NUFFIELD DEPARTMENT OF
CLINICAL NEUROSCIENCES
Medical Sciences Division

Investigating the molecular role of transcription factor MAFG in circadian rhythm regulation

Sejal Kapoor

1490817

Somerville College

Supervisor: Professor Aarti Jagannath

Sleep and Circadian Neuroscience Institute
Nuffield Department of Clinical Neurosciences

University of Oxford

Date: 17th October, 2022

word count:12,600

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Acknowledgements

I would like to begin by thanking my supervisor, Professor Aarti Jagannath. Thank you for allowing me to join your group and for your exceptional support and advice every step of the way. Your knowledge, enthusiasm and attention to detail have been an inspiration, and I have learnt so much throughout my time in the lab.

Next, I'd like to thank the entire Jagannath lab for their input, time, effort and support without which this report would not have been possible. Lewis, Anna and Simona, thank you for patiently putting up with my constant questions about everything, for helping with the data collection, and most importantly, for your invaluable advice.

Finally, I'm eternally grateful to my parents, and my brother, Sujoy. Thank you, Mum and Dad, for the constant love and support that you have given me throughout my life and for pushing me to do my best always. This endeavour would not have been possible without you.

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Abstract

The molecular mechanisms underlying circadian photoentrainment are complex, comprising several regulatory factors. There is evidence to suggest differential chromatin accessibility in the master clock, SCN, in response to light at different times of the day. Several genes identified close to differential regions of chromatin accessibility are already known to play an important role within the molecular clock and are recognized to be important for entrainment. Previous studies in the lab using ATAC-seq (Assay for Transposase Accessible Chromatin) showed that chromatin near the gene *Mafg* (MAF BZIP Transcription Factor G) closed following a nocturnal light pulse (LP), indicating repression of the gene. Additionally, the response element to which MAFG binds, which is the AP-1 motif, was highly over-represented in the light regulated accessible chromatin profile, leading us to hypothesise that MAFG, that is part of the AP-1 transcription factor family, regulates photoentrainment through the regulation of AP-1.

To investigate this, I conducted *in vitro* and *in vivo* analyses to evaluate MAFG function in circadian entrainment. Sections of the *Mafg* promoter that responded by closing in response to light were cloned into a luciferase reporter plasmid and transfected into U2OS cells. Forskolin (which simulates a LP via the AP-1 pathway) reduced reporter expression, and this was further reduced in cells with siRNA-mediated *Mafg* knockdown. Furthermore, knockdown of *Mafg* in BMAL1:Luc U2OS circadian reporter cells showed a reduction in amplitude. *In vivo*, the baseline circadian phenotype, and photic entrainment phenotype of *Mafg* knockout mice was studied by housing *Mafg*+/+ and *Mafg*-/- mice in strict light/dark cycles. Periodogram analysis showed reduced amplitude of circadian period in knockout mice, suggesting that they have weak & fragmented rhythms. *Mafg* knockouts also showed slower re-entrainment in a 6-hour phase-advancing jetlag study and reduced phase shifting in response to a nocturnal LP (CT 16), indicating reduced photic responses in *Mafg* -/- animals. However, qPCR analysis of gene expression in the SCN isolated from CT16 light pulsed *Mafg*+/+ and *Mafg*-/- mice shows very little difference in a subset of the light regulated genes assessed between the genotypes. In summary, a novel role for MAFG in regulating circadian photoentrainment was shown, however discrepancies in the mechanism of action remain to be addressed.

1. Introduction

Almost all life on earth has evolved around a predictable and continuous light/dark cycle, leading to many cellular processes oscillating over an approximate 24 hour period (Roenneberg and Merrow, 2002). This gives an individual their circadian rhythm, which enables them to predict light/dark cycles, preparing them for environmental factors occurring at different times of the day. Circadian rhythms are controlled by the molecular clock (Hardin et al., 1990), a complex transcriptional-translational feedback loop (TTFL), which in addition to controlling the passage of time at the molecular level, governs the expressions of countless clock controlled genes (CCGs).

The intricate functioning of the molecular clock is now well understood, however, there still remain many gaps in our understanding of how it is entrained to the external environment, and how exposure to these external factors influences gene expression.

This project aims to explore the underlying molecular mechanisms that directly control the molecular clocks of cells within the suprachiasmatic nucleus (SCN), the central pacemaker of the circadian system; taking into account how light input has a direct effect on molecular clock gene expression within the SCN. In particular, the role of the transcription factor MAFG in regulating photo entrainment is investigated, both on the molecular and behavioural level.

1.1 The core mammalian molecular clock

The circadian molecular clock is the core TTFL, with a period of around 24 hours, consisting of four clock proteins: Circadian locomotor output cycles kaput (CLOCK), Brain and muscle ARNT like protein 1 (BMAL1), Period (PER) and Cryptochrome (CRY) (Buhr and Takahashi, 2013). BMAL1 and CLOCK heterodimerize (Huang et al., 2012), and act as the positive arm by binding to E-box motifs (CACGTG) in CCGs, including *Per1* and *Cry1* (Figure 1.1). *Per* and *Cry* protein products in turn act as the negative arm by attenuating the action of

CLOCK/ BMAL1, therefore completing the loop. It is the rate of accumulation and subsequent degradation of these proteins that determines the period of circadian oscillation.

The TTFL loops are further complemented by an ancillary loop, consisting of two nuclear receptors REV-ERB (α/ß) and retinoic acid related orphan receptor (ROR-α/ß/γ) (Akashi and Takumi, 2005); these are activated by CLOCK/BMAL1. REV-ERBα represses *Bmal1* transcription, however, in this loop it is counteracted by continuous activation of *Bmal1* by RORα. This alternate action of ROR and REV-ERB allows positive and negative regulation of *Bmal1,* respectively, resulting in the rhythmic expression of *Bmal1* (Figure 1.1).

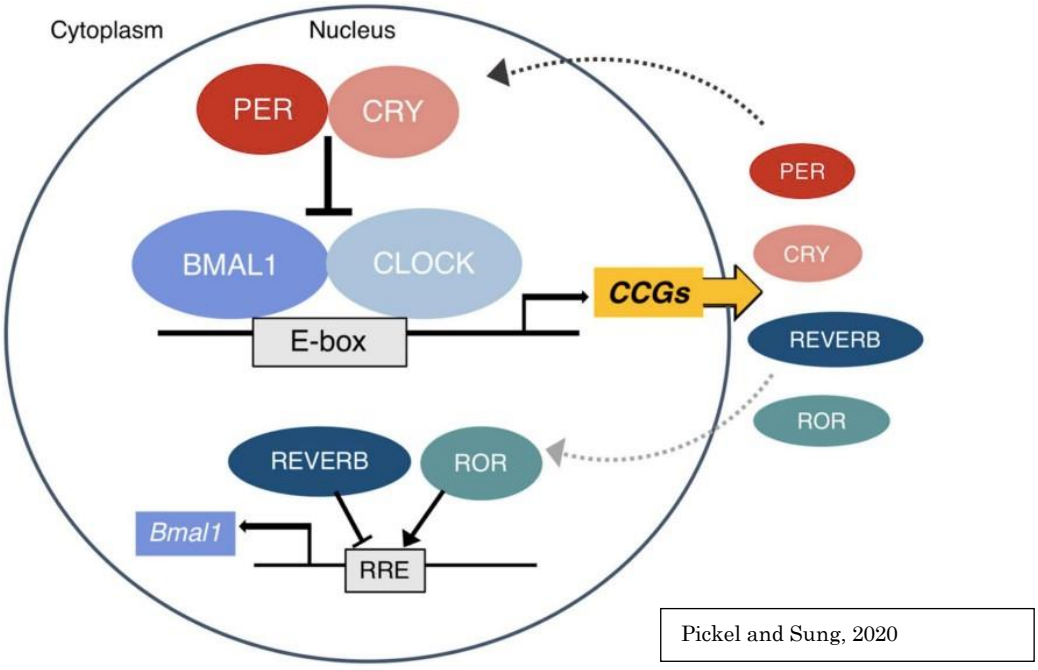


Figure 1.1 The core mammalian clock consisting of the transcriptional-translational feedback loop. BMAL1 and CLOCK heterodimerize to bind the E-box element to positively regulate *Per* and *Cry*. *Per* and *Cry* protein products in turn act as the negative arm by attenuating the action of CLOCK/ BMAL1. REV-ERB/ROR ancillary loop is also shown.

1.2 The synchrony of the circadian clock is important for human health

The BMAL1-CLOCK heterodimer controls the rhythmic expression of genes involved in physiological function, thereby causing processes such as sleep, metabolism, and the immune system to oscillate with a 24-hour period (Rijo-Ferreira and Takahashi, 2019). In fact, increasing evidence suggests that nearly half of all physiological processes follow a daily rhythm that is controlled by the circadian clock (Giri et al., 2021). Light is the clock's preeminent entraining cue (*Zeitgeber*), and the clock responds to light by shifting its phase. The effects of light vary depending on the time of light exposure (Ashton et al., 2022). The molecular clock generates periodic rhythms even in the absence of external stimuli such as light and temperature. Therefore, circadian rhythms will persist even when living in a cave without light or temperature cues. However, in an environment without *zeitgebers*, the circadian rhythm in humans will begin to free-run with a period slightly longer than 24 hours (Walker et al., 2020). Free-running refers to an endogenous rhythm that is not synchronized to the external physical environment (Vitaterna et al., 2001). In the absence of external cues, the human circadian rhythm may also become desynchronized with the external world, meaning that the timing of the biological clock is no longer aligned with the natural day-night cycle. This can result in a number of physiological and behavioural changes, including sleep disturbances, changes in body temperature, hormonal changes and impaired cognitive performance (Fishbein et al., 2021).

Therefore, maintaining synchrony between the internal circadian rhythm and the environment is essential for health and survival. For instance, in an extreme scenario, a nocturnal rodent leaving its burrow during the day due to an asynchronous internal clock risks becoming prey (Vitaterna et al., 2001). In humans, regular circadian rhythm disruption caused by jet lag, shift work, or sleep deprivation is implicated in health problems including obesity, diabetes, cancer, susceptibility to viral infections and Alzheimer's disease (Rijo-Ferreira and Takahashi, 2019).

There remains, however, a very basic understanding of the molecular mechanisms behind how the circadian oscillator, the SCN, entrains to environmental light. Therefore, there are few treatment options available to those individuals whose medical conditions or daily work/routines inevitably lead to circadian disruption. This is despite the fact that the rectification of circadian disruption in numerous cases, will greatly improve the symptoms of many mental illnesses, and more generally significantly improve the health of any individual. For instance, sleep and circadian rhythm disruption (SCRD) both hastens the onset and exacerbates the symptoms in neuropsychiatric conditions (Jagannath et al., 2013). Indeed, SCRD can be a prodromal symptom of bipolar disorder in high risk individuals (Rock et al., 2014) and the stabilisation of SCRD with behavioural therapy can reduce paranoid delusions in schizophrenia (Freeman et al., 2017). Therefore, the maintenance of sleep and circadian rhythms is critical for health.

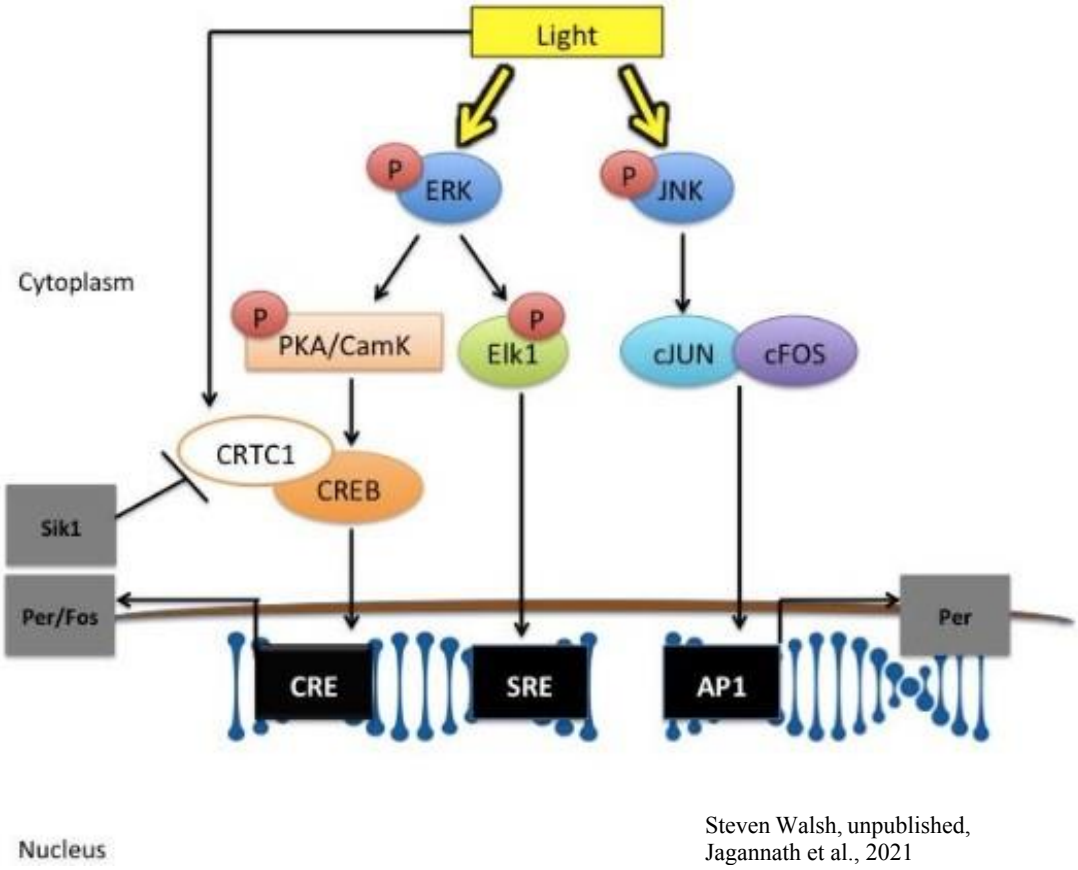
This project aims to explore the underlying molecular pathways that directly control the molecular clock of cells within the SCN by using U2OS cell models *in vitro* and mouse SCN *in vivo*. By gaining a greater understanding of the molecular mechanisms underpinning entrainment of circadian rhythms, it is hoped that new drug targets can be identified. This in-turn will lead to the development of new treatments, to correct circadian rhythm mis-alignment, ensuring correct synchronisation to the external environment.

1.3 Identification of MAFG as a candidate regulating entrainment

The molecular mechanisms underlying circadian photo-entrainment are complex, comprising several regulatory factors that regulate transcription in response to light. Cells of the SCN like all cells contain their own molecular clock. However, SCN neurons have the ability to entrain their own molecular clocks to the outside environment by synaptic input from the eyes via the retinohypothalamic tract (RHT). As previously described, *Per1/2* are core elements of the molecular 24h clock, but remarkably in the SCN, the transcription of *Per1/2* is also activated via

light input (Schwartz et al., 2011). Photic signalling via the RHT releases neurotransmitters, which activate several G-protein coupled receptor (GPCR) signalling pathways and results in an influx of Ca^{2+} and cAMP within SCN neurons. This leads to subsequent activation of two mitogen activated protein kinase (MAPK) pathways; extracellular-signal regulated kinase (ERK) and c-JUN NH2-terminal kinase (JNK) (Obrietan et al., 1998). Phosphorylated ERK activates protein kinase A (PKA) and calmodulin dependent kinase (CamK) which in turn phosphorylates CREB (Lonze and Ginty, 2002), allowing it to bind to CRE elements in promoters of *Per1* and *Fos* to induce their expression. In parallel, ERK also activates ELK-1, which binds to serum response elements (SRE) in promoters of *Per1* and *Fos* to induce their expression independent of CREB.

On the other hand, phosphorylation via the JNK pathway leads to activation of JUN proteins, which heterodimerize with cFOS to form the activating protein-1 (AP-1) transcription factor complex (Halazonetis et al., 1988). This AP-1 factor induces *Per1* expression by binding to the AP-1 binding site within the *Per1*/2 promoters (Kornhauser et al., 1990).



