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Generation of knockout cell lines to investigate the roles of Cklf and Cry2 in the regulation of circadian clock

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GENERATION OF KNOCKOUT CELL LINES TO INVESTIGATE THE ROLES OF *Cklf* AND *Cry2* IN THE REGULATION OF CIRCADIAN CLOCK

by

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Abstract

Many physiological parameters such as body temperature, circulating hormone levels, blood pressure and metabolic activity are rhythmic, and their rhythmicity is governed by the circadian clock. This is a self-sustained ~24-hour cycle, which at the molecular level is determined by negative transcriptional and translational feedback loops controlled by the products of core clock genes. There are at least 10 core clock genes namely *Bmal1, Bmal2, Clock, Npas2, Per1, Per2, Per3, Cry1, Cry2, Nr1d1 and Nr1d2.* In addition to these core clock genes, clock modifier genes exist that modulate the molecular clock by integrating synchronous and asynchronous clues from signal transduction pathways. Nearly 1000 clock modifier genes have been identified and among these is the chemotactic cytokine-like protein CKLF. shRNA mediated down-regulation of *Cklf* conducted in U2OS cell lines exhibited short period length, whereas its knockdown in MMH-D3 reporter cells resulted in decreased amplitude of oscillation, suggesting a role for *Cklf* in regulating the oscillatory function of the clock. Since *Cklf* is an immune mediator, this result suggests a functional interaction between the immune system and the clock. To further investigate the interaction between *Cklf* and the core clock genes and to determine how the circadian clock is integrated with and regulated by immune mediators, we carried out knockout studies using the latest Crispr/Cas9 gene editing tool. Crispr/Cas9 is an efficient genome editing tool that allows for genetic perturbation of individual genes with enhanced specificity and efficacy of gene knockout compared to RNAi approaches. Using Crispr/Cas9 method my work focused on knocking out *Cklf* and one of the core clock genes *Cry2* in MMH-D3 reporter cell lines. We focused on *Cry2* because it is one of the key proteins that controls the period of the clock. We successfully knocked out *Cry2* and showed that the knockout cells exhibited long-period phenotype, confirming that *Cry2* is core clock gene that

functions to maintain the period of the clock to about 24 hours. In contrast, repeated efforts to knock out *Cklf* using the Crispr/Cas9 approach was unsuccessful. Future work aims to obtain *Cklf* knockout cell lines by improving the genome editing efficiency such as higher transfection efficiency, expanding flow cytometry-based single cell sorting and cloning, and designing new PCR genotyping strategies.

Table of contents

List of figures	v
Keys to Abbreviations	vii
Introduction	1
Significance and hypothesis	8
Materials and Methods	9
Results	21
Discussion	33
References	37

List of figures

1. Molecular mechanism of circadian clock 2

2. Expanded clock gene network 5

3. Phenotype of the KD studies of *Cklf* using shRNA 6

4. Primer design to genotype the KO Cry*2* and *Cklf* clones 10

5. Alternative Design to knock out *Cklf* 19

6. Fluorescence image of transfected MMH-D3 reporter cells to check transfection efficiency 26

7. Genotyping of *Cry2* polyclonal clones for primer validation and verifying the presence of deletion amplicons 26

8. Genotyping of *Cry2* polyclonal clones for primer validation and verifying the presence non-deletion amplicons 27

9. Genotyping of individual *Cry2* clones for non-deletion, monoallelic, and biallelic/ KO clones 27

10. Western blot of WT, HZ and KO *Cry2* clones 28

11. Lengthening of period length of HZ and KO *Cry2* clones 28

12. Lengthening of period length of HZ, KO *Cry2* clones in comparison to WT clones 29

13. Genotyping of *Cklf* polyclonal clones for primer validation and verifying the presence deletion amplicons 29

14. Genotyping of *Cklf* polyclonal clones for primer validation and verifying the presence non-deletion amplicons 30

15. Genotyping of single cell clones of *Cklf* 30

16. Transfected MMH-D3 cells screened for possible representative areas receiving Crispr/Cas9 *Cklf* constructs using confocal microscope 31

17. Non-specific bands of polyclonal *Cklf* FACS sorted cells 31

18. Genotyping of *Cklf* polyclonal clones for primer validation and PCR standardization 32

19. Genotyping of *Cklf* single cell clones 32

Keys to Abbreviations

BMAL- Brain and muscle Arnt-like protein

bp- Base pair

Cas9- CRISPR associated protein 9 nucleases

Cklf- Chemokine like factor gene

CKLF- Protein product of the gene *Cklf*

CLOCK- Circadian locomotor output cycles kaput

CRISPR- Clustered Repeatedly Interspaced Short Palindromic Repeats

CRY- Cryptochromes

DMSO- Dimethyl Sulfoxide

DPBS- Dulbecco's phosphate-buffered saline

EDTA- Ethylenediaminetetraacetic acid

gDNA- Genomic deoxynucleic acid

HZ- Heterozygous

KD- Knockdown

KO- Knockout

LB- Lysogeny broth

LPS- Lipopolysaccharide

MMH-D3- Met Murine Hepatocytes Day 3

NHEJ- Non-homologous end joining

PAGE- Polyacrylamide Gel Electrophoresis

PCR- Polymerase chain reaction

PER- Period

Rev-Erb α- Reversal Viral Erythroblastic Oncogene Product α

RIPA- Radioimmunoprecipitation assay

RORE- Retinoic acid-related Orphan Receptor Response Element

RPMI- Roswell Park Memorial Institute media

SCN- Suprachiasmatic nucleus

SDS- Sodium dodecyl sulphate

shRNA- Short hairpin ribonucleic acid

siRNA- Short interference ribonucleic acid

TE- Tris EDTA

WT- Wild type

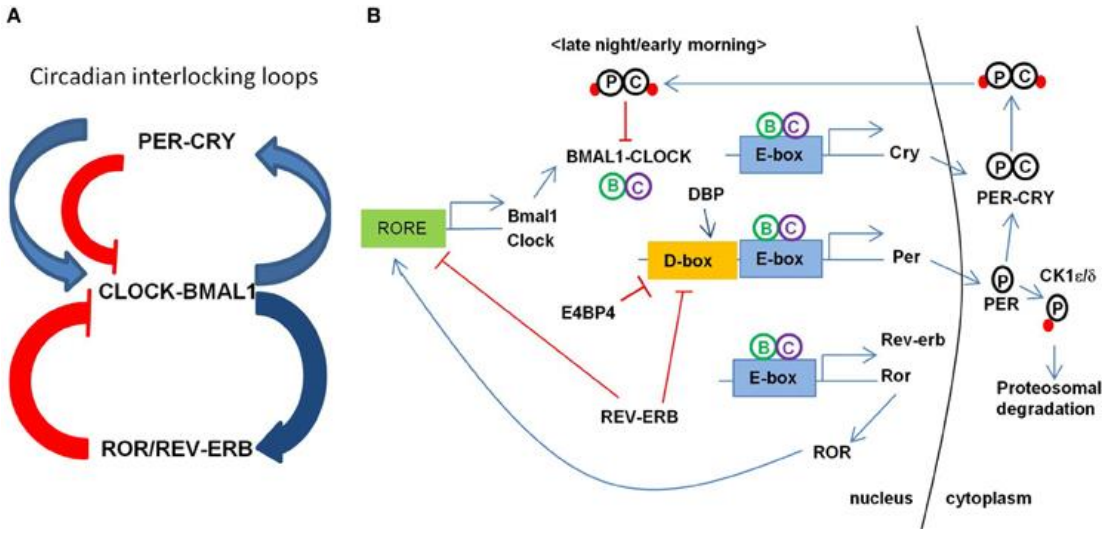
Introduction

Inherent timing mechanisms which control the various physiological processes and behavioral states of organisms are called biological clocks (Birky & Bray, 2014). The biological oscillators occurring over a period of approximately 24 hours are known as circadian rhythms. The oscillations are controlled by the endogenous circadian clocks. The characteristic features of the circadian clock are that its periodicity persists under ambient conditions and in the absence of environmental clues (Buhr & Takahashi, 2013). Another unique feature of the clock is its robustness under different temperature conditions.

The master circadian pacemaker is located in the Suprachiasmatic Nucleus (SCN). Circadian clocks are present in all cell types besides the SCN and are called the peripheral clocks, which are coordinated and synchronized by the SCN clock (Partch, Green, & Takahashi, 2014). The molecular mechanism of the circadian clock in individual cells is based on a negative transcriptional/translational feedback mechanism, known to consist of three feedback loops, each of which are regulated by their own activators and repressors (Figure 1).

In the core feedback loop, the activators are *Bmal1*and *Clock* while the repressors are *Per* and *Cry* which form time-of-day-dependent complexes that function to regulate the E-box cis-element-mediated transcription of genes (Takahashi, 2015). The positive limb of the loop is composed of transcriptional activators BMAL1and CLOCK proteins which form heterodimers and bind to the E-boxes present in the promoter region of target genes including *Cry1,2* and *Per1,2,3.* The PER and CRY proteins are the negative components of the transcriptional/translational feedback loop; they in turn form heterodimers and enter the nucleus to bind to BMAL1-CLOCK complex and inhibit their own transcription (Albrecht, 2012), completing the loop. Since the negative components are involved in the regulation of their own

transcription, the circadian clock mechanism is a negative transcriptional/translational loop. The repression phase ends when PER and CRY are degraded, leading to a new transcription cycle. The period of the clock is determined in part by the stability and the degradation rate of PER and CRY (Buhr & Takahashi, 2013).



(Cho, 2012)

Figure 1. Molecular mechanism of the circadian gene regulation. The core clock loop consists of BMAL1-CLOCK and PER-CRY complexes acting via the E-box. The ROR/REV-ERB form additional feedback loops. RORs as activators and REV-ERBs as repressors regulate the expression of BMAL1. D-box, another accessory loop is necessary to maintain the precision of the clock.

