Regulatory interaction between the ZPBP2-ORMDL3/Zpbp2-Ormdl3 region and the circadian clock

Matthew L. Chang¹, Sanny Moussette¹, Enrique Gamero-Estevez², José Héctor Gálvez³, Victoria Chiwara², Indra R. Gupta¹,²,⁴, Aimee K. Ryan¹,²,⁴, Anna K. Naumova¹,²,⁵*

¹ The Research Institute of the McGill University Health Centre, Montreal, Quebec, Canada, ² Department of Human Genetics, McGill University, Montreal, Quebec, Canada, ³ Canadian Centre for Computational Genomics, Montreal, Quebec, Canada, ⁴ Department of Paediatrics, McGill University, Montreal, Quebec, Canada, ⁵ Department of Obstetrics and Gynecology, McGill University, Montreal, Quebec, Canada

* These authors contributed equally to this work.

*anna.naumova@mcgill.ca

Abstract

Genome-wide association study (GWAS) loci for several immunity-mediated diseases (early onset asthma, inflammatory bowel disease (IBD), primary biliary cholangitis, and rheumatoid arthritis) map to chromosomal region 17q12-q21. The predominant view is that association between 17q12-q21 alleles and increased risk of developing asthma or IBD is due to regulatory variants. ORM sphingolipid biosynthesis regulator (ORMDL3) residing in this region is the most promising gene candidate for explaining association with disease. However, the relationship between 17q12-q21 alleles and disease is complex suggesting contributions from other factors, such as trans-acting genetic and environmental modifiers or circadian rhythms. Circadian rhythms regulate expression levels of thousands of genes and their dysregulation is implicated in the etiology of several common chronic inflammatory diseases. However, their role in the regulation of the 17q12-q21 genes has not been investigated. Moreover, the core clock gene nuclear receptor subfamily 1, group D, member 1 (NR1D1) resides about 200 kb distal to the GWAS region. We hypothesized that circadian rhythms influenced gene expression levels in 17q12-q21 region and conversely, regulatory elements in this region influenced transcription of the core clock gene NR1D1 in cis. To test these hypotheses, we examined the diurnal expression profiles of zona pellucida binding protein 2 (ZPBP2/Zpbp2), gasdermin B (GSDMB), and ORMDL3/Ormdl3 in human and mouse tissues and analyzed the impact of genetic variation in the ZPBP2/Zpbp2 region on NR1D1/Nr1d1 expression. We found that Ormdl3 and Zpbp2 were controlled by the circadian clock in a tissue-specific fashion. We also report that deletion of the Zpbp2 region altered the expression profile of Nr1d1 in lungs and ileum in a time-dependent manner. In liver, the deletion was associated with enhanced expression of Ormdl3. We provide the first evidence that disease-associated genes Zpbp2 and Ormdl3 are regulated by circadian rhythms and the Zpbp2 region influences expression of the core clock gene Nr1d1.
Introduction

Human chromosomal region 17q12-q21 harbors risk alleles for several immunity-mediated diseases, including early onset asthma, inflammatory bowel disease (IBD), primary biliary cholangitis (PBC), and rheumatoid arthritis (RA) [1–7]. This suggests that the disease-associated variants (daVs) in this region are likely to have pleiotropic effects and impact a pathway(s) that is critical for the pathogenesis of not one, but several immunity-mediated diseases (reviewed in [8]). Identification of the gene responsible for the genetic association with disease is complicated by the fact that no polymorphisms that would alter the protein sequence are found in the best gene candidate ORM sphingolipid biosynthesis regulator (ORMDL3) whereas regulatory genetic variants influence expression of at least three protein-coding genes residing in this region, zona pellucida binding protein 2 (ZPBP2), gasdermin B (GSDMB) and ORMDL3, suggesting that transcription of these genes is governed by shared regulatory mechanisms [1, 9, 10]. Our search for functional genetic cis-regulatory variants identified several candidates, the best characterized of which is rs12936231-C/G located in intron 5 of ZPBP2 [10, 11]. It causes loss/gain of the insulator protein CTCC factor (CTCF) binding site [10–12]. Allelic differences in CTCF-binding lead to differences in chromosome conformation [10, 13]. Therefore, it has been proposed that loss of CTCF site (the rs12936231-C allele) in the ZPBP2 gene resulted in chromatin conformation that favored interaction between the promoters of ORMDL3 and GSDMB and a distant enhancer leading to higher transcription rates [10, 13]. Of the three genes, ORMDL3 has been singled out as the best candidate for explaining the link between the 17q21 alleles and asthma due to its strong and highly reproducible eQTL, higher expression levels in asthmatic subjects and its role in sphingolipid metabolism, which has recently come to the fore as a significant modulator of lung hypersensitivity and inflammatory responses [14, 15]. However, the association between lipid levels and the ORMDL3 genotype in humans remains unclear [16–18]. Moreover, conflicting evidence from different mouse studies puts into question the causal relationship between Ormdl3 and asthma [19–22]. Thus, the sum of current data suggests a complex relationship between the 17q12-q21 daVs and disease, such as a cumulative effect from variation in expression of several neighboring genes, impact of modifier loci, epigenetic variation, environmental factors, or an association that is time-dependent. Here, we explored the latter possibility.

The circadian clock system regulates multiple biological processes from homeostasis, metabolism, and immunity to reproduction and behavior (reviewed in [23, 24]). The molecular circadian clock consists of several core clock genes that drive 24h oscillations in the expression of thousands of genes throughout the genome. Mutations in core clock genes lead to phenotypic abnormalities in mice, whereas in humans, factors that disturb circadian rhythms, such as jet lag or shift work, are suspected to increase the risk of developing certain immune-mediated diseases (reviewed in [25]). Remarkably, the core clock gene nuclear receptor subfamily 1, group D, member 1 (NR1D1, also known as REV-ERB-alpha) resides about 200 kb distal to the top asthma and IBD daVs in chromosomal region 17q21. NR1D1 is a transcription factor that regulates thousands of genes throughout the genome and links the circadian clock with lipid metabolism [26, 27]. Evidence from chromatin conformation studies suggests that the ZPBP2-ORMDL3 and NR1D1 regions physically interact [28]. However, whether the ZPBP2-ORMDL3 daVs modify the diurnal expression profile of NR1D1 remains unknown.