Steven Walsh, unpublished,
Jagannath et al., 2021

Figure 1.3a Light induction of PER/FOS, in the SCN. This occurs via two pathways, either through CREB/ELK1 via ERK, or by the AP1 complex via JNK.

However, circadian entrainment is far more complex; indeed the above model suggests the potential for integration of multiple pathways affecting light regulated transcription. Entrainment mechanisms also allow for the modulation of pathways that provide input. As a key example, light does not phase shift the clock equally at all times (Ralph and Menaker, 1988); for a diurnal human the largest shifts occur in the morning at dawn, while for a nocturnal mouse it is mainly at dusk. Furthermore, other physiological factors such as sleep history also modulate the impact of light on the clock (Franken, 2013), allowing circadian timing to be fine-tuned to the multiple demands of the environment and internal physiology. Recent work in the lab showed an important role for adenosine in regulating circadian entrainment. Adenosine, as a key correlate of sleep need, signals directly to the SCN through A_{2A} and A_1 adenosine receptors to regulate circadian responses to light. This was shown to be through the Ca^{+2}-ERK-AP-1 and CREB/CRTC1-CRE pathways to regulate the clock genes *Per1* and *Per2* (Jagannath et al., 2021. It was seen that adenosine, that has been previously known to modulate Ca^{+2} release (Fredholm et al., 2001) (Fredholm et al., 2011), increased intracellular calcium to activate ERK1/2 via multiple pathways including RAS, the protein tyrosine kinase PYK2 and Calmodulin Kinase 1 (Schmitt et al., 2004). Furthermore, ERK-induced phosphorylation increased AP-1 signalling and c-*Jun* transcription, highlighting the important role of AP-1 in circadian regulation (Jagannath et al., 2021).

Interestingly, the induction of the core elements of AP-1, FOS and cJUN, have long been known to occur in response to light in the SCN (Abe et al., 1991), and so has AP-1 binding (Kornhauser et al., 1992), however, as *Fos* knockout mice appear to have little wrong with their photoentrainment phenotype (Honrado et al., 1996), it has been assumed the role of AP-1 complex is redundant. AP-1 complex can be

formed from a combination of over 20-30 factors which include isoforms of FOS, JUN, small MAFs and ATFs (figure 1.4) which dimerise, undergo posttranslational modifications and bind to specific response elements to set off transcription for various processes such as cell differentiation, transformation, immunity, inflammation and so on. It is unlikely that the removal of any 1, such as FOS, can result in a dramatic phenotype. While c-FOS activation is not an absolute requirement for rhythm generation nor photic responses, it is required for normal entrainment of the mammalian biological clock. In the absence of this gene, animals still generate circadian rhythms which respond to light in a manner that is close to normal, indicating that other pathways exist. Because *c-Fos* is one of several related genes whose protein products can dimerize with others as AP-1 DNA transcription factors, it is possible that without the c-FOS protein, other related proteins such as JUNB, or perhaps other biochemical pathways, may be present that can serve similar functions. During development, the circadian system of these animals may compensate for the absence of FOS by upregulating these mechanisms, possibly involving the superfamily of bZIP proteins (Kerppola and Curran 1994), many of which are expressed in the SCN in response to phase shifting light pulses. However, chromatin accessibility studies on diverse tissues (Xu et al., 2017, unpublished SCN work from our group) as well as promoter analysis of the light-regulated transcriptome (Jagannath et al., 2021) (Xu et al., 2021) have confirmed the role of AP-1 as a transcription factor and as core to the light response in the SCN.

In addition, the amount by which the phase of the clock can be shifted at any one time appears to be limited, with re-entrainment to shifted light dark cycles typically taking a number of days. For instance, the clock shifts by around 1 hour in one day when an LD cycle is changed, a 6-hr jetlag takes around 6 days for the clock to re-entrain to the new light dark cycle, and so on. Analysing the light-regulated transcriptome of the SCN identified a key role for Salt Inducible Kinase 1 (SIK1) and CREB-regulated transcription co-activator 1 (CRTC1) in entrainment of the clock. Light causes CRTC1 to co-activate CREB, inducing the expression of *Per1* and *Sik1*. SIK1 then inhibits further shifts of the clock by

phosphorylation and deactivation of CRTC1. Effectively, SIK1 acts as a brake on the molecular clock, preventing large and disrupting shifts of the network (Jagannath et al., 2013).

Given that the TTFL is conserved and functional in all cells, how it achieves temporal and tissue-specific regulation is an important question. It is hypothesised that one way this may be accomplished is by differential regulation of chromatin accessibility of clock protein binding motifs. Recent experiments in the Jagannath lab using Assay for Transposase-Accessible Chromatin sequencing have identified chromatin state changes in the mouse SCN when subjected to a light pulse at specific times of the day. It was discovered that among others such as *Csnk1e* and *Nfil3*, a gene called *Mafg* showed a substantial amount of chromatin that closed near to it when given a 30 minute light pulse at CT 16 (Figure 1.3d). This is interesting as MAFG (a small MAF in the MAF family), is a member of the larger AP-1 transcription factor family and is thus known to dimerise and bind both the AP-1 and the MAREI & MAREII motifs (MAREs are an extended version of CRE and TRE) (Kerppola and Curran, 1994). The dimerisation and binding of the AP-1 complex is explained in the next section. The AP-1 motif is also known to be enriched within the chromatin that was changing its state across the day (Figure 1.3e).

Mafg is ubiquitously expressed in the whole brain (Yue et al., 2014), however its expression within the SCN and circadian profile are not as well characterized. The gene was examined using databases such as SCNseq, Allen Mouse Brain Atlas and CircaDB. The SCNseq database provides gene expression data over a 24-hour cycle from SCN of mice maintained in cycles of 12 hours of light and 12 hours of dark, thereby including additional light driven gene expression (http://www.wgpembroke.com/shiny/SCNseq/) (Pembroke et al. 2015). Figure 1.3b from the SCNseq database (shows us that *Maf* may be rhythmically expressed – upregulated in the light period and downregulated in the dark period; however rhythmic information about *Mafg* in particular was not available. Figure 1.3c is an image obtained from the Allen Institute Mouse Brain Atlas (ABA) and is

available online at http://mouse.brain-map.org/ (Lein et al. 2007). It shows *Mafg* expression patterns in the whole brain and particularly in the dentate gyrus region of the hypothalamus.

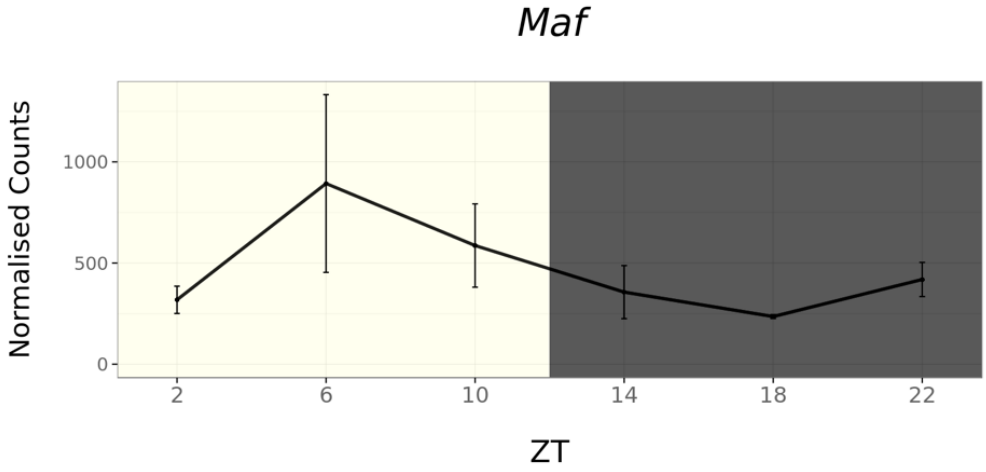


Figure 1.3b *Maf* gene expression in a 24-hour cycle from SCN of mice maintained in cycles of 12 hours of light and 12 hours of dark (obtained from SCNseq).

No information about *Mafg* specifically or transcription factor AP-1 gene expression at time intervals throughout the circadian cycle was available on CircaDB (http://circadb.hogeneschlab.org/) (Pizarro et al. 2013). However, RNA sequencing data after LP at CT17 for 30 minutes, 1hour, and 3hours respectively, showed an upregulation of *Maff,* that encodes the transcription factor MAFF in the same family as MAFG (Xu et al., 2021).

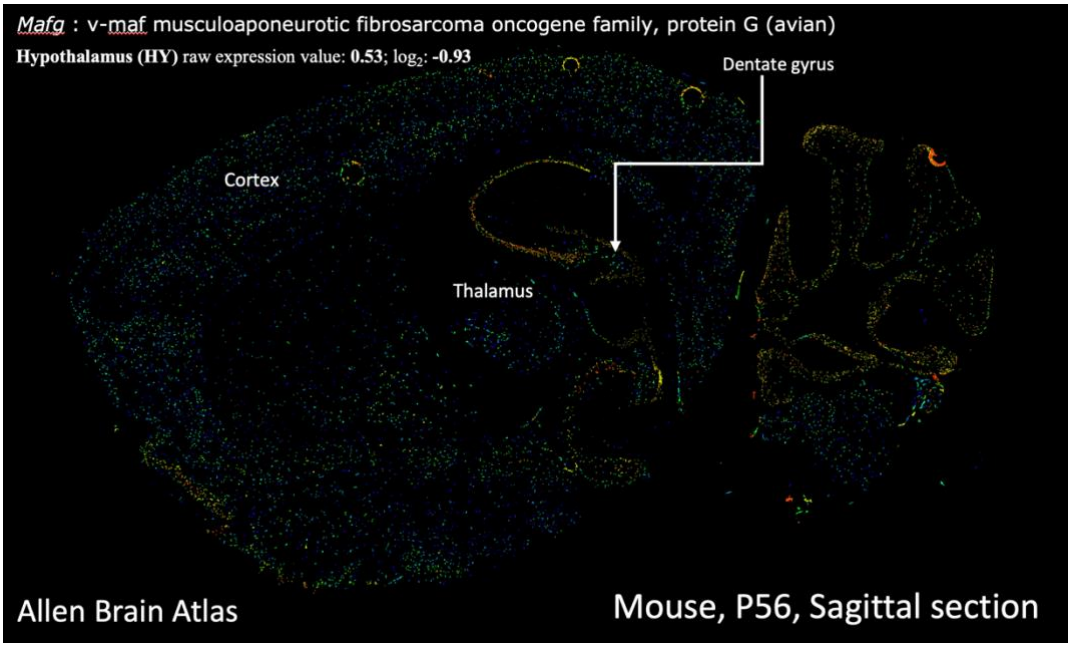


Figure 1.3c Sagittal section showing *Mafg* expression using in situ hybridisation in the adult mouse C57BL/6J brain, Age: 56 days, Sex: male, Orientation: antisense (obtained from Allen Mouse Brain Atlas).

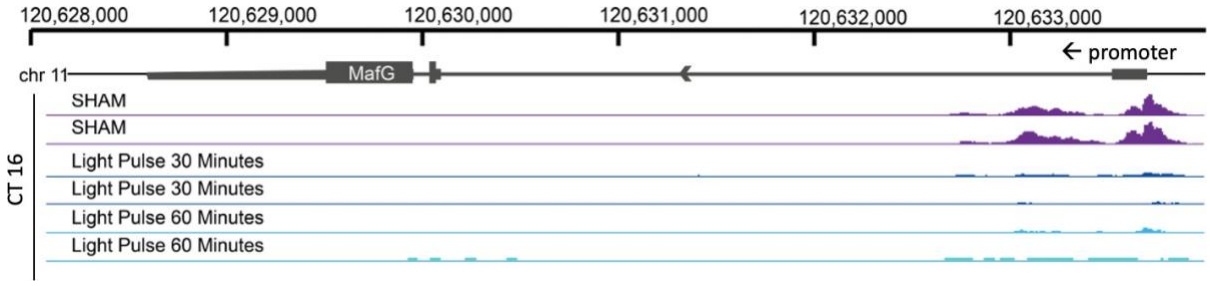


Figure 1.3d Read coverage tracks for *Mafg* from ATAC-seq light pulse experiment indicating that chromatin near to *Mafg* closes following a 30 minute and 60 minute light pulse at CT16. (Figure adapted from thesis of Steven Walsh).

Rank	Motif	P-value	log P-pvalue	% of Targets	% of Background	STD(Bg STD)	Best Match/Details	Motif File
1		1e-570	-1.315e+03	8.94%	0.58%	32.4bp (57.5bp)	BORIS(Zf)/K562-CTCFL-ChIP-Seq(GSE32465)/Homer(0.903) More Information | Similar Motifs Found	motif file (matrix)
2		1e-317	-7.315e+02	6.55%	0.68%	41.6bp (64.8bp)	Rfx1(HTH)/NPC-H3K4me1-ChIP-Seq(GSE16256)/Homer(0.928) More Information | Similar Motifs Found	motif file (matrix)
3		1e-175	-4.030e+02	8.25%	2.22%	50.2bp (62.3bp)	Jun-AP1(bZIP)/K562-cJun-ChIP-Seq(GSE31477)/Homer(0.991) More Information | Similar Motifs Found	motif file (matrix)

Figure 1.3e Known motifs identified within differential chromatin changing across the day including AP-1 whose TFs are involved with the molecular clock and important for entrainment in the SCN.

1.4 Hypothesis and Aims

Currently there is little association of MAFG with mechanisms underlying sleep and circadian rhythms. MAFG is recognised as a small-Maf (sMaf) protein that is characterised by a basic region for DNA binding and a leucine zipper (bZip) structure for dimer formation.

sMafs homodimerise, or heterodimerise with other specific bZIP transcription factors. The homodimers, bind to palindromic DNA sequences called Maf Recognition Element (MARE: TGCTGACTCAGCA) and similar sequences

(Kataoka et al., 1995) and it has been demonstrated that the basic region of a Maf factor recognizes the flanking GC sequences (Kurokawa et al., 2009). The middle parts of these Maf-binding sequences completely match with two binding sequences for AP-1 transcription factor, i.e., phorbol 12-O-tetradecanoate-13-acetate (TPA)-responsive element (TRE) and cyclic AMP responsive element, suggesting partial overlapping of the target genes for MAF and AP-1. Furthermore, MAF efficiently formed heterodimers with the components of AP-1, FOS and JUN, through their leucine zipper structures, and these heterodimers show binding specificities distinct from those for MAF-MAF and JUN-JUN homodimers. The reason for this is due to differences in the DNA-binding domains of these proteins. JUN contains a basic region and a leucine zipper domain that allow it to form homodimers or heterodimers with other AP-1 family members. In contrast, small MAF proteins have a different DNA-binding domain that recognizes the MARE sequence. Therefore, the binding specificity of small MAF proteins and JUN is different, and they do not interact directly with each other. However, they can cooperatively regulate gene expression by binding to adjacent sites on DNA or by interacting with other transcription factors. The bZip dimers recognize partially overlapping DNA-binding sequences related to an AP-1 site and could compete for binding depending on the target DNA sequences. Thus, a multiple combination of the dimers should generate a greatly expanded repertoire of transcriptional regulatory potential. (Kataoka et al., 1994) (Kataoka et al., 1995).

Fos and *Jun* are well known immediate-early genes that are rapidly transcribed in the SCN in response to a light stimulus, and are members of AP-1 transcription factor family. As seen in Figure 1.4, different combinations of the AP-1 transcription factors dimerise and undergo transcriptional posttranslational modifications such as phosphorylation, sumoylation and ubiquitination with the help of co-factors and modification enzymes. The dimer then binds to a specific DNA sequence, namely TRE, CRE or MARE, depending on which factors the dimer is made up of (Bejjani et al., 2019). This sets off transcription for many genes that

are involved in physiological functions like cell division, cell migration, inflammation and apoptosis.