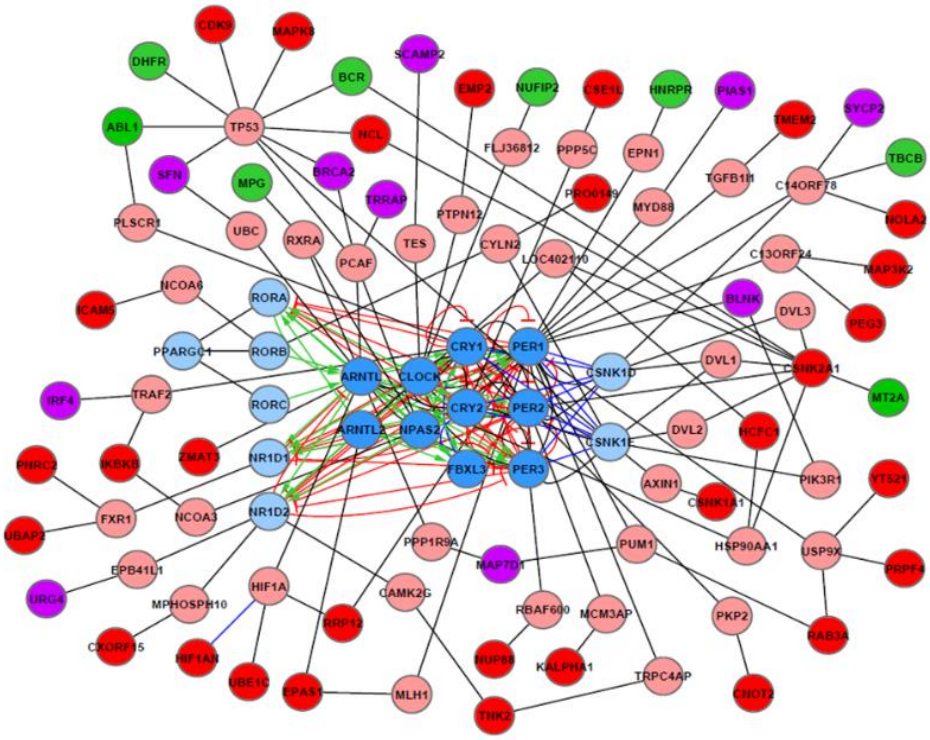
The E-box activation also activates a second feedback loop, in which the Rev-Erbα/β repressors and RORα/β/gamma activators competes for RORE binding sites present in the promoter region of *Bmal1* (Figure 1) (Cho, 2012). ROR proteins initiates the transcription of

Bmal1 while REV-ERB proteins inhibit the transcription of *Bmal1*. This opposing action of RORs and REV-ERBs are necessary for maintaining the period of circadian rhythmicity. The third loop is the D-box loop which is required for the robustness of the clock and maintaining the precision of its period (Takahashi, 2015; Cho, 2012). It is the combined action of the three loops just described that brings about the approximate 24-hour cycle. The binding elements of the three different loops act during different time of the cycle to bring about a circadian cycle with a periodicity of about 24 hours. The E-Box elements act in the morning while the RORE elements during evening and the D-Box during the day.

In addition to the core clock genes acting in different loops to bring about the circadian cycle, numerous modulators affect the function of the clock in different cell types. (Takahashi, 2004). These clock-regulating genes are the clock modifier genes. Zhang et al. (2009) conducted a whole genome screen using RNAi in human U2OS cells to identify clock modifier genes. Knockdown of more than 25,000 genes in the human genome was done using siRNA and the effect on the clock was studied. Changes in the amplitude and period length of the clock was noted for nearly 1,000 knockdown genes. Along with the knockdown study, protein interaction studies showed direct interaction of some of the proteins encoded by these genes with the core clock components while other interactions were indirect through bridging molecules. This study showed the functional interconnectedness of the clock with many other biological pathways (Zhang et al., 2009) (figure 2). This led to the continued study of clock modifier genes in our lab focusing on nearly 300 genes with the strongest knockdown clock phenotypes.

My study focuses on one of these modifiers, *Cklf,* along with *Cry2,* a core clock gene. Cells in which *Cklf* has been downregulated exhibit short period and low amplitude phenotype (figure 3) (Zhang et al., 2009; Ramanathan et al., unpublished). *Cklf* is one of the several

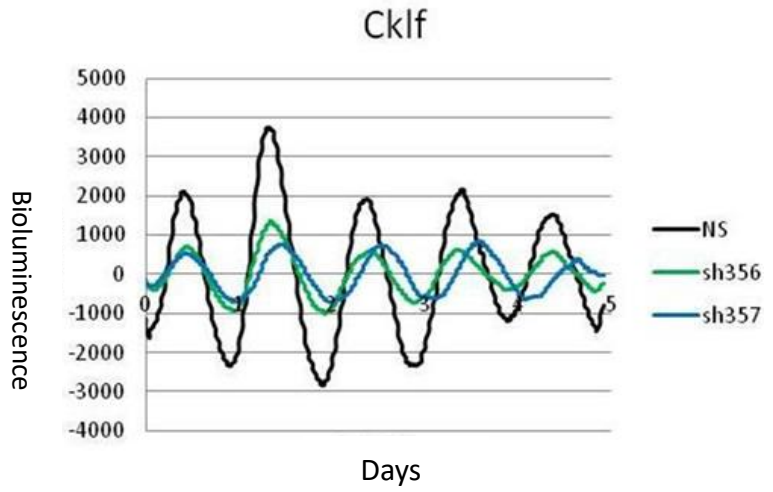
chemokine-like gene. Its chromosomal location is unique as it is one of the three chemokine genes located on human chromosome 16 while most of the 40 or so chemokine genes are located on chromosome 17 (Wenling et al., 2001). *Cklf* is a type of CC chemokine among the four distinct types of chemokines identified. The nearly 40 different chemokines are classified into four different types based on the cysteine motifs in the amino acid sequences. The four types are CXC, CC, CX3 and C. CXC is also called alpha chemokine to indicate that there is an amino acid separating the two cysteine motifs.CC chemokines do not have any amino acid separating the cystines and are also called beta chemokines. The C-type is also called gamma chemokine and has one cysteine at the N-terminus and one at the C-terminus. CX3 has three amino-acids between the two cysteines (Murdoch and Finn, 2000).



(Zhang et al., 2009)

- Green -short period hits
- Red-long period hits
- Purple-high amplitude
- Blue-clock components
- Pink-common interacting proteins

Figure 2- The expanded clock gene network. The interactions of the various clock modifiers (represented by different colors) with the core clock components were identified. Each color represented a phenotype the clock modifier exhibited as shown above



(Ramanathan et al., unpublished)

Figure 3. Bioluminescence data of shRNA downregulation of *Cklf*. shRNA mediated downregulation of *Cklf* caused shortening of period length and decrease in amplitude of circadian bioluminescence rhythms as indicted by green (sh356) and blue (sh357) in MMH-D3 hepatocytes. The difference in the period length and amplitude between the two sh RNA used can also be noted.

However, *Cklf* also differs from other CC chemokines as the mature protein contains only one conserved CC motif but no additional C residues in the C terminus which is present in other CC chemokines. Hence, it is considered a chemokine-like factor rather than a true chemokine (Wenling et al., 2001). The product of the gene is a chemotactic cytokine, a small protein with molecular weight between 5 to15 kDa that acts as a chemoattractant for lymphocytes, monocytes and neutrophils and also stimulates the proliferation of myoblasts. Cytokines are involved in many immune and inflammatory processes. Cytokines include interleukins, interferons, tumor necrosis factors and chemokines. *Cklf* being a chemokine, exerts its action through G- protein coupled receptors. Approximately 20 chemokine receptors have been identified and are named as

CCR (chemokine receptor)1-20. Among these receptor *Cklf* exerts its action through CCR4 receptor. (Mantovani, 1999; Murdoch and Finn, 2000).

The interaction between the circadian and the immune system is bidirectional (Dumbell, Matveeva, & Oster, 2016). Numerous studies have shown that the core clock proteins namely BMAL1, CLOCK, PER and CRY control fundamental aspects of the immune system (Curtis, Bellet, Sassone-Corsi, & O'Neill., 2014). *Bmal1* gene deletion in monocytes, macrophages and granulocytes resulted in these cells losing the rhythms of cytokine response (Gibbs et al., 2012). LPS injection in *Cry1/Cry2* double KO mice increased cytokine expression (Narasimamurthy et al., 2012). Studies have also shown reciprocal interaction. In a study the levels of cytokines exhibited circadian variation (Scheiermann, Kunisaki, & Frenetterring, 2013). Cytokines such as interleukins 1 and 6, and tumor necrotic factor α stimulate the hypothalamic pituitary axis to produce glucocorticoids which in turn act on immune cells to suppress proinflammatory responses (Pollmächer et al., 1996). Inflammation and infection can disrupt the expression of the core clock genes (Logan & Sarkar, 2012). *Cklf,* being a chemotactic cytokine, is involved in immune mediation and is increased in autoimmune and inflammatory diseases such as rheumatoid arthritis and asthma. In the case of asthma, the increase in the inflammatory response is by the stimulation of NF-kB signaling pathway by *Cklf* through activation of CCR4 receptor (Li et al., 2014). This increase in inflammatory response is increased during the wee hours of the morning suggesting a circadian variation (Durrington, Farrow, Loudon & Ray, 2013).

The studies described above show interconnectedness between the immune signaling and the circadian clock pathways. This led us to further study the effect of *Cklf* knockout on the function of the circadian clock, which will advance our understanding of the functional intersection of the circadian clock and *Cklf* pathways. To this end, in this project, we aimed to

create *Cklf* knockout cell lines, as well as *Cry2* knockout as a positive control, using type II Crispr/Cas9 gene editing tool in MMH-D3 cell lines to obtain knockout cell lines with which to study the role of *Cklf* in regulating clock function.