To gain better understanding of the regulatory mechanisms governing gene expression in the 17q12-q21 disease-associated region, we asked two questions: 1) whether the 17q12-q21 disease gene candidates were regulated by circadian rhythms; and 2) whether regulatory elements in the ZPBP2 region influenced transcriptional regulation of NR1D1.

First, using public databases we examined diurnal fluctuations in the expression levels of ZPBP2, ORMDL3 and GSDMB and the mouse orthologs Zpbp2 and Ormd3 and found diurnal...
rhythms in the mouse genes. Second, we tested the hypothesis that the ZPBP2 region harbored regulatory elements that influenced expression of NR1D1 using publicly available data from human lymphoblastoid cell lines. Third, we tested the same hypothesis by comparing expression profiles of Nr1d1 in wild type (WT) and Zpbp2 mutant mice (KO) that carry a deletion of the 5’ portion of the Zpbp2 gene including its promoter and enhancer regions. We find that the Zpbp2 deletion influences diurnal regulation of Nr1d1 in lungs and ileum. We also show that the deletion is associated with enhanced expression of Ormdl3 in liver, supporting previous findings that implicate the human ZPBP2 region in transcriptional regulation of ORMDL3.

Materials and methods

Mice

The B6.129S7-Zpbp2<sup>tmZuk</sup>/Mmjax (MMRRC#42297) strain of mice [29] that carry a deletion of the 5’ region of Zpbp2 (exons 1 to 3) [30] in a C57BL/6J genetic background (referred to as KO from this point on) was maintained in our mouse facility for several years. Control C57BL/6J mice (WT) were born and maintained in the same colony as KO mice. All mice were housed in 12h:12h light/dark cycle and had access to food and water ad libitum. Lungs, liver and small intestine (ileum) from adult KO and WT mice of both sexes were collected every 4 hours (Zeitgeber times ZT2, ZT6, ZT10, ZT14, ZT18, and ZT22) over a 24 h period (n = 3 mice per time point/genotype). Between ZT12 and ZT0 (dark phase) all procedures including organ harvesting were performed under red light. To validate the effect of genotype on expression, independent sets of ZT10 samples from mice of both genotypes and both sexes were assayed (n = 4–7 for WT mice, n = 5–10 for KO mice). All procedures were conducted in accordance with the guidelines set by the Canadian Council of Animal Care (Ottawa, Ontario, Canada) and were approved by the Animal Care Committee of the McGill University Health Center (Montreal, Quebec, Canada).

Expression analysis

RNA was extracted, cDNA synthesized, and expression levels assayed in each mouse sample individually. RNA extraction, cDNA synthesis, and analysis of gene expression levels were performed as previously described [29]. Expression levels of Nr1d1, Ormd3 and Zpbp2 were determined in both WT and KO mice using RT-qPCR and normalized to the levels of eukaryotic translation elongation factor 2 (Eef2) RNA. The reliability of Eef2 as a normalization control has been validated in our previous study [29]. For diurnal expression profiling, the peak level of average expression in WT mice was set at 1.0. For the validation experiments, the mean of WT expression was set at 1. Primers are listed in S1 Table.

RNA-sequencing. Two independent RNA-sequencing experiments were conducted on RNA from lungs. Lungs were collected from 3 Zpbp2 KO (KO1-KO3) and 3 WT (WT1-WT3) mice at ZT7 and 3 Zpbp2 KO (KO4-KO6) and 3 WT (WT4-WT6) mice at ZT10. The sequencing for both experiments was done at the McGill University and Genome Quebec Innovation Centre (Montreal, QC, Canada). Differential gene expression analysis was performed using the GenPipes RNA-seq pipeline [31]. Briefly, reads were trimmed and filtered for quality, then they were aligned to the mouse reference genome (GRCm38) using STAR [32]. The abundance of each transcript was estimated using HT-Seq Count [33]. Differential gene expression was determined with both the EdgeR [34] and DESeq [35] packages. Pathway analysis was done using DAVID Bioinformatics Resources 6.8 software [36].

Use of publicly available expression data. Publicly available expression data were used to assess diurnal or circadian variation in the transcription of human 17q12-q21 genes or their
mouse orthologs, respectively. Human expression data from adipose tissue (GSE87761 [37]), skin biopsies (GSE112660 [38]), peripheral blood cells (GSE107537 [39] and GSE48113, [40]) were used to examine diurnal variation in ZPBP2, ORMDL3 and GSDMB. To determine whether expression of mouse genes Ormdl3 and Zpbp2 was regulated by circadian rhythms in nine different peripheral tissues, expression data from the circadian gene expression atlas assayed by microarray (GSE54652, C57BL/6J male mice kept in constant darkness (DD) [41]), liver from C57BL/6J male mice maintained in light/dark conditions (GSE52333, [42]), distal colon from male mice maintained in light/dark conditions (GSE10644 [43]) were used.

To determine the effect of ZPBP2 genotype on NR1D1 expression levels, expression data for human genes ORMDL3, GSDMB, ZPBP2, and NR1D1 were obtained from the Gene Expression Omnibus (GEO) datasets for lymphoblastoid cell lines (GSE8052 [1]).

