THE PENNSYLVANIA STATE UNIVERSITY
SCHREYER HONORS COLLEGE

DEPARTMENT OF BIOLOGY

# CRISPR-Edited Cell Lines to Identify Specific Transcripts for *Per1* in Learning and Circadian Rhythms

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SPRING 2023

A thesis
submitted in partial fulfillment
of the requirements
for a baccalaureate degree in Biology
with honors in Biology

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## ABSTRACT

Long-term memory is known to require *de novo* transcription. Previous research has determined that the gene *Period 1 (Per1)*, which plays a well-established role in the circadian rhythm, is also needed for long-term memory within multiple memory-relevant brain regions such as the hippocampus. How *Per1* modulates memory, however, is unclear. To address this question, here we aimed to create CRISPR-edited cell lines that have edited promoter regions to allow for the identification of specific transcripts of *Per1*. I investigated: (1) if the binding of the transcription factor *Creb* to CRE sites in the *Per1* promoter region leads to the expression of a learning-specific *Per1* transcript; and (2) if the binding of the heterodimer Clock/Bmal to E-boxes in the *Per1* promoter region leads to the expression of a circadian rhythm-specific *Per1* transcript. To meet this objective, I first designed guide RNAs (sgRNAs) to allow for the editing of the 5.5 kb promoter region. Second, the cutting efficiency of sgRNAs was determined by transfecting a mouse hippocampal cell line (HT22). Third, donor plasmids will be designed and cloned to contain either mutated E-boxes or CRE sites. Fourth, the mouse hippocampal cell line will be transfected with the sgRNAs, Cas9, and the appropriate donor plasmid to create the edit. Fifth, the DNA will then be extracted to analyze the promoter region for the presence of mutations using Sanger sequencing. I will be looking for a homozygous mutant cell line, but as this is a large edit and unlikely to occur in a single editing process, the process will be repeated until a homozygous mutant cell line is achieved. Future work will create two mice lines that will contain the mutant promoter regions specifically in the hippocampus and then their learning behaviors will be evaluated, tested, and analyzed in addition to assessing their circadian rhythm.

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# ACKNOWLEDGEMENTS

This thesis would not have been possible without the help and support of many individuals. I would like to begin by thanking my research supervisor, Dr. Kwapis, who welcomed me into her lab during my freshman year and has taught me so much since. Her valuable guidance has influenced so many of my college and future career choices. In addition, I would like to thank my honors advisor, Dr. Schaeffer, who has been a great teacher and support since my first day at Penn State. He always has his door open to answer all my questions regarding the thesis and to help me navigate through my pre-medical journey. The advice given to me by both Dr. Kwapis and Dr. Schaeffer has been very impactful in the success of my thesis.

I owe my utmost gratitude to Lauren Bellfy for her incredible mentorship throughout the past three years. Her insightful thinking and work ethic have inspired me to become the student and researcher I am today. Her guidance has made me pursue my work independently and confidently. I also want to special thanks Shoko, Chad S., Chad B., Hannah, and Gretchen who are always kind to answer questions I may have. I also would like to thank Derek and Marc for discussing ideas for this thesis with me. Lastly, I would like to thank the entire Kwapis Lab for always being so welcoming and helping make lab my favorite part of the day. I am truly honored to be a part of the Kwapis Lab.

This material is based upon work supported by the NIA under Award No. K99/R00AG056596, the Whitehall Foundation under grant # 2020-0506, and the AFAR under Junior faculty grant # A21105, all to Janine L. Kwapis. Any opinions, findings, and conclusions or recommendations expressed in this publication are those of the author(s) and do not necessarily reflect the views of the NIA, Whitehall Foundation, or AFAR. In addition, The

Schreyer Honors college granted me a scholarship toward expenses so I could work in the lab during the summer of 2021. This was especially important as I was not able to work in the wet-lab for around a year due to the pandemic.

The best support system I could ever hope for is my mother and sister Christina, for whom I will always be grateful. They constantly support me in pursuing my passions and career goals. The person I am today owes a lot to their love and guidance.

# Chapter 1

# Introduction

## 1.1 Circadian Clock

The circadian rhythm is an internal process that drives biological processes across a roughly 24-hour period. It serves as a natural pacemaker that synchronizes internal metabolic, physiological, and behavioral processes in tandem with the external time of day (Jilg et al., 2010; Mieda, 2020). Previous research on the circadian system primarily focuses on its effects on the sleep/wake cycle, but there are many more body processes influenced. Specifically, the circadian clock affects body coordination and strength, feeding behavior, metabolism, body temperature, hormonal levels, memory formation, and many others (Monk et al., 1983; Smies et al., 2022). Recent research has led to the idea that circadian clock genes modulate functions outside of brain's central pacemaker to provide local diurnal control over a range of functions (Hastings et al., 2003; Heyde & Oster, 2019). This local control may impact memory formation and expression.

The circadian clock can be thought of as two separate pieces in the mammalian body; the central clock located in the suprachiasmatic nucleus (SCN) and the peripheral clocks located in other brain regions and organs (Balsalobre et al., 1998; Hastings et al., 2003). The peripheral clocks are proposed to regulate the rhythmic functions that are performed in those individual organs and tissues, such as the pancreas, liver, and lungs (Mieda, 2020). One such peripheral clock has been identified in the dorsal hippocampus, which is a memory-relevant brain region

(Jilg et al., 2010). How the circadian clock is involved in learning and memory is just starting to be understood.