Significance

Previous shRNA down-regulation studies on *Cklf* conducted in our lab have shown that down-regulation of this gene induces low amplitude and short period length phenotypes in U2OS and MMH-D3 reporter cell lines (Zhang et al., 2009) (Ramanathan et al., unpublished, figure3). These results suggest that *Cklf* may influence the core clock components in the generation of oscillatory rhythms and paved the way for me to further understand the relationship between *Cklf* and the clock mechanism. With the interconnectedness between the circadian clock and the immune system, knockout of *Cklf* may provide important insights into the interaction between the chemokine *Cklf*, an immune regulator and the clock gene network, as well as on how the immune system regulates the circadian clock. To progress toward this goal, my work focused on establishing cell lines in which the expression of *Cklf* and *Cry2* (as a known clock gene control) were permanently deleted using the Crispr/Cas9 gene editing tool.

Hypothesis

The Crispr/Cas9 gene editing tool will enable establishment of cell lines in which *Cklf* is deleted from the cell's genome, and these cell lines will be useful for functional studies of the role of *Cklf* in the circadian cycle.

Materials and Methods

Design to knockout *Cklf* and *Cry2* using the Crispr/Cas9 gene editing tool

The Crispr/Cas9 system is an efficient gene editing tool adapted from adaptive immune system in prokaryotic cells which consists of a single guide RNA (sgRNA) that directs the Cas9 endonucleases at specific genomic locus to produce a double strand break (Bauer et al., 2015). The Streptococcus pyogens derived Cas9 is an endonuclease which creates double stranded break as the sgRNA recognizes specific targets in the genomic DNA sequence via Watson and Crick base pairing. The resulting double stranded break is repaired by non-homologous end joining (NHEJ). Non-homologous end joining is the double stranded break repair occurring without a homologous template causing frameshift mutations leading to nonsense mediated decay of the mRNA transcripts (Bauer et al., 2015).

Two sgRNAs were designed manually to target portion of exon 1 and 2 of *Cklf* (mus musculus, accession number NC_000074.6) and exon 7 and 9 of *Cry2* (mus musculus, accession numberNM_009963.4). The sgRNA consists of 20mer protospacer sequence upstream of the NGG sequence which is the protospacer adjacent motif at the genome recognition site of *Cklf* and *Cry2*. Each guide had 24-25 mer oligos along with the reverse complement. (Bauer et al, 2015). The sequence of the sgRNA used are shown in Table 1.

Table 1.

Gene	Exon	sgRNA	Sequence
Cry2	Exon 7	Forward	CACCGCCCCTCCATTCGGTCAAACC
		Reverse	AAACGGTTTGACCGAATGGAGGGGC
	Exon 9	Forward	CACCGCATGATTGACGATGGGCCGT
		Reverse	AAACACGGCCCATCGTCAATCATGC

Table1. (continued)

Cklf	Exon 1	Forward	CACCGCACGGCCGGTCGTAAGCCGT
		Reverse	AAACACGGCTTACGACCGGCCGTGC
	Exon 2	Forward	CACCGTCCACACGTGTATAAGACT
		Reverse	AAACAGTCTTATACACGTGTGGAC

pX459 vector from Addgene (plasmid ID 48139) with BbsI restriction enzyme site was used to ligate the sgRNA designed for each of *Cry2* and *Cklf* after restriction digestion with Bbs1 (5000 U/ml). pX459 also encodes a puromycin-resistance element as a selectable marker. Primers were designed manually to detect the deletion by PCR and verified using freely available MIT Crispr Designing tool. One set of primers for each of *Cry2* and *Cklf* were designed internal to the sequence to be deleted called as the non-deletion band. Another set of primers external that is upstream and downstream to the site of cleavage of *Cry2* and *Cklf* termed as delete band were also designed (figure 4). The sequences to delete region were designed 100 bp away from the site of cleavage so that the small indel at the sgRNA target site would not impact the detection of deletion.

(Bauer et al., 2015)

Figure 4- Primer design to detect KO clones. The red arrows indicate the primers to detect the desired deletion and the blue arrow indicate the primers designed to detect the non-deleted portion for both *Cry2* and *Cklf*.

Table 2.

Gene	Primers	Sequences
Cry2-delete	Forward	5'-GAACCATGGCGATTGGGAGA-3'
	Reverse	5'-ATCTTTTACAGCCTGGGGGC-3'
Cry2-non-delete	Forward	5'-GACGCCATCATGACCCAACT-3'
	Reverse	5'-TAGGGTACCCCAATCCAGGAG-3'
Cklf-delete	Forward	5'-CGCCTACTCCAACAGTGCTT-3'
	Reverse	5'-AAGGTCAGCCATACCTACTGGT-3'
Cklf-non-delete	Forward	5'-GACGCAAGGCATTGATTGCT-3'
	Reverse	5'-CCCTGGCCGTTTGTACTTCT-3'

Cloning of oligos of *Cry2 and Cklf*

Synthetic oligos for Cry*2* and *Cklf* were cloned into pX459 vector as follows. Oligo synthesis was performed by Integrated DNA technologies. The oligos for *Cry2* and *Cklf* were briefly spun and resuspended at a final concentration of 100µM in distilled water. For annealing reaction of 50 ul, 1.5 µl of forward oligo (100 uM), 1.5 µl of reverse oligo (100 uM) for each of *Cry2* and *Cklf,* 5 µl of 10X NEB buffer 2, 42 µl ddH2O was used. Annealing was carried out at 95°C for 5 min followed by 70°C for 10 min and cooled gradually by placing the oligo solution in a water bath, unplugging the machine.

The annealed oligos were then used for serial dilution to 500 nM so that the stoichiometry between the oligo and the vector is captured. pX 459 vector was digested with Bsbs1 enzyme. 20 µl reaction was used for digestion with Bbs1 restriction enzyme 0.4 µl, buffer

2.1 2µl and 3.7 µl dd H20 at 37°C for 4 hours. Fragments were purified by 1% agarose gel. The open vector was extracted by gel purification. The DNA fragments were cut and resuspended in 750 µl of QG buffer (5.5 M guanidine thiocyanate, 20 mM Tris-HCl, pH 6.6 (25ºC), dissolve in water (pH 7) in an eppendorf tube. The tube was placed in heat block at 42ºC for 10 minutes.10 µl sodium acetate was added into the tube and mixed gently till the solution turned yellow. The solution was poured into a mini prep column (Quigen) and allowed to stand for 5 minutes. The column was washed with 500 ml of PE buffer. The column was placed in collecting tube and centrifuged at 14,000 rpm for 1minute. The prep columns were then placed in a clean sterile 1.5ml micro centrifuge tubes. DNA was eluted by adding 40 µl of extraction buffer to the center of the prep spin column and allowed to stand for one minute. The tubes were then centrifuged at 10,000rpm for 1 minute. The elute was run again on 1% agarose gel for confirmation. Ligation was carried out at 16°C for 4 hours with1 ul of 10X ligase buffer, 0.2 ul ATP, 1 ul of annealed oligo pair, 2 ul of vector, 1 ul T4 ligase and H2O to adjust the volume to 20ul.

One µl of the ligation reaction was then transferred to 50µl Mac E coli cells and plated onto lysogeny broth (LB) agar plate with 100µg/ml of ampicillin and incubated overnight at 37 °C. 2-3 colonies for each of the Crispr-Cas9 construct of *Cklf* and *Cry2* were picked and inoculated into mini-prep culture using LB media. 2-3 colonies for each of the crispr-cas9 construct of *Cklf* and *Cry2* were picked and grown in LB media by placing in a shaker overnight. The following day the tubes were removed from shaker and centrifuged at 3,600rpm for 10 minutes. The supernatant was poured out and the precipitate of bacterial cells at the bottom was resuspended in 250 µl of P1 buffer and transferred to a microcentrifuge tube. The content was vortexed. 250µl of buffer P2 and the tubes were gently inverted 4-6 times to mix the content until the solution becomes viscous and slightly clear. Then, 350µl of buffer N3 was added and

the tubes were inverted gently 4-6 times until cloudy. The solution is centrifuged at 15,000rpm for 10 minutes. The supernatant is poured in prep spin column placed on top of a 2-ml collection tube to allow the flow through. The columns are placed in a vacuum column to allow for the solution to flow through. 750 µl PE buffer was then added to the columns. The columns were centrifuged at 15,000 rpm for one minute. The prep columns were then placed in a clean sterile 1.5 ml microcentrifuge tubes. DNA was eluted by adding 40µl of extraction buffer to the center of the prep spin column and allowed to stand for one minute. The tubes were centrifuged at 10,000 rpm for 1 minute. The concentration of the eluted DNA for each of the plasmid of *Cry2* and *Cklf* were measured at 260 nm. The plasmid was digested with Bbs1 restriction enzyme and run on agarose gel. Bands of *Cklf* and *Cry2* constructs were sliced out from the gel and their nucleotide sequences was determined by Sanger sequencing at the University of Tennessee Health Science Center.

Transfection of Crispr/Cas9 constructs into cell of interest

The sequence verified plasmids encoding *Cklf* and *Cry2* were transfected using the liposome-based reagent lipofectamine 2000 (Invitrogen) into 60-70% confluent MMHD3(murine hepatocytes) reporter cell lines grown in RPMI culture media (Hyclone) in 6 well culture plates coated with type I collagen in DPBS(Fischer). (40µl of collagen dissolved in 1ml of DPBS). The RPMI media was supplemented with 50ml of 10% fetal bovine serum, 16 ng/ml insulin like growth factor-II (IGF-II), 55ng/ml of epidermal growth factor (EGF), 10 µg/ml of insulin, 5 ml of 1x streptomycin-glutamine-penicillin. The transfection was carried out at a ratio of 1 µg of DNA:2 µg of the transfecting agent. 2 µg of each Crispr-Cas9 plasmids (total 4µg) of *Cklf* and *Cry2* was used for transfection (n=2 for each of *Cry2* and *Cklf*).