**Statistical analyses**

Comparisons between groups were done using the GraphPad Prizm 8.0 software (one-way or two-way ANOVA followed by post hoc analyses with Sidak’s correction for multiple testing). Effect sizes were calculated using Cohen’s d [44] (http://www.socscistatistics.com). The Jonckheere-Terpstra-Kendall (JTK) p-values for mouse circadian expression data were taken from the Circadian Expression Profiles Database (CircaDB at http://circadb.hogeneschlab.org).

**Results**

**Diurnal/Circadian regulation of 17q12-q21 genes and their mouse orthologs**

To test the hypothesis that ZPBP2, GSDMB, or ORMDL3 were regulated by circadian rhythms in human tissues, we extracted gene-specific data from expression datasets with sample collection covering 24h time periods. Diurnal oscillations in RNA levels were observed for core clock gene NR1D1 (Fig 1). We found no statistically significant effect of time of the day on regulation of ORMDL3, ZPBP2, or GSDMB. Nevertheless, it is worth noting that ORMDL3 tended to have higher expression levels in the afternoon when NR1D1 levels were at their minimum.

Circadian regulation varies between different cell types and human datasets that include time of sample collection are limited to tissues that are easily obtainable from live donors. To get a better idea of the circadian as well as tissue-specific variation in the expression of our genes of interest, we examined expression data from mice that were kept in constant darkness [41]. The mouse orthologs Zpbp2, Ormdl3, and Nr1d1 reside on chromosome 11 with most of the region being conserved. However, the mouse lacks a transcribed GSDMB gene ortholog between Zpbp2 and Ormdl3.

Data from nine peripheral organs (e.g. adrenal, aorta, brown adipose tissue, heart, kidney, liver, lung, skeletal muscle, and white adipose tissue (GSE54650 [41]) from mice kept in constant darkness and livers and distal colon from mice maintained in light/dark cycle ([42, 43]) were examined to determine if Ormdl3 or Zpbp2 transcription was controlled by the circadian clock and if the circadian regulation was tissue-specific. Ormdl3 showed circadian oscillations in expression in the adrenal, aorta, brown adipose tissue, and liver, but not lungs, kidney, white adipose tissue, skeletal muscle, or heart (Fig 2 and data from the Circadian Expression Profiles Database, [41]). Zpbp2 levels oscillated in the adrenal (Fig 2). Nr1d1 levels showed circadian oscillations in all peripheral organs, as expected (Fig 2). It is worth noting that mice are nocturnal animals and the circadian rhythm of mouse Nr1d1 expression is shifted compared to the human NR1D1, which peaks in the early morning and has a trough during the day (Fig 2). Interestingly, Ormdl3 expression in adrenal, aorta, and brown adipose tissue peaked during
the subjective day (see figure legend for detail), whereas in liver it peaked during the subjective
night (Fig 2). *Zpbp2* expression in adrenal peaked during the subjective night when *Nr1d1*
expression was at its trough.

Diurnal oscillations in *Nr1d1*, *Ormdl3* or *Zpbp2* levels were also detected in mice main-
tained in light/dark conditions (S1 Fig) [42, 43]. Thus, analysis of published and publicly avail-
able data demonstrates that *Ormdl3* and *Zpbp2* are controlled by the circadian clock in the
mouse and this regulation is tissue-specific.

Cis-regulatory effects on *NR1D1* expression levels in human LCLs

Genetic variation in the *ZPBP2* gene region is associated with variation in the expression levels
of *ZPBP2*, *GSDMB*, and *ORMDL3* in cis [1, 9, 10]. To test the hypothesis that the same regula-
tory variants also influenced expression of *NR1D1*, we analyzed publicly available expression
data from 336 EBV-transformed LCLs derived from a familial cohort of asthmatic and non-
asthmatic children from the UK [1]. Genotypes for functional SNPs rs4795397 and
rs12936231 were not available in this cohort. Instead, we used genotype data for a closely
linked SNP rs11557467-G/T located in exon 4 of ZPBP2 less than 500 bp proximal to rs12936321 (Fig 3A). We noticed that sex ratios varied between genotype groups (sex ratios of 1.6 for GG, 0.8 for GT and 2.3 for TT). To make sure that sex bias did not mask or enhance the effect of genotype, expression levels of 17q12-q21 genes were analyzed considering genotype alone as well as sex and genotype of the donor (Fig 3B and 3C). Circadian regulation of the ZPBP2-ORMDL3/Zpbp2-Ormdl3 region

https://doi.org/10.1371/journal.pone.0223212.g002

Fig 2. Ormd3 and Zpbp2 are regulated by the circadian clock in mouse organs. Circadian oscillations of Ormd3 or Zpbp2 RNA levels in adrenal, aorta, brown adipose tissue (B-fat), liver, and lungs of wild type C57BL/6J male mice. The x-axis shows time since beginning of DD in hours. The y-axis shows expression levels measured on the individual gene chips. Data extracted from GSE54650 [41]. JTK p-values are taken from the Circadian Expression Profiles Database.

linked SNP rs11557467-G/T located in exon 4 of ZPBP2 less than 500 bp proximal to rs12936321 (Fig 3A). We noticed that sex ratios varied between genotype groups (sex ratios of 1.6 for GG, 0.8 for GT and 2.3 for TT). To make sure that sex bias did not mask or enhance the effect of genotype, expression levels of 17q12-q21 genes were analyzed considering genotype alone as well as sex and genotype of the donor (Fig 3B and 3C). Both sex and genotype influenced expression of ORMDL3 (p<0.05 and p<0.0001, for sex and genotype, respectively, two-way ANOVA) and ZPB2 (p<0.0005, for sex and genotype, two-way ANOVA) (Fig 3C). LCLs carrying rs11557467-G alleles tended to have higher RNA levels of NR1D1 compared to rs11557467-T homozygotes, but these differences did not reach statistical significance (Fig 3B). No differences between males and females with respect to NR1D1 levels were observed (Fig 3C).
Zpbp2 influences Ormdl3 RNA levels in mouse liver