Zeitgebers are any external cues that allow an organism to align with environmental stimuli. Light/dark cues are the most powerful zeitgebers, whether these light cues come from the outside environment, such as the sun, or from an inside environment, such as a lamp that is on a timer (Golombek & Rosenstein, 2010). Many other zeitgebers exist such as eating and drinking patterns, social interactions, and temperature. Essentially, any external stimuli that occurs around the same time every day can serve as a zeitgeber. The SCN is primarily under control by the light/dark cycle, while the peripherical clocks are more influenced by other zeitgebers (Heyde & Oster, 2019).

Most cells in the body can maintain information about the time of day in the form of a rhythmic molecular clock. There are four key circadian clock genes/gene families responsible for driving the molecular clock: Circadian Locomotor Output Cycles Kaput (*Clock*), Period (*Per1*), Brain and Muscle ARNT-Like 1 (*Bmal1*), and Cryptochrome (*Cry1-2*)(Gekakis et al., 1998). These genes, interact in an ~24h negative transcription-translation feedback loop (TTFL), which encodes rhythmic expression that oscillates through every cell to maintain time-of-day information(Gekakis et al., 1998; Mieda, 2020; Sato et al., 2006). Importantly, neurons located in all subregions of the hippocampus have been found to express these genes and gene families (Jilg et al., 2010), suggesting these clock genes may play a role in modulating memory across the day/night cycle.

The circadian system begins when the heterodimer, CLOCK-BMAL1, binds to E-box motifs located upstream of the *Per* and *Cry* gene families, inducing their expression (Alberini, 2009; Hirayama et al., 2007). The *Per* and *Cry* genes are then translated in the cytoplasm where

they dimerize. The heterodimer then returns to the nucleus to inhibit the transcription factors CLOCK and BMAL1, subsequently preventing further transcription of *Per* and *Cry*. The PER and CRY proteins are degraded over time, allowing the CLOCK-BMAL1 complex to restart the loop. The entire TTFL runs roughly in accordance with the light/dark cycle, over an ~24-hour period (Jilg et al., 2010). In addition, these clock genes are known to rhythmically oscillate in neurons outside the SCN, including in the dorsal hippocampus (Hirayama et al., 2007; Mieda, 2020; Sato et al., 2006)

Despite the progress made in the field, we are still unclear on the mechanism through which memory is modulated. According to previous research, the *Per1* gene is specifically known to impact memory (Bellfy et al., 2022; Brunswick et al., 2023; Kwapis et al., 2018; Urban et al., 2021). In recent studies, the knockdown of *Per1* in the dorsal hippocampus caused impairment of spatial memory. Upon local upregulation of *Per1* in aging mice, memory improved (Kwapis et al., 2018). Since *Per1* is well known to regulate memory in the hippocampus, my research investigates this mechanism to understand how memory may be modulated.

### 1.2 Learning and Memory Formation Across the Circadian Day

Long-term memory formation is greatly dependent on the time of day. In many tests mice show optimal memory performance at certain times during the day (Chaudhury & Colwell, 2002; Eckel-Mahan et al., 2008; Gerstner et al., 2009; Rawashdeh et al., 2016; Urban et al., 2021) with some groups showing better memory performance at night (Liu et al., 2022; Loss et al., 2015; Tsao et al., 2022). In accordance with this, knock-down or disruption of clock genes impairs

long-term memory formation in young mice. A study conducted by Kwapis and colleagues (2018) identified *Per1* as a gene of interest for memory formation through RNA-sequencing. In young mice (aged 2-4 months), when *Per1* expression was locally knocked down in the DH, memory formation was disrupted, but local expression of *Per1* in the DH of aged mice (aged 18-20 months) ameliorated age-related impairments in memory. In addition to these findings, they also revealed that the circadian rhythm was unaffected by these manipulations, suggesting that *Per1* in the DH functions autonomously of its role in the SCN. This suggests that *Per1*, as well as other core clock genes, may exert local diurnal control over memory formation (Kwapis et al., 2018).

The brain's ability to process, consolidate, and retain information in the form of memories is a well-known, but complicated process. Organisms, and especially humans, are processing so much new information each day and our brains must be able to sort through this information and decide what to keep (Bellfy et al., 2022; Kwapis et al., 2020). Memories can be stored either in the form of short or long-term memories.

The formation of long-term memories requires three phases: acquisition, consolidation, and retrieval. The acquisition phase is characterized by the initial learning period. After this phase, the memory is either stored transiently, lasting up to a few hours as a short-term memory, or undergoes the next phase, consolidation, into a long-term memory. To consolidate a long-term memory, transcription of memory-relevant genes must occur. The consolidation of long-term memory changes the brain's neural connectivity, producing new connections between synapses or strengthening existing ones (Alberini, 2009; Alberini & Kandel, 2015; Chatterjee et al., 2020). The last phase, retrieval, refers to the ability of the organism to express this memory. Memories can last the entire lifespan of the animal as long as the information continues being used, which

strengthens the synaptic connections (Alberini & Kandel, 2015). If the memory does not remain in use, it may be lost due to weakening of the synaptic connections (Alberini, 2009, 2011).

