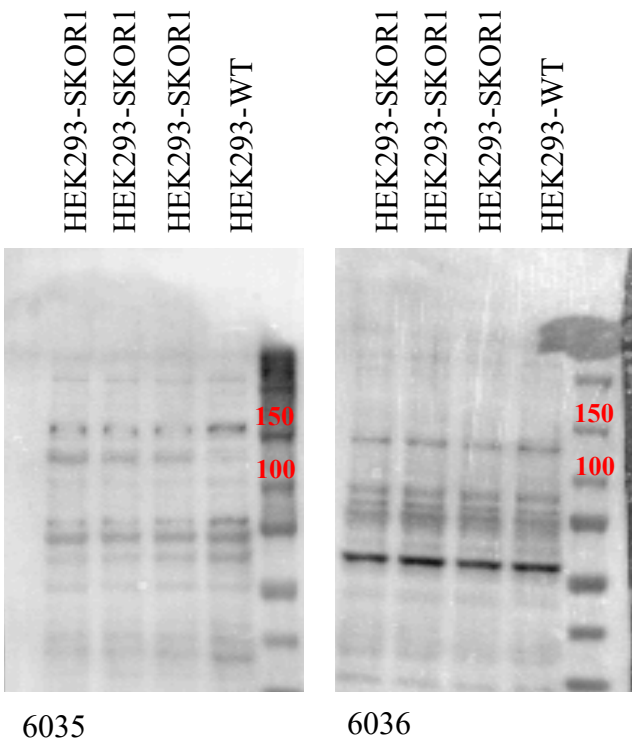


Figure 24. Western blot analyses of the polyclonal anti-SKOR1 generated in New Zealand rabbits. Rabbits #6035 and #6036 were immunized by peptide 2.

Generation of human cells with SKOR1 dysregulation

In order to identify genes that are regulated by SKOR1, two different human cell lines were used: HAP1 and HEK293. The HEK293 cells from the previous section (tested by #6034 anti-SKOR1) were used for the generation of cell lines overexpressing SKOR1 in a stable manner. Moreover, the ORF of *SKOR1* was also overexpressed in HAP1 cells using the same pcDNA3.1+ vector with CMV promoter (in triplicates) and they were characterized by western blot analysis (Figure 25).

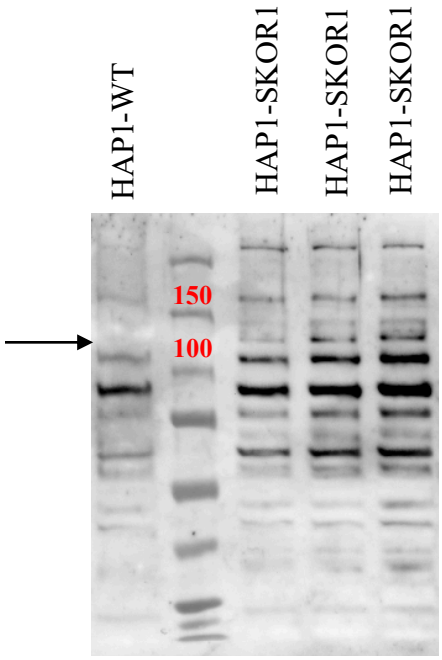


Figure 25. Western blot analysis of HAP1 cells overexpressing SKOR1 using #6034 antibody. The black arrow indicates the SKOR1 overexpression band in the transfected cells compared to their parental wild-type cells.

Both HEK293 and HAP1 stable cell lines showed an overexpression of SKOR1. Albeit the overexpression was stronger in the case of HEK293 derived cells, an observation likely due to these cells being more efficiently transfected than HAP1 cells. The decision to use two different human cell lines was made to minimize the likelihood of identifying expression changes that would be specific to a single cell type. Hence, changes that would be observed in the datasets of the two cell types would be more likely to be linked to *SKOR1* regulatory functions. We considered that genes which are up- or downregulated in these OE cells are possibly activated or repressed by SKOR1 transcriptional activity, respectively. In addition to the RNA-Seq datasets derived from the *SKOR1*-OE cells, datasets derived from *SKOR1* knockout (*SKOR1*-KO) HAP1 cells and their parental wild-type (WT) cells (Horizon) were also generated. The direction of SKOR1 regulation on genes that were up or downregulated in *SKOR1*-OE cells should be observed in the opposing directions in *SKOR1*-KO.

Differential gene expression analysis of human cell lines with SKOR1 dysregulation

RNA-Seq experiment was performed using total RNA samples extracted from the human cell lines in which *SKOR1* expression was dysregulated (OE and KO). Differential gene expression analysis was performed using edgeR algorithm [157] with the following contrast designs: HAP1-OE vs. HAP1-WT, HAP1-KO vs. HAP1-WT and HEK293-OE vs. HEK293-WT. The MDS (multidimensional scale) plots of the RNA-Seq analyses in the two cell lines, separately show clusters of each condition's replicates (Figure 26 and Figure 27). MD (mean-difference) plots also show the distribution of the DEGs, and both HAP1 and HEK293 cells overexpressing *SKOR1* show the data point corresponding to *SKOR1* as the most differentially expressed gene (Figure 26 and Figure 27).

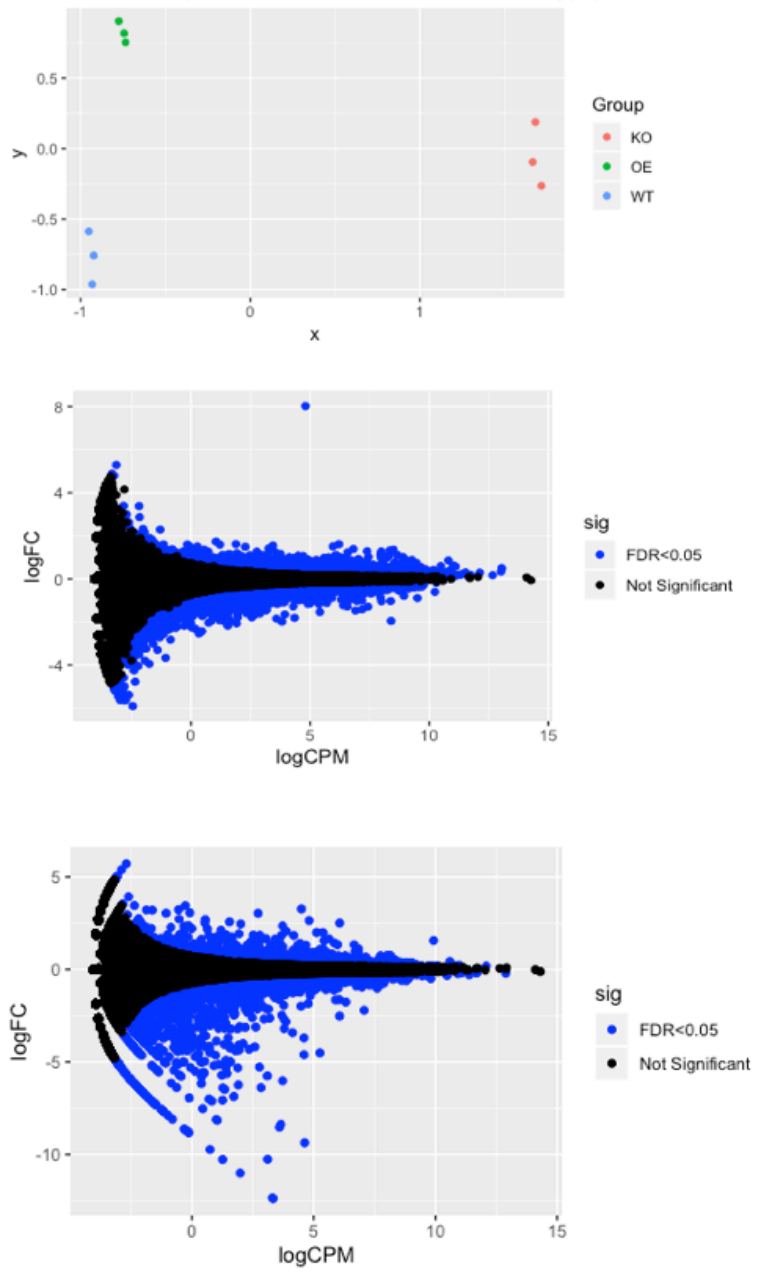


Figure 26. RNA-Seq data analysis of HAP1 cells with SKOR1 gene overexpressed or silenced. The top panel shows MDS plot of the RNA-Seq data analyzed in HAP1 with clusters of HAP1 SKOR1-KO (in red), HAP1 SKOR1-OE (in green) and HAP1 parental WT cells (in blue). The middle panel shows MD plot of the DEGs comparing HAP1 cells overexpressing SKOR1 with the parental wild-type cells; the single data point with a high LogFC indicates SKOR1. The

bottom panel shows the MD plot of the DEGs comparing SKOR1-KO HAP1 cells with their parental WT cells.

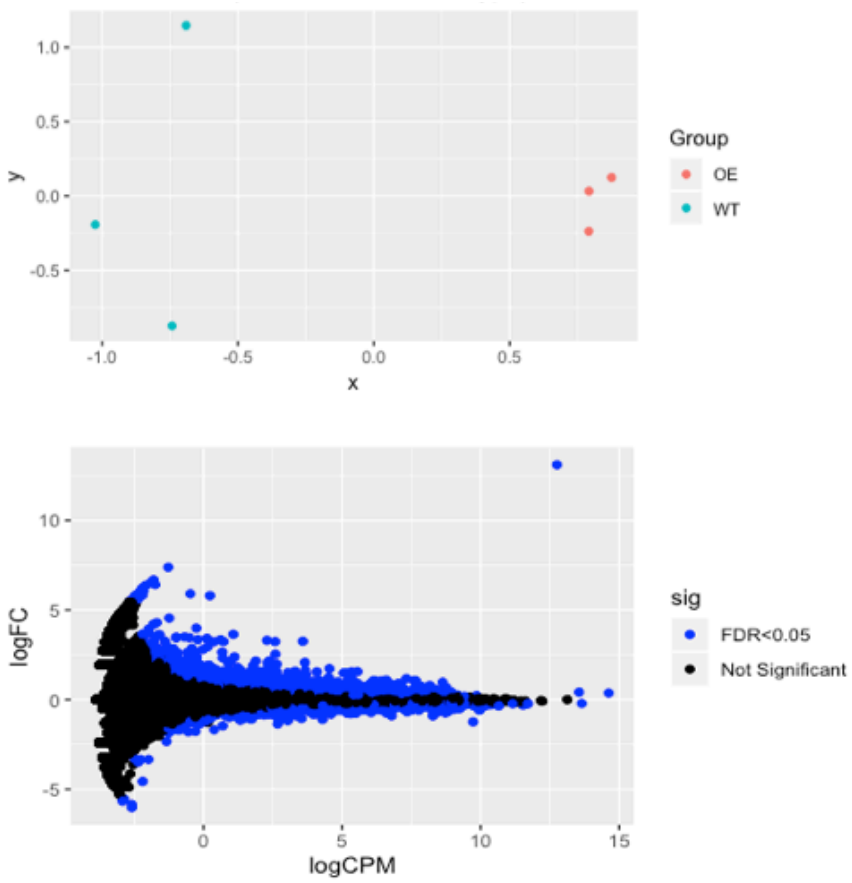


Figure 27. RNA-Seq analysis of HEK293 cells with SKOR1 gene overexpressed. The top panel shows MDS plot of the RNA-Seq data analyzed in HEK293 cells with clusters of HEK293 SKOR1-OE (in red) and HEK293 WT cells (in blue). The bottom panel shows MD plot of the DEGs comparing HEK293 cells overexpressing SKOR1 with the wild-type cells; the single data point with a high LogFC indicates SKOR1.

Cross validation of DEGs in HAP1and HEK293 cells with SKOR1 dysregulation

The DEGs that are upregulated in OE cells and downregulated in KO cells were deemed to be possibly activated by SKOR1 (19 genes), conversely the DEGs that are downregulated in OE cells and upregulated in the KO cells were deemed to be possibly repressed by SKOR1 (44 genes, Figure 28).

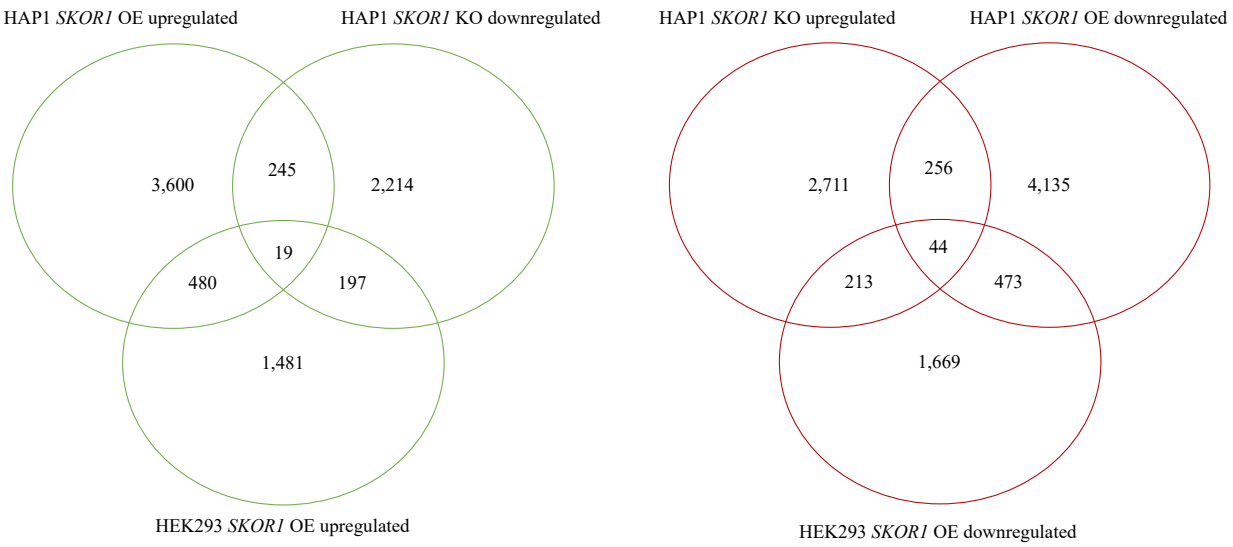


Figure 28. Venn diagram of DEGs common between HAP1 and HEK293 cells. The left panel shows the overlaps between DEGs upregulated in HAP1 SKOR1-OE cells, HEK293 SKOR1-OE cells and downregulated in HAP1 SKOR1-KO; 19 genes common to all the three datasets were considered as possibly regulated by SKOR1. The right panel shows the overlaps between DEGs downregulated in HAP1 SKOR1-OE cells, HEK293 SKOR1-OE cells and upregulated in HAP1 SKOR1-KO; 44 genes common to all the three datasets were considered as possibly repressed by SKOR1.