MAFG is widely expressed in various body tissues including the brain and it's gene expression is induced by oxidative stresses, such as hydrogen peroxide and electrophilic compounds (Crawford et al., 1996) (Katsuoka et al., 2005). The modulation of small-Maf proteins is primarily influenced at the transcriptional level, however, post-translational modifications also have an important role in mediating small-Maf activity. Inhibitor studies provide evidence that repression through MAFG involves histone deacetylase (HDAC) activity containing a repressor complex, thus making sumoylation and acetylation important Maf-modulating mechanisms (Blank, 2008). Because sMafs lack any transcriptional activation domains, sMaf homodimers act as transcriptional repressors (Motohashi et al., 2000). It has been reported that sumoylation at Lysine 14 (K14) within the sMaf sumoylation motif is required for sMaf homodimer-mediated repression (Motohashi et al., 2006). Although the overexpression of wild-type MAFG in bone marrow represses its target gene expression, the overexpression of sumoylation-deficient MAFG (K14 is mutated to arginine) fails to repress target gene expression. One plausible explanation for this observation is that this is an active repression rather than passive repression, in which the homodimer simply occupies the binding site because a HDAC inhibitor blocks the sMaf homodimer-mediated repression (Motohashi et al., 2006).

Structure-function studies have shown that the amino-terminal two-thirds of the Maf molecule, which is absent in the small Maf family proteins, confers the proteins' trans-activating activity. As expected from their structure, the small Maf family proteins act as negative regulators of transcription as homodimers. Conversely, the small Maf proteins can heterodimerize with another bZip protein, NF-E2 p45, crucial for regulating erythroid-specific gene expression, and support active transcription *in vivo*. (Igarashi et al., 1994). Heterodimers of many bZip proteins, including MAF, FOS, JUN, NF-E2 P45, and their related molecules, can recognize sequences related to an AP-1 site. Transactivation studies such as by Kataoka and colleagues in 1995 have shown

that strong endogenous trans-activating activity found in QT6 cells and NIH 3T3 cells with a reporter plasmid might be due to the endogenous activity of such complexes. It could then be hypothesised that MAFG homodimers would inhibit AP-1 transactivation, whereas MAFG heterodimers could activate transcription depending on whether the transcription factor dimerising with MAFG has a canonical transactivation domain or not.

Although *Mafg*−/− animals are viable and fertile, the *Mafg* loss-of-function leads to abnormal megakaryocyte proliferation accompanied by thrombocytopenia, as well as age-dependent behavioural defects, such as response to handling – instead of employing a typically smooth sideways motion to reach upward, as in their wild-type or heterozygous littermates, *Mafg*−/− pups convulsively attempted to throw themselves into an upright position; clasping hindlegs together when inverted, was another uncharacteristic response which is not observed in heterozygous littermates or wild type animals. These highlight the essential role of MAFG in neurological and haematopoietic function (Shavit et a., 1998).

MAFG also interacts with 'cap n collar' CNC proteins such as NRF2 (nuclear factor erythroid 2–related factor 2, an emerging regulator of cellular resistance to oxidants) as a response to oxidative signalling. There is evidence that MAFG plays a role in CNS pathology as multiple sclerosis (MS) was found to be characterised by increased MAFG and decreased NRF2 levels. Furthermore, MAFG interacts with MAT2α (methionine adenosyltransferase IIα – a cofactor that cooperates with sMAFs to modulate gene expression), to promote DNA methylation and subsequently downregulate anti-inflammation and antioxidant transcriptional mechanisms (Wheeler et al., 2020).

Evidence that AP-1 binding to DNA increases after a light-pulse within the SCN can be found in the paper by Kornhauser et al., 1995. Furthermore, a motif analysis of the genes that comprise the SCN light-regulated transcriptome showed enrichment of both CRE and AP-1 response elements, underlining the importance of AP-1 in augmenting the transcriptional responses to light (Jagannath et al., 2021). The aforementioned discovery that chromatin at the promoter of the

transcription factor MAFG showed reduced accessibility following a nocturnal light pulse, indicating repression of the gene, and the fact that the AP-1 motif (TGA G/C TCA) was highly over-represented in the light regulated transcriptome, leads us to hypothesise that MAFG is associated with photoentrainment as a part of an AP-1 transcription factor complex.

In this project, I aim to profile the regulation of MAFG in photoentrainment, by conducting a series of *in vitro* experiments, as well as characterise the photic entrainment phenotype of *Mafg* knockout mice *in vivo*. I also aim to identify molecular targets downstream of MAFG so that it's mechanism of action can be further understood in future.

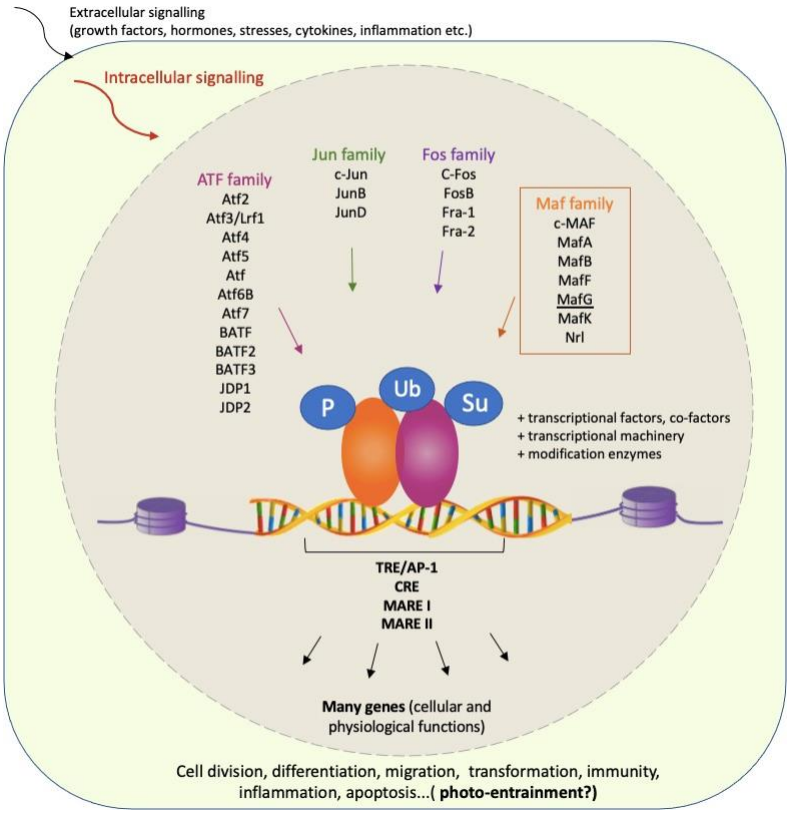


Figure 1.4. AP-1 transcription factor family; AP-1 dimers bind to different response elements to activate several genes involved in cellular and physiological functions, of which we are investigating photoentrainment.

2. Material and methods

2.1 Experimental Methods

2.1.1 Cell Culture Protocol

Cells were incubated in a T75 Corning™ Cell Culture Flask (product no. 430825) with 12ml Dulbecco's Modified Eagle's Medium (DMEM; Thermo Fisher Scientific, product no. BE12-604Q), containing 10% bovine calf serum (product no. 16170078) at 37°c/15% CO2. Cells were split once 70-80% confluent using the following protocol:

i) Remove all culture medium

ii) Wash cells with 12ml phosphate buffered saline (PBS, Thermo Fisher Saline)

iii) Lift cells using TrypLE Express (Life Technologies Corporation, USA, Ref: 12604-013). Cells incubated for 5 minutes with 5ml TrypLE at 37°c.

iv) Deactivate TrypLE and centrifuge: 7ml DMEM added and cells spun at 1500 rpm for 5 minutes.

v) Re-suspend and split cells: Supernatant removed and cells re-suspended in fresh 2ml DMEM; 200µl (10%) of cell suspension returned to flask containing 11.8ml DMEM.

Cells were used for transfection once confluent, remaining cells were split as outlined above and returned to the incubator.

2.1.2 Transfection of cultured cells

A transfection was used to artificially incorporate a number of nucleic sequences of interest into human U2OS cells. By using lipid reagents, a chemical-mediated

transfection was utilized to transfer plasmids containing the sequence of interest along with a luciferase reporter into the cells. When activated, the sequence of interest drives transcription of the luciferase gene, and subsequent luminescence was used to measure the activity of the sequence of interest.

The sequences of interest (available in the Appendix A) were first incorporated into the standard shuttle vector, pMX, a pUC19 derivative with a choice of antibiotic resistance markers, from the Invitrogen GeneArt Kit (https://www.thermofisher.com/uk/en/home/life-science/cloning/gene-synthesis/geneart-gene-synthesis.html). These vectors are intended for delivery of the clonal synthetic DNA. The sequence of interest was then cloned from the GenArt plasmid into the Promega GL4.27 [luc2P/minP/Hygro] plasmid (https://www.promega.co.uk/) containing the luciferase gene (Cloning carried out by Norbert Varga). This plasmid was then transfected into cultured cells to complex with lipid reagents. Invitrogen Lipofectamine® 3000 was used as the transfection reagent. This process enables negatively charged DNA molecules to pass through the negatively charged cell membrane. The cells were incubated for 24 hours following transfection before being stimulated by luciferin containing recording media and/or Forskolin/DMSO (see section 3.1), and the subsequent luminescence was recorded using a BMG plate reader for upto 72 hours. All *in vitro* experiments were conducted in 96-well plates. Where the cells were co-transfected with both *Mafg* DNA and siRNA, the cells were harvested after they had been in the plate reader and the efficiency of *Mafg* knockdown was validated using qPCR.

The following protocol was followed for transfection:

i) Cloning sequences of interest into plasmid using Invitrogen Gene ART: Sequences were determined from the Integrated Genome Browser (IGB), a restriction enzyme cutting site was added to either end of the sequence before submission to company for cloning.

ii) Cloning into plasmid with luciferase gene: Sequences of interest were cloned

from GeneArt plasmid into the Promega GL4.27 [luc2P/minP/Hygro] plasmid. (carried out by Norbert Varga)

iii) Plasmid DNA/lipid complexes:

1. Per sample: 9.4µl Invitrogen Lipofectamine™ 3000 (LP3000) Transfection Reagent (ThermoFisher: L3000015) diluted 1:10 with 0.6µl ibc Opti-MEM Reduced Serum Medium (ThermoFisher: 31985070).
2. Plasmid DNA concentration determined using NanoDrop 2000/2000c Spectrophotometers (ThermoFisher: ND-2000).
3. Plasmid DNA diluted with Opti-Mem and Invitrogen Lipofectamine™ 3000 (P3000) transfection reagent (ThermoFisher: L3000015) solution to 20ng/µl.
4. 10µl of (1) mixed with 10µl (3) to give final plasmid DNA/lipid complex containing 200ng DNA.

iv) Cultured cell counting: Cells spun at 500g for 5 minutes and pellet re-suspended in 3ml of growth medium. 10 uL of cell suspension added to 50 uL Trypan blue (ThermoFisher: 15250061) and 40 uL of PSB, cells were counted using a haemocytometer.

v) Preparing 96-well plate: 10µl DNA/lipid complex and 20,000 cells in 90 µl of growth medium added to each well of the plate.

vi) Cells and DNA plasmid/lipid complex left to incubate for 24 hours

In the experiments where *Mafg* was knocked down using siRNA, the cells were co-transfected with the sequences of interest, diluted with Opti-MEM as above but without the P3000 reagent, along with si-*Mafg* (Horizon Discovery Biosciences Limited: D-042610-01-0002; siGENOME Mouse Mafg (17134) siRNA). A final concentration of 20nM siRNA per well was used. For these experiments, the DNA/siRNA/lipid complex was left to incubate for 48 hours; the rest of the steps are the same as above. siRNA sequences are available in Appendix B.

The following protocol was followed for treatment of cells and recording of bioluminescence:

i) Mix medium for recording: Per sample, 1:50 dilution B-27 Supplement (ThermoFisher: 17504044) and 1:1000 dilution Luciferin were added to 100uL of recording media.

ii) Prepare cells for plate reader: Growth medium removed from all cells and 100µl (1) added to each well

iii) Cells placed in BMG plate reader: The cells were left for 3 hours to record a baseline level of luminescence

iv) Treatment added to cells: 10µl recording medium removed, and the same volume of relevant treatment added to each well.

v) Cells returned to plate reader

BMAL::LUC/ ASSAY PROTOCOL
Plating & reverse transfecting cells

1. Dilute the lipofectamine RNAiMAX reagent in Opti-MEM medium: dilute
2. 0.2 µl of RNAiMAX (Life Technologies Limited : 13778075) in 10 µl of Opti-MEM medium per well to make up a master-mix for the total number of wells to be transfected.

3. Dilute the siRNA in Opti-MEM medium. dilute 0.2 µl of the 20 µM siRNA stock in 10 µl of Opti-MEM medium per well to make up a master-mix for the total number of wells to be transfected.

4. Trypsinise cells and transfer cell suspension to a falcon tube. Centrifuge at 1000 rpm for 5 minutes.

5. Remove supernatant and resuspend cell pellet in antibiotic-free culture medium.

6. Count cells and dilute to 375, 000 cells/ml (to plate 30k cells/well).

7. Add the RNAiMAX solution from step #1 to the siRNA solution from step #2. Combine equal volumes of the RNAiMAX solution and the siRNA solution for a 1:1 ratio. Make up a master-mix for the total number of wells to be transfected and incubate for 10 minutes at room temperature.*The complex will degrade over time so must be added to cells after max 30 minutes*
8. Add 20 µl of the RNAiMAX/siRNA complex to each well of the 96-well plate
9. Add 80 µl of cell suspension to each well. Mix gently and incubate for ~24 h at 37°C.

Synchronise cells and start recording

1. Synchronise cells with 100 nM dexamethasone for 1 h. To do this, first prepare the dexamethasone by diluting it 1:1000 in the culture media (dexamethasone stock = 100 µM). For example, add 11 µl dexamethasone to 11 ml media for one whole plate.

2. Remove the spent media from the plate and add 100 µl of the prepared dexamethasone per well. Incubate for 1 h at 37°C.
3. Prepare recording media as described above

4. Wash cells twice with 100 µl PBS.

5. Add 200 µl recording medium to each well.

6. Leave in the incubator for ~1h to allow CO2 levels to equilibrate.

7. Set up plate on plate reader to record luminescence. Plate reader settings: 36°C

2.1.3 Animal Work - *In vivo* methods

C57BL/6 mice of both sexes were used for all *in vivo* experiments; animals were housed in light tight chambers (LTC), with light/dark cycles being strictly controlled, and were fed a standard diet. The knockout mice were generated by a

full removal of the *Mafg* gene (Shavit et al., 1998, Genes Dev). Mice were housed under artificial light-dark cycles for the purpose of entrainment not earlier than 4-7weeks old, where they were each housed in a single cage with a wheel, and were subsequently culled at approximately 12-24 weeks old after circadian screening/ light manipulation. All experiments were carried out in the dark; ensuring mice were culled in circadian time (CT), rather than *Zeitgeber* time (ZT), Figure 2.1.3 a illustrates the difference between ZT and CT. Prior to all experiments, mice were subject to their current light dark cycle for a minimum of 10 days, to make certain all animals were fully entrained. Mice were culled as quickly as possible using cervical dislocation to preserve the molecular profile.

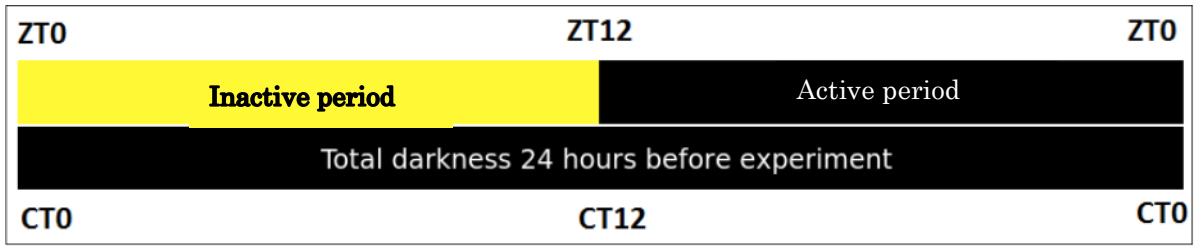


Figure 2.1.3a: *Zeitgeber* time vs circadian time. Lights are turned off prior to all experiments guaranteeing mice oscillate by their internal molecular clocks (Circadian Time), rather than by any external *Zeitgeber* such as light.