EGFP expressing construct was used as control to check for the transfection efficiency. EGFP constructs were transfected using liposome-based transfection method into WT MMH-D3 reporter cell lines (n=1). The day following transfection fresh RPMI media with 10% serum was replaced into each well containing the transfected and the control cells and incubated at 37°C overnight. On day 2 following transfection, the transfection efficiency was evaluated in the EGFP control cells using fluorescence microscopy. When the cells reached 100% confluence, the cells transfected with *Cry2* and *Cklf* were detached with trypsin and transferred into a 10 cm type I collagen coated culture plate and grown in RPMI media containing 10% serum. Puromycin (1µg/ml of RPMI media) was added to the medium to select for transfected cells. A kill curve was plotted to determine the concentration of puromycin to be used. Different concentrations of puromycin ranging from 0.5 µg/ml to 5 µg/ml were initially used to determine the concentration of puromycin to be used as selection marker. The lowest concentration that was effective in killing all the cells on day 5 was selected. The selected concentration was 1µg/ml.

Single cell sorting

On day 5 after antibiotic selection, the puromycin-resistant cells of both *Cry2* and *Cklf* which could represent possible cells which have received the CRISPR-Cas9 construct were used for sorting into 96 well plates. The cells in the 10 cm plate of Cry*2* and *Cklf* were washed with 2 ml of DPBS and 1 ml of trypsin was added into each of the plate and incubated at 37°C for 3-4 minutes. 2 ml of RPMI media with 10% serum was added into each of the plate to stop trypsinization. The cells were collected in a 15 ml tube and centrifuged at 2,500rpm for 5minutes.The trypsin with RPMI media was aspirated out. The pellet was resuspended in 6 ml of RPMI media and the number of cells were counted using a cell counter. Approximately 300,000 cells/ml of *Cry2* and *Cklf* were serial diluted to achieve 1 cell per well into a 96 well plate. The

plate was coated with type I collagen (n=5-15 plates/ gene). 150 microliters of RPMI media with 10% serum was used for each well to grow single cell clones. The single cell clones were allowed to grow for 7-8 days with a change of media on day 4 (n= 4-6 clones per 96 well plate). The possible single cell clones were then collected after 8-10 days (cells were 100% confluent) by trypsinizing with 20l of 0.05% trypsin-EDTA solution after aspiration of the media and 100 µl was transferred to two 96 well plates containing 150µl of RPMI media per well. One 96 well plate was used for growing the cells for further passaging into larger dishes while the other plate was stored using storage media for MMH-D3 cells (90% serum and 10%Di methyl sulfoxide DMSO) at 80°C for further use. Each of these single cell clones of *Cry2* and *Cklf* from 96 well plate was then transferred to 48 well plate once 100% confluent. The cells could grow till 100% confluent and then transferred to two 24-well plate. One for genotyping and the plate for further use in immunoblots and bioluminescence recording. Once the cells in 24 well plate were 100% confluent, the cells were trypsinized and seeded into 12-well and later into 6-well plates. The 100% confluent cells in one of the 6 well plate was used for genomic DNA extraction (see below). While the cells in the second plate were grown for immunoblotting and bioluminescence studies.

Genomic DNA extraction

The 100% confluent cells were washed with DPBS, trypsinised using 20µl of 0.05% trypsin-EDTA solution and collected into 1 ml Eppendorf tube prior to centrifugation at 1,000 rpm for 1 minute. The solution was aspirated, and the cells were washed with DPBS and centrifuged at 1,000 rpm for one minute. The DPBS above the cell pellet was aspirated with a pipette connected to a vacuum line. The cell pellet was then used for DNA extraction. The DNA extraction solution was made of 25 ml of 20 mM of Tris chloride pH 8.8 and 250 microliter of

Tween 20. 250µl of DNA extraction solution and of proteinase K were added to the cell pellet and mixed thoroughly by pipetting up and down. The cell lysate was then placed on heat block at 55°C for 45 minutes and for 95°C for 10 minutes to extract the genomic DNA. The cell lysate was cooled at room temperature and stored at -20^{0}C for screening of Crispr/Cas9 mediated deletion (Bauer et al., 2015).

Screening of Crispr/Cas9 mediated deletion and clone selection

Five µl of genomic DNA of each clone was taken after centrifuging at 1,000 rpm for 1 minute into a single eppendorf 1.5 ml tube to get polyclonal population of the clones. This polyclonal population of clones was used for standardizing the PCR conditions for each pair of primers designed for *Cry2* and *Cklf* clones. Primers were validated from bulk sorted cells composed of polyclonal population of cells exposed to the sgRNA. This was done to verify the presence of intended genomic deletion in addition to standardizing the PCR conditions. A 10 µl PCR reaction with the forward and reverse primer (1µl each), taq polymerase (0.2µl) genomic DNA of the polyclonal population (2 µl), 5x buffer (2µl) and water was added to a final volume of 10 µl. PCR was then carried out for 40 cycles using the following parameter 95°C for 15 min, 95°C for 30 sec, 60°C for 1 min, 72°C for 1 min, and 72°C for 10 min. The samples were then analyzed on 2% agarose gel using 1X Tris-acetate-EDTA (TAE) buffer.

Screening of the monoclonal cell lines for *Cry2* and *Cklf* deletion was carried out with the standardized PCR condition described above for non-deletion and deletion bands. The samples were run on 2% agarose gel using 1X Tris-acetate-EDTA (TAE) buffer.

Bioluminescence recording

Three 35 mm culture dishes for each of the KO or biallelic deletion clones, monoallelic deletion or HZ (heterozygous clones), WT clones of *Cry2* were used for bioluminescence recording. Each dish was type I collagen coated and the KO, monoallelic deletion, WT cells were seeded and grown in RPMI medium with 10% serum. These dishes were incubated at 37°C until the cells in each of the 35 mm dish were 100% confluent. The confluent cells were synchronized with synchronizing media (RPMI with dexamethasone) and incubated for 2 hours at 37°C. Next, cells were placed in recording media. The recording media was made of 10X DMEM (Hyclone), 25 mM HEPES, sodium bicarbonate, 1x Pen/Strep/Gln, 1 mM luciferin, sodium hydroxide, B-27, 1 mM Luciferin, p-27 and hypure water.

The dishes were then sealed with 40 mm thick sterile coverslips using vacuum grease to prevent evaporation. The dishes were loaded into lumicycle luminometer (version 2.31, Actimetrics) kept inside an incubator set at 36°C without water or CO_2. Real-time bioluminescence recording was performed for 1 week to determine the amplitude and periodicity of the circadian circle of the KO, HZ and WT cell lines of *Cry2*. The lumicycle uses photomultiplier tubes for light detection emitted from the cells. These photomultipler tubes have the advantage of detecting very dim luciferase bioluminescence. The data on the first day is excluded because of high transient bioluminescence on change of media. Lumicycle Analysis program version 2.53 (*Actimetrics*) was used for data analysis of circadian parameters.

Immunoblots

The KO, HZ and WT single cell clones of *Cry2* were further characterized by immunoblotting to evaluate *Cry2* protein levels. The KO, HZ and WT cells of Cry2 were grown

in 6 well plate in RPMI media until 100% confluent. The confluent cells were washed with DPBS and lysed using ice cold lysis buffer RIPA. The cell suspension of the KO, HZ and WT was then transferred into a cooled microcentrifuge tube and agitated constantly for 30 minutes at 4°C. The cells were centrifuged at 12,000 rpm for 10 minutes after which the supernatant was removed and placed in a new tube and the pellet discarded. The samples were denatured by placing at 100°C for 5 minutes. 10µg proteins were loaded and separated using SDS-PAGE along with a molecular marker. The gel was run for 2 hours. Once the proteins got separated on the gel due to the negative charge imparted on the protein by the ionic SDS detergent, transfer onto nitrocellulose membrane was done by the sandwich technique. In this technique the gel is directly in contact with the nitrocellulose paper. The transfer involves the use of porous pads and filter paper. The transfer of proteins from the gel onto the nitrocellulose paper takes place on application of current. The membrane is washed with dd water and blocked using blocking buffer. (1X TBST with 5% nonfat dry milk) for one hour. Cry2 primary antibody (1:1000 dilution) (Santa Cruz) is added and the membrane incubated overnight at 4°C. The following day the membrane is washed with three washes of TBST, 5 minutes each time. Secondary antibody conjugated to peroxidase is diluted in blocking buffer (1:1000 dilution) was added to the membrane and incubated for one hour. Chemiluminescence is used to detect the required signals.

Alteration in the design of Crispr/Cas9

To improve the efficiency of detection of KO clones, we modified the design of *Cklf* Crispr/Cas9 approach (figure5). We used two fluorescence proteins each one attached to one sgRNA Px458 vector, in which sgRNA was ligated at the BbsI restriction enzyme site, and fluorescence proteins were used as FACS sorting marker. Two sgRNAs were designed to knockout the entire coding portion of *Cklf* by Dr. Stephane Pelletier, in collaboration with Dr.

Doug Green, at St Jude Children's Research Hospital as previously described (Pelletier et al., 2015). One sgRNA had GFP as a selectable marker and the other had m-cherry as a selectable marker. This gave higher chance of detecting the possible knockout gene by screening the cells which received both GFP and m-cherry Crispr/Cas9 constructs (figure 8). The cloning, transfection method and screening of the Crispr/Cas9 mediated deletion and non-deletion was similar as described above. However, the transfection efficiency was evaluated using confocal microscopy at the Integrated Microscopy Center at the University of Memphis. The transfected cells of *Cklf* were screened individually for GFP and m-cherry. The preparation was screened for cells which fluoresced both in the red and green channels, making them appear orange-yellow, because these were the possible good candidates for having successfully incorporated the Crispr/Cas9 construct.