To determine if the Zpbp2 region was involved in regulation of Ormdl3 or Nr1d1 expression in mice, we used a mouse strain that lacks Zpbp2 and focused on lungs, small intestine (ileum) and liver, the three organs that are affected in human diseases with association to the ZPBP2-ORMDL3 region, i.e. asthma, IBD and PBC.
The mouse Zpbp2 region is orthologous to the human \textit{ZPB2} region that harbors the functional cis-regulatory SNPs rs4795397 and rs12936231 (Fig 4A and 4B). The Zpbp2 knock-out mice (KO) carry a \textasciitilde 1.5 kb deletion of the Zpbp2 exons 1 to 3 and do not express Zpbp2 \cite{29,30}. The deletion removes part of the Zpbp2 promoter and enhancer regions and is also associated with hypermethylation of the remainder of the Zpbp2 promoter, DNAse I hypersensitive sites and putative enhancer (Fig 4B) \cite{29}.

In the liver of WT mice, expression levels of Zpbp2 and Ormdl3 varied over time with peak levels during the night (ZT18) and trough during the day (ZT6) (\(p<0.0001\) and \(p<0.005\), respectively, one-way ANOVA) similar to the pattern observed in mice in other studies (Fig 2, S1 Fig) \cite{41–43}. \textit{Nr1d1} RNA levels peaked during the day (ZT6) and dropped during the night (ZT18), as expected (\(p<0.0001\), one-way ANOVA).

If the deleted region harbored DNA elements that influenced \textit{Nr1d1} regulation, we would observe differences in \textit{Nr1d1} expression between the KO mice and WT controls. If the Zpbp2 region was involved in the regulation of Ormdl3, as suggested by human data \cite{10, 11}, we would expect to find changes in Ormdl3 levels. The Zpbp2 deletion did not alter the liver \textit{Nr1d1} expression profile. However, Ormdl3 RNA levels were higher in the KO mice compared to controls (Fig 4C) (\(p<0.005\) and \(p<0.0001\), for the effects of time and genotype, respectively, two-way ANOVA).

The Zpbp2 deletion is associated with changes in the regulation of \textit{Nr1d1} in lungs and ileum

In lungs, Zpbp2 levels changed with time (\(p<0.05\), one-way ANOVA), whereas Ormdl3 levels did not show time-dependent oscillations. Our WT mice had two Zpbp2 peaks during the day. \textit{Nr1d1} was expressed rhythmically with a peak during the day (ZT6-ZT10) and low expression at night (ZT18-ZT22). KO mice had lower \textit{Nr1d1} expression at ZT10 (\(p<0.05\), two-way ANOVA followed by multiple comparisons and Sidak’s correction for multiple testing) (Fig 4C). The effect of the deletion on ZT10 \textit{Nr1d1} levels was confirmed in an independent set of WT and KO samples (\(p<0.0001\), t-test statistics). KO mice also had a minor albeit statistically significant increase in Ormdl3 RNA levels (\(d=0.73, p<0.05\), for the effect of genotype, two-way ANOVA) (Fig 4C and S2 Fig).

In ileum, no circadian oscillations in expression of Zpbp2 or Ormdl3 were observed. KO mice had \textasciitilde25% lower Ormdl3 levels compared to WT mice (\(d=0.92, p<0.05\) for the effect of genotype, two-way ANOVA) (Fig 4C and S2 Fig). \textit{Nr1d1} levels were lower at ZT10 in KO mice compared to WT controls, similar to the decrease observed in lungs. Lower ZT10 \textit{Nr1d1} levels in KO mice were confirmed in an independent set of mice (\(p<0.005\), t-test statistics) (Fig 4C).

The Zpbp2 deletion impacts the PPAR and cytokine signalling pathways in the mouse lung

Deletion of Zpbp2 changes the expression profile of the core clock gene \textit{Nr1d1} in lungs. We therefore expected that differences between the lung transcriptomes of KO and WT mice would affect multiple genes and pathways regulated by the circadian clock. To identify the pathways affected by the Zpbp2 deletion in lungs at two different time points, RNA-seq was performed. At ZT7, 40 differentially expressed genes (DEGs) with absolute fold difference | logFC| \(\geq 1.5\) and adjusted p-value \(< 0.05\) were identified and used for pathway analysis (S2 Table). At ZT10, 42 DEGs were identified and used for pathway analysis (Fig 5, S2 Table). The deletion influenced genes in the PPAR, adipocytokine and chemokine signalling pathways (Table 1).
Fig 4. The Zpbp2 deletion alters expression profiles of Ormdl3 and Nr1d1. A. The human ZPBP2 region harbors several regulatory features, i.e. enhancer marks H3K4me and H3K27ac, DNAse I hypersensitive sites, and regulatory polymorphisms that influence expression of ORMDL3 and GSDMB. All features are shown in the context of the UCSC genome browser (hg38) (https://genome.ucsc.edu). B. The mouse Zpbp2 region. The deletion (red box) and highly methylated region (black box) of the Zpbp2 promoter overlap with DNAse I hypersensitive sites in liver, lungs and small intestines (ileum) similar to the enrichment of enhancer marks in the human ZPBP2 region. All features are shown in the context of the UCSC genome browser (mm9) (https://genome.ucsc.edu). C. Diurnal profiles of gene expression levels in mouse liver, lungs, and ileum. Expression levels of Zpbp2, Ormdl3 and Nr1d1 were determined in WT and KO mice at 6 time points (n = 3 per time point) and normalized to housekeeping gene Eef2 and the expression maximum in WT mice was set to 1. Error bars for diurnal expression levels show standard error of the mean (SEM). Nr1d1 expression differences between the KO and WT mice at ZT10 were validated in an independent set of mice and average expression in WT mouse was set at 1. Error bars show standard deviation (SD). Significant differences in expression levels between WT and Zpbp2 KO mice are indicated by asterisks: * p<0.05, ** p<0.01, *** p<0.001.