Pathway and gene set enrichment analysis shows pathways related to known RLS mechanisms

Gene Ontology (GO) annotation and Kyoto encyclopedia of genes and genomes (KEGG) pathway enrichment information was compiled for the 44 downregulated by SKOR1 and 19 SKOR1 upregulated genes, using Enrichr [161, 162]. The 44 DEGs which appear to be repressed by SKOR1 showed a significant enrichment in pathways involved in neurodevelopment processes (Table 6). These pathways include, axon guidance, spinal cord development, glial cell development and post-synapse assembly. This result is noticeably fitting with previous observations made by Mizuhara et al. who profiled the expression of SKOR1 in mice and observed it to include the developing spinal cord [143]. Skor1 is selectively co-expressed with Lbx1 transcription factor and they cooperatively repress transcription. It is hypothesized that Skor1 acts as a transcriptional corepressor for Lbx1 in regulating cell fate determination in the dorsal horn spinal cord [143].

Table 6. Significantly enriched pathways in the DEGs possibly repressed by SKOR1.

Pathway	**Description**	**p-value**	**FDR**	**Genes**
KEGG:04360	Axon guidance	2.33E-06	1.28E-4	*NTNG2, WNT5A, SLIT2, NGEF, NFATC4, NTN3*
GO:0021782	Glial cell development	1.20E-05	7.8E-3	*MXRA8, DLL1, SOX4*
GO:0016055	Wnt signaling pathway	9.06E-05	0.02	*DDX3X, WNT5A, CELSR2, SOX4*
GO:0060828	Regulation of canonical Wnt signaling pathway	9.25E-05	0.02	*PRKN, DDX3X, WNT5A, SOX4, RBMS3*
GO:0099068	Post-synapse assembly	2.93E-4	0.04	*NLGN2, WNT5A*
GO:0021510	Spinal cord development	2.93E-4	0.04	*DLL1, SOX4*

In addition to the neurodevelopmental pathway, other pathways were observed and among them ferroptosis pathway showed a significant enrichment in the 19 DEGs possibly activated by

SKOR1 (Table 7). Ferroptosis is an iron related form of programmed cell death, where the accumulation of reactive oxygen species (ROS) leads to oxidative stress and death. Ferroptosis is an iron dependent non-apoptotic cell death process [183]. This process is only dependent on intracellular iron and not the other metals. Inhibiting ferroptosis can protect the organism from neurodegenerative disorders [184]. Its role has been implicated in several neurological diseases including Parkinson's and Alzheimer's disease [185-187]. As mentioned earlier in this thesis, brain iron abnormality is the principal well-accepted dysregulation in RLS. Moreover, *MEIS1* as another RLS-associated gene plays roles in regulation of iron management proteins [124] and also positively regulates *SKOR1* expression [2]. The interactions between *MEIS1* and *SKOR1* and their individual roles in regulation of iron metabolism can be subject to future RLS studies to decipher other possible interactors and to further explain the RLS underlying pathways in the brain.

Table 7. Significantly enriched pathways in the DEGs activated by SKOR1

Pathway	**Description**	**p-value**	**FDR**	**Genes**
GO:0015175	Neutral amino acid transmembrane transporter activity	2.2E-4	0.01	*SLC1A4, SLC3A2*
GO:0015807	L-amino acid transport	2.4E-4	0.03	*SLC1A4, SLC3A2*
GO:1901342	Regulation of vasculature development	3.1E-4	0.03	*WARS, HMOX1*
GO:0043043	Peptide biosynthetic process	4.9E-4	0.03	*WARS, EEF1A2, SARS*
GO:0045765	Regulation of angiogenesis	5.1E-4	0.03	*WARS, SARS, HMOX1*
KEGG:04216	Ferroptosis	5.8E-4	0.02	*HMOX1, SLC3A2*
GO:0043039	tRNA aminoacylation	6.1E-4	0.03	*WARS, SARS*
GO:0004812	Aminoacyl-tRNA ligase activity	6.4E-4	0.01	*WARS, SARS*
GO:0006418	tRNA aminoacylation for protein translation	8.1E-4	0.03	*WARS, SARS*
GO:0006865	Amino acid transport	8.8E-4	0.03	*SLC1A4, SLC3A2*
GO:0003924	GTPase activity	9.9E-4	0.01	*RAB15, EEF1A2, RAB39A*
GO:0046942	Carboxylic acid transport	9.8E-4	0.03	*SLC1A4, SLC3A2*
GO:0006412	Translation	1.1E-3	0.03	*WARS, EEF1A2, SARS*

GO:0015171	Amino acid transmembrane transporter activity	1.2E-3	0.01	*SLC1A4, SLC3A2*
GO:0031400	Negative regulation of protein modification process	1.5E-3	0.04	*WARS, FAM129A*
KEGG:00970	Aminoacyl-tRNA biosynthesis	1.6E-3	0.03	*WARS, SARS*
GO:0032482	Rab protein signal transduction	1.8E-3	0.04	*RAB15, RAB39A*
GO:0071705	Nitrogen compound transport	2E-3	0.04	*SLC1A4, SLC3A2*
GO:0042326	Negative regulation of phosphorylation	2.1E-3	0.04	*WARS, FAM129A*
GO:0006812	Cation transport	2.3E-3	0.04	*SLC1A4, SLC3A2*
GO:0022603	Regulation of anatomical structure morphogenesis	2.5E-3	0.04	*WARS, HMOX1*
GO:0042803	Protein homodimerization activity	2.5E-3	0.02	*WARS, SARS, HMOX1, ABAT*
GO:0034613	Cellular protein localization	2.8E-3	0.05	*RAB15, ID1, RAB39A*
GO:0017111	Nucleoside-triphosphatase activity	6.1E-3	0.03	*RAB15, EEF1A2, RAB39A*
GO:0005432	Calcium:sodium antiporter activity	6.2E-3	0.03	*SLC3A2*
GO:0015193	L-proline transmembrane transporter activity	7.1E-3	0.03	*SLC1A4*
GO:0015180	L-alanine transmembrane transporter activity	7.1E-3	0.03	*SLC1A4*
GO:0022858	Alanine transmembrane transporter activity	7.1E-3	0.03	*SLC1A4*
GO:0015368	Calcium:cation antiporter activity	0.01	0.05	*SLC3A2*
GO:0003746	Translation elongation factor activity	0.01	0.05	*EEF1A2*
GO:0008483	Transaminase activity	0.01	0.05	*ABAT*

Genes common between SKOR1 and MEIS1 regulatory network

Following the identification of ferroptosis as an enriched pathway for the DEGs that are possible downstream target of SKOR1, we noted that *HMOX1* (heme oxygenase 1) is also present in the ferroptosis pathway [184]. This gene has been suggested to play roles in brain aging and neurodegeneration [188]. *HMOX1* was also reported to have a small association with RLS in a Spanish cohort [175]. Furthermore, *HMOX1* was previously observed to be regulated by MEIS1

(Chapter 4). Given that MEIS1 also showed a transcriptional regulatory role on *SKOR1* (Chapter 3) it would seem that this action of MEIS1 takes place, at least partially, through SKOR1. Observing the regulation of *HMOX1,* a gene highly related to iron metabolism, by the two transcription factors strongly associated with RLS is significant as it suggests that a transcriptional regulatory network is at play between these three genes. This network could be the pathogenic route at play in a subset of RLS cases. In a search for other genes that might also be common between SKOR1 and MEIS1 regulatory network, we also looked for the overlaps between the data obtained from Chapters 4 and 5. This resulted in the identification of seven genes (Figure 29). Among these genes, *CDC20, LANCL1* and *NTN3* show functions related to nervous system and can be implicated in the future studies. We believe that investigating the regulatory functions, and possible networks between the various DEGs identified for MEIS1 and SKOR1, represents a valid approach to shed light on the pathogenic event underlying the onset and progression of RLS in a subset of patients. The dysregulations and networks identified for these two RLS genes might also be contributing to RLS cases not associated with these two genes and this could warrant further investigations in other RLS cases with or without the currently established genetic risk factors.

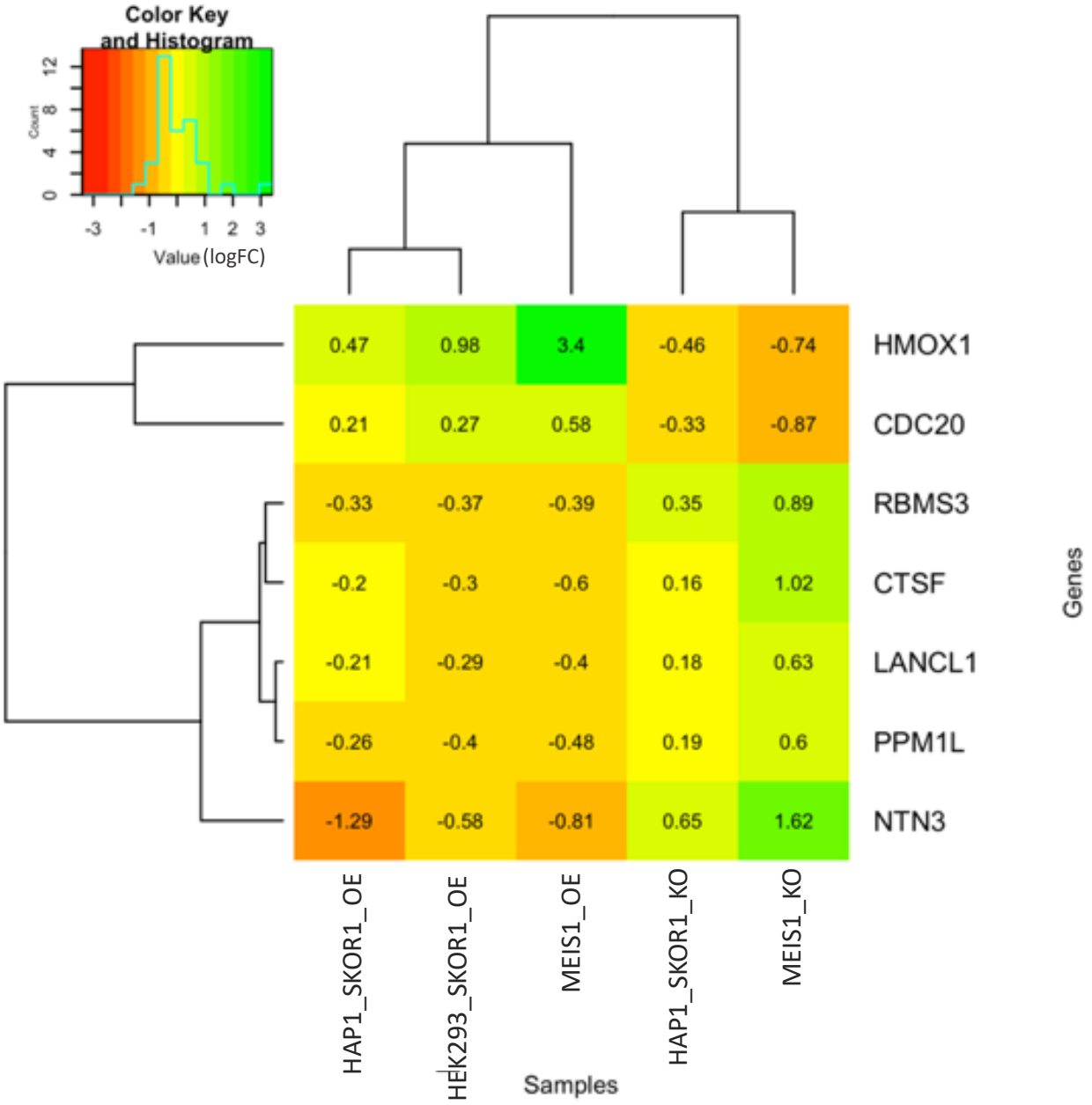


Figure 29. Integrating the data obtained from cell line transcriptomic analyses from Chapters 4 and 5 (MEIS1 and SKOR1 regulatory network). Seven genes are present in the transcriptomic data obtained from both SKOR1 and MEIS1. HMOX1 and CDC20 are positively regulated by both transcription factors. RBMS3, CTSF, LANCL1, PPM1L and NTN3 are negatively regulated. The values represent the logFC of the gene expression.

Chapter 6 Discussion

The focus of this thesis is the study of restless legs syndrome (RLS), a complex sleep-related sensory-motor disorder with a strong genetic component. RLS is known to be most prevalent in European descent populations and to occur in genetically predisposed individuals. Specifically, the symptoms of RLS occur during the rest and sleep phases, and depending on their severities they will decrease the quality of life and affect mental health of those affected [189].

Despite the high prevalence of RLS (ranked among the most common movement disorders), its biology remained somewhat poorly defined. It was only after 1945, when the Swedish neurologist Karl Ekbom provided a detailed description of this new disease, that RLS became the focus of more attention by the research and medical community [190]. Following its early descriptions, RLS investigations were primarily centered on its clinical characterization and the evaluation of therapy. However, since the early 2000s, several studies were launched to decipher the contribution of genetic risk factors. Between 2001 and 2012, linkage studies were conducted with the assumption that RLS was a monogenic mendelian disorder, an approach which identified eight RLS candidate loci (mostly with autosomal dominant mode of inheritance) [92]. In 2007, the first genome-wide association study on a common sleep-related disorder was performed on RLS [96]. This approach was made to assess the contribution of common risk factors [96]. Since this first GWAS, a number of studies using this approach have been reported and altogether have established a total of 19 genetic loci associated with RLS, each locus with a low to moderate effect size [100]. Such genetic findings have shed a much-needed light on the nature of the genetic architecture underlying RLS. As a complex trait a combination of several genetic factors, each probably interacting with the other, in addition to environmental factors are deemed to contribute to the development and/or progression of the symptoms.

Two of the 19 RLS predisposing genetic loci, *MEIS1* and *SKOR1*, are the focus of the work presented in this thesis. The association of these two genes with RLS was replicated in the follow-up studies and was reported to be valid for individuals from different populations. The common variants in *MEIS1* noncoding regions (intron 8) showed the most significant association signals with RLS. Our group has previously performed investigations on lymphoblastoid cell lines (LCLs) and postmortem brain tissues (thalamus and pons) which were obtained from individuals who had received an RLS diagnostic from a physician specialized in sleep disorders [108]. That study revealed that LCLs and thalamus obtained from the subset of patients who carried the intronic risk variants located within *MEIS1* (rs12469063/rs2300478: GG/GG), expressed significantly reduced levels of *MEIS1* mRNA and protein [108]. Such an observation suggested a regulatory role for the common variants located within *MEIS1*. It also suggested that *MEIS1* insufficiency could be a key underlying event at play in the onset and/or progression of RLS in the subset of the patients carrying the associated variants.