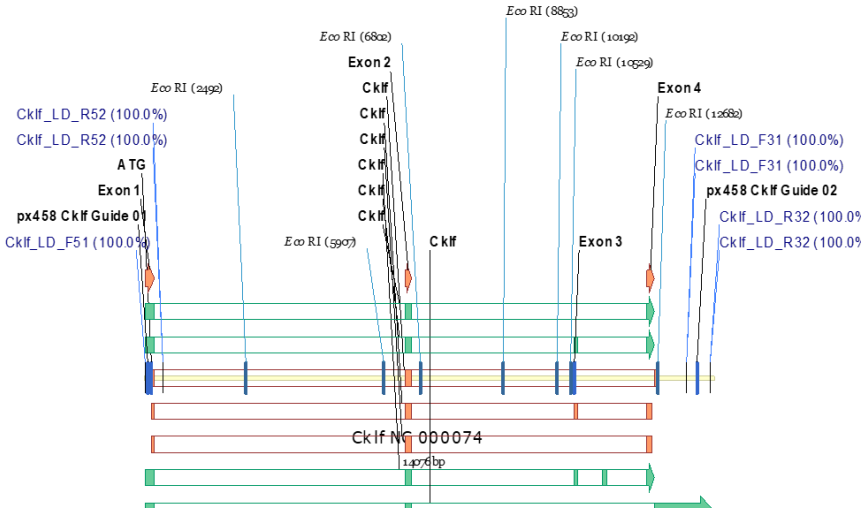


Figure 5. Design to knock out *Cklf*. One guide (Cklf guide 1) has m-cherry as selection marker and the other guide (Cklf guide2) has GFP as the selection marker. F51 and F31 are forward primers to detect the KO and the WT clones. R52 and R32 are reverse primers to detect KO and WT clones of *Cklf*. The design includes the variants of Cklf along with all the 4 exons.

Fluorescence activated cell sorting (FACS) was used to sort the transfected cells. FACS enriches the cells that received high levels of the Crispr/Cas9 constructs into 96 well plate containing 150 µl of RPMI media (n=20). The single cell clones were then transferred into a single 96 well plate which were further transferred to 48-well plate and then to 24-well plate and 12-well plates followed by 6-well plates. This was followed by gDNA extraction as described above, followed by genotyping as described for *Cry2*. But as the results were not consistent we changed the method of DNA extraction.

Alternative method of DNA extraction

The cells were washed with DPBS and trypsinised. RPMI media was added to stop trypsinization and the cells were collected in 1 ml microcentrifuge tubes. The tubes were centrifuged and the trypsin along with RPMI media was aspirated by an aspirator. The cells were again washed with DPBS and centrifuged. DPBS was aspirated with a pipette connected to a vacuum line and the pellet was suspended in DNA lysis buffer with proteinase K. We used DNA lysis buffer made of 100 mM Tris, pH 8, 5 mM EDTA, 0.2% SDS, 200 mM sodium chloride. 300 microliters of this DNA lysis buffer with 5 microliter of proteinase K was used for a 100% confluent cells in a 6-well plate. The cells were placed at 55°C in a heat block for one hour. The tubes were then centrifuged at 13,000 rpm for 10 minutes. The supernatant was collected in a fresh tube to which equal amount of isopropanol as the DNA lysis buffer was added and mixed gently. These tubes were centrifuged at 13,000rpm for 10 minutes. The supernatant was discarded, and the pellet was mixed 300 microliters of 70% ethanol. This was again centrifuged at13,000 rpm for 10 minutes. The supernatant was discarded with the DNA pellet at the bottom. This was then air dried for 10-15 minutes. The pellet was resuspended in 30 microliters of TE

buffer. These tubes were then incubated at55°C for 30 minutes and were used for genotyping as described above. The extracted DNA was stored at -20°C for further use.

Results

Screening of Crispr/Cas9 *Cry2* and *Cklf* clones for deletion and clone selection

As an initial effort to develop knockout cell lines of *Cry2* and *Cklf* we used the latest Crispr/Cas9 system (Bauer et al., 2015). We attempted to develop these cell lines to study the functional interaction between the circadian clock and the immune mediator *Cklf*.

We first identified an appropriate target site for *Cry2* represented by exons 7 and 9 which are functionally important exons for regulating the circadian clock (NCBI website) and for *Cklf*, exon 1 and 2 which are the exons common for all the 4 variants of *Cklf* (NCBI website). MMH-D3 cells were transiently transfected with liposome-based reagent with pX459-Cry2 sgRNA plasmid and Px459-Cklf sgRNA plasmid in two separate wells of a 6 well culture plate. A transfection efficiency of 70% was obtained for the generation of *Cry2* and *Cklf* KO clones which was evaluated using EGFP transfected into MMH-D3 cells (figure 6). The transiently transfected *Cry2* and *Cklf* puromycin resistant MMH-D3 cells were sorted into 96 well plate for single cell cloning. The expanded single cell clones of *Cry2* and *Cklf* were used for gDNA extraction and a polyclonal cell population was derived by mixing 5 µg of each of the single cell clones in an eppendorf tube. This polyclonal population of cells of *Cry2* and *Cklf* was used for PCR standardization. The *Cry2* gDNA polyclonal cells had a delete band at 370 bp which is for the wild type allele and a non-delete band at 546 bp representing a possible knockout allele (figures 7 and 8).

Screening of single cell clones of *Cry2*

The gDNA of single cell clones were subjected to screening for *Cry2* KO cell lines once PCR was standardized. We screened 150 single cell clones of *Cry2.* The single cell clones of *Cry2* were screened for the presence or absence of the delete band (370 bp) and the non-delete band (546 bp) (figure 9). The clones which gave only delete bands(370bp) without non-delete bands were considered as KO clones while the clones considered as heterozygous or monoallelic deletion had both the delete (370 bp) and the non-delete (546 bp) bands. This indicates that Crispr/Cas9 non-deleted allele is imperfectly edited by indel formations meaning one allele of the gene was deleted while the other allele of the gene was not deleted during non-homologous end joining repair. The single cell clones with biallelic deletion or the KO clones of *Cry2* had a band at 370bp and did not have the non-delete band at 546 bp. We obtained 7 Crispr/Cas9 homozygous knockout (KO) and 2 heterozygous (HZ) *Cry2* clones. Each of the 7 KO clones and 2 HZ clones of *Cry2* were reconfirmed for the presence or absence of the delete and non-delete bands by repeating the PCR and running the PCR amplicons on agarose gel (3 times). The 7 KO and 2 HZ clones of *Cry2* were allowed for expansion for 5 days in RPMI media. In all cases the PCR results confirmed the results of the indel analysis. The gel extraction of KO clones and the HZ clones was used for characterizing the clones using Sanger sequencing. Sequencing results confirmed the KO, HZ and WT clones of *Cry2*. The KO sequences exhibited deletion which encompassed the target site.

Western blots analysis of *Cry2* KO, HZ and WT clones

The seven KO clones and two HZ clones along with WT *Cry2* clones were probed for protein expression by Western blots (Figure 10). The decrease in the amount of protein in the HZ *Cry2* clones and the absence of proteins in the KO *Cry2* clones was noted relative to the WT.

This result validated the Crispr/Cas9 KO, HZ *Cry2* clones and shows that in the KO clones *Cry2* expression was completely abrogated.

Crispr/Cas9 KO of *Cry2* in MMH-D3 cell lines caused lengthening of period length

Seven *Cry2* KO clones and one HZ *Cry2* clone along with WT clone were used for recording the circadian rhythms in these cells using Lumicycle luminometer (Actimetrics). The lumicycle analysis program version 2.53 (Actimetrics) was used to determine circadian parameters. Circadian period phenotype of monoallelic deleted clones and KO *Cry2* clones in comparison to the WT cells were recorded using real time bioluminescence (Figure 9). The period lengths for the KO clones were 1.5-3.5 hours longer than the WT clones (approximately 24 hrs). These individual KO clones exhibited variation from 25.5to 27.5, compared to approximately 24 hours for the WT clones (Figure 8 and 9).

The magnitude of period change in homozygous KO clones of *Cry2* was significantly longer (27 .5) than WT (approximately24 to 24.5). However, the HZ KO clones exhibited modest change in period (long period phenotype (26 .5) in comparison to the KO clones (figures 11,12). This data is consistent with the essential role of *Cry2* in maintaining the period length of the clock to approximately 24 hours.

Screening of single cell clones of *Cklf*

Attempt 1

We obtained 600 single cell clones of *Cklf* gene deletion clones. A polyclonal population of cells of *Cklf* was used for screened for the presence or absence of delete band (560 bp) and the non-delete band (429 bp) (Figures 13,14) for PCR standardization. This was followed by single cell clone screening of *Cklf* clones which gave us one KO clone and 4 heterozygous or the monoallelic deleted clones (figure 15) and the remaining were non-delete clones. The single KO

clone and the 4 HZ clones were again genotyped, but the clones gave us smears which could have been due to possible contamination. To this end we grew single cell clones of Cklf again but were unsuccessful to detect a KO clone. This led us to alter the Crispr/Cas9 design by using two fluorescence proteins, each attached to one sg RNA as described above and targeted the entire coding portion of *Cklf*

Developing an alternative Crispr/Cas9 protocol to obtain *Cklf* KO cells

Attempt 2

With the Crispr/Cas9 design described above we were able to successfully knock out *Cry2*, but not *Cklf*. We improved the experimental design used to obtain and detect KO clones of *Cklf*. Two sgRNAs were ligated with two different fluorescence markers. One sgRNA was ligated with GFP and the other with m-cherry. This was designed so that the cells receiving both the sgRNA would have yellow fluorescence. The use of two markers would increase the chance of detecting cells transfected with Crispr/Cas9 *Cklf* constructs by using FACS which screens for cells receiving yellow fluorescence. Liposome-based transfection was used as described above. Two wells of the 6-well plate were used for transfection. Each well was screened for GFP (figure16) and m-cherry (figure16) fluorescence individually. The cells which expressed both fluorescence markers appear yellow (figure 17) and were considered to have received Crispr/Cas9 *Cklf* construct. The transfection efficiency was calculated as described above and the transfection efficiency of 30% was obtained (figure 16). The cells were then sorted into 96 well plates (n=20) using FACS to enrich for the top 3% of the cells positive for both fluorescent markers. Each plate had 15-20 single cell clones which were expanded over a 7 days period. 350 single cell clones from 20 plates were used for gDNA extraction. These single cell clones of *Cklf*

were screened for deletion (no band) and non-deletion (463bp and 603bp) bands. Polyclonal cells were pooled as described above for *Cry2* and *Cklf* and used for PCR standardization.