Jet Lag *in vivo* procedures

Mice were kept on a 12 hour light/dark cycle (7am lights on to 7pm lights off) for at least 7 days before the experiment, or until they were fully entrained. They were then subjected to a 6 hour phase advancing jet lag by pushing their sleep and wake times 6 hours earlier than normal (1am lights on to 1pm lights off). The light intensity was kept at a 100 lux.

Light Pulse *in vivo* procedures

Mice were kept on a 12 hour light/dark cycle; the timing of this cycle was varied depending on the experimental plan to ensure mice were culled at the correct CT, Table 2.1.3 summarises the different light cycles that were used. Lights within LTCs were adjusted to ensure CT16 occurred during working hours, meaning that the original light dark cycle was reversed (Table 2.1.3). All light pulsing was

carried out at either 10 or 100 lux for a period of either 30 or 60 minutes, if multiple mice were culled, light timings were staggered to allow time for dissection. Mice from the same experimental condition were not culled sequentially in an attempt to mitigate batch effects. After all animals seemed entrained, mice were left in DD (constant darkness) for one cycle before they were light pulsed at CT 16 and culled immediately after.

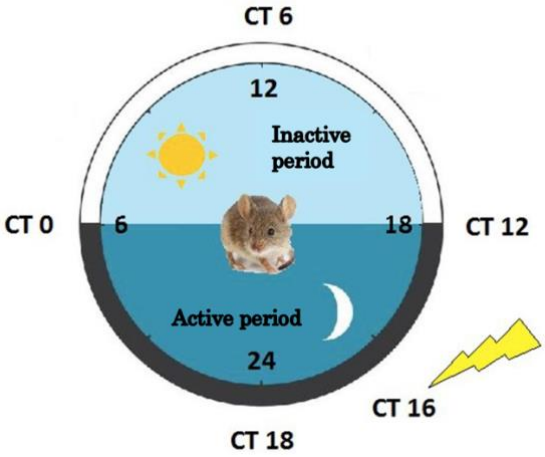


Figure 2.1.3b Mice were put into total darkness 24 hours before the experiment, to ensure mice were light pulsed in CT time at CT16.

Experiment time	Lights On	Lights Off
CT16 at 10am for tissue collection	6pm	6am
CT16 at 11am for tissue collection	7pm	7am
CT16 at 9pm for phase shift	6am	6pm

Table 2.1.3c The different light/dark cycles used. The LD time used depended on experimental timing. The mice are first entrained to an LD cycle (ZT) but since the light pulse occurred after a day of DD, it is characterized in CT.

2.1.4 Tissue Collection

The mice were culled and brain tissue (SCN and Cortex) was collected from them as follows.

Brain removal and Cortex punch: Brains were removed and placed into a brain matrix (Kent Scientific, Torrington CT, USA). A skin graft blade (Swann-Morton, Sheffield, UK) was positioned at Bregma −0.10 mm (Figure S1). A second blade was placed 1 mm (Bregma −1.10) caudal from the first, and a 1 mm thick brain

slice was dissected. SCN and Cortex punches were taken using a sample corer (1 mm internal diameter, Fine Science Tools GmbH, Heidelberg, Germany) from the brain slice (n = 3 to 5for each condition), flash frozen on dry ice and stored at −80°C prior to RNA extraction. All procedures were performed in accordance with the UK Home Office Animals (Scientific Procedures) Act 1986 and the University of Oxford's Policy on the Use of Animals in Scientific Research.

2.1.5 RNA extraction and cDNA library preparation

RNA was first extracted using the following protocol:

i) Brain removal and SCN punch: Protocol from section 2.1.4 followed, with 1 punch taken per sample.

ii) Tissue homogenization and RNA stabilisation: Tissue was homogenised in 100µl Qiagen QIAzol Lysis Reagent (Cat. No. 79306) using a Microtube Homogenizer (Bel-Art F65000- 0000) for 10 seconds, and incubated for 5 minutes at room temperature.

iii) Phase separation using chloroform: An additional 400µl Qiagen QIAzol Lysis Reagent added, along with 100µl chloroform, sample shaken vigorously, incubated for 10 minutes at room temperature, and spun at 12,000 RCF for 15 minutes.

iv) RNA purification: Clear band containing RNA carefully removed and purified using the Qiagen RNeasy Micro Kit (Cat. No. 74004); genomic DNA was removed using DNase.

v) RNA quantitation: Total RNA extracted following the manufacturer's instructions and was eluted in water. RNA concentration and quality was determined using a NanoDrop™ ND-1000 spectrophotometer.

vi) cDNA was synthesized from the purified RNA according to the qScript cDNA Synthesis Kit (Quantabio). mRNA was quantified using the QuantiFast SYBR® Green PCR Kit (QIAGEN) in a StepOnePlus™ thermal cycler (Applied

Biosystems).

2.1.6 qRT-PCR validation

Primer sequences are listed in Appendix C. Cycling conditions were 95 °C for 5 min, and 40 cycles of 95 °C for 10 s, 60 °C for 30 s, 72 °C for 12 s. The cycle thresholds for each gene were normalized to *Rps9*, *GusB*, and *Tbp* housekeeping genes. The relative gene expression between LP and control samples was calculated using the $2^{-\Delta Ct}$ method. To ensure that punches of the defined areas were accurately dissected, qPCR of the SCN-specific mRNA *Egr1*, *Fos* and *Per1* and *Per2* were conducted (Conte et al., 2005), revealing selective enrichment.

2.2 Statistical analysis

All data presented in this thesis were analysed using GraphPad Prism (Version 9.4, La Jolla, CA). All data are expressed as mean ± SEM, as detailed in each figure legend. For comparisons between two groups only, a Student's t-test was applied. For multiple comparisons with only a single variable, a one-way ANOVA with Dunnett's multiple comparisons correction was applied. For comparisons with two variables, a two-way ANOVA with Sidak's multiple comparisons correction was applied. The test used for each individual data set is specified in the corresponding figure legend. A P value of <0.05 was taken to be statistically significant. The preliminary investigation of potential MAFG targets was done on Microsoft Excel by comparing gene lists from published data. The functions used were 'IF' and 'COUNTIF'.

3. Results

To investigate whether MAFG, a member of the AP-1 transcription factor family, is involved in photo-entrainment, we conducted a series of *in vitro* experiments to profile the regulation of MAFG and profiled the photic entrainment phenotype of *Mafg* knockout mice *in vivo*. Furthermore, potential MAFG targets in the mouse SCN were investigated by conducting RT-PCRs.

3.1 *In vitro* analysis of *Mafg* promoter region using a luciferase reporter construct

Here I sought to validate the results of the ATAC-seq light pulse experiment conducted previously in the Jagannath lab wherein chromatin at the promoter region of *Mafg* was observed as being variably accessible after a light pulse (Jagannath et al., unpublished data). I hypothesised that the *Mafg* promoter, when isolated and tagged to a reporter, would result in reduced expression of the reporter when light pulse like conditions were created *in vitro*. This is as reduced accessibility of chromatin should ultimately lead to reduced transcription from the regulated regions. A section of the *Mafg* promoter region was chosen for a luciferase reporter assay.

To evaluate the link between chromatin accessibility and other epigenetic markers/modifications, areas of differential chromatin were compared to chromatin immunoprecipitation sequencing (CHIP-seq) coverage tracks for common epigenetic marks. The methylation of DNA or modification of histone proteins will have a direct effect on chromatin structure; therefore areas of the genome identified by ATACs-seq to alter in chromatin state were anticipated to contain epigenetic markers which have enabled these changes to take place.

Data was taken from the Encode consortium for mouse mid-brain CHIP-seq (Davis et al., 2018), data included marks for histone methylation protein H3K4me3 (Encode ID: ENCSR203KIB), and histone acetylation proteins H3K9ac (Encode ID: ENCSR286OKN) and H3K27 (Encode ID: ENCSR088UKA). Epigenetic marks were found to be abundantly present where ATAC-seq has been shown to change chromatin state, including near to MAFG following a light pulse at CT16. In contrast chromatin close to *Fos* where chromatin did not change under any sample condition investigated by ATAC-seq showed less abundant epigenetic marks (Steven Walsh, Jagannath lab, unpublished data).

This clearly demonstrates that areas of the genome where chromatin accessibility is dynamic and changes state following external stimuli such as light pulsing, are frequently bound by epigenetic marks such as histone acetylation proteins to facilitate this change.

These regions were found to contain numerous TF binding sites using JASPAR, a transcription factor binding site prediction tool (Fornes et al., 2020). Three smaller sections (namely - full peak, first peak and second peak regions – Figure 3.1) of this *Mafg* promoter region containing binding sites for TFs such as AP-1, CREB and EG1 motifs, as shown in Figure 3.1a were also separately cloned, to identify which parts of the promoter were most relevant to changing reporter expression.

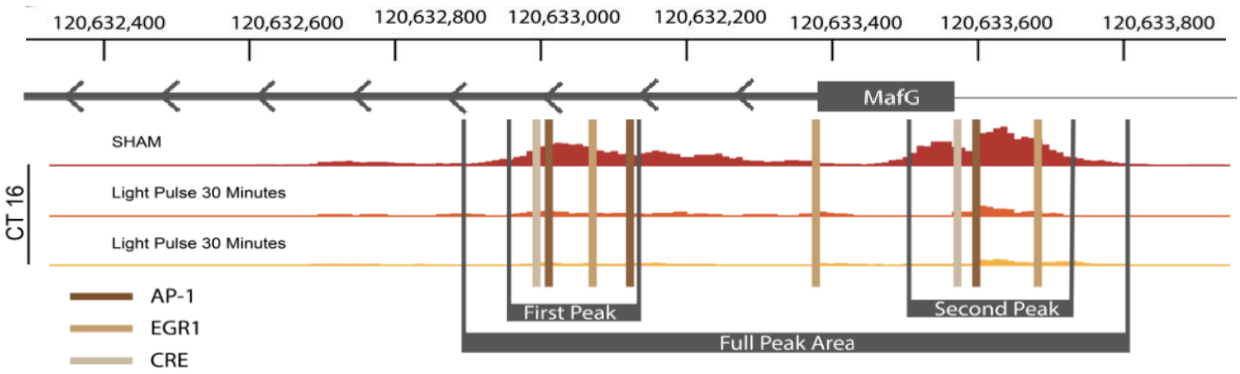


Figure 3.1a ATAC-Seq tracks showing the *Mafg* promoter after a light pulse in the SCN with specific response elements highlighted, times as indicated. Sections of the *Mafg* promoter (entire region – Full peak or FP; first peak – 1P, second peak – 2P) were cloned into a reporter luciferase plasmid (pGL2.4). (Figure adapted from thesis of Steven Walsh).

For the luciferase reporter assay, the above genomic sequences were cloned into pGL4.27 plasmids by a previous member of the lab. This plasmid encodes the luciferase reporter gene luc2P, and contains multiple cloning regions upstream of the promoter and the luc2P gene, where response elements of interest can be inserted.

Human U2OS osteosarcoma cells were cultured as outlined in section 2.1.1 and the pGL4.27 plasmids containing the sequences of interest were transfected into these cells as described in section 2.1.2. A human cell line was selected because even though the ATAC-seq experiments were conducted in mice, there is evidence that the regulatory pathways of circadian entrainment, the mechanisms that drive the phasic expression of the genes involved (the TTFL) are highly conserved across species (Monaco et al., 2015) (Allada and Chung, 2010). We chose U2OS cells for

the *in vitro* assay as this model has been extensively characterized by functional studies examining knockdown effects of all known clock components with cellular and behavioral phenotypes in knockout mice (Baggs et al., 2009; Liu et al., 2007). In addition, this model was used in small molecule screening and limited-scale RNAi screening to find modifiers of amplitude and periodicity (Hirota et al., 2008; Maier et al., 2009; Baggs et al., 2009).

This recapitulation of *in vivo* circadian behaviours provided evidence that U2OS cells serve as robust models to study features of the circadian clock. We were interested to determine whether a response was detected in human cells and if so, it could potentially be significant in explaining the relevance of the *Mafg* promoter showing reduced accessibility in response to light.

As we hypothesise *Mafg* to regulate its own expression, based on the presence of AP-1 elements in its promoter, the cloned and transfected cells were further subjected to siRNA mediated *Mafg* knockdown and treated with Forskolin as described in section 2.1.2. Forskolin was chosen as it simulates a light pulse through its activation of CRE via the cAMP-CREB pathway (Yagita and Okamura, 2000), and AP-1 through the induction of cFOS (Sharma et al., 2000). The primary action of Forskolin is the activation of protein kinase A (PKA); then, this kinase phosphorylates CREB (Gonzalez et al., 1989). While the PKA pathway can be activated by forskolin in many different cell types, the specific downstream effects can vary depending on the cellular context (Kaneko et al., 2020). The control for the efficiency of the reporter plasmid consisted of cells that were transfected with a pGL4.27 plasmid containing only the luciferase reporter. Alongside the siMafg mediated *Mafg* knockdown, samples transfected with non targeting siRNA were used as controls. Following treatment, luminescence through as reported by luciferase was recorded. A baseline measurement of 3 hours was used and the output data was collected for 48 hours after the treatment of cells (n=4 wells for each condition).

All three genomic sequences, without any treatment, drove the expression of luciferase via the reporter gene to a greater extent than the controls, as seen in

Figures 3.1c-h. Figure 3.1b shows a comparison between the transcription induced by the three promoter sections alongside transcription induced by cells where the sequences of interest have been knocked down using siRNA. It is seen that transcription is reduced in the presence of *Mafg,* These findings confirm that the sequences of interest can induce transcription and are likely essential for the initiation of transcription of genes further downstream to the sequences. In addition to this, Forskolin seemed to reduce luciferase signal (Figures 3.1d, 3.1f, 3.1h), demonstrating that the *Mafg* promoter region studied was regulated by light-like stimuli.

It was observed that that the knockdown of *Mafg* decreased luciferase activity (figure 3.1b), and Forskolin treatment caused a further depression in luciferase signals, implying that the promoter region may be closed to input stimuli and is itself further repressed in response to *Mafg* siRNA mediated knockdown (Figures 3.1b-h). Thus, *in vitro* analysis corroborates the ATAC-Seq finding that *Mafg* accessibility is reduced in response to stimuli that mimic light. This experiment was repeated to validate results and data from one of the experiments has been presented below. However, more experiments would need to be conducted as the isolated effect of *Mafg* knockdown cannot be inferred from figures 3.1d, 3.1f and 3.1h.

3.1b.