Follow-up investigations aiming to identify the role of *MEIS1* in RLS were conducted in model organisms, with a focus on possible links with iron metabolism because of the evident role of iron dysregulation in RLS. The literature indicates a higher prevalence of RLS in populations with iron deficiency [49]. MRI examinations of RLS patients' brains revealed a reduced levels of iron in this organ, by comparison to observations in control individuals without RLS [191]. Moreover, iron treatments has been reported to be an effective therapy for a subset of the RLS patients [192]. A study of the links between *MEIS1* and iron metabolism made by our group in a *C. elegans* model also revealed the downregulation of *Unc-62* (*MEIS1* orthologue in worms) to cause changes in the expression of iron management proteins (e.g. ferritin and *DMT1*). This study supported the existence of a regulatory role for MEIS1 over iron metabolism proteins.

Other investigations made using a mouse model showed that *MEIS1* haploinsufficiency can result in hyperactivity and this effect was more specific to the beginning of the rest phase of the animal, a phenotype resembling the RLS symptoms [118]. Moreover, mice and zebrafish models showed that the noncoding regions of *MEIS1* harboring the RLS-associated risk variants have cis-regulatory roles during neurodevelopment [114]. These follow-up studies of the RLS GWAS were significant at various levels:

- They opened the possibility of a regulatory role for the other RLS-associated common variants on their own gene expression; an event which could be similar to the regulatory role observed for *MEIS1* common variants on its expression level.
- They put an emphasis on the biological function of MEIS1 protein in the development of RLS symptoms, in addition to the highly significant genetic associations of its noncoding variants.
- They suggested some connections between MEIS1 functions and the iron pathways relevant to RLS.
- They raised the possibility of a regulatory role for MEIS1 as a transcription factor on other genes that might be more directly involved in RLS pathways.

The experimental designs and findings of this thesis are divided in three approaches. Chapter 3 investigates the RLS underlying mechanisms in two aspects:

1. It examines the effects of the common RLS-associated variants on the expression levels of their corresponding genes as regulatory single nucleotide polymorphisms (rSNP). A

majority of post-GWAS follow-up research has focused on the regulatory role of common SNPs found by GWAS [193]. These common association signals have low to moderate effect size on the development of the symptoms; some of them are located within highly conserved noncoding regions (HCNRs) and are composed of cis-regulatory elements (CREs). Thus, raising the possibility for these noncoding variants to affect the regulatory function of the CREs harboring them. A previous study from our group had investigated the noncoding variants of *MEIS1 and* confirmed such a regulatory effect (Xiong et al. [108]). We also conducted similar examinations to assess the effects of common variants from the noncoding regions of *SKOR1, BTBD9* and *MAP2K5* levels (at that time these were the only strong associations) on the mRNA expression of their respective genes but no significant effect was revealed. We also measured the effects of these common variants on the expression levels of the other RLS-associated genes [96]. Among all combinations of the variants and gene expressions, a significant positive correlation was observed between *MEIS1* risk haplotype and *SKOR1* mRNA expression levels. This reduced expression of *SKOR1* followed a pattern similar to the one observed for MEIS1 in individuals with the *MEIS1* risk haplotype; i.e. the samples that carried the *MEIS1* risk haplotype had lower expression of both *MEIS1* and *SKOR1*. This regulatory effect might be directly due to the action of the MEIS1 protein because it is a transcription factor and was also observed to regulate additional proteins (e.g. *FTN* and *DMT1*) that were deemed to be relevant to RLS [124].

2. We investigated the regulatory effect of MEIS1 transcription factor on *SKOR1* using human cell lines [2]. When the expression of *MEIS1* was downregulated using siRNA,

we observed a 70% reduction of *SKOR1* mRNA level. An *in silico* analysis revealed the presence of three consensus MEIS1 specific DNA binding sites in the promoter region and intron 2 of *SKOR1*. The possibility of a direct protein-DNA binding was assessed using an electrophoretic mobility shift assay (EMSA) designed to separately look at these three predicted MEIS1 binding sites. EMSA results showed that MEIS1 has a direct binding to the two predicted sites located in the promoter region of *SKOR1*. EMSA revealed no binding to the third predicted site, which was located in the second intron of *SKOR1*. Following the observation of these EMSA results, a luciferase reporter assay was designed to examine the enhancer activity of the *SKOR1* promoter regions with positive MEIS1-DNA binding results. The regulatory role of MEIS1 over SKOR1 was confirmed by this reporter assay in the two promoter regions with positive EMSA results. Furthermore, the luciferase assay enabled us to observe that one of the MEIS1 DNA binding regions harbored an RLS-associated variant in its close proximity and we reported that the binding of MEIS1 to this region only happens when the risk allele is present.

Based on the findings from Chapter 3, we suggest existence of a link between two RLS-associated genes where the reduced expression of *MEIS1* is associated with the reduced expression of *SKOR1*. A link we confirmed in material (LCLs and thalamus) obtained from RLS cases (this link was however not observed in the pons brain region). Furthermore, we hypothesized this direct binding and regulatory effect of MEIS1 to be part of the pathogenic route underlying RLS. Moreover, the direct binding of MEIS1 to one of its binding sites in the *SKOR1* promoter region is dependent on the presence of an RLS risk variant near this binding site. Interpretation of the results presented in Chapter 3 suggests that in addition to a plausible transcriptional regulatory role

for MEIS1 in RLS pathogenesis, in the RLS-associated intergenic region of *MAP2K5/SKOR1, SKOR1* should be prioritized for further functional follow-up studies.

In Chapter 4 we studied the regulatory role of MEIS1 as a transcription factor in RLS in a transcriptome-wide context. The rational for this chapter was based on the fact that *MEIS1* has a significant association signal with RLS (to our knowledge the most significant association thus far reported for any common condition). However, its product had no direct involvement in the RLS pathways, and it deemed critical. The previous functional studies of its links with RLS suggested some regulatory roles on other genes involved in RLS relevant pathways (e.g. iron management and neurodevelopment). We hypothesized that identifying genes that are regulated by MEIS1, and are linked to RLS relevant pathways, would be a key advancement toward a better characterization of molecular mechanisms underlying this disease. We generated human cell lines in which *MEIS1* expression was dysregulated. *MEIS1* was overexpressed in one set, and it was knocked out in another set of human cell lines. The transcriptome profiles of these cells were subsequently generated using an RNA-Seq approach. Differential gene expression analysis made across these sets of human cell lines identified genes and pathways which appear to be regulated by MEIS1, either in a direct or indirect manner. Some of the identified DEGs are relevant to pathways previously reported to be involved in RLS. For instance, *HMOX1* is a heme degrading protein that produces free iron in addition to carbon monoxide and biliverdin. *VDR* (vitamin D receptor) is another RLS relevant gene because past studies suggested RLS to be more prevalent in vitamin D deficient populations [177-179]. Associations between RLS and variants within *HMOX1* and *VDR* were also previously reported in a small Spanish cohort [175, 176]. Pathway analysis of the RNA-Seq results in Chapter 4 also showed that mineral absorption is the only significantly enriched

pathway in the DEGs activated in the *MEIS1* manipulated cell lines. In this pathway, in addition to *HMOX1* and *VDR*, members of metallothionein (MT) family of proteins are also present.

In Chapter 4, we also used postmortem brain tissues from RLS patients to build on our earlier report where a reduced *MEIS1* expression level was observed in the LCL and thalamus of the subset of RLS patients carrying the *MEIS1* risk haplotype. RNA-Seq data was generated from 10 thalamus and 10 pons samples showing highly contrasting levels of *MEIS1* expression (with risk vs. non-risk RLS *MEIS1* haplotype). The brain tissues obtained from RLS patients enabled us to look into the effect of *MEIS1* dysregulations in actual RLS brains and to validate the results from our cell models. Pathway analysis of the RNA-Seq results derived from the RLS brains also revealed a significant enrichment of mineral absorption pathway, with MTs as a consistent family of proteins also present in thalamus RNA-Seq dataset. MTs are small proteins that bind to intracellular metals [180]. Among the five types of MTs, MT1 and MT2 expression is localized in the spinal cord and brain. In these regions, they regulate the cellular homeostasis of essential metals and protect cells from free radicals generated by the oxidative stress. MT3 have been mainly found in the neurons [180]. In Parkinson's disease, low levels of antioxidants and high levels of free iron make grey matter vulnerable to reactive oxygen species (ROS) attack. It has been argued that MTs (more specifically MT2A) released from astrocytes may play a role in protecting dopaminergic neurons from oxidative stress damage. To our knowledge, the possible role of MTs in RLS pathogenesis is reported for the first time in the work in Chapter 4 (published at *PLoS ONE* [3]).

The findings from Chapter 4 demonstrate an enrichment of the mineral absorption pathway in RLS and links it to the most significant genetic risk factor of RLS (*MEIS1*). This pathway is highly relevant to the currently known RLS mechanisms, because of the evident role of iron

metabolism in the disease. The detailed links between RLS and mineral absorption pathway and the intricate roles that MTs probably play in the pathogenesis of RLS would remain to be elucidated and it should be more extensively investigated in the future RLS research.

Chapter 5 of the thesis moved its focus to *SKOR1*. Common variants within *SKOR1* noncoding regions significantly contribute to RLS genetic [100]. The research on the identification of the functions of SKOR1 is very limited and based on the current knowledge, *SKOR1* has its highest expression in the central nervous system (CNS). This protein acts as a corepressor for a transcription factor called Lbx1 in mice (*SKOR1* was previously called *LBXCOR1*). These two genes cooperatively act in regulation of cell fate in the dorsal horn interneurons of the spinal cord. The necessity of Lbx1/Skor1 for generation of GABAergic phenotypes in dorsal horn interneurons of spinal cord potentially implicate a role for SKOR1 in the modulation of pain and sensory input of RLS [115, 194, 195]. Due to the lack of a good-quality antibody specific to SKOR1, examination of its role in RLS through functional assays were not immediately possible. Consequently, *SKOR1* studies were mostly limited to RNA expression. The most informative data about the links between *SKOR1* and RLS are the published data from Chapter 3 of this thesis [2]. The links between MEIS1 and *SKOR1* and the reduced expression of *SKOR1* in one RLS brain region point to the possibility of an effect from *SKOR1* dysregulation in RLS development. Based on the corepressor transcriptional activity of SKOR1 in the nervous system, we sought to assess its regulatory action in a transcriptome-wide context in RLS, an approach similar to the experiments performed on MEIS1 in Chapter 4.

In Chapter 5, we first designed and produced specific antibodies for SKOR1 so that this would enable new avenues for SKOR1 investigations. Then we generated human cell lines in

which *SKOR1* is either highly overexpressed or knocked out. RNA-Seq datasets were obtained from these lines and followed up with analyses focused on differential gene expression to identify genes regulated by SKOR1. These DEGs would likely be indirect targets of SKOR1 transcriptional activity, since this protein does not present binding features that would enable it to directly interact with DNA. Then we sought to elucidate the pathways enriched in the genes regulated by SKOR1. Interestingly, the pathway analyses revealed enrichments in terms that are relevant to RLS biology. Among the enriched pathways on which SKOR1 appears to have a positive regulatory action, ferroptosis was relevant to RLS. Ferroptosis is an iron related form of programmed cell death, where the accumulation of reactive oxygen species (ROS) leads to oxidative stress and death [184]. A role for ferroptosis has been implicated in neurological diseases including Parkinson's and Alzheimer's disease [185-187]. As introduced in the above sections, brain iron dysregulation is the most widely accepted abnormality in RLS and the identification of this iron related pathway in the SKOR1 regulated genes is meaningful. In this pathway, *HMOX1* (heme oxygenase 1) is also present. This gene has been suggested to play roles in brain aging and neurodegeneration [188] and was also present in the MEIS1 regulatory network (Chapter 4). It is noteworthy to reiterate that we also observed a positive regulatory role of MEIS1 over *SKOR1* (Chapter 3). These results altogether suggest that a regulatory network might exist across these three genes, a network which we believe should warrant investigation in a broader range of RLS cases.

Additionally, the functional annotation of genes onto which SKOR1 appears to have a repressor action suggests an enrichment of pathways and terms that are highly related to neurodevelopment (e.g. axon guidance, glial cell development, spinal cord development and post-synaptic assembly). Similar enrichment analyses that were performed in the latest meta-analysis of RLS GWAS (15,126 cases and 95,725 controls of European ancestry) by Schormair et al. in

2017 [100] also puts an emphasis on neurodevelopmental genes, specifically genes with roles in axon guidance, synapse formation and neuronal specification [100]. The observation of a similar enrichment across the independent RLS GWAS meta-analysis and our functional annotation of SKOR1 regulated genes certainly strengthens these pathways as relevant to the onset and/or progression of RLS. The meta-analysis also warrants the investigation of these pathways in a broader range of RLS cases, and not just cases associated with *MEIS1* or *SKOR1*.

Overall, RLS is a highly prevalent condition for which very limited biological explanations are fully ascertained, and for which much still remains to be discovered. This is due to the complex nature of the disorder and the contribution of several environmental factors, as well as interactions between several predisposing genes, each with a minor contribution. A major issue of the post-GWAS era is that very limited aspects of the roles of association signals of complex traits have been investigated and RLS is not an exception. The findings presented in this thesis were shared with the research community through peer review journals, hence they represent an important contribution to our knowledge of RLS underlying mechanisms. Our in-depth examination about the roles of two significant RLS genetic contributors provided novel insight in regard to their relevance with the disease.

The links between two genes (*MEIS1* and *SKOR1*) strongly associated with RLS, their regulatory actions toward novel genes and pathways previously not known to be related to this condition, open new avenues for RLS investigations. It is also noteworthy to remark that common genes (e.g. *HMOX1*) were observed to be downstream the regulatory activities of the two RLS-associated genes.

Chapter 7 Conclusions and Future Directions

Restless legs syndrome (RLS) is a complex neurological condition. A combination of genetic and environmental susceptibility factors contributes to its development. So far, RLS research has been mainly focused on the identification of genetic risk factors, but the actual roles of the genes associated with the disorder have not been widely understood, a situation similar to what is observed in other complex disorders. The work presented in this thesis implemented a combination of *in vitro* protein-DNA binding and reporter assays as well as high-throughput transcriptomic and pathway analyses in human cell lines and postmortem RLS brain tissues to investigate the intricate roles of two RLS candidate genes, *MEIS1* and *SKOR1*. The novel outcomes of this thesis include identification of a link between *MEIS1* and *SKOR1*, as well as an enrichment in pathways involved in iron metabolism (mineral absorption and ferroptosis) and neurodevelopment. Furthermore, *HMOX1* which is involved in iron metabolism showed significant enrichment in the transcriptomic data obtained from both *MEIS1* and *SKOR1* dysregulated cells. This implicates a possible network between these three genes that might be playing roles in RLS mechanisms.

These findings shared with the scientific community are some of very few functional and expression studies performed on RLS, to date. In the future, the possible actions of other RLS susceptibility genetic factors in this disorder could be studied using approaches similar to those that were performed here (e.g. *in vitro* cell models, RNA-Seq from postmortem brain tissues, etc.). Mending together studies conducted at different levels (e.g. the integration of genomic and transcriptomic data) could drive the development of a much-needed therapy and give the affected individuals the rest they deserve.