At the molecular level, memory consolidation is relatively well-understood, although the mechanisms are very complicated as we continue to find correlations between memory processes and other body processes. Many of the major molecular cascades involved in memory consolidation oscillate across the molecular day (Gerstner et al., 2009). In addition, many of the brain structures important to memory formation, including the prefrontal cortex, amygdala, retrosplenial cortex, and hippocampus, present oscillating patterns within the core clock genes described previously (Rawashdeh et al., 2016; Smies et al., 2022).

There are many cascades involved in memory consolidation, but I will be focusing on the cAMP-binding protein (CREB) cascade, as it is a major signaling pathway necessary to form long-term memories. The cellular transcription factor CREB also shows direct relations with the circadian system. According to Rawashdeh et al., the cascade exhibits a rhythmic diurnal oscillation, showing a peak in CREB during the day. Studies also revealed CREB phosphorylation was induced by spatial learning only during the day, and this mechanism is dependent on the presence of *Per1* (Rawashdeh et al., 2016). The role of memory and learning is undoubtedly closely linked to the circadian clock through various processes.

### 1.3 Per1 Promoter Region

As established throughout this review, *Per1* is a critical component of the molecular clock and also functions locally in the dorsal hippocampus to modulate memory, however, we understand very little about how *Per1* modulates memory. Therefore, the goal of

this study was to further investigate how *Per1* affects learning and memory. The promoter region (the location where RNA polymerase and many transcription factors bind to initiate transcription) of *Per1* is fairly well-characterized. Found within this region are numerous CRE and E-box sites. There are five E-box and four CRE sites found within chr11: 69094823-69100227 (Hida et al., 2000).

The E-box motif (CACGTG) is the most well-known regulatory element in the *Per1* promoter region. Motifs are short DNA sequences that specify protein binding. The E-box motif serves as the binding site of the heterodimer, CLOCK-BMAL1, which serves as a positive regulatory mechanism. Other transcription factors that bind to these sites are negative regulatory elements which include the PERs and CRYs. In addition to these mechanisms, the E-box sites influence other clock genes and clock-controlled genes (Alberini, 2009; Hida et al., 2000; Nakahata et al., 2008).

The CRE site (CGCTA) is found in four locations in the *Per1* promoter region. The CREB nuclear transcription factor binds to these CRE sites to influence neuronal processes. The binding specifically drives metabolism, cell survival, and the expression of different transcription factors and growth factors (Alberini, 2009; Hirayama et al., 2007; Nakahata et al., 2008).

## 1.4 CRISPR/Cas9

The clustered regularly interspaced short palindromic repeats (CRISPR)-Cas system is a natural defense mechanism found in bacterial and archaeal genomes to fight off invading viruses and plasmids. CRISPR are segments of DNA sequences that are found in the genomes of these prokaryotic organisms. When a bacterium is infected by a virus, the bacteria

captures pieces of the viral DNA and incorporates them into its own genome. It inserts these pieces of DNA in a particular pattern to form segments known as CRISPR arrays. The bacteria *Streptococcus pyogenes* only relies on one protein, which is the nuclease Cas9, and two noncoding RNAs to reach its DNA target and incorporate the new piece of DNA.

This natural mechanism is now used as a tool to manipulate genomes in other species, including mice. This technology is very new, as it was first used in a mammalian cell line in 2013. The two noncoding regions needed to reach the target site are crRNA and tracrRNA, which can be fused into one single guide RNA (sgRNA). sgRNAs are designed based on where in the genome an edit is desired. Cas9 drives genome editing by generating a double-stranded break (DSB) at the desired location. The DSB can be repaired through the imprecise method of non-homologous end joining (NHEJ) which can introduce small insertions or deletions potentially knocking out function of the region of interest or through the precise method of homology-directed repair (HDR) which utilizes a template that has a desired edit to make specific changes to the region of interest. This template will contain the desired edits with sequences homologous to the endogenous DNA flanking the edit, which are referred to as homology arms. The larger the edited sequence, the larger the homology arms must be. Then the completed strand is ligated, and the resultant strand now has the desired DNA sequence (Ran et al., 2013). I am utilizing this CRISPR technology to edit the *Per1* genome to better understand Per1 and how it impacts learning and memory formation.

# Chapter 2

# Materials and Methods

## 2.1 Designing sgRNAs

The first part of the experiment is designing the sgRNAs. First, I obtained the coordinates CRE and E-box sites in the promoter region of the gene Per1 using the University of California Santa Cruz (UCSC) Genome Browser (chr11:69094823-69100227, genome build mm10). Due to the size of the promoter region that needed to be edited, 5.5kb, I decided to create two cuts, one upstream of the region and the other downstream, flanking the target region (Hida et al., 2000). Following the identification of the upstream and downstream regions, I utilized a different database, The Wellcome Sanger Institute to pick two candidate sgRNAs for each region. My criteria for picking the best candidates were the least number of off-targets, and choosing two forward sgRNAs, and two reverse sgRNAs.

To create each sgRNA, I utilized two complementary strands of DNA called oligonucleotides. I used these oligos to create the four sgRNAs L1, L2, R1, and R2. I combined OligoA and OligoB for each sgRNA and annealed them together using the Hifi program. First, I combined Oligo A, Oligo B, HiFi Mix, and Q, then annealed the samples.