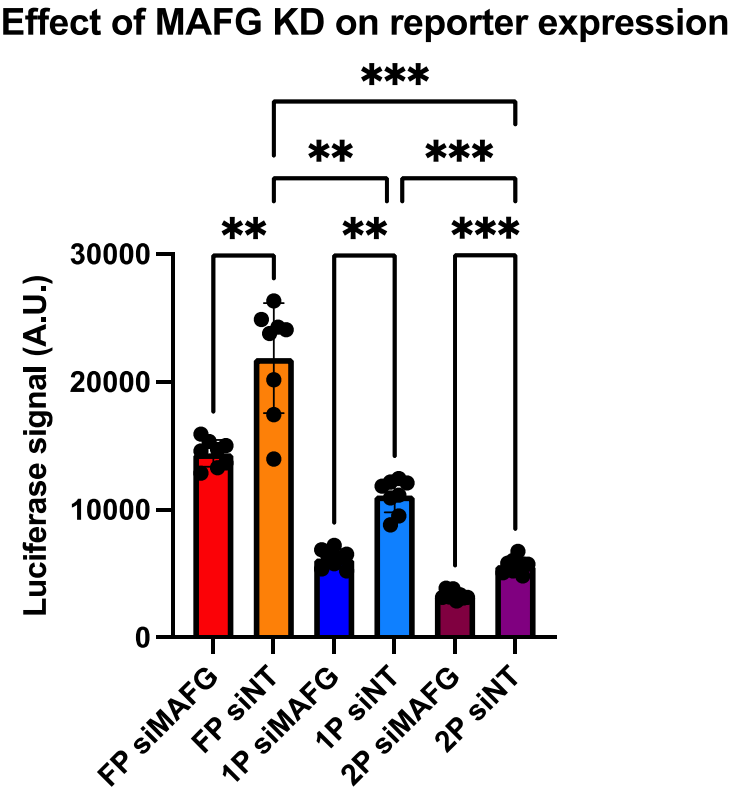
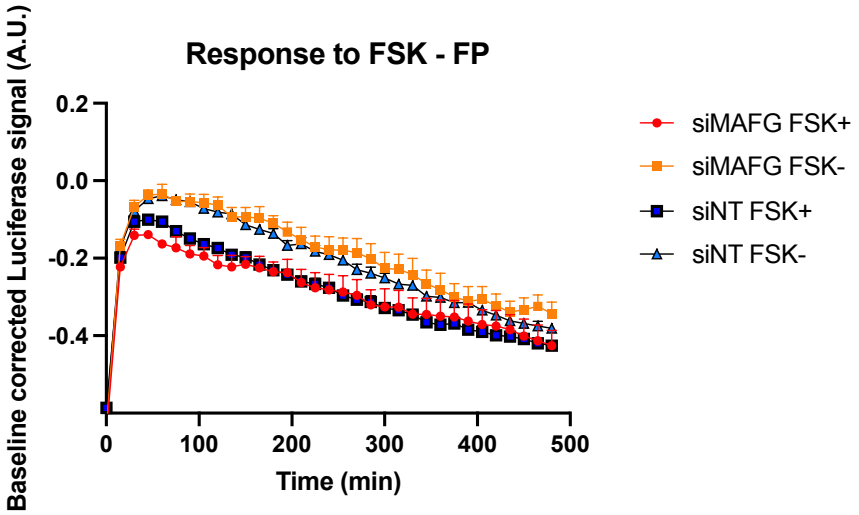


Figure 3.1b U2OS cells transfected with the sequences of interest (full peak-FP, first peak-1P and second peak-2P) and after siRNA mediated *Mafg* knockdown. Levels of luciferase expression from each reporter indicated, in combination with knockdown of *Mafg* (siMafg) or control (siNT); n=4 wells for each condition. Luciferase expression was downregulated in *Mafg*-silenced cells across all three sequences, suggesting reduced transcription when the gene is silenced. Tukey's multiple comparisons in a one-way ANOVA analysis showed the following adjusted p values : FP siMAFG vs. FP siNT (0.0095); 1P siMAFG vs. 1P siNT (0.0011); 2P siMAFG vs. 2P siNT (0.0002). Data is presented as mean +/- S.E.M.

3.1c



3.1d

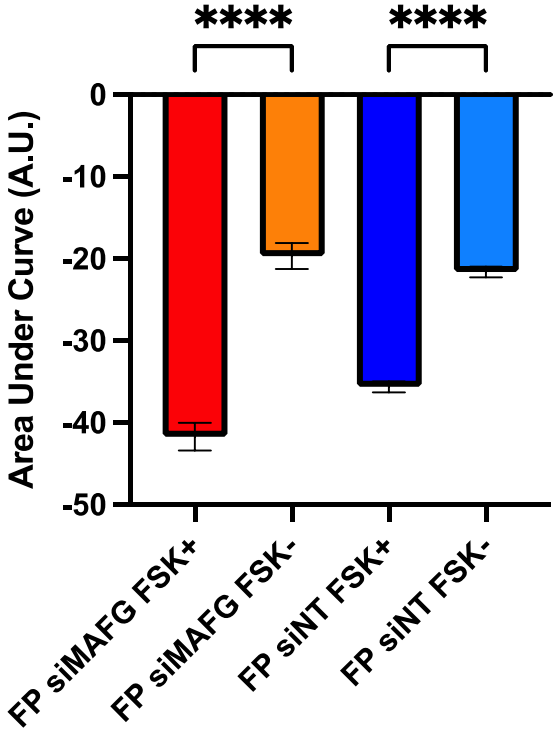
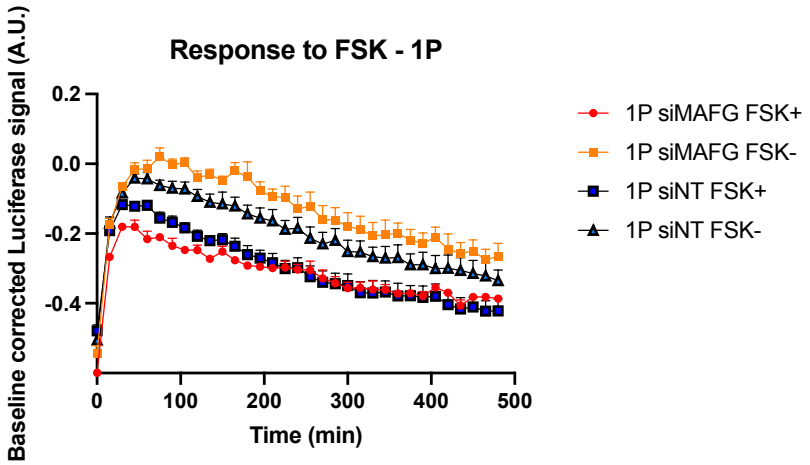


Figure 3.1c and 3.1d U2OS cells transfected with the full peak *Mafg* promoter region and following siRNA mediated *Mafg* knockdown. Levels of luciferase expression indicated, in combination with knockdown of *Mafg* (siMafg) or control (siNT); n=4 wells for experimental and control siRNA. Figure 3.1c shows baseline corrected luciferase. The luciferase signal response to treatment with forskolin (10 uM) applied before time point 0 shown alongside in figure 3.1d by plotting corresponding area under curve. Forskolin depresses the luciferase signal significantly, compared to DMSO control, and this expression is further downregulated in *Mafg* KD cells. Data is presented as mean +/- S.E.M.

3.1e



3.1f

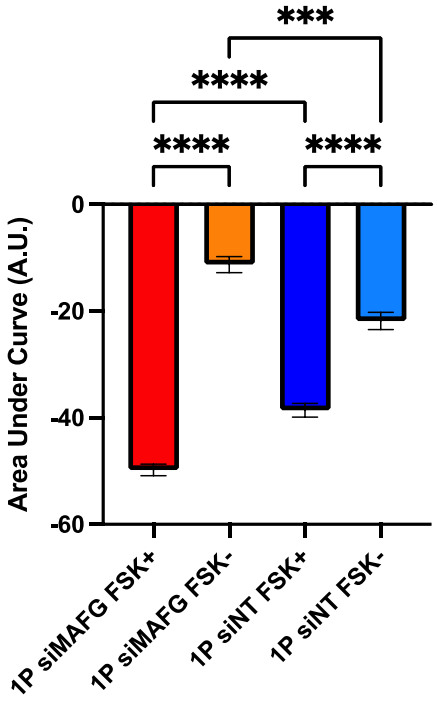
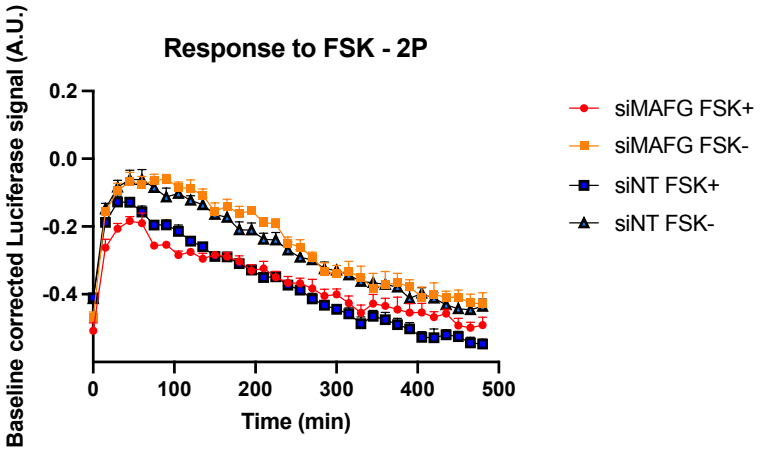


Figure 3.1e and 3.1f U2OS cells transfected with the first peak *Mafg* promoter region and following siRNA mediated *Mafg* knockdown. Levels of luciferase expression indicated, in combination with knockdown of *Mafg* (siMafg) or control (siNT); n=4 wells for experimental and control siRNA. Figure 3.1e shows baseline corrected luciferase. The luciferase signal response to treatment with forskolin (10 uM) applied before time point 0 shown alongside in figure 3.1f by plotting corresponding area under curve. Forskolin dampens the luciferase signal significantly compared to DMSO control, and this expression is further downregulated in *Mafg* silencd cells. Data is presented as mean +/- S.E.M.

3.1g



3.1h

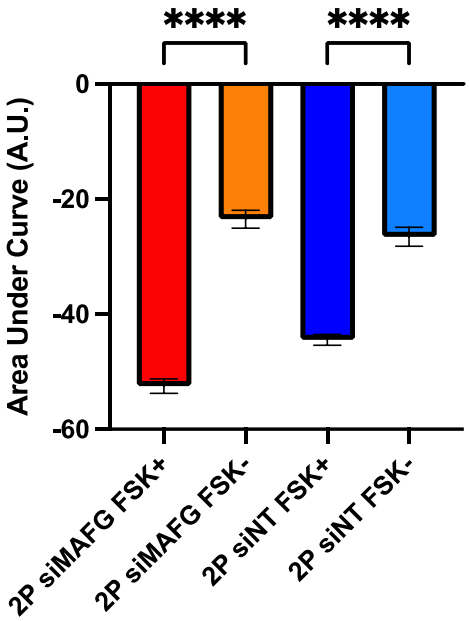


Figure 3.1fg and 3.1h U2OS cells transfected with the second peak *Mafg* promoter region and following siRNA mediated *Mafg* knockdown. Levels of luciferase expression indicated, in combination with knockdown of *Mafg* (siMafg) or control (siNT); n=4 wells for experimental and control siRNA. Figure 3.1g shows baseline corrected luciferase. The luciferase signal response to treatment with forskolin (10 uM) applied before time point 0 shown alongside in figure 3.1h by plotting the corresponding area under the curve. Forskolin significantly dampens the luciferase signal compared to DMSO control and this expression is further downregulated in *Mafg* KO cells. Data is presented as mean +/- S.E.M.

BMAL:Luc U2OS Reporter Analysis

To assess the role of *Mafg* in regulating circadian rhythmicity, *Mafg* was knocked down with siRNA in BMAL1:Luc stably transfected U2OS cells where the circadian oscillation of BMAL1 can be monitored through luciferase readouts. SIK1 knockdown was used as positive control alongside the non-targeting siRNA where SIK1 was expected to lengthen the period. Periodogram analysis of these results on Biodare2, 2019 (Zielinski et al, 2014) indicates that the absence of *Mafg* led to a reduced amplitude of rhythm but the period did not change. The same was also evident in the *in vivo* results in section 3.2, thus suggesting a weakened circadian rhythm. However, SIK1 knockdown, that was expected to show an increase in the period length, as seen previously in the lab using the same reporter assay, surprisingly did not have an effect. This would need to be investigated further, such as by checking SIK1 knockdown levels with qPCR.

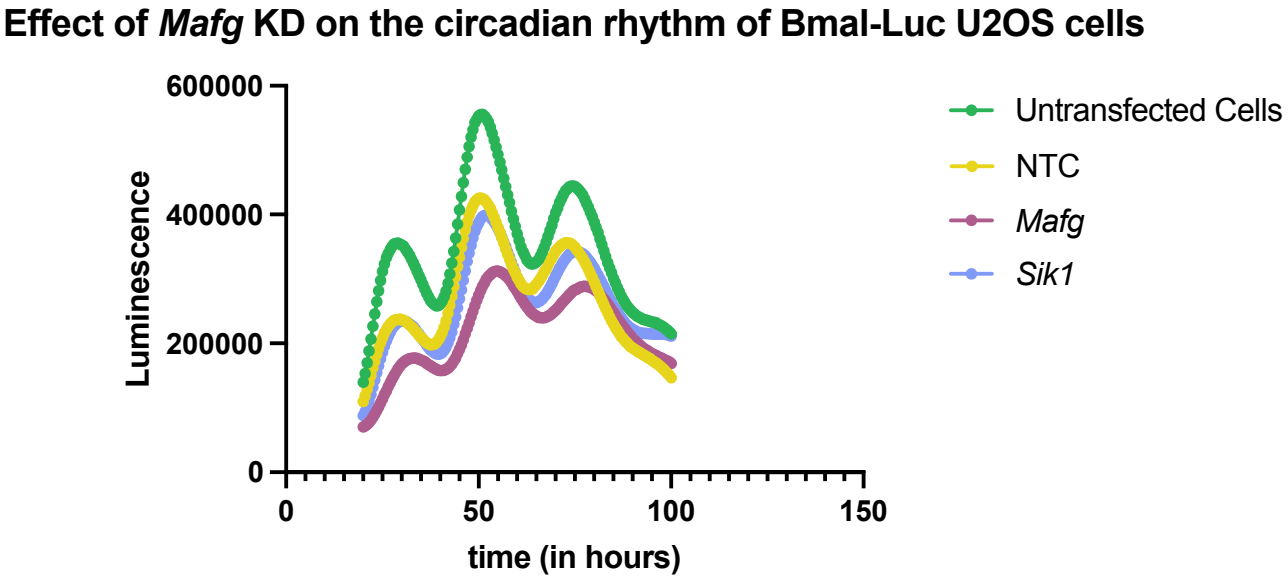


Figure 3.1i
Luminescence plot of U2OS BMAL:Luc reporter cells after siRNA mediated *Mafg* knockdown to visualise its oscillations compared to cells with non-targeting siRNA.

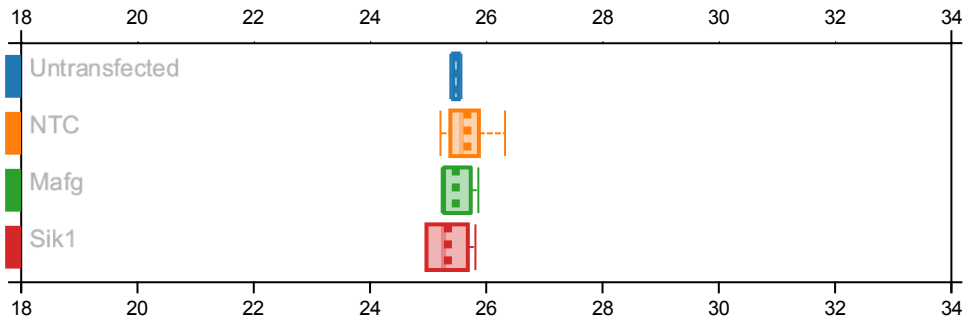


Figure 3.1j
Biodare analysis using the FFT NLLS method showing a trend of reduced period for *Mafg* knockdown cells depicted by 'Mafg'. No significant change in period can be seen. The period and amplitudes of the 4 conditions respectively, Untransfected period=25.49, amp.= 4.23e+4; NTC period =25.69, amp. = 3.34e+4; *Mafg* period= 25.49, amp.= 1.39e+4; *Sik1* period = 25.36, amp.= 3.25e+4; n=8 wells for each condition.

3.2 Circadian phenotypes of the *Mafg* Knockout mouse

I next investigated *in vivo* how MAFG affects entrainment. The behaviour and baseline circadian phenotype of *Mafg* knockout mice was studied by observing their running wheel activity under altered light dark cycles (Jud et al., 2005). In addition, their photic entrainment phenotypes were studied using two main paradigms - jetlag and light pulse induced phase shifting.