Chapter 8 Reference

1. Sarayloo F, Dion PA, Rouleau GA. MEIS1 and Restless Legs Syndrome: A Comprehensive Review. Front Neurol. 2019;10:935. Epub 2019/09/26. doi: 10.3389/fneur.2019.00935. PubMed PMID: 31551905; PubMed Central PMCID: PMCPMC6736557.

2. Catoire H, Sarayloo F, Mourabit Amari K, Apuzzo S, Grant A, Rochefort D, et al. A direct interaction between two Restless Legs Syndrome predisposing genes: MEIS1 and SKOR1. Scientific reports. 2018;8(1):12173. doi: 10.1038/s41598-018-30665-6. PubMed PMID: 30111810.

3. Sarayloo F, Dionne-Laporte A, Catoire H, Rochefort D, Houle G, Ross JP, et al. Mineral absorption is an enriched pathway in a brain region of restless legs syndrome patients with reduced MEIS1 expression. PloS one. 2019;14(11):e0225186. Epub 2019/11/15. doi: 10.1371/journal.pone.0225186. PubMed PMID: 31725784.

4. Koo BB. Restless Leg Syndrome Across the Globe: Epidemiology of the Restless Legs Syndrome/Willis-Ekbom Disease. Sleep Med Clin. 2015;10(3):189-205, xi. Epub 2015/09/04. doi: 10.1016/j.jsmc.2015.05.004. PubMed PMID: 26329429.

5. Allen RP, Picchietti DL, Garcia-Borreguero D, Ondo WG, Walters AS, Winkelman JW, et al. Restless legs syndrome/Willis-Ekbom disease diagnostic criteria: updated International Restless Legs Syndrome Study Group (IRLSSG) consensus criteria--history, rationale, description, and significance. Sleep medicine. 2014;15(8):860-73. doi: 10.1016/j.sleep.2014.03.025. PubMed PMID: 25023924.

6. Burtscher C, Baxmann A, Kassubek J, Hornyak M, Matuja W, Schmutzhard E, et al. Prevalence of restless legs syndrome in an urban population of eastern Africa (Tanzania). J Neurol

Sci. 2014;346(1-2):121-7. Epub 2014/09/03. doi: 10.1016/j.jns.2014.08.007. PubMed PMID: 25179877.

7. Winkler AS, Trendafilova A, Meindl M, Kaaya J, Schmutzhard E, Kassubek J. Restless legs syndrome in a population of northern Tanzania: a community-based study. Movement disorders : official journal of the Movement Disorder Society. 2010;25(5):596-601. Epub 2010/03/04. doi: 10.1002/mds.22806. PubMed PMID: 20198689.

8. Cho SJ, Hong JP, Hahm BJ, Jeon HJ, Chang SM, Cho MJ, et al. Restless legs syndrome in a community sample of Korean adults: prevalence, impact on quality of life, and association with DSM-IV psychiatric disorders. Sleep. 2009;32(8):1069-76. Epub 2009/09/04. PubMed PMID: 19725258; PubMed Central PMCID: PMCPMC2717197.

9. Cho YW, Shin WC, Yun CH, Hong SB, Kim JH, Allen RP, et al. Epidemiology of restless legs syndrome in Korean adults. Sleep. 2008;31(2):219-23. Epub 2008/02/16. doi: 10.1093/sleep/31.2.219. PubMed PMID: 18274269; PubMed Central PMCID: PMCPMC2225579.

10. Kim WH, Kim BS, Kim SK, Chang SM, Lee DW, Cho MJ, et al. Restless legs syndrome in older people: a community-based study on its prevalence and association with major depressive disorder in older Korean adults. Int J Geriatr Psychiatry. 2012;27(6):565-72. Epub 2011/08/10. doi: 10.1002/gps.2754. PubMed PMID: 21826739.

11. Kim J, Choi C, Shin K, Yi H, Park M, Cho N, et al. Prevalence of restless legs syndrome and associated factors in the Korean adult population: the Korean Health and Genome Study. Psychiatry Clin Neurosci. 2005;59(3):350-3. Epub 2005/05/18. doi: 10.1111/j.1440-1819.2005.01381.x. PubMed PMID: 15896230.

12. Park YM, Lee HJ, Kang SG, Choi HS, Choi JE, Cho JH, et al. Prevalence of idiopathic and secondary restless legs syndrome in Korean Women. Gen Hosp Psychiatry. 2010;32(2):164-8. Epub 2010/03/23. doi: 10.1016/j.genhosppsych.2009.11.016. PubMed PMID: 20302990.

13. Ulfberg J, Bjorvatn B, Leissner L, Gyring J, Karlsborg M, Regeur L, et al. Comorbidity in restless legs syndrome among a sample of Swedish adults. Sleep medicine. 2007;8(7-8):768-72. Epub 2007/09/11. doi: 10.1016/j.sleep.2006.11.015. PubMed PMID: 17825613.

14. Erer S, Karli N, Zarifoglu M, Ozcakir A, Yildiz D. The prevalence and clinical features of restless legs syndrome: a door to door population study in Orhangazi, Bursa in Turkey. Neurol India. 2009;57(6):729-33. Epub 2010/02/09. doi: 10.4103/0028-3886.59467. PubMed PMID: 20139500.

15. Hadjigeorgiou GM, Stefanidis I, Dardiotis E, Aggellakis K, Sakkas GK, Xiromerisiou G, et al. Low RLS prevalence and awareness in central Greece: an epidemiological survey. Eur J Neurol. 2007;14(11):1275-80. Epub 2007/10/25. doi: 10.1111/j.1468-1331.2007.01966.x. PubMed PMID: 17956448.

16. Vogl FD, Pichler I, Adel S, Pinggera GK, Bracco S, De Grandi A, et al. Restless legs syndrome: epidemiological and clinicogenetic study in a South Tyrolean population isolate. Movement disorders : official journal of the Movement Disorder Society. 2006;21(8):1189-95. Epub 2006/05/11. doi: 10.1002/mds.20922. PubMed PMID: 16685686.

17. Celle S, Roche F, Kerleroux J, Thomas-Anterion C, Laurent B, Rouch I, et al. Prevalence and clinical correlates of restless legs syndrome in an elderly French population: the synapse study. J Gerontol A Biol Sci Med Sci. 2010;65(2):167-73. Epub 2009/11/17. doi: 10.1093/gerona/glp161. PubMed PMID: 19914971.

18. Tison F, Crochard A, Leger D, Bouee S, Lainey E, El Hasnaoui A. Epidemiology of restless legs syndrome in French adults: a nationwide survey: the INSTANT Study. Neurology.

2005;65(2):239-46. Epub 2005/07/27. doi: 10.1212/01.wnl.0000168910.48309.4a. PubMed PMID: 16043793.

19. Kerkhof GA. Epidemiology of sleep and sleep disorders in The Netherlands. Sleep medicine. 2017;30:229-39. Epub 2017/02/22. doi: 10.1016/j.sleep.2016.09.015. PubMed PMID: 28215254.

20. Pekmezovic T, Jovic J, Svetel M, Kostic VS. Prevalence of restless legs syndrome among adult population in a Serbian district: a community-based study. Eur J Epidemiol. 2013;28(11):927-30. Epub 2013/10/23. doi: 10.1007/s10654-013-9857-0. PubMed PMID: 24146048.

21. Benediktsdottir B, Janson C, Lindberg E, Arnardottir ES, Olafsson I, Cook E, et al. Prevalence of restless legs syndrome among adults in Iceland and Sweden: Lung function, comorbidity, ferritin, biomarkers and quality of life. Sleep medicine. 2010;11(10):1043-8. Epub 2010/10/22. doi: 10.1016/j.sleep.2010.08.006. PubMed PMID: 20961808.

22. Juuti AK, Laara E, Rajala U, Laakso M, Harkonen P, Keinanen-Kiukaanniemi S, et al. Prevalence and associated factors of restless legs in a 57-year-old urban population in northern Finland. Acta Neurol Scand. 2010;122(1):63-9. Epub 2009/12/03. doi: 10.1111/j.1600-0404.2009.01262.x. PubMed PMID: 19951275.

23. Bjorvatn B, Leissner L, Ulfberg J, Gyring J, Karlsborg M, Regeur L, et al. Prevalence, severity and risk factors of restless legs syndrome in the general adult population in two Scandinavian countries. Sleep medicine. 2005;6(4):307-12. Epub 2005/06/01. doi: 10.1016/j.sleep.2005.03.008. PubMed PMID: 15923140.

24. Broman JE, Mallon L, Hetta J. Restless legs syndrome and its relationship with insomnia symptoms and daytime distress: epidemiological survey in Sweden. Psychiatry Clin Neurosci.

2008;62(4):472-5. Epub 2008/09/10. doi: 10.1111/j.1440-1819.2008.01825.x. PubMed PMID: 18778446.

25. Lavigne GJ, Montplaisir JY. Restless legs syndrome and sleep bruxism: prevalence and association among Canadians. Sleep. 1994;17(8):739-43. Epub 1994/12/01. PubMed PMID: 7701186.

26. Froese CL, Butt A, Mulgrew A, Cheema R, Speirs MA, Gosnell C, et al. Depression and sleep-related symptoms in an adult, indigenous, North American population. J Clin Sleep Med. 2008;4(4):356-61. Epub 2008/09/04. PubMed PMID: 18763428; PubMed Central PMCID: PMCPMC2542493.

27. Lee HB, Hening WA, Allen RP, Earley CJ, Eaton WW, Lyketsos CG. Race and restless legs syndrome symptoms in an adult community sample in east Baltimore. Sleep medicine. 2006;7(8):642-5. Epub 2006/10/07. doi: 10.1016/j.sleep.2006.03.015. PubMed PMID: 17023211.

28. Winkelman JW, Finn L, Young T. Prevalence and correlates of restless legs syndrome symptoms in the Wisconsin Sleep Cohort. Sleep medicine. 2006;7(7):545-52. Epub 2006/06/03. doi: 10.1016/j.sleep.2006.01.004. PubMed PMID: 16740407.

29. Ram S, Seirawan H, Kumar SK, Clark GT. Prevalence and impact of sleep disorders and sleep habits in the United States. Sleep & breathing = Schlaf & Atmung. 2010;14(1):63-70. Epub 2009/07/25. doi: 10.1007/s11325-009-0281-3. PubMed PMID: 19629554.

30. Sawanyawisuth K, Palinkas LA, Ancoli-Israel S, Dimsdale JE, Loredo JS. Ethnic differences in the prevalence and predictors of restless legs syndrome between Hispanics of Mexican descent and non-Hispanic Whites in San Diego county: a population-based study. J Clin Sleep Med. 2013;9(1):47-53. Epub 2013/01/16. doi: 10.5664/jcsm.2338. PubMed PMID: 23319904; PubMed Central PMCID: PMCPMC3525987.

31. Allen RP, Bharmal M, Calloway M. Prevalence and disease burden of primary restless legs syndrome: results of a general population survey in the United States. Movement disorders : official journal of the Movement Disorder Society. 2011;26(1):114-20. Epub 2011/02/16. doi: 10.1002/mds.23430. PubMed PMID: 21322022.

32. Persi GG, Etcheverry JL, Vecchi C, Parisi VL, Ayarza AC, Gatto EM. Prevalence of restless legs syndrome: a community-based study from Argentina. Parkinsonism Relat Disord. 2009;15(6):461-5. Epub 2009/01/03. doi: 10.1016/j.parkreldis.2008.11.012. PubMed PMID: 19119052.

33. Castillo PR, Kaplan J, Lin SC, Fredrickson PA, Mahowald MW. Prevalence of restless legs syndrome among native South Americans residing in coastal and mountainous areas. Mayo Clin Proc. 2006;81(10):1345-7. Epub 2006/10/14. doi: 10.4065/81.10.1345. PubMed PMID: 17036560.

34. Castillo PR, Mera RM, Fredrickson PA, Zambrano M, Del Brutto VJ, Del Brutto OH. Psychological distress in patients with restless legs syndrome (Willis-Ekbom disease): a population-based door-to-door survey in rural Ecuador. BMC Res Notes. 2014;7:911. Epub 2014/12/17. doi: 10.1186/1756-0500-7-911. PubMed PMID: 25510187; PubMed Central PMCID: PMCPMC4302128.

35. Eckeli AL, Gitai LL, Dach F, Ceretta H, Sander HH, Passos AD, et al. Prevalence of restless legs syndrome in the rural town of Cassia dos Coqueiros in Brazil. Sleep medicine. 2011;12(8):762-7. Epub 2011/08/10. doi: 10.1016/j.sleep.2011.01.018. PubMed PMID: 21824818.

36. Ohayon MM, O'Hara R, Vitiello MV. Epidemiology of restless legs syndrome: a synthesis of the literature. Sleep medicine reviews. 2012;16(4):283-95. doi: 10.1016/j.smrv.2011.05.002. PubMed PMID: 21795081; PubMed Central PMCID: PMC3204316.

37. Trenkwalder C, Allen R, Hogl B, Paulus W, Winkelmann J. Restless legs syndrome associated with major diseases: A systematic review and new concept. Neurology. 2016;86(14):1336-43. doi: 10.1212/WNL.0000000000002542. PubMed PMID: 26944272; PubMed Central PMCID: PMC4826337.

38. Chesson AL, Jr., Wise M, Davila D, Johnson S, Littner M, Anderson WM, et al. Practice parameters for the treatment of restless legs syndrome and periodic limb movement disorder. An American Academy of Sleep Medicine Report. Standards of Practice Committee of the American Academy of Sleep Medicine. Sleep. 1999;22(7):961-8. Epub 1999/11/24. doi: 10.1093/sleep/22.7.961. PubMed PMID: 10566915.

39. Earley CJ, Allen RP. Pergolide and carbidopa/levodopa treatment of the restless legs syndrome and periodic leg movements in sleep in a consecutive series of patients. Sleep. 1996;19(10):801-10. Epub 1996/12/01. doi: 10.1093/sleep/19.10.801. PubMed PMID: 9085489.

40. Allen RP, Earley CJ. Augmentation of the restless legs syndrome with carbidopa/levodopa. Sleep. 1996;19(3):205-13. Epub 1996/04/01. doi: 10.1093/sleep/19.3.205. PubMed PMID: 8723377.

41. Garcia-Borreguero D. Dopaminergic Augmentation in Restless Legs Syndrome/Willis-Ekbom Disease: Identification and Management. Sleep Med Clin. 2015;10(3):287-92, xiii. Epub 2015/09/04. doi: 10.1016/j.jsmc.2015.05.020. PubMed PMID: 26329438.

42. Muramatsu K, Chikahisa S, Shimizu N, Sei H, Inoue Y. Rotigotine suppresses sleep-related muscle activity augmented by injection of dialysis patients' sera in a mouse model of restless legs syndrome. Scientific reports. 2019;9(1):16344. Epub 2019/11/11. doi: 10.1038/s41598-019-52735-z. PubMed PMID: 31704978; PubMed Central PMCID: PMCPMC6841937.