However, during the standardization procedure, we obtained only non-specific bands and the results for the delete region and non- delete region were inconsistent (figure 17). This prompted us to take a critical look at the properties of the primers used. One of the primers used had GC content greater than 60% and so we selected new primers manually and verified their properties with the software Primer Blast. The newly designed primers were used for standardization of the polyclonal cell population of *Cklf* (figure 18). The polyclonal clones gave us both delete and non-delete bands. Once standardization was achieved, PCR was carried out to screen *Cklf* single cell clones. The PCR amplicons exhibited nonspecific bands and inconsistent results were obtained for the delete and the non-delete regions each time the PCR was repeated (figure 19).

Attempt 3

We once again carried out single cell seeding into 96 well plates using FACS to obtain more clones. We obtained 400 clones and performed genotyping to detect delete and non- delete *Cklf* clones.in this attempt we tried an alternative method of DNA extraction as described above to our surprise, the results were again inconsistent with non-specific bands and variation in the results of the delete and non-delete regions each time the PCR was repeated (figure 19). The variation consists of either smear, absence of the delete and the non-delete band or non-specific bands. This result suggests standardizing the PCR conditions for further screening of single cell clones of *Cklf*.

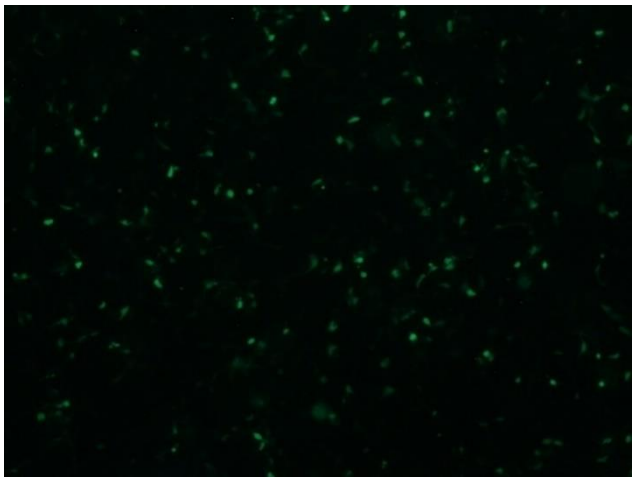


Figure 6. Fluorescent image of transfected EGFP expressing MMH-D3 reporter cells. This was used to check for the transfection efficiency as indicated by the EGFP expression in these cells.

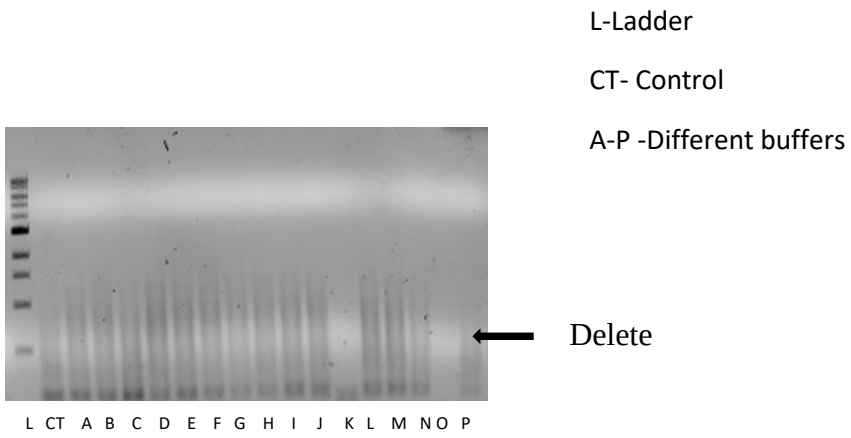


Figure 7. Genotyping of *Cry2* polyclonal clones for primer validation and verifying the presence of deletion amplicons. Polyclonal *Cry2* clones were used with different buffers. Each lane represents a different buffer. The different buffers are marked in alphabetical order. The band at 370 bp represents the deletion amplicons.

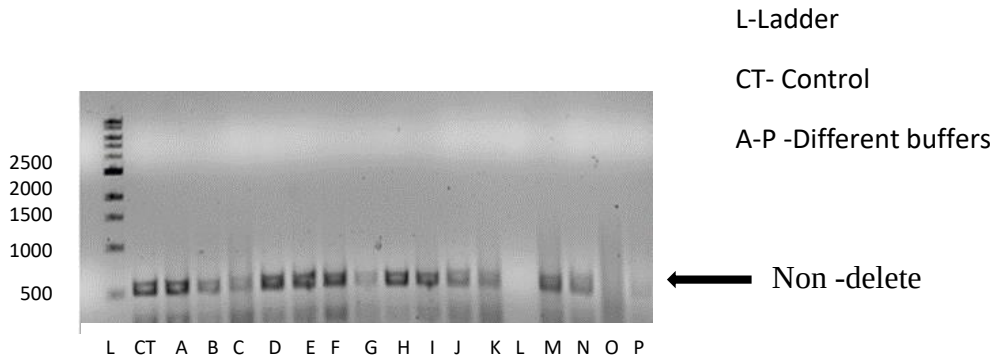


Figure 8. Genotyping of *Cry2* polyclonal clones for primer validation and verifying the presence non-deletion amplicons. Polyclonal *Cry2* clones were used with different buffers. Each lane represents a different buffer. The different buffers are marked in alphabetical order. The bands at 546 bp represents non-deletion amplicons.

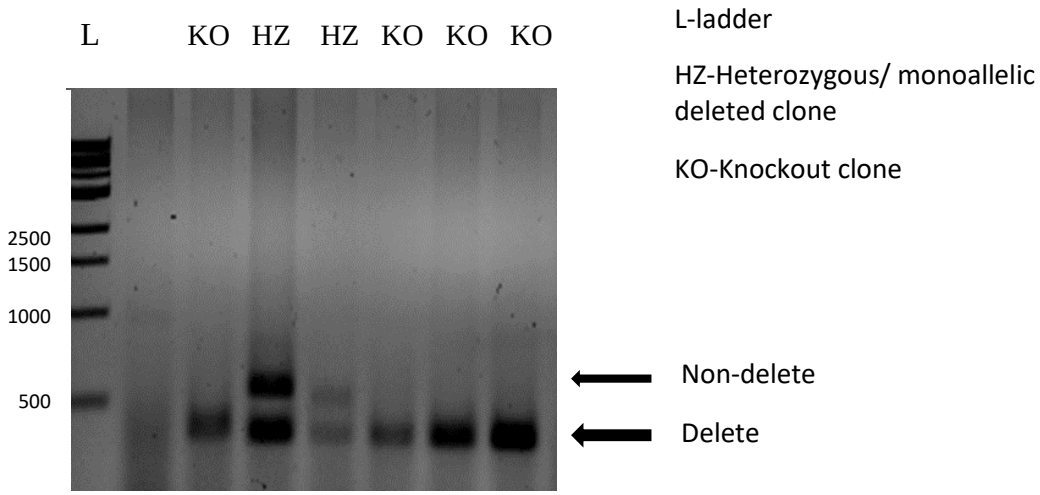


Figure 9. Genotyping of individual *Cry2* clones for non-deletion, monoallelic, and biallelic clones. Each lane represents individual clones amplified by PCR and labelled as KO for knockout/biallelic clones and HZ for monoallelic deletion clones screened using primers. The band at 370 bp represents the KO/ biallelic deletion amplicons of *Cry2* while the band at 546 bp along with the band at 370 bp represents the represents the monoallelic amplicons.

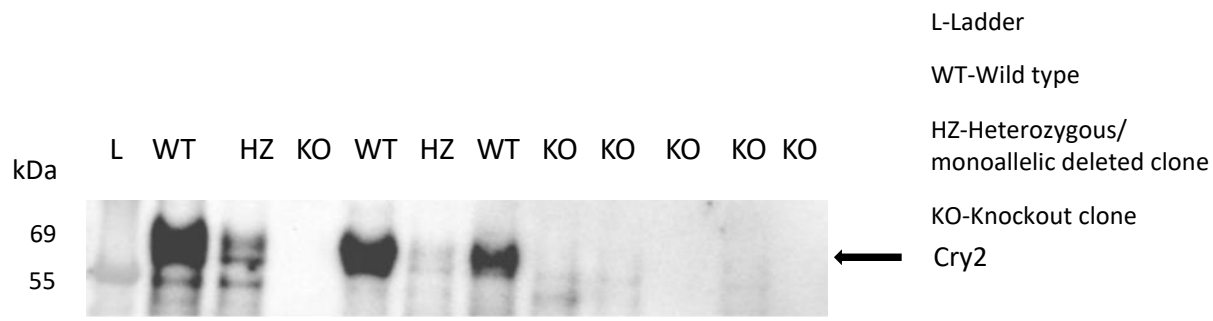


Figure 10. Western blot of WT, HZ and KO Cry2 clones. Data shown is representative of three independent experiments. Cry2 WT, HZ and KO protein in 10µg of whole cell lysate from MMH-D3 hepatocytes. A decrease in the amount of protein in the monoallelic (HZ) *Cry2* clones and the absence of proteins in the KO *Cry2* clones was noted relative to the WT.