https://doi.org/10.1371/journal.pone.0223212.g004
Discussion

We demonstrate that in the mouse Zpbp2 and Ormdl3 are regulated by the circadian clock in a tissue-specific fashion. Moreover, deletion of part of the mouse Zpbp2 gene that removes the region orthologous to the human cis-regulatory region is associated with significant changes in the diurnal expression profile of the core clock gene Nr1d1 in lungs and ileum, but not liver. Nr1d1 encodes a transcription factor that influences regulation of thousands of genes throughout the genome [26, 45]. Therefore, by modifying Nr1d1 expression the Zpbp2 deletion affects

Table 1. Biological pathways affected by the Zpbp2 deletion in mouse lungs.

| ZT7          | ZT10                                    |
|--------------|-----------------------------------------|
| mmu03320: PPAR signaling pathway | mmu04062: Chemokine signaling pathway  |
| mmu04920: Adipocytokine signaling pathway | mmu04060: Cytokine-cytokine receptor interaction |
| mmu04010: MAPK signaling pathway     | mmu04713: Circadian entrainment        |
| mmu04915: Estrogen signaling pathway  | mmu04152: AMPK signaling pathway       |

https://doi.org/10.1371/journal.pone.0223212.t001
expression of multiple genes in the lungs. At ZT7, PPAR signaling, which is known to play a critical role in aligning the circadian clock and lipid metabolism, is one of the most pronounced changes. This is particularly interesting since the Zpbp2 KO mice are prone to obesity as they age and show changes in sphingolipid metabolism, i.e. lower levels of sphingosine-1-phosphate (SIP) and very long chain ceramides [29]. Reduced levels of SIP are consistent with the increase in the expression of Ormdl3, which is an inhibitor of sphingolipid metabolism. At ZT10, Zpbp2 KO mice have higher expression levels of genes involved in chemokine signaling, inflammation and immunity, i.e. processes that are intertwined with circadian rhythms (reviewed in [24]). NR1D1 is a transcriptional repressor and controls cytokine production and inflammation [24, 46, 47]. It is therefore plausible that a modest decrease in Nr1d1 levels in Zpbp2 KO mice is associated with higher expression of several immunity-related genes, e.g. interferon regulatory factor 4 (Irf4), C-C motif chemokine ligand 8 (Ccl8), toll-like receptor 13 (Tlr13), C-X-C motif chemokine ligand 9 (Cxcl9), and chemokine (C-X3-C motif) receptor 1 (Cx3cr1). Several orthologs of genes implicated in susceptibility to inflammatory conditions in humans, e.g. solute carrier family 11 (proton-coupled divalent metal ion transporters), member 1 (Slc11a1, also known as Nramp) and amphiregulin (Areg) are among the ZT10 DEGs. It is also worth keeping in mind that we used rather stringent criteria for DEG selection. Including genes with smaller than 3-fold difference in expression levels dramatically increases the number of DEGs (Fig 5A and 5B).

Several lines of evidence suggest that the human ZPBP2 region harbors an enhancer and a CTCF-binding site whose functions are modified by genetic variants associated with predisposition to asthma and several other chronic inflammatory diseases [10, 11]. Genetic variants in the ZPBP2 gene are also associated with changes in the expression levels of ORMDL3 in human cells [10, 11]. Our data support the role of the ZPBP2 region in the regulation of ORMDL3, as deletion of the mouse orthologous region is associated with changes in Ormdl3 RNA levels in mouse liver, lungs and ileum. Moreover, the deletion has opposite effects in liver and ileum, i.e. leads to upregulation of Ormdl3 in liver and reduced expression in ileum. Our data show that the Zpbp2 region is involved in the regulation of Nr1d1. These findings are novel and demonstrate the importance of the inclusion of the time axis in GWAS follow-up studies, including eQTL analyses and mouse model-based experiments. Our data are consistent with published results that show chromatin interactions between the Zphp2 and Nr1d1 genic regions [28] and data from 4C-seq experiments done by other groups that demonstrate interactions between the Nr1d1 “super-enhancer” and the Zpbp2 and Ormdl3 regions [48]. This may explain why this deletion leads to changes in the diurnal expression profile of Nr1d1, which is located 200 kb away.

Remarkably, the Zpbp2 deletion altered Nr1d1 expression levels only at one of the tested time points, at ZT10. We speculate that such a time specificity points to a time-dependent interaction between the Zpbp2 and Nr1d1 genes. Interestingly, levels of the CLOCK protein and its association with chromatin peak at ZT10 [27, 49]. Given that Nr1d1 transcription is tightly regulated by the CLOCK/BMAL1 complex [50], it is possible that the effect of the deletion on Nr1d1 expression is mediated through changes in CLOCK binding. Whether the deletion changes the configuration of chromatin loops or it is the Zpbp2 gene product that is critical for Nr1d1 regulation will have to be determined in future studies.