43. Casoni F, Galbiati A, Ferini-Strambi L. D3 receptor agonist efficacy in restless legs syndrome. Adv Pharmacol. 2019;84:21-35. Epub 2019/06/24. doi: 10.1016/bs.apha.2019.01.005. PubMed PMID: 31229173.

44. Garcia-Borreguero D, Silber MH, Winkelman JW, Hogl B, Bainbridge J, Buchfuhrer M, et al. Guidelines for the first-line treatment of restless legs syndrome/Willis-Ekbom disease, prevention and treatment of dopaminergic augmentation: a combined task force of the IRLSSG, EURLSSG, and the RLS-foundation. Sleep medicine. 2016;21:1-11. Epub 2016/07/28. doi: 10.1016/j.sleep.2016.01.017. PubMed PMID: 27448465.

45. Salminen AV, Winkelmann J. Restless Legs Syndrome and Other Movement Disorders of Sleep-Treatment Update. Current treatment options in neurology. 2018;20(12):55. Epub 2018/11/10. doi: 10.1007/s11940-018-0540-3. PubMed PMID: 30411165.

46. Garcia-Borreguero D, Larrosa O, de la Llave Y, Verger K, Masramon X, Hernandez G. Treatment of restless legs syndrome with gabapentin: a double-blind, cross-over study. Neurology. 2002;59(10):1573-9. Epub 2002/11/27. doi: 10.1212/wnl.59.10.1573. PubMed PMID: 12451200.

47. Inoue Y, Hirata K, Uchimura N, Kuroda K, Hattori N, Takeuchi M. Gabapentin enacarbil in Japanese patients with restless legs syndrome: a 12-week, randomized, double-blind, placebo-controlled, parallel-group study. Curr Med Res Opin. 2013;29(1):13-21. Epub 2012/11/06. doi: 10.1185/03007995.2012.746217. PubMed PMID: 23121149.

48. Trenkwalder C, Benes H, Grote L, Garcia-Borreguero D, Hogl B, Hopp M, et al. Prolonged release oxycodone-naloxone for treatment of severe restless legs syndrome after failure of previous treatment: a double-blind, randomised, placebo-controlled trial with an open-label extension. The Lancet Neurology. 2013;12(12):1141-50. Epub 2013/10/22. doi: 10.1016/S1474-4422(13)70239-4. PubMed PMID: 24140442.

49. Allen RP, Auerbach S, Bahrain H, Auerbach M, Earley CJ. The prevalence and impact of restless legs syndrome on patients with iron deficiency anemia. Am J Hematol. 2013;88(4):261-4. Epub 2013/03/16. doi: 10.1002/ajh.23397. PubMed PMID: 23494945.

50. Godau J, Klose U, Di Santo A, Schweitzer K, Berg D. Multiregional brain iron deficiency in restless legs syndrome. Movement disorders : official journal of the Movement Disorder Society. 2008;23(8):1184-7. doi: 10.1002/mds.22070. PubMed PMID: 18442125.

51. Koo BB, Bagai K, Walters AS. Restless Legs Syndrome: Current Concepts about Disease Pathophysiology. Tremor Other Hyperkinet Mov (N Y). 2016;6:401. doi: 10.7916/D83J3D2G. PubMed PMID: 27536462; PubMed Central PMCID: PMCPMC4961894.

52. Earley CJ, Connor J, Garcia-Borreguero D, Jenner P, Winkelman J, Zee PC, et al. Altered brain iron homeostasis and dopaminergic function in Restless Legs Syndrome (Willis-Ekbom Disease). Sleep medicine. 2014;15(11):1288-301. Epub 2014/09/10. doi: 10.1016/j.sleep.2014.05.009. PubMed PMID: 25201131.

53. Connor JR, Ponnuru P, Wang XS, Patton SM, Allen RP, Earley CJ. Profile of altered brain iron acquisition in restless legs syndrome. Brain : a journal of neurology. 2011;134(Pt 4):959-68. Epub 2011/03/15. doi: 10.1093/brain/awr012. PubMed PMID: 21398376; PubMed Central PMCID: PMCPMC3069701.

54. Connor JR, Patton SM, Oexle K, Allen RP. Iron and restless legs syndrome: treatment, genetics and pathophysiology. Sleep medicine. 2017;31:61-70. Epub 2017/01/07. doi: 10.1016/j.sleep.2016.07.028. PubMed PMID: 28057495; PubMed Central PMCID: PMCPMC5334282.

55. Davis BJ, Rajput A, Rajput ML, Aul EA, Eichhorn GR. A randomized, double-blind placebo-controlled trial of iron in restless legs syndrome. Eur Neurol. 2000;43(2):70-5. Epub 2000/02/25. doi: 10.1159/000008138. PubMed PMID: 10686463.

56. Grote L, Leissner L, Hedner J, Ulfberg J. A randomized, double-blind, placebo controlled, multi-center study of intravenous iron sucrose and placebo in the treatment of restless legs syndrome. Movement disorders : official journal of the Movement Disorder Society. 2009;24(10):1445-52. Epub 2009/06/03. doi: 10.1002/mds.22562. PubMed PMID: 19489063.

57. Allen RP, Adler CH, Du W, Butcher A, Bregman DB, Earley CJ. Clinical efficacy and safety of IV ferric carboxymaltose (FCM) treatment of RLS: a multi-centred, placebo-controlled preliminary clinical trial. Sleep medicine. 2011;12(9):906-13. Epub 2011/10/08. doi: 10.1016/j.sleep.2011.06.009. PubMed PMID: 21978726.

58. Cho YW, Allen RP, Earley CJ. Clinical efficacy of ferric carboxymaltose treatment in patients with restless legs syndrome. Sleep medicine. 2016;25:16-23. Epub 2016/11/09. doi: 10.1016/j.sleep.2016.06.021. PubMed PMID: 27823710.

59. Manconi M, Govoni V, De Vito A, Economou NT, Cesnik E, Mollica G, et al. Pregnancy as a risk factor for restless legs syndrome. Sleep medicine. 2004;5(3):305-8. Epub 2004/05/29. doi: 10.1016/j.sleep.2004.01.013. PubMed PMID: 15165540.

60. Lee KA, Zaffke ME, Baratte-Beebe K. Restless legs syndrome and sleep disturbance during pregnancy: the role of folate and iron. J Womens Health Gend Based Med. 2001;10(4):335-41. Epub 2001/07/11. doi: 10.1089/152460901750269652. PubMed PMID: 11445024.

61. Neau JP, Marion P, Mathis S, Julian A, Godeneche G, Larrieu D, et al. Restless legs syndrome and pregnancy: follow-up of pregnant women before and after delivery. Eur Neurol. 2010;64(6):361-6. Epub 2010/11/23. doi: 10.1159/000322124. PubMed PMID: 21088424.

62. Cesnik E, Casetta I, Turri M, Govoni V, Granieri E, Strambi LF, et al. Transient RLS during pregnancy is a risk factor for the chronic idiopathic form. Neurology. 2010;75(23):2117-20. Epub 2010/12/08. doi: 10.1212/WNL.0b013e318200d779. PubMed PMID: 21135386.

63. Ondo WG. Common comorbidities and differential diagnosis of restless legs syndrome. J Clin Psychiatry. 2014;75(3):e06. Epub 2014/04/11. doi: 10.4088/JCP.12074nr2c. PubMed PMID: 24717387.

64. Mucsi I, Molnar MZ, Rethelyi J, Vamos E, Csepanyi G, Tompa G, et al. Sleep disorders and illness intrusiveness in patients on chronic dialysis. Nephrology, dialysis, transplantation : official publication of the European Dialysis and Transplant Association - European Renal Association. 2004;19(7):1815-22. Epub 2004/05/27. doi: 10.1093/ndt/gfh130. PubMed PMID: 15161955.

65. Kutner NG, Bliwise DL. Restless legs complaint in African-American and Caucasian hemodialysis patients. Sleep medicine. 2002;3(6):497-500. Epub 2003/11/01. PubMed PMID: 14592144.

66. La Manna G, Pizza F, Persici E, Baraldi O, Comai G, Cappuccilli ML, et al. Restless legs syndrome enhances cardiovascular risk and mortality in patients with end-stage kidney disease undergoing long-term haemodialysis treatment. Nephrology, dialysis, transplantation : official publication of the European Dialysis and Transplant Association - European Renal Association. 2011;26(6):1976-83. Epub 2010/11/09. doi: 10.1093/ndt/gfq681. PubMed PMID: 21056943.

67. Mehmood T, Auerbach M, Earley CJ, Allen RP. Response to intravenous iron in patients with iron deficiency anemia (IDA) and restless leg syndrome (Willis-Ekbom disease). Sleep medicine. 2014;15(12):1473-6. Epub 2014/12/03. doi: 10.1016/j.sleep.2014.08.012. PubMed PMID: 25441748.

68. Trenkwalder C, Allen R, Hogl B, Paulus W, Winkelmann J. Restless legs syndrome associated with major diseases: A systematic review and new concept. Neurology. 2016;86(14):1336-43. Epub 2016/03/06. doi: 10.1212/WNL.0000000000002542. PubMed PMID: 26944272; PubMed Central PMCID: PMCPMC4826337.

69. Satija P, Ondo WG. Restless legs syndrome: pathophysiology, diagnosis and treatment. CNS Drugs. 2008;22(6):497-518. Epub 2008/05/20. doi: 10.2165/00023210-200822060-00004. PubMed PMID: 18484792.

70. Gemignani F, Brindani F, Vitetta F, Marbini A, Calzetti S. Restless legs syndrome in diabetic neuropathy: a frequent manifestation of small fiber neuropathy. J Peripher Nerv Syst. 2007;12(1):50-3. Epub 2007/03/22. doi: 10.1111/j.1529-8027.2007.00116.x. PubMed PMID: 17374101.

71. Hattan E, Chalk C, Postuma RB. Is there a higher risk of restless legs syndrome in peripheral neuropathy? Neurology. 2009;72(11):955-60. Epub 2008/11/29. doi: 10.1212/01.wnl.0000336341.72621.db. PubMed PMID: 19038854.

72. Ondo W, Jankovic J. Restless legs syndrome: clinicoetiologic correlates. Neurology. 1996;47(6):1435-41. Epub 1996/12/01. doi: 10.1212/wnl.47.6.1435. PubMed PMID: 8960723.

73. Walters AS, Hickey K, Maltzman J, Verrico T, Joseph D, Hening W, et al. A questionnaire study of 138 patients with restless legs syndrome: the 'Night-Walkers' survey. Neurology. 1996;46(1):92-5. Epub 1996/01/01. doi: 10.1212/wnl.46.1.92. PubMed PMID: 8559428.

74. Montplaisir J, Boucher S, Poirier G, Lavigne G, Lapierre O, Lesperance P. Clinical, polysomnographic, and genetic characteristics of restless legs syndrome: a study of 133 patients diagnosed with new standard criteria. Movement disorders : official journal of the Movement Disorder Society. 1997;12(1):61-5. Epub 1997/01/01. doi: 10.1002/mds.870120111. PubMed PMID: 8990055.

75. Winkelmann J, Wetter TC, Collado-Seidel V, Gasser T, Dichgans M, Yassouridis A, et al. Clinical characteristics and frequency of the hereditary restless legs syndrome in a population of 300 patients. Sleep. 2000;23(5):597-602. Epub 2000/08/18. PubMed PMID: 10947027.

76. Xiong L, Montplaisir J, Desautels A, Barhdadi A, Turecki G, Levchenko A, et al. Family study of restless legs syndrome in Quebec, Canada: clinical characterization of 671 familial cases. Archives of neurology. 2010;67(5):617-22. Epub 2010/05/12. doi: 10.1001/archneurol.2010.67. PubMed PMID: 20457962.

77. Allen RP, La Buda MC, Becker P, Earley CJ. Family history study of the restless legs syndrome. Sleep medicine. 2002;3 Suppl:S3-7. Epub 2003/11/01. PubMed PMID: 14592159.

78. Trenkwalder C, Seidel VC, Gasser T, Oertel WH. Clinical symptoms and possible anticipation in a large kindred of familial restless legs syndrome. Movement disorders : official journal of the Movement Disorder Society. 1996;11(4):389-94. Epub 1996/07/01. doi: 10.1002/mds.870110407. PubMed PMID: 8813218.

79. Babacan-Yildiz G, Gursoy E, Kolukisa M, Karakose F, Akkoyunlu EM, Celebi A, et al. Clinical and polysomnographic features of a large Turkish pedigree with restless leg syndrome and periodic limb movements. Sleep & breathing = Schlaf & Atmung. 2013;17(2):741-6. Epub 2012/08/03. doi: 10.1007/s11325-012-0756-5. PubMed PMID: 22855331.

80. Esteves AM, Pedrazzoli M, Bagnato M, Moreira F, Mello MT, Tufik S. Two pedigrees with restless legs syndrome in Brazil. Braz J Med Biol Res. 2008;41(2):106-9. Epub 2008/02/26. doi: 10.1590/s0100-879x2008000200005. PubMed PMID: 18297190.

81. Lazzarini A, Walters AS, Hickey K, Coccagna G, Lugaresi E, Ehrenberg BL, et al. Studies of penetrance and anticipation in five autosomal-dominant restless legs syndrome pedigrees. Movement disorders : official journal of the Movement Disorder Society. 1999;14(1):111-6. Epub 1999/01/26. PubMed PMID: 9918352.

82. Ondo WG, Vuong KD, Wang Q. Restless legs syndrome in monozygotic twins: clinical correlates. Neurology. 2000;55(9):1404-6. Epub 2000/11/23. doi: 10.1212/wnl.55.9.1404. PubMed PMID: 11087794.

83. Desai AV, Cherkas LF, Spector TD, Williams AJ. Genetic influences in self-reported symptoms of obstructive sleep apnoea and restless legs: a twin study. Twin Res. 2004;7(6):589-95. Epub 2004/12/21. doi: 10.1375/1369052042663841. PubMed PMID: 15607009.

84. Xiong L, Jang K, Montplaisir J, Levchenko A, Thibodeau P, Gaspar C, et al. Canadian restless legs syndrome twin study. Neurology. 2007;68(19):1631-3. doi: 10.1212/01.wnl.0000261016.90374.fd. PubMed PMID: 17485653.

85. Desautels A, Turecki G, Montplaisir J, Sequeira A, Verner A, Rouleau GA. Identification of a major susceptibility locus for restless legs syndrome on chromosome 12q. American journal of human genetics. 2001;69(6):1266-70. doi: 10.1086/324649. PubMed PMID: 11704926; PubMed Central PMCID: PMC1235538.

86. Bonati MT, Ferini-Strambi L, Aridon P, Oldani A, Zucconi M, Casari G. Autosomal dominant restless legs syndrome maps on chromosome 14q. Brain : a journal of neurology. 2003;126(Pt 6):1485-92. PubMed PMID: 12764067.

87. Chen S, Ondo WG, Rao S, Li L, Chen Q, Wang Q. Genomewide linkage scan identifies a novel susceptibility locus for restless legs syndrome on chromosome 9p. American journal of human genetics. 2004;74(5):876-85. doi: 10.1086/420772. PubMed PMID: 15077200; PubMed Central PMCID: PMC1181982.