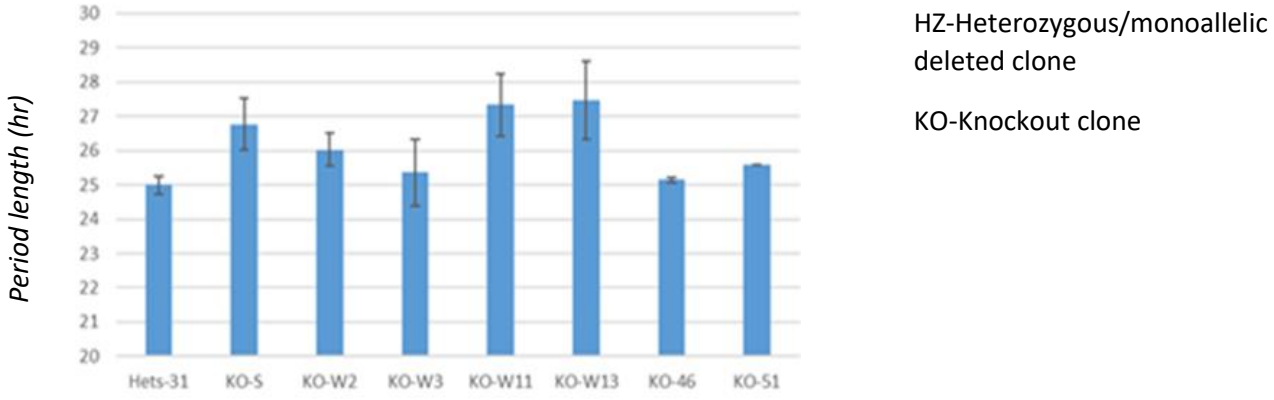
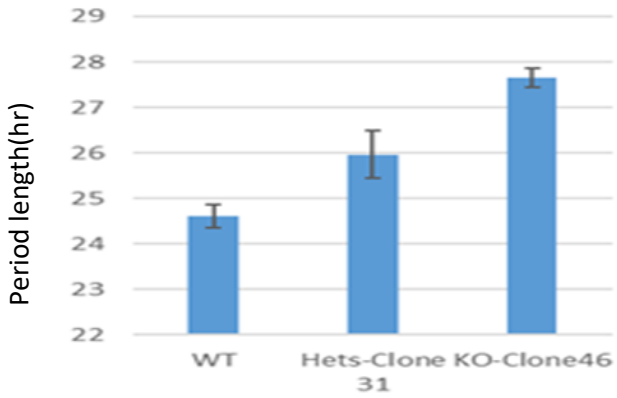


Figure 11. Lengthening of period length of HZ and KO *Cry2* clones. Crispr/Cas9 knockout studies on *Cry2* lengthens the period length of circadian bioluminescence rhythms in MMH-D3 hepatocytes. Bioluminescence data are presented as an average of three independent experiments for each clone. The magnitude of period change in homozygous KO clones of *Cry2* was significantly longer (period phenotype (27.5)) comparison to WT (24.5). However, the monoallelic KO clones exhibited modest change in period (long period phenotype (26.5) in comparison to the KO clones (27.5).



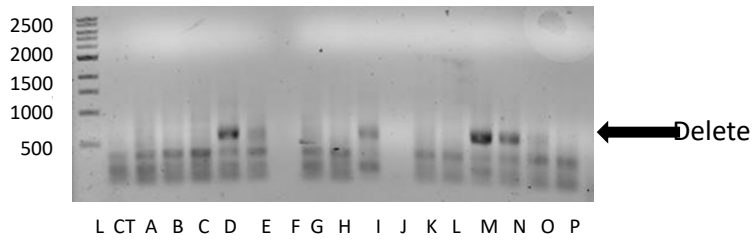
WT- Wild type

Hets-Heterozygous/monoallelic deleted clone

KO-Knockout clone

Figure 12. Lengthening of period length of HZ and KO *Cry2* in comparison to WT clones.

The magnitude of period change in homozygous KO clones of *Cry2* was significantly longer (period phenotype (27.5) comparison to WT (24.5). However, the monoallelic KO clones exhibited modest change in period (long period phenotype (26.5) in comparison to the KO clones (27.5).



L-Ladder

CT- control

A-P -Different buffers

Figure 13. Genotyping of *Cklf* polyclonal clones for primer validation and verifying the presence deletion amplicons. Polyclonal *Cklf* clones were used with different buffers. Each lane represents a different buffer. The different buffers are marked in alphabetical order. The band at 560 bp represents delete amplicons.

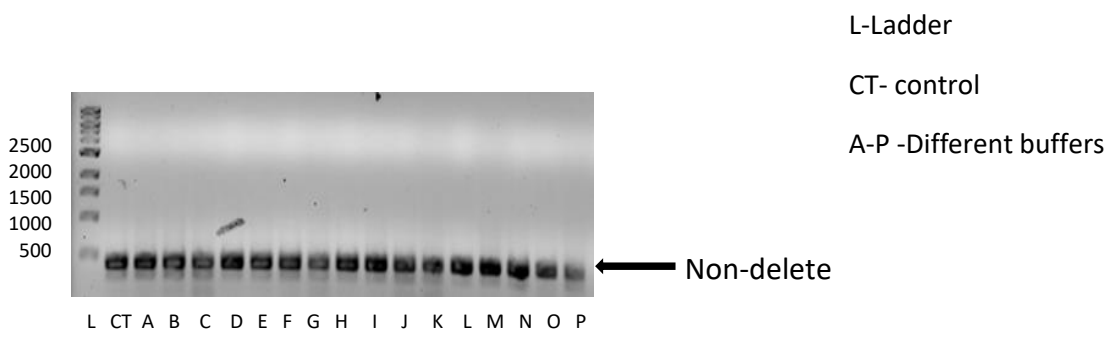


Figure 14. Genotyping of *Cklf* polyclonal clones for primer validation and verifying the presence non-deletion amplicons. Polyclonal *Cklf* clones were used with different buffers. Each lane represents a different buffer. The different buffers are marked in alphabetical order. The band at 429 bp represents delete amplicons.

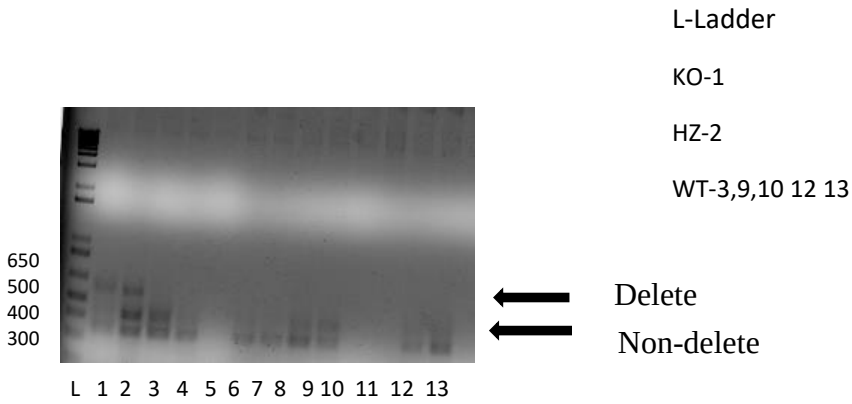


Figure 15. Genotyping of single cell clones of *Cklf*. Each lane represents individual single cell clones. A single band at 546bp represents deletion amplicon/KO *Cklf* clone while the band at 429bp(delete) along with the band at 546bp (non-delete) represents a monoallelic or HZ deletion clones of *Cklf* while the lanes with only band at 429bp represents a WT clone**.**

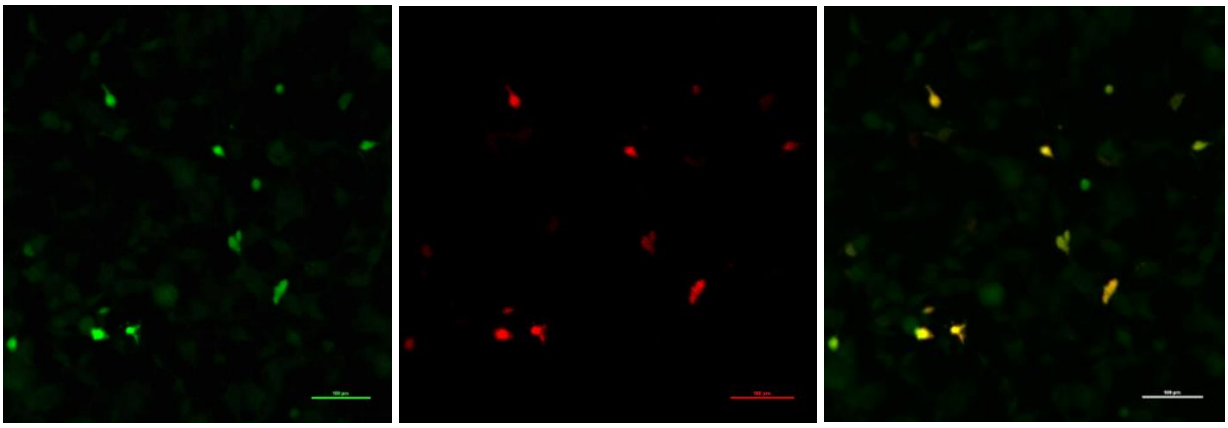


Figure 16. Transfected MMH-D3 cells screened for possible representative areas receiving Crispr/Cas9 *Cklf* constructs using confocal microscope. Green fluorescence on the left of the figure represents the possible cells receiving the GFP Crispr/Cas 9 *Cklf* construct. Red fluorescence in the middle of the figure represents the possible cells receiving the m- cherry Crispr/Cas 9 *Cklf* construct. Yellow fluorescence represents cells which have possibly received both GFP and m-cherry Crispr/Cas9 *Cklf* construct.