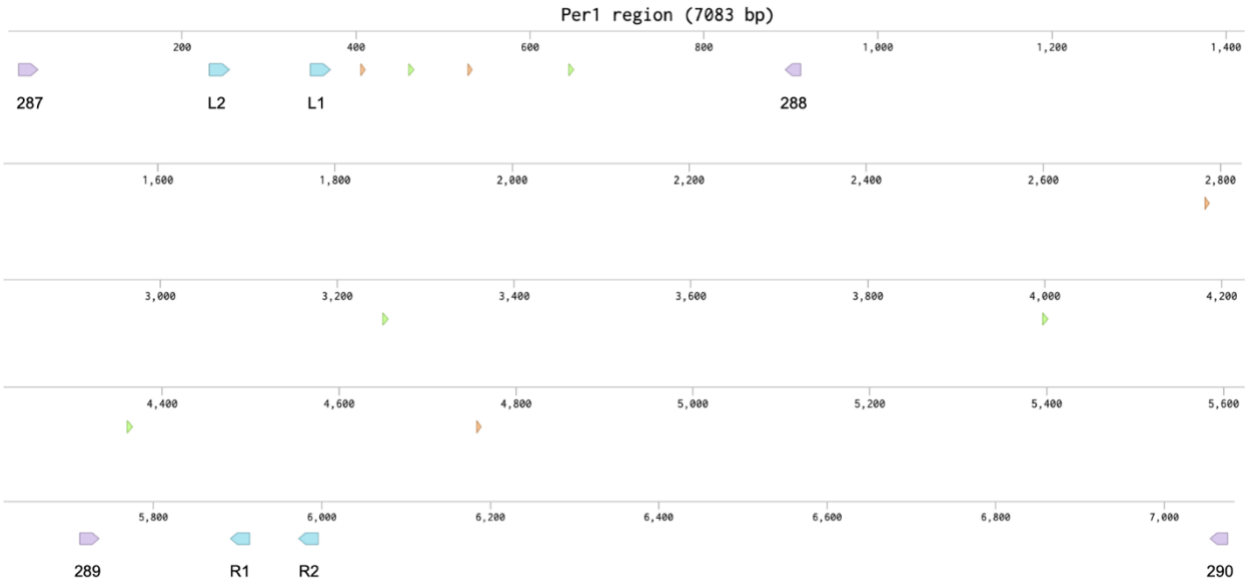


**Figure 1 Location of primers, sgRNA sequences, E-box and CRE sites**

**Key:**

| | |
|---|---|
| | **Primers** |
| | **sgRNAs** |
| | **CRE sites** |
| | **E-box sites** |

**Table 1 sgRNAs**

| **Name** | **Sequence** | **Oligo A** | **Oligo B** | **Coordinates** | **Projected Off-targets** |
|---|---|---|---|---|---|
| L1 | ACGCCTGCGTAC AAGTCGCTCGG | 276 | 277 | 11:69094769-69094791 | {0: 1, 1: 0, 2: 0, 3: 0, 4: 10} |

| | | | | | |
|---|---|---|---|---|---|
| L2 | GTGCTCAGATTTTTAAGCTTTGG | 278 | 279 | 11:69094653-69094675 | {0: 1, 1: 1, 2: 0, 3: 11, 4: 167} |
| R1 | GGTAGAAAGGCAGCTCTCGTAGG | 280 | 281 | 11:69100314-69100336 | {0: 1, 1: 0, 2: 0, 3: 8, 4: 89} |
| R2 | GCCCCTCACTGTTGTGACTCAGG | 282 | 283 | 11:69100395-69100417 | {0: 1, 1: 0, 2: 0, 3: 7, 4: 129} |