Figures 3.2a (i-iv) show the running wheel activity profiles of wild-type as well as knockout animals. From figures 3.2a (i) and (ii) it can be seen that the *Mafg* deficient mice have much lower activity counts, compared to wild-type mice, and it is fragmented mostly in the dark period with some activity falling in the light period as well. Similarly, figures 3.2a (iii) and (iv) show that the knockout mice have reduced activity that is similarly sparse and fragmented in constant dark. Furthermore, periodogram analysis was conducted and although period differences (figure 3.2b) between wild-type and knockout mice were not seen, the amplitudes of *Mafg* -/- mice seemed to be significantly lower than *Mafg* +/+ mice when they were under an LD cycle as well as when they were in constant darkness (figures 3.2c and 3.2d). The sexes were combined for the above analysis as no

difference between sexes was seen. To rule out the possibility that *Mafg* KO animals showed a lower amplitude due to their lower activity the amplitudes were normalised for activity (where *Mafg*-/- activity was reduced by half, a fourth and an eighth) in a separate analysis and it was still found that the amplitude reduction was evident. The reduced amplitude in the knockout mice corresponds to the results obtained using the reporter cells in the *in vitro* analysis. This suggests a potential role of MAFG in the circadian clock in addition to its role in entrainment. Overall, this analysis suggests that the lack of *Mafg* in these animals leads to a weak and fragmented rhythm.

3.2a

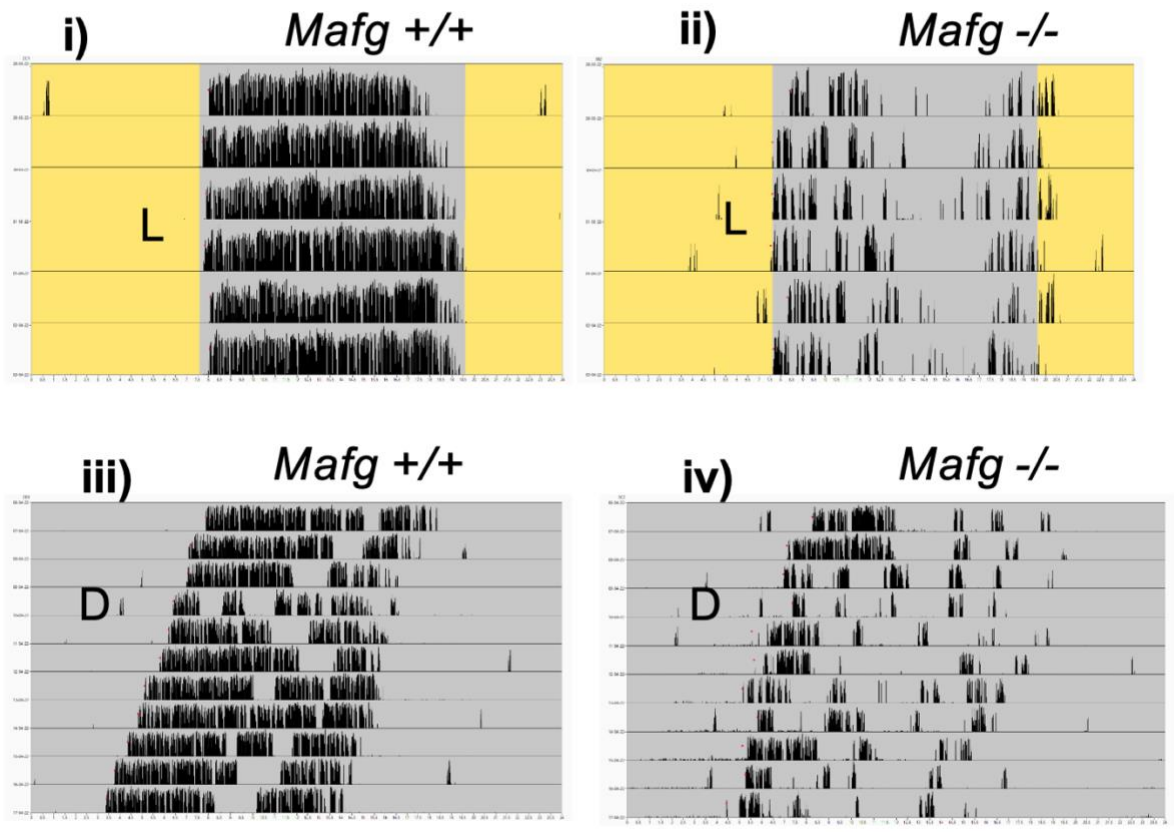


Figure 3.2a: Baseline circadian phenotype of the *Mafg* KO mouse. *Mafg* WT and KO (n=16 to 18 per group) mice were housed in a 12:12 LD (Light Dark) cycle, yellow indicates lights on, grey lights off, and black indicates wheel running activity. Represented actograms depict baseline phenotypes (i) and (iii) shown for WT mice and (ii) and (iv) shown for KO animals. *Mafg*-/- mice show a reduced and scattered activity profile compared to WT mice in both, the LD cycle and constant darkness (D).

3.2b

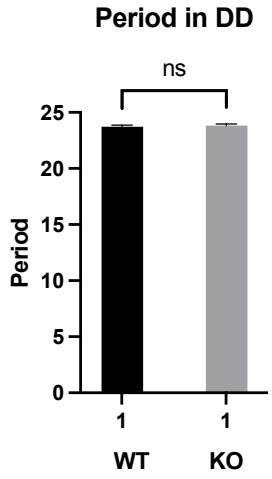
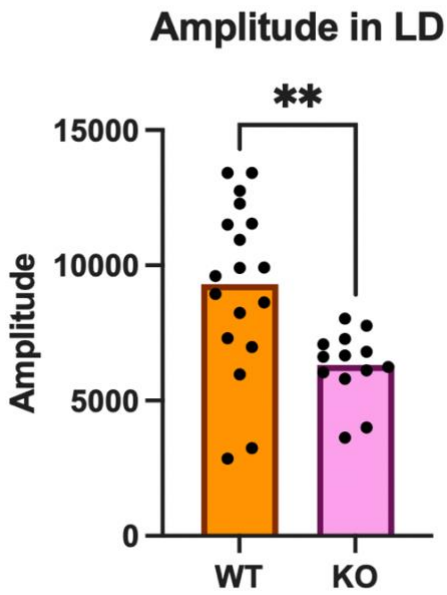
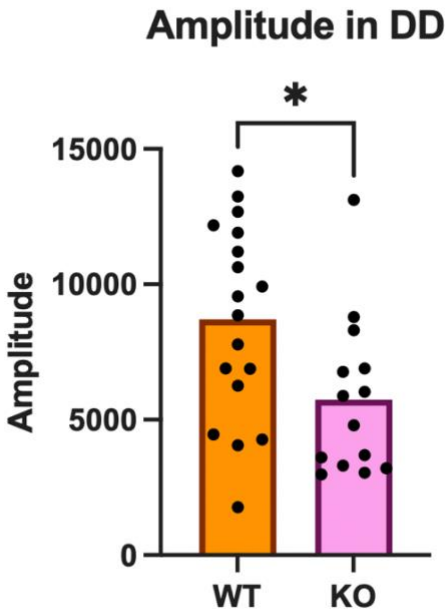


Figure 3.2b Period analysis of *Mafg* WT and KO mice housed in DD cycle (n=6-9) using a t-test showed no significant difference (p value = 0.574) across both genotypes. WT mean = 23.71 hours, KO mean = 23.83 hours

3.2c



3.2d



Figures 3.2c Amplitudes of *Mafg* WT and KO mice housed in LD cycle (n=6-9), two-tailed student t-test showing a significant affect (p=0.0031) across both genotypes where *Mafg* -/- have a reduced amplitude, no difference in amplitude between sexes.
Figure 3.2d Amplitude of *Mafg* WT and KO mice housed in DD cycle (n=6-9), two-tailed student t-test showing a significant affect (p=0.0182) across both genotypes where *Mafg* -/- have a reduced amplitude, no difference in amplitude between sexes.

As described earlier, AP-1 is a core factor in the photic entrainment pathway (Kornhauser et al., 1992) (Abe et al., 1991) (Kornhauser et al., 1990). We hypothesized that the absence of *Mafg* would compromise photic entrainment by altering AP-1 related transcription. To test this, *Mafg*-/- (*Mafg* knockout) mice were first entrained to a 12:12 hour light dark cycle for a few days. They were then subjected to a 6 hour phase advancing jet lag and their ability to entrain to their new light dark cycle was compared to that of *Mafg*+/+ (wild-type) mice that had been subject to the same light conditions (figure 3.2e i) and ii)). Based on the finding that MAFG is typically a repressive factor (Kannan et al., 2012), it was

hypothesised that the *Mafg*-/- mice would have reduced repression on AP-1 and thus faster re-entrainment to the new light dark cycle. Surprisingly, from the phase (Onset or wake-time minus lights off time) analysis (figure 3.2f) using a 3-way ANOVA, it was found that the *Mafg*-/- mice of both sexes re-entrained much slower than the wild type control mice, leading us to question whether MAFG acts as a transcriptional repressor or activator. Additionally, females across both genotypes re-entrained faster than their respective male counterparts.

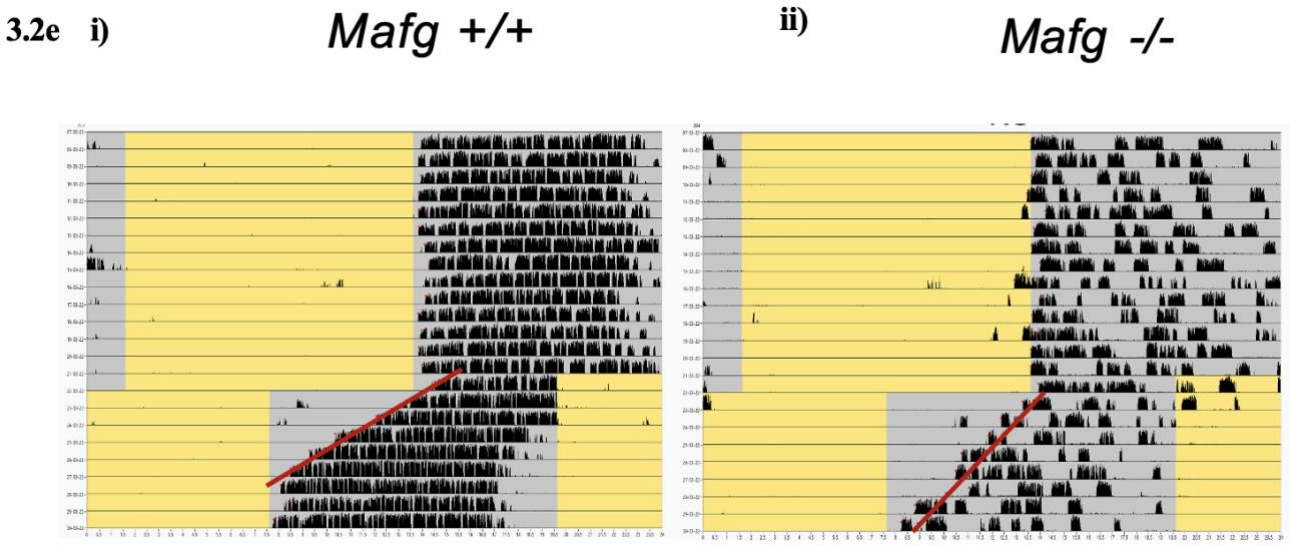


Figure 3.2e Actograms of *Mafg* WT and KO mice housed on running wheels, in 12:12hr LD cycles before and after a 6 hour LD cycle advance. Yellow = lights on, grey = lights off, black bars = wheel running activity in i) *Mafg*+/+, ii) *Mafg* -/-.
WT animals show consistent activity and appear to entrain faster than the KO mice. The KO mice seem to have lower and more scattered activity compared to WT. Approximate phase of re-entrainment shown by red line.

3.2f

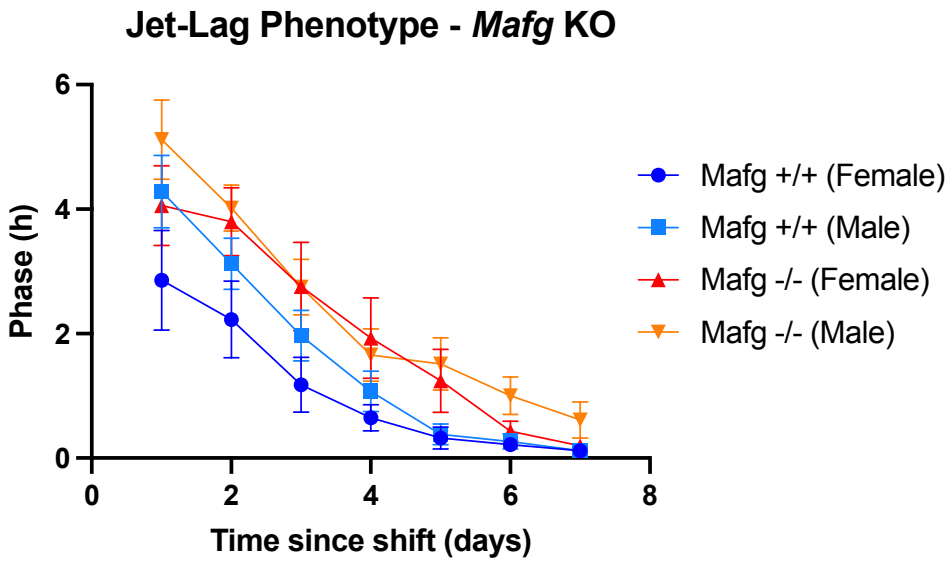


Figure 3.2f Phase (in hours) of onset of activity of male and female mice housed in a 12:12 LD (Light Dark) cycle, relative to lights off on days following LD cycle shift by 6 hours, n=7 to 9 for each condition and sex. Each day plotted and data analysed by Two-way ANOVA. A significant effect of both genotype ($p<0.0001$) and sex ($p=0.041$) was observed. Multiple comparisons using Sidak's multiple comparisons test reveals a significant difference ($p<0.0001$) in WT vs KO mice on every single day. *Mafg*+/+ mice appear to entrain quicker than *Mafg*-/- mice for both sexes. Across both the genotypes, the female mice entrain faster than the male mice. Data is presented as mean +/- S.E.M.

To further assess the photic entrainment function of *Mafg*, control and knockout mice were subject to a 30 minute light pulse of 10 lux intensity at CT16. This time of day was chosen based on the ATAC-seq experiment showing differential chromatin accessibility in the *Mafg* promoter region. Figure 3.2 i) and ii) show the timepoint where the two groups of animals were light pulsed, as indicated by the red dot. The phase shift, that is the delay in onset or wake-up time, on the day after light pulse administration, can be seen. Analysis using a one-way ANOVA revealed significantly reduced phase shifting in knockout mice after the 30 minute light pulse. This implies that perhaps the knockout animals were less affected by the light pulse and along with the jet lag analysis, goes on to show overall reduced photic responses in mice that lack the *Mafg* gene.

3.2g

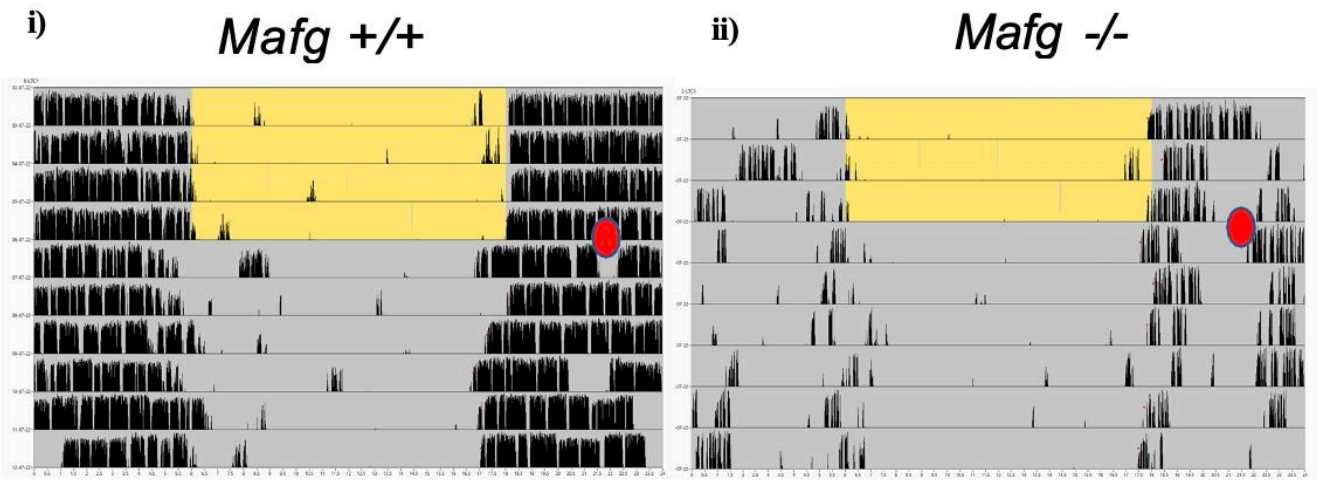


Figure 3.2g Actograms of *Mafg* WT and KO male and female mice housed on running wheels, in 12:12hr LD cycles. Yellow = lights on, grey = lights off, black bars = wheel running activity. Mice exposed to a 10 lux 30min light pulse at CT16 (indicated by red dot) in i) *Mafg* +/+ and ii) *Mafg* -/-, showing that *Mafg* -/- show smaller phase shifts than *Mafg* +/+ animals. The KO mice seem to have lower and more scattered activity compared to WT. Approximate phase angle shown by red line.