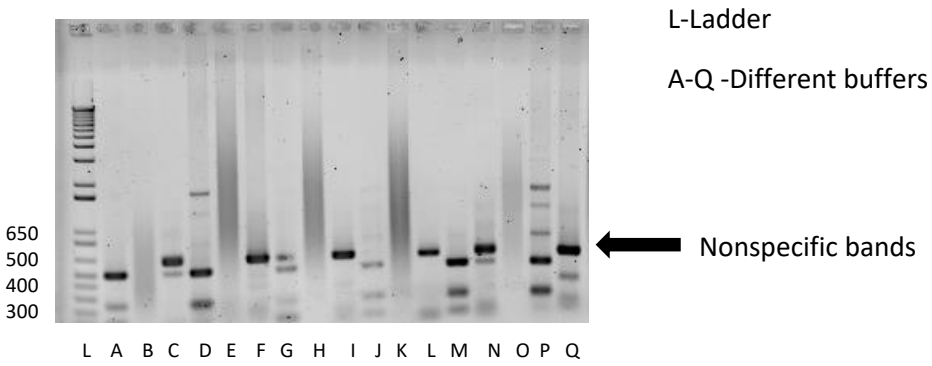


Figure 17. Non-specific bands of polyclonal *Cklf FACS* sorted cells. Each lane represents the polyclonal population of *Cklf* clones screened for deletion and non-deletion amplicons for primer validation and PCR standardization. The arrow represents nonspecific bands. The different buffers are represented with different alphabetics below the figure.

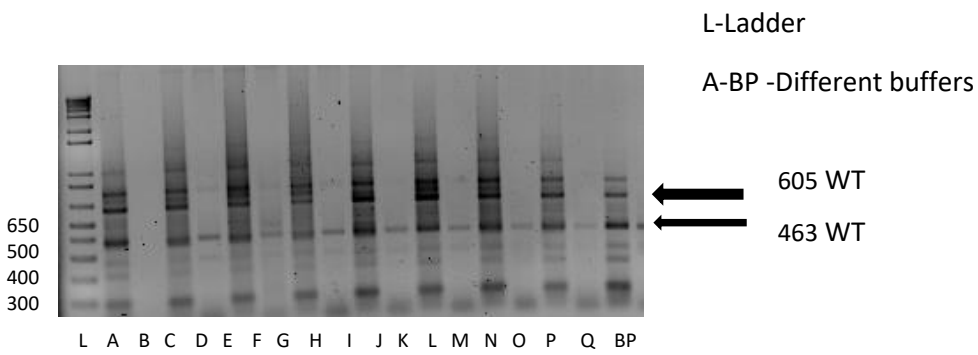


Figure 18. Genotyping of *Cklf* polyclonal clones for primer validation and PCR standardization. Polyclonal *Cklf* clones were used with different buffers. Each lane represents a different buffer indicated by the different alphabets. The different buffers are marked in alphabetical order. The expected WT bands were at 463 bp and 605bp which was achieved.

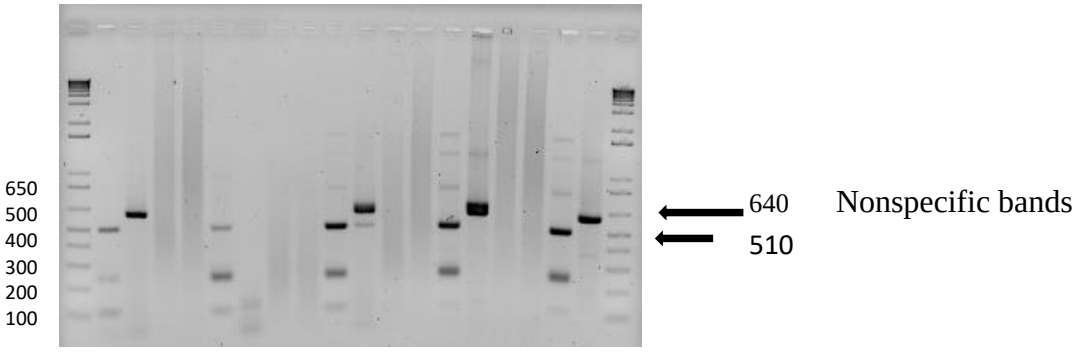


Figure 19. Genotyping of *Cklf* single cell clones. Each lane represents a **s**ingle cell clone of *Cklf* screened for KO and WT band (463, 605). All the single cell clones either were smears or had non-specific bands.

Discussion

In this study we used the Crispr/Cas9 method in MMH-D3 reporter cell lines to create KO cell line of *Cry2* and *Cklf.* Our workflow is adapted from previously published methods (Bauer et al., 2015) and can be divided into four phases:1) guide sequence design and sgRNA construction, 2) screening of sgRNA, 3) isolation of single cell clones, 4) screening of single cell clones and validation. Thus, the workflow can be applied to any other cell lines which are used to study the circadian clock functions.

The rapidly developing RNA programmable gene editing technology can be applied to knockout a single gene as in this study or can be harnessed for multiple genome engineering (Cong et al., 2013; Mali et al., 2013). sgRNA targeting distinct sites within the genome can be co-expressed to knockout multiple genes simultaneously and eliminate redundancy in signaling pathways. This approach can be used to eliminate other repressor clock genes such as *Cry1, Per1, 2* or clock activator genes such as *Bmal1* and *Clock* and to define the physiological roles of specific clock network components. The Crispr/Cas 9 system can also be applied to knock-in point mutations or new sequences into genes by providing a donor DNA template with desired modification of the sequence. The workflow is the same as described except the introduction of donor template (Mali et al., 2013). Crispr/Cas9 is an expanding tool currently available for in vivo and in vitro gene modification. However, the system does have certain caveats such as the potential off target gene disruption (Kuscu, Arslan, Singh, Thorpe, & Adli, 2014). The short 20 bases guide sequence that directs Cas9 to the target sites could bind to other sequences in the genome. Hence it is very important to design the guide sequence with a high target specificity. The seeding sequence which is the 10-12 base pairs adjacent to the PAM or 3' end of the guide is a key component adding to the specificity of the target region (Zhang,Tee,Wang, Huang, &

Yang, 2015). In one of the studies performed using Crispr-Cas9, non-deletion clones were frequently observed due to indel formation in one of the two alleles and was not just due to the transfection efficiency to the Crispr plasmids into the cells (Canver et al., 2014). Although Crispr offers advantages in the simplicity of the design and is a rapidly developing gene editing tool, several studies have shown that it is not without limitations and drawbacks (Zhang et al., 2015).

Using the above-mentioned method, we successfully generated KO single cell clones of *Cry2* using the Crispr/Cas9 system. Our data, indicate that a sgRNA programmed to a target site in exon 6 and 7 of *Cry2* gene triggers sequence specific Cas9 mediated NHEJ, resulting in ablation of *Cry2* protein expression. The KO cell lines of *Cry2* exhibited long period phenotype.

Regarding *Cklf* we used two approaches to detect for *Cklf* deletion. In our study we were successful in obtaining delete and non-delete bands from polyclonal population of *Cklf* clones using two different approach as described above. We achieved 4 monoclonal and one KO *Cklf* cell lines but the results were inconsistent. The repeated PCR either gave smears or nonspecific bands which we thought could be due to DNA contamination or degradation of DNA over a period. This led us to rescreening of new single cell clones but again with inconsistent data. The low percentage of KO and the screening of non-delete clones of *Cklf* with inconsistency in the results obtained may be due to technical difficulties. This can be overcome by improving the genome editing efficiency such as higher transfection efficiency, expanding flow cytometry-based single cell sorting and cloning, and designing new PCR genotyping strategies. Confirmation that dysregulation of *Cklf* affects the circadian clock awaits obtaining *Cklf* KO cells. These cells will be important to gain a mechanistic understanding of the interactions between circadian clock and *Cklf* and to determine whether there may be uncoupling of the

activity of the peripheral clock during *Cklf* induced periods of immune response and inflammation.

Our data showing that the downregulation of the immune mediator *Cklf,* induces short period length (speeding up the clock oscillation) and low amplitude phenotypes (Ramanathan et al., unpublished) suggest that *Cklf* and the circadian clock are connected as alterations in rhythm amplitude and period length are considered to be reliable indicators of clock fidelity. Marked circadian disruption in the downregulation of *Cklf* supports the role for *Cklf* in timekeeping. This data supports the idea that *Cklf* has a possible input into circadian loops acting as a critical link between immune regulation and circadian time keeping. This result may serve as a starting point to understand the functional interaction between two important biological pathways, namely the circadian clock and signaling through the immune mediator *Cklf*.

This observation remains to be investigated in further studies. We believe that *Cklf* is the mechanistic conduit interconnecting these two processes. These findings emphasize the significance of molecular clock and its regulation of rhythmic production by *Cklf*, a clock modifier gene which subsequently influence different biochemical pathways involved in various pathophysiolog**y.**

One of the studies done on *Cklf,* showed that it helped in selective recruitment of leukocytes by activation of the NF-kB pathway and triggers immunological changes in asthma (Lin, Staahl, Alla, & Doudna, 2014). However, the timing of the asthmatic attack by the immune system varies, with the attack worsening in the wee hours of the morning suggesting circadian dependency (Durrington et al., 2013). Studies done in our lab on NF-kB indicated that this pathway plays a significant role in regulation of the clock gene network. These findings suggest

that *Cklf* and circadian clock may act via NF-kB as a common pathway. This finding requires further studies to be carried out.

Generation of KO cell lines of *Cklf* would be a step in the process of understanding the interaction of *Cklf* and circadian clock. However, delineating a precise regulating mechanism of action of *Cklf* would require additional work in further studies.

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