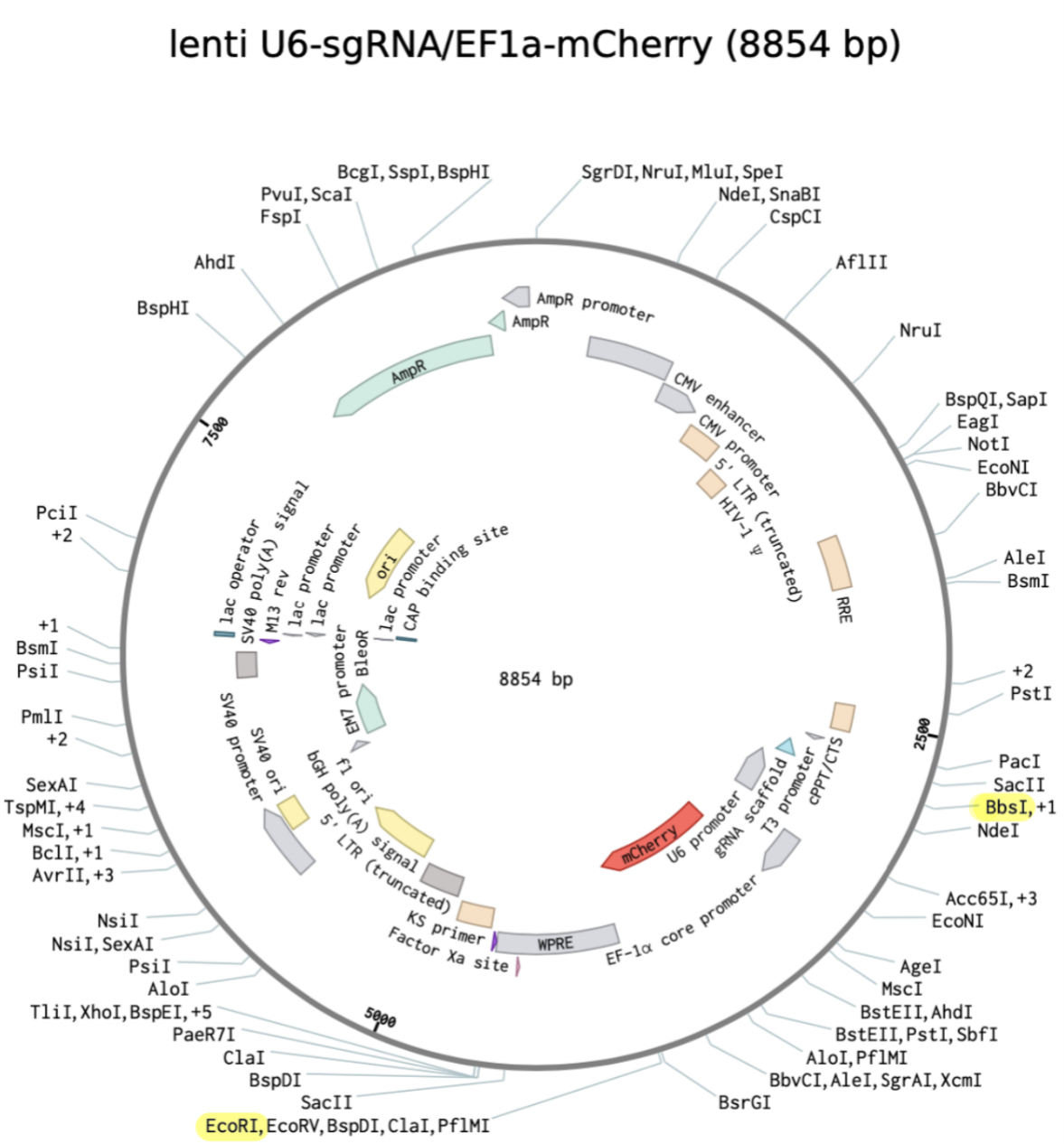


**Figure 2 JK35 Plasmid**

The JK35 plasmid was digested, by BbsI-HF, and linearized to ligate in the sgRNAs. Based on the plasmid sequence, I found that the BbsI-HF would be a good enzyme to use to have matching sticky ends with the designed sgRNAs and is only present once in the plasmid. I then

ran a 1% agarose gel with the linearized plasmid and extracted the DNA from the gel using the Monarch DNA Gel Extraction Kit. I excised the DNA from the gel and then heated the sample to elute the DNA contents. Finally, I ligated the sgRNAs into plasmids. To do this, I combined the sgRNA, plasmid, Q, and Geneart. Then I mixed the sample and allowed it to incubate at room temperature for 25 minutes.

## 2.2 Transformation

The next part of my experiment is the transformation of the sgRNAs into Stbl3-competent cells. First, I LB Agar carbenicillin plates to enable selection of the transformed plasmid. To provide the bacteria with optimal starting conditions for transformation, I pre-heated the carbenicillin plates in a 37°C incubator. The next steps were carried out next to an open flame to prevent contamination from microbes that may be present in the laboratory space. Next, I thawed stbl3-competent cells and added 2.5 µl of the ligated mixture. I incubated the sample for 30 minutes on ice, followed by a 45-second incubation at 42°C, followed by another 2 minutes on ice. Next, I added 250 µl of Luria-Bertani (LB) broth to the samples and then incubated them for 50 minutes at 37°C while shaking. I created two plates for each sgRNA. I plated one with 50 µl of cells and one with 250 µl. Finally, I incubated the plates overnight at 37°C.

## 2.3 Miniprep

To see if I successfully cloned the plasmid, I amplified and isolated the plasmid from a single colony. The next steps were carried out next to an open flame to prevent contamination from microbes that may be present in the laboratory space. To amplify the colony, I combined LB broth and carbenicillin in two glass tubes for each sgRNA to increase the downstream yield. Then I selected a colony from the corresponding plate and swirled it into the tubes using an autoclaved wooden stick. I made sure that the colony was isolated, circular, and an average size to the rest, to ensure a homogenous plasmid population. After adding the colony to the tubes, I placed the tubes in the incubator at 37°C while shaking for 16 hours.

After the incubation, I created a glycerol stock for future use if there is any contamination. To isolate the plasmid, I used the QIAprep Spin Miniprep Kit following the manufacturer's protocol with minor changes. First, I combined the bacterial solutions for each sgRNA into a 15 mL tube. I spun the samples down for 2 minutes at 6800 relative centrifugal force (rcf). I discarded the supernatant to reform the pellet. All subsequent steps follow the protocol with no further changes. Finally, I nanodropped the samples to check DNA quality and analyze their concentrations.