88. Levchenko A, Provost S, Montplaisir JY, Xiong L, St-Onge J, Thibodeau P, et al. A novel autosomal dominant restless legs syndrome locus maps to chromosome 20p13. Neurology. 2006;67(5):900-1. doi: 10.1212/01.wnl.0000233991.20410.b6. PubMed PMID: 16966564.

89. Pichler I, Marroni F, Volpato CB, Gusella JF, Klein C, Casari G, et al. Linkage analysis identifies a novel locus for restless legs syndrome on chromosome 2q in a South Tyrolean population isolate. American journal of human genetics. 2006;79(4):716-23. doi: 10.1086/507875. PubMed PMID: 16960808; PubMed Central PMCID: PMC1592574.

90. Kemlink D, Plazzi G, Vetrugno R, Provini F, Polo O, Stiasny-Kolster K, et al. Suggestive evidence for linkage for restless legs syndrome on chromosome 19p13. Neurogenetics. 2008;9(2):75-82. doi: 10.1007/s10048-007-0113-1. PubMed PMID: 18193462; PubMed Central PMCID: PMC2757615.

91. Levchenko A, Montplaisir JY, Asselin G, Provost S, Girard SL, Xiong L, et al. Autosomal-dominant locus for Restless Legs Syndrome in French-Canadians on chromosome 16p12.1. Movement disorders : official journal of the Movement Disorder Society. 2009;24(1):40-50. doi: 10.1002/mds.22263. PubMed PMID: 18946881.

92. Jimenez-Jimenez FJ, Alonso-Navarro H, Garcia-Martin E, Agundez JAG. Genetics of restless legs syndrome: An update. Sleep medicine reviews. 2018;39:108-21. Epub 2017/10/17. doi: 10.1016/j.smrv.2017.08.002. PubMed PMID: 29033051.

93. Winkelmann J, Schormair B, Xiong L, Dion PA, Rye DB, Rouleau GA. Genetics of restless legs syndrome. Sleep medicine. 2017;31:18-22. Epub 2017/01/10. doi: 10.1016/j.sleep.2016.10.012. PubMed PMID: 28065402.

94. Ghosh S, Collins FS. The geneticist's approach to complex disease. Annu Rev Med. 1996;47:333-53. Epub 1996/01/01. doi: 10.1146/annurev.med.47.1.333. PubMed PMID: 8712786.

95. Gallagher MD, Chen-Plotkin AS. The Post-GWAS Era: From Association to Function. American journal of human genetics. 2018;102(5):717-30. Epub 2018/05/05. doi: 10.1016/j.ajhg.2018.04.002. PubMed PMID: 29727686; PubMed Central PMCID: PMCPMC5986732.

96. Winkelmann J, Schormair B, Lichtner P, Ripke S, Xiong L, Jalilzadeh S, et al. Genome-wide association study of restless legs syndrome identifies common variants in three genomic regions. Nature genetics. 2007;39(8):1000-6. doi: 10.1038/ng2099. PubMed PMID: 17637780.

97. Kemlink D, Polo O, Frauscher B, Gschliesser V, Hogl B, Poewe W, et al. Replication of restless legs syndrome loci in three European populations. J Med Genet. 2009;46(5):315-8. Epub 2009/03/13. doi: 10.1136/jmg.2008.062992. PubMed PMID: 19279021; PubMed Central PMCID: PMCPMC2695663.

98. Winkelmann J, Czamara D, Schormair B, Knauf F, Schulte EC, Trenkwalder C, et al. Genome-wide association study identifies novel restless legs syndrome susceptibility loci on 2p14 and 16q12.1. PLoS genetics. 2011;7(7):e1002171. doi: 10.1371/journal.pgen.1002171. PubMed PMID: 21779176; PubMed Central PMCID: PMCPMC3136436.

99. Yang Q, Li L, Chen Q, Foldvary-Schaefer N, Ondo WG, Wang QK. Association studies of variants in MEIS1, BTBD9, and MAP2K5/SKOR1 with restless legs syndrome in a US population. Sleep medicine. 2011;12(8):800-4. Epub 2011/09/20. doi: 10.1016/j.sleep.2011.06.006. PubMed PMID: 21925394; PubMed Central PMCID: PMCPMC3514407.

100. Schormair B, Zhao C, Bell S, Tilch E, Salminen AV, Putz B, et al. Identification of novel risk loci for restless legs syndrome in genome-wide association studies in individuals of European ancestry: a meta-analysis. The Lancet Neurology. 2017;16(11):898-907. doi: 10.1016/S1474-4422(17)30327-7. PubMed PMID: 29029846; PubMed Central PMCID: PMC5755468.

101. Welter D, MacArthur J, Morales J, Burdett T, Hall P, Junkins H, et al. The NHGRI GWAS Catalog, a curated resource of SNP-trait associations. Nucleic acids research. 2014;42(Database issue):D1001-6. Epub 2013/12/10. doi: 10.1093/nar/gkt1229. PubMed PMID: 24316577; PubMed Central PMCID: PMCPMC3965119.

102. Gabriel SB, Schaffner SF, Nguyen H, Moore JM, Roy J, Blumenstiel B, et al. The structure of haplotype blocks in the human genome. Science. 2002;296(5576):2225-9. Epub 2002/05/25. doi: 10.1126/science.1069424. PubMed PMID: 12029063.

103. Edwards SL, Beesley J, French JD, Dunning AM. Beyond GWASs: illuminating the dark road from association to function. American journal of human genetics. 2013;93(5):779-97. Epub 2013/11/12. doi: 10.1016/j.ajhg.2013.10.012. PubMed PMID: 24210251; PubMed Central PMCID: PMCPMC3824120.

104. Schaub MA, Boyle AP, Kundaje A, Batzoglou S, Snyder M. Linking disease associations with regulatory information in the human genome. Genome research. 2012;22(9):1748-59. Epub 2012/09/08. doi: 10.1101/gr.136127.111. PubMed PMID: 22955986; PubMed Central PMCID: PMCPMC3431491.

105. Maurano MT, Humbert R, Rynes E, Thurman RE, Haugen E, Wang H, et al. Systematic localization of common disease-associated variation in regulatory DNA. Science. 2012;337(6099):1190-5. Epub 2012/09/08. doi: 10.1126/science.1222794. PubMed PMID: 22955828; PubMed Central PMCID: PMCPMC3771521.

106. Vilarino-Guell C, Chai H, Keeling BH, Young JE, Rajput A, Lynch T, et al. MEIS1 p.R272H in familial restless legs syndrome. Neurology. 2009;73(3):243-5. Epub 2009/07/22. doi: 10.1212/WNL.0b013e3181ae7c79. PubMed PMID: 19620614; PubMed Central PMCID: PMCPMC2715576.

107. Schulte EC, Kousi M, Tan PL, Tilch E, Knauf F, Lichtner P, et al. Targeted resequencing and systematic in vivo functional testing identifies rare variants in MEIS1 as significant contributors to restless legs syndrome. American journal of human genetics. 2014;95(1):85-95. Epub 2014/07/06. doi: 10.1016/j.ajhg.2014.06.005. PubMed PMID: 24995868; PubMed Central PMCID: PMCPMC4085638.

108. Xiong L, Catoire H, Dion P, Gaspar C, Lafreniere RG, Girard SL, et al. MEIS1 intronic risk haplotype associated with restless legs syndrome affects its mRNA and protein expression

levels. Human molecular genetics. 2009;18(6):1065-74. doi: 10.1093/hmg/ddn443. PubMed PMID: 19126776; PubMed Central PMCID: PMC2722232.

109. Moens CB, Selleri L. Hox cofactors in vertebrate development. Developmental biology. 2006;291(2):193-206. doi: 10.1016/j.ydbio.2005.10.032. PubMed PMID: 16515781.

110. Azcoitia V, Aracil M, Martinez AC, Torres M. The homeodomain protein Meis1 is essential for definitive hematopoiesis and vascular patterning in the mouse embryo. Developmental biology. 2005;280(2):307-20. Epub 2005/05/11. doi: 10.1016/j.ydbio.2005.01.004. PubMed PMID: 15882575.

111. Hisa T, Spence SE, Rachel RA, Fujita M, Nakamura T, Ward JM, et al. Hematopoietic, angiogenic and eye defects in Meis1 mutant animals. The EMBO journal. 2004;23(2):450-9. Epub 2004/01/10. doi: 10.1038/sj.emboj.7600038. PubMed PMID: 14713950; PubMed Central PMCID: PMCPMC1271748.

112. Mercader N, Leonardo E, Azpiazu N, Serrano A, Morata G, Martinez C, et al. Conserved regulation of proximodistal limb axis development by Meis1/Hth. Nature. 1999;402(6760):425-9. Epub 1999/12/10. doi: 10.1038/46580. PubMed PMID: 10586884.

113. Barber BA, Liyanage VR, Zachariah RM, Olson CO, Bailey MA, Rastegar M. Dynamic expression of MEIS1 homeoprotein in E14.5 forebrain and differentiated forebrain-derived neural stem cells. Ann Anat. 2013;195(5):431-40. Epub 2013/06/13. doi: 10.1016/j.aanat.2013.04.005. PubMed PMID: 23756022.

114. Spieler D, Kaffe M, Knauf F, Bessa J, Tena JJ, Giesert F, et al. Restless legs syndrome-associated intronic common variant in Meis1 alters enhancer function in the developing telencephalon. Genome research. 2014;24(4):592-603. doi: 10.1101/gr.166751.113. PubMed PMID: 24642863; PubMed Central PMCID: PMC3975059.

115. Mignot E. A step forward for restless legs syndrome. Nature genetics. 2007;39(8):938-9. doi: 10.1038/ng0807-938. PubMed PMID: 17660808.

116. Dasen JS, Tice BC, Brenner-Morton S, Jessell TM. A Hox regulatory network establishes motor neuron pool identity and target-muscle connectivity. Cell. 2005;123(3):477-91. Epub 2005/11/05. doi: 10.1016/j.cell.2005.09.009. PubMed PMID: 16269338.

117. Meneely S, Dinkins ML, Kassai M, Lyu S, Liu Y, Lin CT, et al. Differential Dopamine D1 and D3 Receptor Modulation and Expression in the Spinal Cord of Two Mouse Models of Restless Legs Syndrome. Front Behav Neurosci. 2018;12:199. Epub 2018/09/21. doi: 10.3389/fnbeh.2018.00199. PubMed PMID: 30233336; PubMed Central PMCID: PMCPMC6131574.

118. Salminen AV, Garrett L, Schormair B, Rozman J, Giesert F, Niedermeier KM, et al. Meis1: effects on motor phenotypes and the sensorimotor system in mice. Dis Model Mech. 2017;10(8):981-91. Epub 2017/06/25. doi: 10.1242/dmm.030080. PubMed PMID: 28645892; PubMed Central PMCID: PMCPMC5560065.

119. Salminen AV, Lam DD, Winkelmann J. Role of MEIS1 in restless legs syndrome: From GWAS to functional studies in mice. Adv Pharmacol. 2019;84:175-84. Epub 2019/06/24. doi: 10.1016/bs.apha.2019.03.003. PubMed PMID: 31229170.

120. Allen RP, Donelson NC, Jones BC, Li Y, Manconi M, Rye DB, et al. Animal models of RLS phenotypes. Sleep medicine. 2017;31:23-8. Epub 2016/11/15. doi: 10.1016/j.sleep.2016.08.002. PubMed PMID: 27839945; PubMed Central PMCID: PMCPMC5349858.

121. Harrison PM, Arosio P. The ferritins: molecular properties, iron storage function and cellular regulation. Biochimica et biophysica acta. 1996;1275(3):161-203. Epub 1996/07/31. PubMed PMID: 8695634.

122. Skjorringe T, Burkhart A, Johnsen KB, Moos T. Divalent metal transporter 1 (DMT1) in the brain: implications for a role in iron transport at the blood-brain barrier, and neuronal and glial pathology. Front Mol Neurosci. 2015;8:19. Epub 2015/06/25. doi: 10.3389/fnmol.2015.00019. PubMed PMID: 26106291; PubMed Central PMCID: PMCPMC4458610.

123. Gunshin H, Mackenzie B, Berger UV, Gunshin Y, Romero MF, Boron WF, et al. Cloning and characterization of a mammalian proton-coupled metal-ion transporter. Nature. 1997;388(6641):482-8. Epub 1997/07/31. doi: 10.1038/41343. PubMed PMID: 9242408.

124. Catoire H, Dion PA, Xiong L, Amari M, Gaudet R, Girard SL, et al. Restless legs syndrome-associated MEIS1 risk variant influences iron homeostasis. Annals of neurology. 2011;70(1):170-5. doi: 10.1002/ana.22435. PubMed PMID: 21710629.

125. Wade QW, Chiou B, Connor JR. Iron uptake at the blood-brain barrier is influenced by sex and genotype. Adv Pharmacol. 2019;84:123-45. Epub 2019/06/24. doi: 10.1016/bs.apha.2019.02.005. PubMed PMID: 31229168.

126. Gonzalez-Latapi P, Malkani R. Update on Restless Legs Syndrome: from Mechanisms to Treatment. Current neurology and neuroscience reports. 2019;19(8):54. Epub 2019/06/30. doi: 10.1007/s11910-019-0965-4. PubMed PMID: 31250128.

127. Salminen AV, Rimpila V, Polo O. Peripheral hypoxia in restless legs syndrome (Willis-Ekbom disease). Neurology. 2014;82(21):1856-61. Epub 2014/05/03. doi: 10.1212/WNL.0000000000000454. PubMed PMID: 24789861.

128. Patton SM, Ponnuru P, Snyder AM, Podskalny GD, Connor JR. Hypoxia-inducible factor pathway activation in restless legs syndrome patients. Eur J Neurol. 2011;18(11):1329-35. Epub 2011/10/12. doi: 10.1111/j.1468-1331.2011.03397.x. PubMed PMID: 21985026.

129. N Silver RA, CJ Earley. MEIS1 as a potential mediator of the RLS-iron pathology. Movement Disorders. 2010;25:S513–4.

130. Guo S, Huang J, Jiang H, Han C, Li J, Xu X, et al. Restless Legs Syndrome: From Pathophysiology to Clinical Diagnosis and Management. Front Aging Neurosci. 2017;9:171. Epub 2017/06/20. doi: 10.3389/fnagi.2017.00171. PubMed PMID: 28626420; PubMed Central PMCID: PMCPMC5454050.

131. Roychoudhury J, Clark JP, Gracia-Maldonado G, Unnisa Z, Wunderlich M, Link KA, et al. MEIS1 regulates an HLF-oxidative stress axis in MLL-fusion gene leukemia. Blood. 2015;125(16):2544-52. Epub 2015/03/06. doi: 10.1182/blood-2014-09-599258. PubMed PMID: 25740828; PubMed Central PMCID: PMCPMC4400291.