To detect a time-specific change in the human, one would have to analyze NR1D1 expression in both genotype- and time-dependent fashion in different cell types. Therefore, we speculate that lack of a significant cis-effect of the ZPBP2 rs11557467 genotype on NR1D1 expression in human LCLs as shown here as well as in GTEx data [51] is insufficient to refute the hypothesis of regulatory interactions between the two genes in human cells and that genetic variants influence the NR1D1 expression profile.
Our recent studies found interaction between 17q12-q21 alleles, DNA methylation levels, sex, and asthma with genetic association being more pronounced in males and DNA methylation of the \textit{ZPBP2} promoter region being associated with reduced risk of asthma in females [52, 53]. Therefore, we asked if sex was also a modifier of gene expression levels. In human LCLs, we observed a sex bias in the levels of \textit{ORMDL3} (higher in females) and \textit{ZPBP2} (higher in males), but not \textit{NR1D1}. In mice, no evidence for sex bias in the regulation of \textit{Ormdl3}, \textit{Zpbp2}, or \textit{Nr1d1} was found ([29] and present study). The ensemble of published works suggests that daVs and variation in DNA methylation influence transcriptional regulation of several genes residing in the 17q12-q21 region with \textit{ORMDL3} and \textit{GSDMB} having been investigated in greater detail as potential disease gene candidates (reviewed in [54]). Based on our new data, we propose that \textit{NR1D1} should be added to the list of gene candidates underlying the GWAS associations in the \textit{ZPBP2-ORMDL3} region. Further studies in humans are necessary to test this hypothesis and clarify the importance of diurnal variation for the genetic association between 17q12-q21 alleles and disease.

**Supporting information**

\textbf{S1 Fig.} Diurnal oscillations in the expression levels of \textit{Nr1d1}, \textit{Ormdl3}, and \textit{Zpbp2} in the (A) distal colon and (B) liver of mice housed in LD (light/dark) conditions (data from [42, 43]). (TIF)

\textbf{S2 Fig.} \textit{Ormdl3} expression levels in KO and WT mice in different organs. The y-axis shows \textit{Ormdl3} RNA levels normalized by \textit{Eef2}. Error bars represent SD. Significant differences in expression levels between WT and \textit{Zpbp2} KO mice are indicated by asterisks * \(p<0.05\), **** \(p<0.0001\). (TIF)

\textbf{S1 Table.} Primers used for qPCR experiments (5'- 3'). (DOCX)

\textbf{S2 Table.} List of lung differentially expressed genes and transcripts. (DOCX)

**Acknowledgments**

The authors are grateful to Nicolas Cermakian for helpful discussions and critical review of the manuscript and to Donovan Chan for help with certain bioinformatics tools.

**Author Contributions**

**Conceptualization:** Anna K. Naumova.

**Formal analysis:** Matthew L. Chang, Sanny Moussette, Enrique Gamero-Estevez, José Héctor Galvèz, Anna K. Naumova.

**Funding acquisition:** Anna K. Naumova.

**Investigation:** Matthew L. Chang, Sanny Moussette, Enrique Gamero-Estevez, Victoria Chiwara, Anna K. Naumova.

**Methodology:** Matthew L. Chang, Sanny Moussette.

**Resources:** Aimee K. Ryan.
Supervision: Indra R. Gupta, Aimee K. Ryan, Anna K. Naumova.

Validation: Matthew L. Chang, Sanny Moussette.

Writing – original draft: Anna K. Naumova.

Writing – review & editing: Matthew L. Chang, Sanny Moussette, Enrique Gamero-Estevez, José Héctor Gálvez, Victoria Chiwara, Indra R. Gupta, Aimee K. Ryan.

References

1. Moffatt MF, Kabesch M, Liang L, Dixon AL, Strachan D, Heath S, et al. Genetic variants regulating ORMDL3 expression contribute to the risk of childhood asthma. Nature. 2007; 448(7152):470–3. https://doi.org/10.1038/nature06014 PMID: 17611496

2. Moffatt MF, Gut IG, Demenais F, Strachan DP, Bouzigon E, Heath S, et al. A large-scale, consortium-based genomewide association study of asthma. N Engl J Med. 2010; 363(13):1211–21. https://doi.org/10.1056/NEJMoa0906312 PMID: 20860503

3. Ellinghaus D, Jostins L, Spain SL, Cortes A, Bethune J, Han B, et al. Analysis of five chronic inflammatory diseases identifies 27 new associations and highlights disease-specific patterns at shared loci. Nat Genet. 2016; 48(5):510–8. https://doi.org/10.1038/ng.3528 PMID: 26974007

4. Qiu F, Tang R, Zuo X, Shi X, Wei Y, Zheng X, et al. A genome-wide association study identifies six novel risk loci for primary biliary cholangitis. Nature communications. 2017; 8:14828. https://doi.org/10.1038/ncomms14828 PMID: 28425483

5. Stahl EA, Raychaudhuri S, Remmers EF, Xie G, Eyre S, Thomson BP, et al. Genome-wide association study meta-analysis identifies seven new rheumatoid arthritis risk loci. Nat Genet. 2010; 42(6):508–14. https://doi.org/10.1038/ng.582 PMID: 20453842

6. Kurreeman FA, Stahl EA, Okada Y, Liao K, Diogo D, Raychaudhuri S, et al. Use of a multiethnic approach to identify rheumatoid arthritis-susceptibility loci, 1p36 and 17q12. Am J Hum Genet. 2012; 90(3):524–32. https://doi.org/10.1016/j.ajhg.2012.01.010 PMID: 22365150

7. Lafer VA, Tiwari HK, Reynolds RJ, Danila MI, Wang J, Edberg JC, et al. Genetic Influences on Susceptibility to Rheumatoid Arthritis in African-Americans. Hum Mol Genet. 2019, 28(5):858–874. https://doi.org/10.1038/s41419-018-0120-8 PMID: 30423114

8. Solovieff N, Cotsapas C, Lee PH, Purcell SM, Smoller JW. Pleiotropy in complex traits: challenges and strategies. Nature reviews Genetics. 2013; 14(7):483–95. https://doi.org/10.1038/nrg3461 PMID: 23752797

9. Ge B, Pokholok DK, Kwan T, Grundberg E, Morcos L, Verlaan DJ, et al. Global patterns of cis variation in human cells revealed by high-density allelic expression analysis. Nat Genet. 2009; 41(11):1216–22. https://doi.org/10.1038/ng.473 PMID: 19838192