Next, I conducted a double digestion to confirm the sgRNA presence. I first determined what restriction enzymes to use based on the plasmid sequence, which I visualized with a program called Benchling. I used the enzymes BbsI-HF and EcoRI-HF. I completed the double digestion reaction on ice by combining 1 μg of DNA and 0.5 μl of each enzyme. Then I incubated the samples for 10 minutes at 37°C. Next, I made a 2% agarose gel to analyze the results. Upon seeing these results, I found that the DNA sizes on the gel appeared to be the correct sizes. Based on the plasmid sequence, as seen in Figure 2, I identified that BbsI cuts after

245bp and the EcoRI enzyme cuts after 6,187bp. If the sgRNA is present, the BbsI cut site is no longer there as the DNA sequence is replaced with the desired sequence. If the sgRNA is present, then only one cut is made at the EcoRI site, and the circular plasmid will become linear. Upon viewing a singular band in the gel, I sent the samples for sanger sequencing. The samples came back, and I analyzed the results using FinchTV and Benchling.

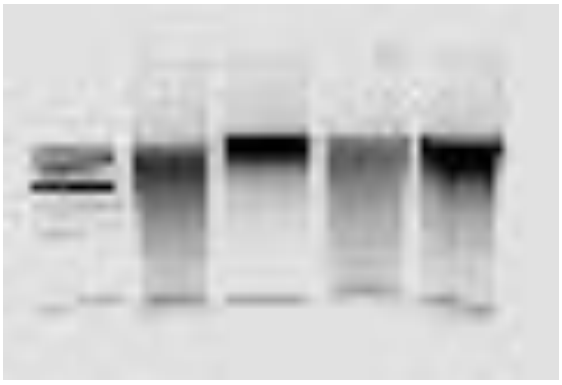

**Figure 3 Gel image of L1 (2nd well)**

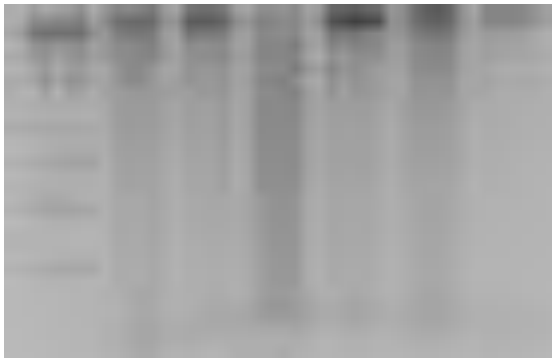

**Figure 4 Gel image of L2 and R1 (2nd and 5th wells respectively)**

## 2.4 Maxiprep

In the next part of the experiment, I conducted a Maxiprep of the Cas9 plasmid, which was a gift from Santhosh Girirajan, and of the plasmids that had a positive presence of the sgRNA. The first steps were the same as above in section 3.2, with the differences of adding 1 µl to the cells, 50 µl of LB, and only making one plate for each sgRNA.

The next day, I added 100 mL of LB broth and 100 µl of carbenicillin to a 1000 mL Erlenmeyer flask. An Erlenmeyer flask was created for each of the samples: Cas9, L1, L2, and R1. Next, I used an autoclaved wooden stick to select a colony from the plate and then swirl it into the solution. After adding the colony to the flasks, I placed them in the incubator at 37°C while shaking for 16 hours. I isolated the plasmid using the QIAGEN Plasmid Maxi Kit following manufacturer's protocol, specifically using the protocol that utilizes a vacuum. Finally, I nanodropped the samples to check DNA quality and analyze their concentrations.

## 2.5 Transfection

The next step of my project is to transfect hippocampal cell lines with the plasmids I have created. For my experiment, I am using HT-22 cells, which are an immortalized line of mouse hippocampal neurons. I grew cells in DMEM with 10% fetal bovine serum (FBS), 1% penicillin/streptomycin (Pen/Strep), and GlutaMAX which is a substitute for L-glutamine (will be referred to as media) in a large 10 cm dish and then split them for transfection. Before splitting, the cells had a confluency of around 90%. I analyzed the cell health and determined the media to have a good red color, the cells were not overcrowded, and a few dead cells. First, I

aspirated the media from the 10 cm dish and washed the cells with 10 mL 1xPBS. I next added 2 mL of Tryp-EDTA to cells and incubated them for 2 mins at 37°C with 5% $CO_2$. Then I added 8 mL of media to deactivate the Tryp-EDTA. To dissociate all cell clumps, I triturated the cells and moved the pipette around the plate to remove any stuck to the plate, all while being cautious to not create any bubbles. I then transferred the solution was transferred to a 15 mL Conical tube. I loaded 10 µl of the cell solution onto a hemocytometer and counted the number of cells. I then calculated the volume of cells needed to plate 0.37 x 10^6 cells on three 35mm dishes (see example 1) with a final volume of 2 mL of media.