132. Hammerschlag AR, Stringer S, de Leeuw CA, Sniekers S, Taskesen E, Watanabe K, et al. Genome-wide association analysis of insomnia complaints identifies risk genes and genetic overlap with psychiatric and metabolic traits. Nature genetics. 2017;49(11):1584-92. Epub 2017/06/13. doi: 10.1038/ng.3888. PubMed PMID: 28604731; PubMed Central PMCID: PMCPMC5600256.

133. Lane JM, Jones SE, Dashti HS, Wood AR, Aragam KG, van Hees VT, et al. Biological and clinical insights from genetics of insomnia symptoms. Nature genetics. 2019;51(3):387-93. Epub 2019/02/26. doi: 10.1038/s41588-019-0361-7. PubMed PMID: 30804566; PubMed Central PMCID: PMCPMC6415688.

134. Lane JM, Liang J, Vlasac I, Anderson SG, Bechtold DA, Bowden J, et al. Genome-wide association analyses of sleep disturbance traits identify new loci and highlight shared genetics with neuropsychiatric and metabolic traits. Nature genetics. 2017;49(2):274-81. Epub 2016/12/20. doi: 10.1038/ng.3749. PubMed PMID: 27992416; PubMed Central PMCID: PMCPMC5491693.

135. El Gewely M, Welman M, Xiong L, Yin S, Catoire H, Rouleau G, et al. Reassessing GWAS findings for the shared genetic basis of insomnia and restless legs syndrome. Sleep. 2018;41(11). Epub 2018/09/15. doi: 10.1093/sleep/zsy164. PubMed PMID: 30215811.

136. Moore Ht, Winkelmann J, Lin L, Finn L, Peppard P, Mignot E. Periodic leg movements during sleep are associated with polymorphisms in BTBD9, TOX3/BC034767, MEIS1, MAP2K5/SKOR1, and PTPRD. Sleep. 2014;37(9):1535-42. Epub 2014/08/22. doi: 10.5665/sleep.4006. PubMed PMID: 25142570; PubMed Central PMCID: PMCPMC4153066.

137. Stefansson H, Rye DB, Hicks A, Petursson H, Ingason A, Thorgeirsson TE, et al. A genetic risk factor for periodic limb movements in sleep. The New England journal of medicine. 2007;357(7):639-47. doi: 10.1056/NEJMoa072743. PubMed PMID: 17634447.

138. Winkelman JW, Blackwell T, Stone K, Ancoli-Israel S, Tranah GJ, Redline S, et al. Genetic associations of periodic limb movements of sleep in the elderly for the MrOS sleep study. Sleep medicine. 2015;16(11):1360-5. Epub 2015/10/27. doi: 10.1016/j.sleep.2015.07.017. PubMed PMID: 26498236; PubMed Central PMCID: PMCPMC4621485.

139. Haba-Rubio J, Marti-Soler H, Marques-Vidal P, Tobback N, Andries D, Preisig M, et al. Prevalence and determinants of periodic limb movements in the general population. Annals of neurology. 2016;79(3):464-74. Epub 2015/12/26. doi: 10.1002/ana.24593. PubMed PMID: 26703954.

140. Aurora RN, Kristo DA, Bista SR, Rowley JA, Zak RS, Casey KR, et al. The treatment of restless legs syndrome and periodic limb movement disorder in adults--an update for 2012: practice parameters with an evidence-based systematic review and meta-analyses: an American Academy of Sleep Medicine Clinical Practice Guideline. Sleep. 2012;35(8):1039-62. Epub 2012/08/02. doi: 10.5665/sleep.1988. PubMed PMID: 22851801; PubMed Central PMCID: PMCPMC3397811.

141. Royo JL, Bessa J, Hidalgo C, Fernandez-Minan A, Tena JJ, Roncero Y, et al. Identification and analysis of conserved cis-regulatory regions of the MEIS1 gene. PloS one. 2012;7(3):e33617.

Epub 2012/03/27. doi: 10.1371/journal.pone.0033617. PubMed PMID: 22448256; PubMed Central PMCID: PMCPMC3308983.

142. Consortium GT. The Genotype-Tissue Expression (GTEx) project. Nature genetics. 2013;45(6):580-5. Epub 2013/05/30. doi: 10.1038/ng.2653. PubMed PMID: 23715323; PubMed Central PMCID: PMCPMC4010069.

143. Mizuhara E, Nakatani T, Minaki Y, Sakamoto Y, Ono Y. Corl1, a novel neuronal lineage-specific transcriptional corepressor for the homeodomain transcription factor Lbx1. The Journal of biological chemistry. 2005;280(5):3645-55. doi: 10.1074/jbc.M411652200. PubMed PMID: 15528197.

144. Stark R, Grzelak M, Hadfield J. RNA sequencing: the teenage years. Nature reviews Genetics. 2019. Epub 2019/07/26. doi: 10.1038/s41576-019-0150-2. PubMed PMID: 31341269.

145. Zhao S, Zhang Y, Gamini R, Zhang B, von Schack D. Evaluation of two main RNA-seq approaches for gene quantification in clinical RNA sequencing: polyA+ selection versus rRNA depletion. Scientific reports. 2018;8(1):4781. Epub 2018/03/21. doi: 10.1038/s41598-018-23226-4. PubMed PMID: 29556074; PubMed Central PMCID: PMCPMC5859127.

146. Purcell S, Neale B, Todd-Brown K, Thomas L, Ferreira MA, Bender D, et al. PLINK: a tool set for whole-genome association and population-based linkage analyses. American journal of human genetics. 2007;81(3):559-75. doi: 10.1086/519795. PubMed PMID: 17701901; PubMed Central PMCID: PMC1950838.

147. Li MZ, Elledge SJ. Harnessing homologous recombination in vitro to generate recombinant DNA via SLIC. Nat Methods. 2007;4(3):251-6. Epub 2007/02/13. doi: 10.1038/nmeth1010. PubMed PMID: 17293868.

148. Biedler JL, Helson L, Spengler BA. Morphology and growth, tumorigenicity, and cytogenetics of human neuroblastoma cells in continuous culture. Cancer Res. 1973;33(11):2643-52. Epub 1973/11/01. PubMed PMID: 4748425.

149. Essletzbichler P, Konopka T, Santoro F, Chen D, Gapp BV, Kralovics R, et al. Megabase-scale deletion using CRISPR/Cas9 to generate a fully haploid human cell line. Genome research. 2014;24(12):2059-65. Epub 2014/11/06. doi: 10.1101/gr.177220.114. PubMed PMID: 25373145; PubMed Central PMCID: PMCPMC4248322.

150. Cao JY, Poddar A, Magtanong L, Lumb JH, Mileur TR, Reid MA, et al. A Genome-wide Haploid Genetic Screen Identifies Regulators of Glutathione Abundance and Ferroptosis Sensitivity. Cell reports. 2019;26(6):1544-56 e8. Epub 2019/02/07. doi: 10.1016/j.celrep.2019.01.043. PubMed PMID: 30726737; PubMed Central PMCID: PMCPMC6424331.

151. Arndt S, Poser I, Moser M, Bosserhoff AK. Fussel-15, a novel Ski/Sno homolog protein, antagonizes BMP signaling. Molecular and cellular neurosciences. 2007;34(4):603-11. doi: 10.1016/j.mcn.2007.01.002. PubMed PMID: 17292623.

152. Wang Z, Gerstein M, Snyder M. RNA-Seq: a revolutionary tool for transcriptomics. Nat Rev Genet. 2009;10(1):57-63. Epub 2008/11/19. doi: nrg2484 [pii]
10.1038/nrg2484. PubMed PMID: 19015660; PubMed Central PMCID: PMC2949280.

153. Schuierer S, Carbone W, Knehr J, Petitjean V, Fernandez A, Sultan M, et al. A comprehensive assessment of RNA-seq protocols for degraded and low-quantity samples. BMC genomics. 2017;18(1):442. Epub 2017/06/07. doi: 10.1186/s12864-017-3827-y. PubMed PMID: 28583074; PubMed Central PMCID: PMCPMC5460543.

154. Cieslik M, Chugh R, Wu YM, Wu M, Brennan C, Lonigro R, et al. The use of exome capture RNA-seq for highly degraded RNA with application to clinical cancer sequencing.

Genome research. 2015;25(9):1372-81. doi: 10.1101/gr.189621.115. PubMed PMID: 26253700; PubMed Central PMCID: PMC4561495.

155. Dobin A, Davis CA, Schlesinger F, Drenkow J, Zaleski C, Jha S, et al. STAR: ultrafast universal RNA-seq aligner. Bioinformatics. 2013;29(1):15-21. doi: 10.1093/bioinformatics/bts635. PubMed PMID: 23104886; PubMed Central PMCID: PMC3530905.

156. Bourgey M, Dali R, Eveleigh R, Chen KC, Letourneau L, Fillon J, et al. GenPipes: an open-source framework for distributed and scalable genomic analyses. Gigascience. 2019;8(6). Epub 2019/06/12. doi: 10.1093/gigascience/giz037. PubMed PMID: 31185495; PubMed Central PMCID: PMCPMC6559338.

157. Robinson MD, McCarthy DJ, Smyth GK. edgeR: a Bioconductor package for differential expression analysis of digital gene expression data. Bioinformatics. 2010;26(1):139-40. doi: 10.1093/bioinformatics/btp616. PubMed PMID: 19910308; PubMed Central PMCID: PMC2796818.

158. Gallego Romero I, Pai AA, Tung J, Gilad Y. RNA-seq: impact of RNA degradation on transcript quantification. BMC Biol. 2014;12:42. Epub 2014/06/03. doi: 10.1186/1741-7007-12-42. PubMed PMID: 24885439; PubMed Central PMCID: PMCPMC4071332.

159. Huang da W, Sherman BT, Lempicki RA. Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. Nature protocols. 2009;4(1):44-57. doi: 10.1038/nprot.2008.211. PubMed PMID: 19131956.

160. Huang da W, Sherman BT, Lempicki RA. Bioinformatics enrichment tools: paths toward the comprehensive functional analysis of large gene lists. Nucleic acids research. 2009;37(1):1-13. doi: 10.1093/nar/gkn923. PubMed PMID: 19033363; PubMed Central PMCID: PMC2615629.

161. Chen EY, Tan CM, Kou Y, Duan Q, Wang Z, Meirelles GV, et al. Enrichr: interactive and collaborative HTML5 gene list enrichment analysis tool. BMC bioinformatics. 2013;14:128. doi: 10.1186/1471-2105-14-128. PubMed PMID: 23586463; PubMed Central PMCID: PMC3637064.

162. Kuleshov MV, Jones MR, Rouillard AD, Fernandez NF, Duan Q, Wang Z, et al. Enrichr: a comprehensive gene set enrichment analysis web server 2016 update. Nucleic acids research. 2016;44(W1):W90-7. doi: 10.1093/nar/gkw377. PubMed PMID: 27141961; PubMed Central PMCID: PMC4987924.

163. Bindea G, Mlecnik B, Hackl H, Charoentong P, Tosolini M, Kirilovsky A, et al. ClueGO: a Cytoscape plug-in to decipher functionally grouped gene ontology and pathway annotation networks. Bioinformatics. 2009;25(8):1091-3. Epub 2009/02/25. doi: 10.1093/bioinformatics/btp101. PubMed PMID: 19237447; PubMed Central PMCID: PMCPMC2666812.

164. Longobardi E, Penkov D, Mateos D, De Florian G, Torres M, Blasi F. Biochemistry of the tale transcription factors PREP, MEIS, and PBX in vertebrates. Developmental dynamics : an official publication of the American Association of Anatomists. 2014;243(1):59-75. Epub 2013/07/23. doi: 10.1002/dvdy.24016. PubMed PMID: 23873833; PubMed Central PMCID: PMCPmc4232920.

165. Chang CP, Jacobs Y, Nakamura T, Jenkins NA, Copeland NG, Cleary ML. Meis proteins are major in vivo DNA binding partners for wild-type but not chimeric Pbx proteins. Molecular and cellular biology. 1997;17(10):5679-87. Epub 1997/10/07. PubMed PMID: 9315626; PubMed Central PMCID: PMC232416.

166. Shanmugam K, Green NC, Rambaldi I, Saragovi HU, Featherstone MS. PBX and MEIS as non-DNA-binding partners in trimeric complexes with HOX proteins. Molecular and cellular

biology. 1999;19(11):7577-88. Epub 1999/10/19. PubMed PMID: 10523646; PubMed Central PMCID: PMC84774.

167. Zhang X, Rowan S, Yue Y, Heaney S, Pan Y, Brendolan A, et al. Pax6 is regulated by Meis and Pbx homeoproteins during pancreatic development. Developmental biology. 2006;300(2):748-57. Epub 2006/10/20. doi: 10.1016/j.ydbio.2006.06.030. PubMed PMID: 17049510.

168. Penkov D, Mateos San Martin D, Fernandez-Diaz LC, Rossello CA, Torroja C, Sanchez-Cabo F, et al. Analysis of the DNA-binding profile and function of TALE homeoproteins reveals their specialization and specific interactions with Hox genes/proteins. Cell reports. 2013;3(4):1321-33. doi: 10.1016/j.celrep.2013.03.029. PubMed PMID: 23602564.

169. Mahe EA, Madigou T, Serandour AA, Bizot M, Avner S, Chalmel F, et al. Cytosine modifications modulate the chromatin architecture of transcriptional enhancers. Genome research. 2017;27(6):947-58. doi: 10.1101/gr.211466.116. PubMed PMID: 28396520; PubMed Central PMCID: PMC5453328.

170. Nicol JW, Helt GA, Blanchard SG, Jr., Raja A, Loraine AE. The Integrated Genome Browser: free software for distribution and exploration of genome-scale datasets. Bioinformatics. 2009;25(20):2730-1. doi: 10.1093/bioinformatics/btp472. PubMed PMID: 19654113; PubMed Central PMCID: PMC2759552.

171. Gan-Or Z, Zhou S, Ambalavanan A, Leblond CS, Xie P, Johnson A, et al. Analysis of functional GLO1 variants in the BTBD9 locus and restless legs syndrome. Sleep medicine. 2015;16(9):1151-5. doi: 10.1016/j.sleep.2015.06.002. PubMed PMID: 26298793.

172. Lek M, Karczewski KJ, Minikel EV, Samocha KE, Banks E, Fennell T, et al. Analysis of protein-coding genetic variation in 60,706 humans. Nature. 2016;536(7616):285-91. doi: 10.1038/nature19057. PubMed PMID: 27535533; PubMed Central PMCID: PMC5018207.

173. Vasconcelos FF, Sessa A, Laranjeira C, Raposo A, Teixeira V, Hagey DW, et al. MyT1 Counteracts the Neural Progenitor Program to Promote Vertebrate Neurogenesis. Cell reports. 2016;17(2):469-83. doi: 10.1016/j.celrep.2016.09.024. PubMed PMID: 27705795; PubMed Central PMCID: PMCPMC5067283.