3.2h

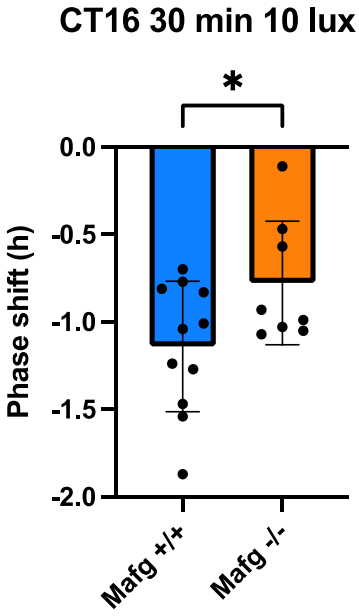


Figure 3.2h One-way ANOVA indicating reduced phase shifting in the knockout mice after the 30-min, 10 lux LP at CT 16.

3.3 qPCR analysis of MAFG targets

The final aspect of this project was to profile changes in the light-regulated transcriptome that were affected in the absence of *Mafg*. Since a large number of genes in the light-regulated transcriptome are repressed at night, due to the disruption of the MAPK pathway disruption (Alzate-Correa et al., 2021), it can be hypothesized that *Mafg* null mutants would show an upregulation of genes involved in photoentrainment located downstream to *Mafg*, since MAFG itself has been hypothesized to be a repressive factor (Motohashi et al., 2000). To investigate this, published data were used to identify putative MAFG targets. Wheeler and colleagues (Wheeler et al., 2020) conducted a study where *Mafg* was knocked down in astrocytes using a lentiviral vector to study the effect on experimental autoimmune encephalomyelitis (EAE). The genes that came up as differential following the knockdown of *Mafg*, were compared to the list of differential genes following the light pulsing of neurons in the SCN for several durations (30 minutes, 1 hour, 3 hour etc.) at CT 17, published in the 2021 paper by Xu and colleagues (Xu et al., 2021). The aim was to detect any overlap between genes that were differential due to *Mafg* knockdown and those due to exposure to a nocturnal light stimulus. Since the knockdown study was in glial cells, limited overlap was expected but given the time constraints, these targets were chosen for further analysis. In the future, this would be done by targeted promoter analysis and/or and RNA-Seq approach. Figure 3.3a shows the genes common to both lists.

To validate these genes, SCN tissue samples were collected from *Mafg* +/+ and *Mafg* -/- mice (n=3 to 5) for each condition) following a 1 hour light pulse at CT 16 at 100 lux intensity. This tissue from light pulsed (LP) and sham/control animals was investigated for an up or downregulation of the genes identified in figure 3.3a using qRT-PCR. In addition to these genes, the expression of some immediate early genes (IEGs) including *Egr1* and *Fos*, previously shown to respond to light in the SCN (Jagannath et al., 2013) as well as clock genes like *Per1* and *Per2*, and lastly *Sik1*, was measured (Figure 3.3b). It is known that *Per1* and *Sik1* mRNA peak after ~1 hr and *Per1* then returns to baseline (Vitaterna et al., 2006). These genes were used as controls to check the quality of the tissue samples.

Housekeeping genes - *GusB*, *Rps9*, and *Tbp* were run as controls alongside, as they are stably expressed and are thus used to normalise mRNA levels between different samples.

Only *Fos* nd *Egr1* from the control genes depicted a significant response in WT mice before and after a LP. However, all of them showed a clear trend of increased expression on light exposure. *Egr1*, *Fos* and *Sik1* also showed significantly increased gene expression on light exposure in knockout animals. This is presumably due to the limited number of samples used; an n of 6-8 would be desirable for this study.

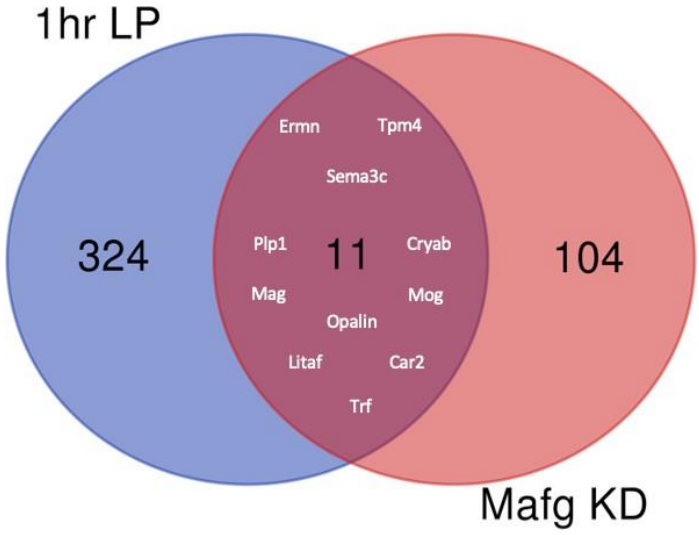


Figure 3.3a Venn-diagram depicting the 11 genes that were differential following *Mafg* knockdown in astrocytes as well as following a 1 hour light pulse at CT17.

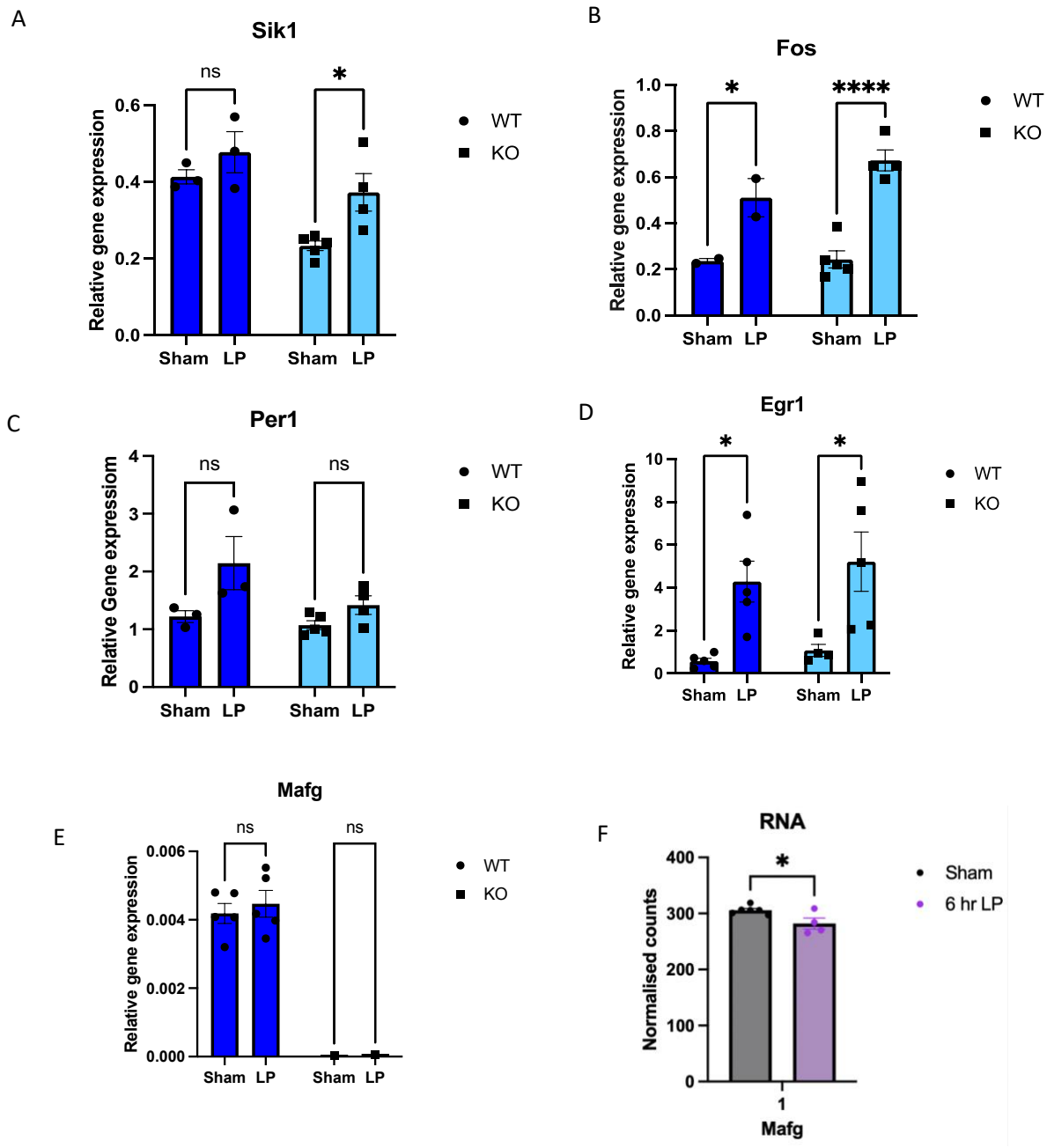
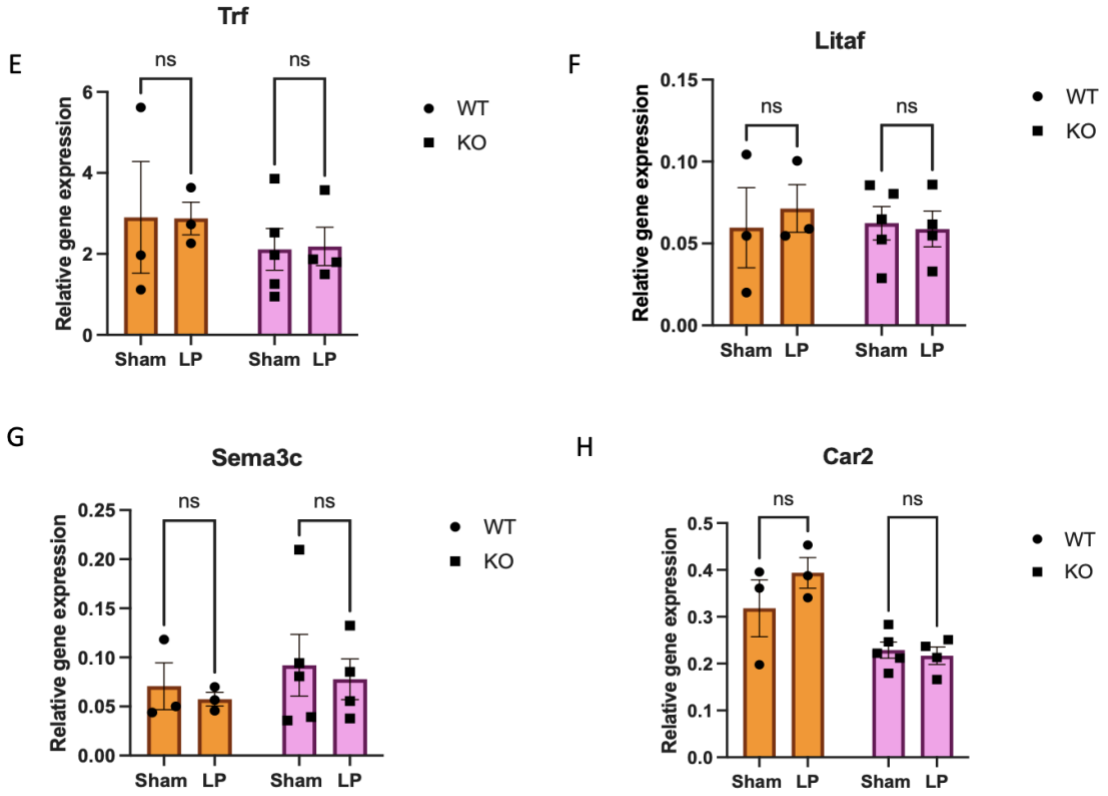


Figure 3.3b A-E qPCR data showing the fold changes for the gene expression of control genes *Egr1*, *Per1*, *Sik1* and *Fos*, and for the gene *Mafg,* calculated by the 2^-ΔCT method. Analysis using a 2-way ANOVA shows that light stimulation (a 1 hour light pulse) tends to increase gene expression across all five genes, for sham samples of mouse SCN, although not significant. *Fos, Egr1,* and *Sik1* show significant upregulation in KO samples after the LP. Data is presented in +- SEM.

Figure 3.F – normalized RNA-sequencing counts for *Mafg* after a 6 hour LP ZT18-0 (gray=control, purple=6hr LP) showing *Mafg* downregulation.

From the qPCR results for the *Mafg* gene itself, it can be seen that there is no change in *Mafg* expression in WT mice after an hourlong LP. Perhaps, it was due to low n numbers and that it was too early in time to see a change in *Mafg* levels, i.e. perhaps differences would show up later in time such as after a 6 hr LP, as was the case in a previously conducted RNA-seq dataset that showed a downregulation of *Mafg* after a 6 hour light stimulus in WT mice (see Figure 3.3F) (Lewis Taylor, unpublished).

After analysis using the ΔCT method, it was found that *Tpm4* (see Figure 3.3c.F) was the only gene that was significantly upregulated (p value for Sham:WT vs. LP:WT = 0.0085) after the light pulse in the sham mice. However, in the LP animals, its expression levels did not increase to the same extent after the light input. This shows that *Tpm4* did not respond to nocturnal light in the same way as sham animals in the absence of *Mafg*, suggesting that MAFG could be a likely transcriptional regulator of the TPM4 protein. The other genes showed a varied response, with some being up or down regulated but not to a significant extent.