**Example 1:**

$Dilution\ Factor: 1$

$Total\ Cells\ Counted: 152$

$$\frac{Cells}{mL} = \left(\frac{152}{4}\right) * DF(1) = 38\ x \frac{10^4 cells}{mL}$$

$$Convert\ 38\ x \frac{10^4 cells}{mL}\ to\ 0.38\ x\ 10^6$$

$$Cells\ for\ plating: 0.37\ x\ 10^6 cells$$

I plated the cells and placed them in the incubator overnight at 37 °C with 5% $CO_2$. The following day I proceeded with the first day of the transfection using the Lipofectamine 3000 kit (Invitrogen). I first created Mix1 which consisted of 250 µl of Optimem, 3 µg of DNA (1.5 µg of Cas9 and 1.5 µg of sgRNA), and 3 µl of Reagent. I created this mix for each of the sgRNAs: L1, L2, and R1. I mixed the solution well and then incubated it at room temperature for five minutes. I next created Mix2 which consists of 825 µl of Optimem and 6.88 µl of Lipofectamine. Then I

added 256.25 µl of Mix2 to each sample of Mix1. I lightly flick mixed Mix1 and Mix2 and then incubated the samples for 30 minutes at room temperature. Finally, I added 500 µl of each solution in a dropwise fashion to each of their own plates.

The following day I changed the media. On the third day, I extracted the cells. First, I added 500 µl of PBS to wash the cells. I added 200 µl of trypsin, and I placed the plates in the incubator for 2 minutes at 37°C. Next, I added 800 µl of DMEM, and transferred the sample to a 1.5 mL tube. I spun down the samples at 500 rcf for five minutes. I discarded the supernatant and stored the samples at -80 °C.

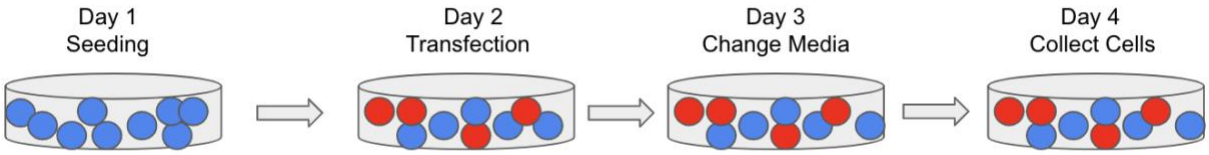


**Figure 5. Transfection procedure**

## 2.6 DNA Extraction

The next step of my project was to extract the DNA from the cells. First, I thawed the cells to room temperature and resuspended the pellet in 200 µl PBS. Next, I added 20 µl of Proteinase K and 4 µl of RNase A to degrade both protein and RNA in the sample. I vortexed the samples and then they incubated at room temperature for two minutes. I added 200 µl Buffer AL and mixed the samples immediately and thoroughly by vortexing. I transferred the mixture into the DNeasy Mini spin column and placed it in a 2 mL collection tube. Next, using the DNeasy

Kit (QIAGEN) I followed the manufacturer's protocol to extract and purify the DNA. Finally, I nanodropped the samples to check DNA quality and analyze their concentrations. I also conducted DNA extraction on wild-type cells to compare them to the altered DNA.

### 2.7 Determining Cutting Efficiency

For this part of my project, I am utilizing the EnGen Mutation kit (New England BioLabs) to determine the cutting efficiency of the sgRNAs I designed. To do this I set up a mixture of the forward and reverse primers (Table 2), Mili Q purified water (Q), Q5 Hot Start High-Fidelity 2X Master Mix, and the template DNA.

**Table 2 Primer sequences for T7E1 cutting efficiency assay**

| **Primers** | **Primer Name** | **Primer Sequence** |
|---|---|---|
| Left forward | LB287 | GAGACCTGGGTTCAGACTTTAG |
| Left reverse | LB288 | TCGACGGCTCCAGAGTAA |
| Right forward | LB289 | GCCTAAATATAGGAGGCGATCAG |
| Right reverse | LB290 | GAAGGAACAGGCTAAGGTGAG |

I created the first solution and then it went through the PCR machine overnight following specific thermocycling conditions: initial denaturation at 98°C for 30 seconds, then 35 cycles of denaturation at 98°C for 5 seconds, annealing at 65°C for 10 seconds, extension at 72°C for 20 seconds, and finally one round of a final extension at 72°C for 2 minutes. Next, I analyzed a

small amount of the PCR product on a 1.5% agarose gel to verify the amplification of a single product. I used a 100bp DNA marker to help estimate the amplicon concentration size.

After successfully creating the PCR product, I conducted a reaction for the heteroduplex formation. For this reaction, I combined the PCR product, 10X NEBuffer 2, and Q. I denatured the solution and then annealed it slowly using a specific protocol in the thermocycler: initial denaturation at 98°C for 5 minutes, annealing from 95°C - 85°C at a rate of -2°C/second, annealing continued from 85°C – 25°C at a rate of -0.1°C/second.

For the heteroduplex digestion, the reaction consisted of combining the annealed heteroduplex product and EnGen T7 Endonuclease I. I followed the EnGen Mutation Kit manufacturer's protocol without any changes. I made a 2% agarose gel and added the samples to verify the amplification of a single product of the correct size. I used a 100bp DNA marker to help estimate the amplicon size. I analyzed the gel according to band number and size, where there should be three bands visible for each sample.

# Chapter 3

# Results

## 3.1 sgRNAs

After analyzing the sgRNAs in the agarose gel, I would expect a single band for each sample. When a single band was viewed, I confirmed the samples via sanger sequencing to confirm the desired sequence was achieved. The L1 sgRNA was achieved successfully the first time through, but L2 and R1 were not achieved until repeating cloning many times. I was unable to achieve a clean, homogenous clone of R2 and therefore only proceeds with L1, L2, and R1 for all subsequent steps. The concentration and purity levels are seen in **Table 3** below.