10. Verlaan DJ, Berlivet S, Hunninghake GM, Madore AM, Lariviere M, Moussette S, et al. Allele-specific chromatin remodeling in the ZPB2/GSDMB/ORMDL3 locus associated with the risk of asthma and autoimmune disease. Am J Hum Genet. 2009; 85(3):377–93. https://doi.org/10.1016/j.ajhg.2009.08.007 PMID: 19732864

11. Berlivet S, Moussette S, Ouimet M, Verlaan DJ, Koka V, Al Tuwaijri A, et al. Interaction between genetic and epigenetic variation defines gene expression patterns at the asthma-associated locus 17q12-q21 in lymphoblastoid cell lines. Hum Genet. 2012; 131(7):1161–71. https://doi.org/10.1007/s00439-012-1142-x PMID: 22271045

12. Moussette S, Al Tuwaijri A, Kohan-Ghadr HR, Elzein S, Farias R, Berube J, et al. Role of DNA methylation in expression control of the IKZF3-GSDMA region in human epithelial cells. PLoS One. 2017; 12 (2):e0172707. https://doi.org/10.1371/journal.pone.0172707 PMID: 28210653

13. Schmiedel BJ, Seumois G, Samaniego-Castriuata D, Cayford J, Schulten V, Chavez L, et al. 17q21 asthma-risk variants switch CTCF binding and regulate IL-2 production by T cells. Nature communications. 2016; 7:13426. https://doi.org/10.1038/ncomms13426 PMID: 27848966

14. Worgall TS, Veerappan A, Sung B, Kim BI, Weiner E, Bholah R, et al. Impaired sphingolipid synthesis in the respiratory tract induces airway hyperreactivity. Science translational medicine. 2013; 5 (186):166ra67. https://doi.org/10.1126/scitranslmed.3005765 PMID: 23693880

15. Espalat MP, Kew RR, Obeid LM. Sphingolipids in neutrophil function and inflammatory responses: Mechanisms and implications for intestinal immunity and inflammation in ulcerative colitis. Advances in biological regulation. 2017; 63:140–55. https://doi.org/10.1016/j.ibior.2016.11.001 PMID: 27866974
16. Zhakupova A, Debeuf N, Krols M, Toussaint W, Vanhoucke L, Alecu I, et al. ORMDL3 expression levels have no influence on the activity of serine palmitoyltransferase. FASEB J. 2016; 30(12):4289–300. https://doi.org/10.1096/fj.201600398 PMID: 27645259

17. Kelly RS, Chawes BL, Bligh K, Virkud YV, Croteau-Chonka DC, McGeachie MJ, et al. An Integrative Transcriptomic and Metabolomic Study of Lung Function in Children With Asthma. Chest. 2018; 154(2):335–48. https://doi.org/10.1016/j.chest.2018.05.038 PMID: 29908154

18. Siow D, Sunkara M, Dunn TM, Morris AJ, Wattenberg B. ORMDL3/serine palmitoyltransferase stoichiometry determines effects of ORMDL3 expression on sphingolipid biosynthesis. Journal of lipid research. 2015; 56(4):898–908. https://doi.org/10.1194/jlr.M057539 PMID: 25691431

19. Miller M, Rosenthal P, Beppu A, Mueller JL, Hoffman HM, Tam AB, et al. ORMDL3 Transgenic Mice Have Increased Airway Remodeling and Airway Responsiveness Characteristic of Asthma. J Immunol. 2014; 192(8):3475–87. https://doi.org/10.4049/jimmunol.1303047 PMID: 24623133

20. Löser S, Gregory LG, Zhang Y, Schaefer K, Walker SA, Buckley J, et al. Pulmonary ORMDL3 is critical for induction of Alternaria induced allergic airways disease. J Allergy Clin Immunol. 2017, 139(5):1496–1507. https://doi.org/10.1016/j.jaci.2016.07.033 PMID: 27623174

21. Miller M, Tam AB, Mueller JL, Rosenthal P, Beppu A, Gordillo R, et al. Cutting Edge: Targeting Epithelial ORMDL3 Increases, Rather than Reduces, Airway Responsiveness and Is Associated with Increased Sphingosine-1-Phosphate. J Immunol. 2017; 198(8):3017–22. https://doi.org/10.4049/jimmunol.1601848 PMID: 28275141

22. Debeuf N, Zhakupova A, Steiner R, Van Gassen S, Deswarte K, Fayazpour F, et al. The ORMDL3 asthma susceptibility gene regulates systemic ceramide levels without altering key asthma features in mice. J Allergy Clin Immunol. 2019 (in press).

23. Bass J, Takahashi JS. Circadian integration of metabolism and energetics. Science. 2010; 330(6009):1349–54. https://doi.org/10.1126/science.1195027 PMID: 21127246

24. Labrecque N, Cermakian N. Circadian Clocks in the Immune System. Journal of biological rhythms. 2015; 30(4):277–90. https://doi.org/10.1177/0748730415577723 PMID: 25900041

25. Preitner N, Damiafo N, Lopez-Molina L, Zakany J, Duboule D, Albrecht U, et al. The orphan nuclear receptor REV-ERBalpha controls circadian transcription within the positive limb of the mammalian circadian oscillator. Cell. 2002; 110(2):251–60. https://doi.org/10.1016/s0092-8674 (02)00825-5 PMID: 12150932

26. Feng D, Liu T, Sun Z, Bugge A, Mullican SE, Alenghat T, et al. A circadian rhythm orchestrated by histone deacetylase 3 controls hepatic lipid metabolism. Science. 2011; 331(6022):1315–9. https://doi.org/10.1126/science.1198125 PMID: 21393543

27. He B, Chen C, Teng L, Tan K. Global view of enhancer-promoter interactome in human cells. Proc Natl Acad Sci U S A. 2014; 111(2):E2191–9. https://doi.org/10.1073/pnas.1320308111 PMID: 24821768