174. Su AI, Wiltshire T, Batalov S, Lapp H, Ching KA, Block D, et al. A gene atlas of the mouse and human protein-encoding transcriptomes. Proceedings of the National Academy of Sciences of the United States of America. 2004;101(16):6062-7. doi: 10.1073/pnas.0400782101. PubMed PMID: 15075390; PubMed Central PMCID: PMC395923.

175. Garcia-Martin E, Jimenez-Jimenez FJ, Alonso-Navarro H, Martinez C, Zurdo M, Turpin-Fenoll L, et al. Heme Oxygenase-1 and 2 Common Genetic Variants and Risk for Restless Legs Syndrome. Medicine. 2015;94(34):e1448. doi: 10.1097/MD.0000000000001448. PubMed PMID: 26313808; PubMed Central PMCID: PMC4602895.

176. Jimenez-Jimenez FJ, Garcia-Martin E, Alonso-Navarro H, Martinez C, Zurdo M, Turpin-Fenoll L, et al. Association Between Vitamin D Receptor rs731236 (Taq1) Polymorphism and Risk for Restless Legs Syndrome in the Spanish Caucasian Population. Medicine. 2015;94(47):e2125. Epub 2015/12/04. doi: 10.1097/MD.0000000000002125. PubMed PMID: 26632733; PubMed Central PMCID: PMCPMC5059002.

177. Balaban H, Yildiz OK, Cil G, Senturk IA, Erselcan T, Bolayir E, et al. Serum 25-hydroxyvitamin D levels in restless legs syndrome patients. Sleep medicine. 2012;13(7):953-7. doi: 10.1016/j.sleep.2012.04.009. PubMed PMID: 22704399.

178. Cakir T, Dogan G, Subasi V, Filiz MB, Ulker N, Dogan SK, et al. An evaluation of sleep quality and the prevalence of restless leg syndrome in vitamin D deficiency. Acta neurologica Belgica. 2015;115(4):623-7. doi: 10.1007/s13760-015-0474-4. PubMed PMID: 25904436.

179. Wali S, Shukr A, Boudal A, Alsaiari A, Krayem A. The effect of vitamin D supplements on the severity of restless legs syndrome. Sleep & breathing = Schlaf & Atmung. 2015;19(2):579-83. doi: 10.1007/s11325-014-1049-y. PubMed PMID: 25148866.

180. Juarez-Rebollar D, Rios C, Nava-Ruiz C, Mendez-Armenta M. Metallothionein in Brain Disorders. Oxid Med Cell Longev. 2017;2017:5828056. Epub 2017/11/01. doi: 10.1155/2017/5828056. PubMed PMID: 29085556; PubMed Central PMCID: PMCPMC5632493.

181. Karayannis T, Au E, Patel JC, Kruglikov I, Markx S, Delorme R, et al. Cntnap4 differentially contributes to GABAergic and dopaminergic synaptic transmission. Nature. 2014;511(7508):236-40. doi: 10.1038/nature13248. PubMed PMID: 24870235; PubMed Central PMCID: PMCPMC4281262.

182. Sleeman MW, Anderson KD, Lambert PD, Yancopoulos GD, Wiegand SJ. The ciliary neurotrophic factor and its receptor, CNTFR alpha. Pharm Acta Helv. 2000;74(2-3):265-72. Epub 2000/05/17. PubMed PMID: 10812968.

183. Xie Y, Hou W, Song X, Yu Y, Huang J, Sun X, et al. Ferroptosis: process and function. Cell Death Differ. 2016;23(3):369-79. Epub 2016/01/23. doi: 10.1038/cdd.2015.158. PubMed PMID: 26794443; PubMed Central PMCID: PMCPMC5072448.

184. Dixon SJ, Lemberg KM, Lamprecht MR, Skouta R, Zaitsev EM, Gleason CE, et al. Ferroptosis: an iron-dependent form of nonapoptotic cell death. Cell. 2012;149(5):1060-72. Epub 2012/05/29. doi: 10.1016/j.cell.2012.03.042. PubMed PMID: 22632970; PubMed Central PMCID: PMCPMC3367386.

185. Do Van B, Gouel F, Jonneaux A, Timmerman K, Gele P, Petrault M, et al. Ferroptosis, a newly characterized form of cell death in Parkinson's disease that is regulated by PKC. Neurobiol

Dis. 2016;94:169-78. Epub 2016/05/18. doi: 10.1016/j.nbd.2016.05.011. PubMed PMID: 27189756.

186. Hambright WS, Fonseca RS, Chen L, Na R, Ran Q. Ablation of ferroptosis regulator glutathione peroxidase 4 in forebrain neurons promotes cognitive impairment and neurodegeneration. Redox Biol. 2017;12:8-17. Epub 2017/02/18. doi: 10.1016/j.redox.2017.01.021. PubMed PMID: 28212525; PubMed Central PMCID: PMCPMC5312549.

187. Weiland A, Wang Y, Wu W, Lan X, Han X, Li Q, et al. Ferroptosis and Its Role in Diverse Brain Diseases. Mol Neurobiol. 2019;56(7):4880-93. Epub 2018/11/09. doi: 10.1007/s12035-018-1403-3. PubMed PMID: 30406908; PubMed Central PMCID: PMCPMC6506411.

188. Schipper HM. Heme oxygenase-1: role in brain aging and neurodegeneration. Exp Gerontol. 2000;35(6-7):821-30. Epub 2000/10/29. PubMed PMID: 11053673.

189. Becker PM. The biopsychosocial effects of restless legs syndrome (RLS). Neuropsychiatric disease and treatment. 2006;2(4):505-12. Epub 2006/12/01. doi: 10.2147/nedt.2006.2.4.505. PubMed PMID: 19412498; PubMed Central PMCID: PMCPMC2671952.

190. Teive HA, Munhoz RP, Barbosa ER. Professor Karl-Axel Ekbom and restless legs syndrome. Parkinsonism Relat Disord. 2009;15(4):254-7. Epub 2008/10/03. doi: 10.1016/j.parkreldis.2008.07.011. PubMed PMID: 18829374.

191. Allen RP, Barker PB, Wehrl FW, Song HK, Earley CJ. MRI measurement of brain iron in patients with restless legs syndrome. Neurology. 2001;56(2):263-5. Epub 2001/02/13. doi: 10.1212/wnl.56.2.263. PubMed PMID: 11160969.

192. Allen RP, Earley CJ. The role of iron in restless legs syndrome. Movement disorders : official journal of the Movement Disorder Society. 2007;22 Suppl 18:S440-8. doi: 10.1002/mds.21607. PubMed PMID: 17566122.

193. Hudson TJ. Wanted: regulatory SNPs. Nature genetics. 2003;33(4):439-40. Epub 2003/04/01. doi: 10.1038/ng0403-439. PubMed PMID: 12665861.

194. Cheng L, Samad OA, Xu Y, Mizuguchi R, Luo P, Shirasawa S, et al. Lbx1 and Tlx3 are opposing switches in determining GABAergic versus glutamatergic transmitter phenotypes. Nature neuroscience. 2005;8(11):1510-5. doi: 10.1038/nn1569. PubMed PMID: 16234809.

195. Sehgal A, Mignot E. Genetics of Sleep and Sleep disorders. Cell. 2011;146(2):194-207. doi: 10.1016/j.cell.2011.07.004. PubMed PMID: PMC3153991.

Appendix

Copyright permissions

ELSEVIER LICENSE
TERMS AND CONDITIONS

Nov 25, 2019

This Agreement between Mrs. Faezeh Sarayloo ("You") and Elsevier ("Elsevier") consists of your license details and the terms and conditions provided by Elsevier and Copyright Clearance Center.

License Number	4707130476682
License date	Nov 13, 2019
Licensed Content Publisher	Elsevier
Licensed Content Publication	Sleep Medicine Clinics
Licensed Content Title	Restless Leg Syndrome Across the Globe Epidemiology of the Restless Legs Syndrome/Willis-Ekbom Disease
Licensed Content Author	Brian B. Koo
Licensed Content Date	Sep 1, 2015
Licensed Content Volume	10
Licensed Content Issue	3
Licensed Content Pages	17
Start Page	189

ELSEVIER LICENSE
TERMS AND CONDITIONS

Nov 25, 2019

This Agreement between Mrs. Faezeh Sarayloo ("You") and Elsevier ("Elsevier") consists of your license details and the terms and conditions provided by Elsevier and Copyright Clearance Center.

License Number	4707131466415
License date	Nov 13, 2019
Licensed Content Publisher	Elsevier
Licensed Content Publication	The American Journal of Human Genetics
Licensed Content Title	The Post-GWAS Era: From Association to Function
Licensed Content Author	Michael D. Gallagher,Alice S. Chen-Plotkin
Licensed Content Date	3 May 2018
Licensed Content Volume	102
Licensed Content Issue	5
Licensed Content Pages	14
Start Page	717
End Page	730

Ethics certificates

MUHC REB – NEUPSY / CÉR CUSM – NEUPSY
3801, rue University, # 686
Montréal (Québec) H3A 2B4
Tel. 514.398.1046
reb.neuro@mcgill.ca
www.leneuro.ca

Annual renewal submission

Submit date: **2019-03-12 08:58**
Project's REB approbation date: **2015-03-20**
Project number: **2015-164, MP-CUSM-14-051**
Form status: **Approved**

Submitted by: **Zaharieva, Vessela**
Nagano identifier: **ROU BANK**
Form: **F9 - 40329**

Administration

1. **MUHC REB Panel & Co-chair(s):**
   Neurosciences-Psychiatry (NEUPSY)
   Co-chairs: Judith Marcoux, Marie-Josée Brouillette

2. **REB Decision:**
   Approved - REB delegated review

3. **Comments on the decision:**

   Please note that this Annual Renewal Form was submitted prior to the REB approval expiry. Furthermore, it applies for all the participating institutions.

4. **Renewal Period Granted:**
   Until 2020-03-19

5. **Date of the REB final decision & signature**
   2019-05-08
   Signature

Mme Kathy Laperrière
MUHC REB Coordinator
For MUHC Co-chair mentioned above

6. FWA 00000840 - FWA 00004545

7. **Local REB number**
IRB00010120

8. **Note:**
In order to be in compliance with Good Clinical Practices, the MUHC REB (when acting as the Reviewing REB), and the PM of the MUHC does not directly communicate with sponsors. The communication channels existing between the PIs and the sponsors will continue to ensure the transmission of documents.

A. General information

1. **Indicate the full title of the research study**

Rou Bank.

2. **If relevant, indicate the full study title in French**

3. **Indicate the name of the Principal Investigator in our institution (MUHC)**

Rouleau, Guy

4. **Are there local co-investigators & collaborators involved in this project?**
No

5. **For each participating centre part of the Québec health and social services network (RSSS), indicate the name of the external investigator**

Sylvain Chouinard

What is the name of the participating center(s)?
CHU-Montréal

Nicolas Dupré

What is the name of the participating center(s)?
CHU-de-Québec

Jacques Michaud

What is the name of the participating center(s)?
CHU-SJ

6. **Indicate the name and the affiliation of the external collaborator(s),(if any)**

Voir la liste des sections 5 & 9

7. **Identify the study coordinator(s)**

Zaharieva, Vessela

Indicate the role of the collaborator(s)

Administrative agent

Mirarchi, Cathy

Indicate the role of the collaborator(s)

Administrative agent

B. Project development

1. **Study start date:**

2006-09-21

2. **Expected ending date of the study:**

☐ Determined date
☑ Undetermined date

3. **Date of recruitment of the first participant?**

☑ 1st enrollment date is...
☐ No participant enrolled

1st participant enrollment date:

2006-09-22

4. **Indicate the current study status at MUHC.**

Study and recruitment in progress

5. **Add a brief statement on the study status**

Study is still in progress

6. **Information about the participants at this institution, since the beginning of the project**

Number of participants who have been recruited

16680

Number of minors

0

Number of participants who have not yet completed the study (still in progress)

0

Number of participants who've completed the study

16680

Number of participants who were recruited to the study, but who were then excluded or withdrawn:

0

Number of participants who dropped out (voluntary withdrawal):

0

7. **Information about the participants at this institution (MUHC) since the previous REB approval**

Number of participants who have been recruited

1305

Number of minors

0

Number of participants who have not yet completed the study (still in progress)

0

Number of participants who've completed the study

1305

Number of participants who were recruited to the study, but who were then excluded or withdrawn:

0

Number of participants who dropped out (voluntary withdrawal):

0

8. **Since the previous REB approval (annual renewal or initial approval):**

Were there any changes to the protocol (or to the databank management framework) ?

No

Specify the current version/date:

version 1, March 14, 2016

Date approved by the REB:

2016-03-22

Were there any changes to the information and consent form?

No

Specify the current version/date:

Version1.0(March14, 2016)

REB approval date:

2016-03-22

Were there any reportable adverse events at this site (or, for multi-center projects, at an institution under the jurisdiction of our REB) that should be reported to the REB under section 5.2.1 of " SOP-REB- 404001 " ?

https://muhc.ca/cae/page/standard-operating-procedures-sops

No

Has there has been any new information likely to affect the ethics of the project or influence the decision of a participant as to their continued participation in the project ?

No

Were there any deviations / major violations protocol (life -threatening or not meeting the inclusion / exclusion criteria)?

No

Was there a temporary interruption of the project?

No

Have the project results been submitted for publication, presented or published?

No

Has the REB been notified of a conflict of interest - (apparent , potential or actual), of one or more members of the research team - that was not known when it was last approved project?

No

Do you want to bring any other info to the REB's attention?

No

9. **For all external participating institutions, please answer the following questions:**

Please select the name of the institution concerned and attach the "Formulaire de renouvellement annuel pour les projets sites externes - Projets multicentriques":

CHU-Montréal

Please print a copy of the "Formulaire de renouvellement annuel pour les sites externes" (see link below), have it completed by other institutions and attach it here.

Formulaire de renouvellement pour les sites externes (MP project)

This form is accessible to external researchers, via our web page.

Demande de renouvellement annuel pour les sites externes-CHUM 2019.pdf

CHU-SJ

Please print a copy of the "Formulaire de renouvellement annuel pour les sites externes" (see link below), have it completed by other institutions and attach it here.

Formulaire de renouvellement pour les sites externes (MP project)

This form is accessible to external researchers, via our web page.

Demande de renouvellement annuel pour les sites externes-CHUSJsigne.pdf

CHU-de-Québec

Please print a copy of the "Formulaire de renouvellement annuel pour les sites externes" (see link below), have it completed by other institutions and attach it here.

Formulaire de renouvellement pour les sites externes (MP project)

This form is accessible to external researchers, via our web page.

AADemande de renouvellement annuel pour les sites externes-CHUdu Quebec.pdf

Is there any institution's info (pdf form) missing?

No

10. **Is there a data safety monitoring committee analyzing data on the safety and efficacy of the treatment?**

No

C. Signature

1. **I confirm that all information is complete & accurate.**

First & last name of person who completed the submission

Vessela Zaharieva