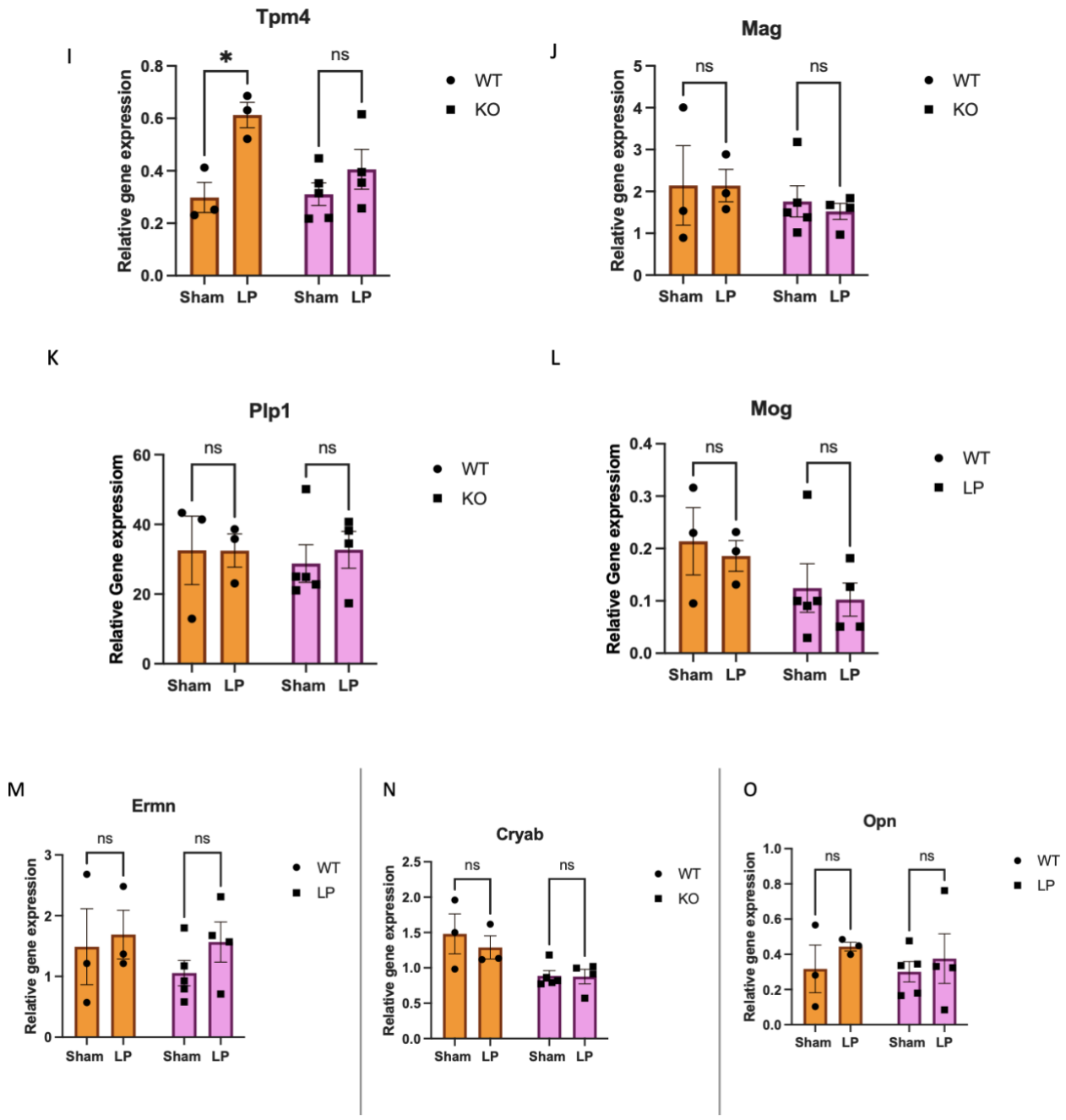


Figure 3.3c (E-O) qPCR data showing the fold changes for the gene expression of potential MAFG transcriptional targets, highlighted in figure 3.3a, calculated by the 2^-ΔCT method. Analysis using a 2-way ANOVA shows the result of nocturnal light (a 1 hour light pulse) on gene expression, for both sham and KO tissue samples of mouse SCN. *Tpm4* (figure I) was highly upregulated in sham mice but showed dampened gene expression when in KO animals. Data is presented in +- SEM.

4. Discussion and Future Work

Light signalling to the SCN is known to cause wide ranging changes in gene expression, but how these changes might be controlled through modifications of chromatin accessibility remains elusive. The aim of this project was to investigate the transcription factor MAFG as a candidate in circadian rhythm regulation and using a variety of *in vitro* and *in vivo* experiments. Some positive conclusions can be drawn. The results from the *in vitro* analysis in section 3.1 proved to be highly significant where the *Mafg* promoter region drove luciferase expression thus suggesting that it is capable of driving transcription in U2OS cells. Furthermore, knockdown of *Mafg* significantly reduced luciferase expression, thus confirming our previous result, and this was further down regulated when subjected to Forskolin, that is known to set off the same molecular pathway as a light pulse. Forskolin is known to elevate the cAMP levels, and through the protein kinase A (PKA) signaling pathway, it enhances the phosphorylation and activation of Ca2+/cAMP responsive element binding protein (CREB) (Yagita and Okamura., 2000). It is important to note that calcium signalling via the cAMP pathway is a common signaling cascade that is behind the induction of numerous cellular and physiological genes. However, the function of Forskolin as pharmacologically induced photic stimuli can be validated with an induction of circadian genes such as *Per1*, *Per2* and *Dbp*, with acute induction of the *Per1* gene and phosphorylation of CREB, following forskolin treatment (Yagita and Okamura, 2000).

This down regulation of *Mafg* transcription as a possible inference from the reduced luciferase expression in response to Forskolin also validates our hypothesis that *Mafg* is repressed when subjected to continued light. This is because it corresponds to the earlier ATAC-seq finding where the chromatin in the promoter region of *Mafg* closed following a nocturnal light pulse. The successful transfection results also strengthen the fact that AP-1 / CRE / EGR-1 are potential binding sites for factors that are influenced by light, thus playing an important role in photo entrainment and the molecular clock. In future, these transfection experiments could be repeated with more controls such as by running a positive

control CMV (cytomegalovirus) plasmid vector to promote constitutive expression of cloned inserts, and an empty vector negative control.

The second aspect of this project where I looked at the circadian phenotypes of *Mafg* knockout mice, I found that the knockout animals had a period similar to WT animals, i.e. approximately 24 hours, but the strength of their circadian rhythm could be inferred to be slightly weaker, owing to their reduced amplitudes in the *in vivo* study. This particular phenotype was backed up by the *in vitro* reporter cell analysis, leading us to believe that MAFG is involved in the circadian clock. Furthermore, it was surprising to see that a 6 hour phase advancing jet lag had an effect that was opposite to what was expected. The faster re-entrainment to the new LD cycle by WT mice compared to KO mice contradicted our hypothesis that MAFG was a potential repressor of phase shifting responses. It would be interesting to investigate this further and to know whether this could be attributed to any other molecular pathway that MAFG potentially acts on, in addition to CREB signalling. Analysis of the phase shifts, that were also in agreement with the jetlag results, where mice that lacked *Mafg* showed smaller phase shifts following a nocturnal light pulse at CT 16, suggest the fact that CREB signalling was not repressed as *Mafg* was absent. It could be inferred that the repression of MAFG may be required for a larger phase shift. By removing that repression, MAFG target genes should have been downregulated, but they did not, perhaps explaining the slower entrainment phenotypes. Since the majority of the light regulated transcriptome is repressed (Alzate-Correa et al., 2021), and AP-1 binding is also likely to be a major part of what is driving the repression, we believe that this could be enabled by MAFG. MAFG could be negatively regulating expression at AP-1 elements. A potential way to investigate this would be to run a luciferase reporter assay by knocking down two members of the sMAF family and comparing the results with that of a co-transfection and knockdown experiment using MAFG and another AP-1 transcription factor family member, from the FOS, JUN or ATF subfamilies. This could shed light on the different effects of MAFG homodimerization and heterodimerisation. From these results it could be inferred that repression was actually needed to facilitate photic

entrainment. Furthermore, despite the induction of light inducing molecular clock genes such as *Per1* within the SCN, the effect of light on molecular clock proteins is not consistent across the whole day. Indeed, entrainment occurs primarily in the morning (Khalsa et al., 2003) for diurnal individuals such as humans, and primarily in the evening for nocturnal animals such as mice; with any light received by nocturnal animals in the morning having a very diminished effect. Additionally, even when light is encountered at the optimum time of the day, entrainment is limited to around two hours (Vitaterna et al., 2006), and any light received after this will not result in further shifts to the molecular clock (Minors et al., 1991). For this reason, the time point of CT 16 was selected for all our experiments. A limitation of the above *in vivo* experiments is that the running wheel activity gives us an approximate measurement of the time that the animal spends in the awake-state. It is difficult to account for any sleep changes as running wheel activity is known to change the sleep landscape in mice (Fisher et al., 2016).

The final aim of this project was to uncover any targets which were in turn regulated by MAFG. The qPCR analysis of the differential genes identified from published data provided a preliminary glance at potential MAFG targets. Tropomyosin 4 (*Tpm4*), that is well known for its role in the muscle contractile system was the only gene observed to be affected by the absence of *Mafg*. However, it's relevance to light entrainment would need to be investigated. The PCR analysis also seemed to show a slight trend in the increase of some of the MAFG targets (Figures 3.3c-I, K, M, N, O) when *Mafg* itself has been removed, but this was not to a significant extent. Although this is difficult to interpret, it could perhaps be due to the repressive nature of MAFG, suggesting that the absence of MAFG would inhibit the repression of the targets, resulting in their increased expression. I also expected to see a change in the expression levels of *Mafg*, but this was not the case, likely due to low n numbers. To cross-check this qPCR result for *Mafg,* I looked at previously conducted RNA-seq experiments on WT mouse SCNs for a 6 hour LP (ZT18-0) within the Jagannath lab, which showed the downregulation of *Mafg* (Lewis Taylor, unpublished data). This tells us that perhaps, 1 hour was too early to see differential levels of *Mafg,* and I aim to test for this using longer light pulses in future. The target identification experiment

did not provide compelling enough evidence, and this could be attributed to the limited number of samples used and the large age-range of the mice used (12 weeks to 23 weeks). Thus, it would ideally have to be repeated with a large enough and equal number of animals for each condition.

In the future, the best way to identify these leads would be through a promoter analysis using software such as HOMER or G-profiler, and choosing those gene candidates that have a strong MAFG element (TGCTGACTCAGCA, characterised by flanking GC motifs). Furthermore, a full RNA-Sequencing analysis of light pulsed mouse SCNs would give a better picture of the molecular changes occurring in the SCN transcriptome, but given the time constraints, it could not be completed. In addition to this, the next step would be to conduct a ChIP-Seq (Chromatin Immunoprecipitation Sequencing) experiment to identify the DNA binding sites for MAFG. Literature suggests that a biotin-ligase recognition peptide (BLRP)-tagged *Mafg* construct was used to conduct ChIP-seq on mouse liver tissue to study metabolism. An anti-BLRP antibody was used after confirming that the BLRP epitope did not interfere with MAFG function (Aguiar-Vallim et l., 2015). A similar approach for the SCN would enable us to narrow down on its targets and how they are regulated, to better understand this novel gene.

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Appendices

A.Cloned Sequences Of Interest

>MAFG Full region

AAAAAAGGTACCCCTGTCTCCCGACTAGACCGGCCGATAgcgggccccggccccccgcccTG
GCCCAGCGTCAGCAGGAGCACCGAGTGACCTCATGCTGATGACTCAGCACGTCTCCGCTGGG
CTCGCACCCGCGTGGCGAGCCCAAGCCGCGCAGCAGGGTCTCCCCGCCGCCTCCCCTGTggg
cccgcgcgcgaccccgcccgcgcggAAGCCGACGGACACCTGATGAGTCAGCAGCTCGGTGC
GGCCACCCCGCTGACCCGGGCAGACGCGCGCAGCTCGCCCTTAAATCGCGCCCCCCGCAGGG
CACCCGGGGTCCGCCGCCGCGCGCACGCCCGTCCCCAGCCTCGCGGTCGCCCGGTCCGCCGG
CTCTAGGGGGACACAGGCGAACAGCCGGCTGGCGGGGAAACCAAGGCACGCGCTGCCGTCGG
GTGCGCCGCAACCCCGAGGCCAggccccgccgcccgccccagccccgcgcACTCACCACACT
Gcgggcccgggcgggccaaggcggggctggggcggggccgcgcgcgcgctccggggctgc
gcggggctggggcgggcgcgaagggtccgggccgggggcttaggaggactggccgcctgcgc
gggccgggccaagcgcgctgggaaggccgggccggaccgggcTCAGAATCCGCCGCCGCCGC
CGCTCCTCAGACGTCACATGATGTTTGGTCACGTGGGTTCCATTCATGAAGCGGCGACGCGG
CTGGCCTAAGCTTAAAGGGAAAGGCGAGGCGGCAGCAAGGCGAGCGCGCCCCCATCACGCCT
GCGCAGCCCCGCGCCGTTGCCCTTGGCAACGCCCGCCGCGCCCTGGCAATGTCTCGCGGGAG
TCGTCCCGGCGCTCGCCGGGTTGACCGTGCTCCTGCAGAGACAGGTTCT**CTCGAGAAAAAA**

>MAFG - First Peak

AAAAAAGGTACCtgcgcgggccgggccaagcgcgctgggaaggccgggccggaccgggcTCA
GAATCCGCCGCCGCCGCCGCTCCTCAGACGTCACATGATGTTTGGTCACGTGGGTTCCATTC
ATGAAGCGGCGACGCGGCTGGCCTAAGCTTAAAGGGAAAGGCGAGGCGGCAGCAAGGCGAGC
GCGCCCCCATCACGCCTGCGCAGCCCCGCGCCGTTGCCCTTGGCAACGCCCGCCGCGCCCTG
GCAATGTCTCGCGGGAGTCGTCCCGGCGCTCGCCGGGTTGACCGTGCTCCTGCAGAGACAGG
TT**CTCGAGAAAAAA**

>MAFG - Second Peak

AAAAAAGGTACCCCGGATCGAGCCCTGTCTCCCGACTAGACCGGCCGATAgcgggccccggc
cccccgcccTGGCCCAGCGTCAGCAGGAGCACCGAGTGACCTCATGCTGATGACTCAGCACG
TCTCCGCTGGGCTCGCACCCGCGTGGCGAGCCCAAGCCGCGCAGCAGGGTCTCCCCGCCGCC
TCCCCTGTgggcccgcgcgcgaccccgcccgcgcggAAGCCGACGGACACCTGATGAGTCAG
CAGCTCGGTGCGGCCACCCCGCTGACCCGGGCAGACGCGCGCAGCTCGCCCTTAAATCGCGC
CCCCCGCAGGGCACCCGGGGTCCGCCGCCGCGCGCACGCCCGTCCCCAGCCTCGCGGTCGCC
CGGTCCGCCGGCTCTA**CTCGAGAAAAAA**

B.siRNA sequences for gene knockdown

Mouse Mafg siRNA	GCAUGAAGCUGGAGCUCGA
Mouse SIK1 siRNA	ACGAUUAGAUUCAAGCAAU
Mouse Non-targeting Control	AUGUAUUGGCCUGUAUUAG

C.Primer Sequences for qRT-PCT

Gene symbol	Forward primer (5'–3')	Reverse primer (5'–3')
Mafg	GACCCCCAATAAAGGAAACAA	TCAACTCTCGCACCGACAT
Tpm4	ACTGAGTCTGCTCCACACGGC	ACTTGAGTTGGCCCCGCACT
Car2	TCGGTGCTCACAATGGGGCTG	CTCCGCTTCTGCGTCCGACC
Sema3c	TGGGGGCATTCAGCCACTCG	CCTCGCTCAACCGAGGCTGT
Litaf	AGGGTGACCACTCTGCCGTG	GCCTGAGGTGTGGGTCAGGAAG
Trf	CCACCTGGCCCAAGCTCCAA	GCCACGGGAGGGAGCATCTG
Opalin	CTGCCGGACACCTACCGTCC	GGACACTCGGGAGCCAAGCC
Cryab	AACAAGGATGGGGTGGGTGC	CCCAGCTCACCGCCAGTTCAT
Mog	ATGGAGGTTGGGCTTGGGCAG	GTCCTCCGGCTGCATGTCCC
Mag	AGGATGATGGGGAATACTGGT	AAGGATTATGGGGGCAAACT
Ermn	ACTGAGCGGGACAGAGTGCAA	TCAAGCTGTTGCTGGGCAGT
Plp1	CTCCAAAAACTACCAGGACTATGAG	AGGGCCCCATAAAGGAAGA
Rps9	CGTCTCGACCAGGAGCTAAAG	GCACCCCAATGCGAACAAG
Sik1	TGGACGTCTGGAGCCTCGGT	AGAGTGGGGTCGGCCTGCAT
Egr1	GAGCGAACAACCCTATGAGC	AGCGGCCAGTATAGGTGATG
Per1	AGTTCCTGACCAAGCCTCGTTAG	CCTGCCCTCTGCTTGTCATC
GusB	CCAGAGCGAGTATGGAGCAG	TCGTCATGAAGTCGGCGAAA
Tbp	CCTTGTACCCTTCACCAATGAC	ACAGCCAAGATTCACGGTAGA