28. Kanagaratham C, Chiwara V, Ho B, Moussete S, Youssef M, Venuto D, et al. Loss of the zona pellucida-binding protein 2 (Zpbp2) gene in mice impacts airway hypersensitivity and lung lipid metabolism in a sex-dependent fashion. Mamm Genome. 2018; 29(3–4):281–98. https://doi.org/10.1007/s00335-018-9743-x PMID: 29536159

29. Lin YN, Roy A, Yan W, Burns KH, Matzuk MM. Loss of zona pellucida binding proteins in the acrosomal matrix disrupts acrosome biogenesis and sperm morphogenesis. Mol Cell Biol. 2007; 27(19):6794–805. https://doi.org/10.1128/MCB.01029-07 PMID: 17664265

30. Bourgey M, Dali R, Eveleigh R, Chen KC, Letournau L, Dillon J, et al. GenPipes: an open-source framework for distributed and scalable genomic analyses. GigaScience. 2019; 8(6).

31. 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. https://doi.org/10.1093/bioinformatics/bts635 PMID: 23104886

32. Anders S, Pyl PT, Huber W. HTSeq—a Python framework to work with high-throughput sequencing data. Bioinformatics. 2015; 31(2):166–9. https://doi.org/10.1093/bioinformatics/btu638 PMID: 25260700

33. 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. https://doi.org/10.1093/ bioinformatics/btp616 PMID: 19910308

34. Anders S, Huber W. Differential expression analysis for sequence count data. Genome Biol. 2010; 11(10):R106. https://doi.org/10.1186/gb-2010-11-10-r106 PMID: 20979621
36. 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. https://doi.org/10.1038/nprot.2008.211 PMID: 19131956

37. Christou S, Wehrens SMT, Ishenwood C, Moller-Levet CS, Wu H, Revell VL, et al. Circadian regulation in human white adipose tissue revealed by transcriptome and metabolic network analysis. Scientific reports. 2019; 9(1):2681. https://doi.org/10.1038/s41598-019-39688-3 PMID: 30800433

38. Wu G, Ruben MD, Schmidt RE, Franscy LJ, Smith DF, Anafi RC, et al. Population-level rhythms in human skin with implications for circadian medicine. Proc Natl Acad Sci U S A. 2018; 115(48):12313–8. https://doi.org/10.1073/pnas.1809442115 PMID: 30377266

39. Kervezee L, Cuesta M, Cermakian N, Boivin DB. Simulated night shift work induces circadian misalignment of the human peripheral blood mononuclear cell transcriptome. Proc Natl Acad Sci U S A. 2018; 115(21):E5540–5. https://doi.org/10.1073/pnas.1720719115 PMID: 29735673

40. Archer SN, Laing EE, Moller-Levet CS, van der Veen DR, Bucca G, Lazar AS, et al. Mistimed sleep disrupts circadian regulation of the human transcriptome. Proc Natl Acad Sci U S A. 2014; 111(6):E682–91. https://doi.org/10.1073/pnas.1316335111 PMID: 24498876

41. Zhang Y, Fang B, Emmett MJ, Damle M, Sun Z, Feng D, et al. GENE REGULATION. Discrete functions of nuclear receptor Rev-erbalpha couple metabolism to the clock. Science. 2015; 348(6242):1488–92. https://doi.org/10.1126/science.aab3021 PMID: 26044300

42. Gibbs JE, Blakley J, Beesley S, Matthews L, Simpson KD, Boyce SH, et al. The nuclear receptor REV-ERBalpha mediates circadian regulation of innate immunity through selective regulation of inflammatory cytokines. Proc Natl Acad Sci U S A. 2012; 109(2):582–7. https://doi.org/10.1073/pnas.1106750109 PMID: 22184247

43. Wang S, Lin Y, Yuan X, Li F, Guo L, Wu B. REV-ERBalpha integrates colon clock with experimental colitis through regulation of NF-kappaB/NLRP3 axis. Nature communications. 2018; 9(1):2446. https://doi.org/10.1038/s41467-018-06585-6

44. Kojima H, Nishida Y, Harada N, Seki M, Nakajima S, et al. Long-Range Chromosome Interactions Mediated by Cohesin Shape Circadian Gene Expression. PLoS Genet. 2016; 12(5):e1005992. https://doi.org/10.1371/journal.pgen.1005992 PMID: 27135601

45. Partch CL, Green CB, Takahashi JS. Molecular architecture of the mammalian circadian clock. Trends in cell biology. 2014; 24(2):90–9. https://doi.org/10.1016/j.tcb.2013.07.002 PMID: 23916625

51. Human genomics. The Genotype-Tissue Expression (GTEx) pilot analysis: multitissue gene regulation in humans. Science. 2015; 348(6235):648–60. https://doi.org/10.1126/science.1262110 PMID: 25954001

52. Naumova AK, Al Tuwaijri A, Morin A, Vailancourt VT, Madore AM, Berlit S, et al. Sex- and age-dependent DNA methylation at the 17q12-q21 locus associated with childhood asthma. Hum Genet. 2013; 132(7):811–22. https://doi.org/10.1007/s00439-013-1298-z PMID: 23546690

53. Al Tuwaijri A, Gagne-Ouellet V, Madore AM, Laprise C, Naumova AK. Local genotype influences DNA methylation in two asthma-associated regions, 5q31 and 17q21, in a founder effect population. Journal of medical genetics. 2016; 53(4):232–41. https://doi.org/10.1136/jmedgenet-2015-103313 PMID: 26671913

54. Stein MM, Thompson EE, Schoettler N, Helling BA, Magnaye KM, Stanhope C, et al. A decade of research on the 17q12-21 asthma locus: Piecing together the puzzle. J Allergy Clin Immunol. 2018, 142(3):749–764. https://doi.org/10.1016/j.jaci.2017.12.974 PMID: 29307657