**Table 3 sgRNA concentration and purity analysis**

| Sample | Concentration (ng/µl) | 260/280 | 260/230 |
|---|---|---|---|
| **L1** | **540.2** | **1.89** | **2.02** |
| **L2** | **487.1** | **1.87** | **2.07** |
| **R1** | **432.7** | **1.88** | **2.10** |

I conducted a Maxiprep of the sgRNAs after confirming the correct sequence through Sanger Sequencing. This is done to amplify the samples to have larger amounts to continue to work with through the rest of the experiments. Upon viewing these results, I confirmed the samples via Sanger Sequencing and found that the desired sequence was accomplished for each sample. The concentration and purity levels are seen in **Table 4** below. These samples were utilized throughout the rest of the experiment.

**Table 4 Maxiprep results**

| Sample | Concentration (ng/µl) | 260/280 | 260/230 |
|---|---|---|---|
| **L1** | **1119.3** | **1.23** | **3.77** |
| **L2** | **1933.8** | **0.71** | **4.29** |
| **R1** | **1691.7** | **0.81** | **4.19** |
| **Cas9** | **744.8** | **1.91** | **2.35** |

## 3.2 DNA Extraction

After transfection of the HT-22 cells with the sgRNA and Cas9, I collected the cells and extracted the DNA. Following protocol, I nanodropped the samples and received the resultant DNA. The concentration of the obtained DNA and purity levels are seen in **Table 5** below.

**Table 5 DNA Extraction results**

| Sample | Concentration (ng/µl) | 260/280 | 230/260 |
|---|---|---|---|
| **L1** | **48.9** | **1.91** | **0.71** |
| **L2** | **66.4** | **2.07** | **2.05** |
| **R1** | **50.9** | **1.93** | **0.83** |
| **WT DNA** | **82.4** | **1.95** | **1.75** |

## 3.3 Cutting Efficiency

Through transfection, the Cas9 and guide RNAs were implemented into the HT-22 cell line. I then collected these cells and extracted the DNA from them. To understand how effective these inserts are, I tested the cutting efficiency of Cas9 on the target regions. The CRISPR cutting of DNA results in DNA segments. After analyzing the gel, I found that these bands were visible, but they were very faint. I utilized the program Image Studio to quantify the bands to determine the cutting efficiency. Once I obtained these quantifications, the cutting efficiency was calculated utilizing the following equation:

$$\%\ Modification = 100\ x\ [\ 1 - (\sqrt{1 - fraction\ cleaved})]$$

$$Fraction\ cleaved = \frac{concentration\ of\ digested\ products}{conc.of\ digested\ products + conc.of\ undigested\ band)}$$

**Example 2: (for L1)**

$$Fraction\ cleaved = \frac{(0.42 + 0.386)}{(0.42 + 0.386 + 32.8)} = 0.024$$

$$\%\ Modification = 100\ x\ \left[\ 1 - \left(\sqrt{1 - 0.024}\right)\right] = 1.2\%$$

**Table 6 Cutting Efficiency Results**

| Sample | Cutting Efficiency |
|---|---|
| L1 | 1.2% |
| L2 | 0.94% |
| R1 | 1.005% |

## Chapter 4 Discussion

The circadian clock guides many internal functions of our body in accordance with the external environment. *Per1*, a major molecular clock gene, has been identified to be closely linked to learning and memory mechanisms. To better understand these mechanisms, I have sought to design an altered DNA sequence that specifically manipulates the CRE and E-box sites within the promoter region of the Per1 gene. I have successfully designed the sgRNAs and Cas9 and have transfected them into cells. I have extracted this DNA and it is ready to be utilized in the next pieces of the project.

There were several limitations I encountered during this project that prevented me from completing further steps. The first steps of this project were to design and create sgRNAs. To do this, I combined the selected oligos and grew bacterial colonies to contain these fragments. The first time I did this I chose a different colony for each tube. However, using two colonies produced a homogenous sample for L1 and a heterogeneous sample for L2, R1, and R2, so I proceeded to use only one colony from each plate.

Another limitation was found during the double digestion. To create the double digestion, I first used the enzymes KoRI-HF and PacI, but after attempting the double digestion a few times, I found that these enzymes were not successful. After further investigating these enzymes, I found that they were not working likely due to one of them being expired. After switching to the EcoRI and BbsI enzymes, the double digestion worked successfully.

Another issue arose during the cutting efficiency experiment. The CRISPR cutting of DNA results in DNA segments. Therefore, a gel analysis of the samples should reveal three bands for each sample. If the Cas9 cutting efficiency is low, then the amount of corresponding DNA will also be low. After analysis of the gel, the three bands were visible, however, they were

very faint indicating poor cutting efficiency. Therefore, it's likely the DNA was successfully extracted and cut at the proper sites, but only small amounts were going through this process. Studies by Davis and Maher reveal that a high cell passage number introduces issues with transfection. Cells at a higher passage number experience changes in morphology, growth rates, protein expression, responses to stimuli, and overall, the efficiency of the transfection. The cells seen in this thesis were obtained from passage number 27. According to protocols by Sigma-Aldrich, HT-22 mouse hippocampal cells are best used under passage 10, without significantly altering the expression and functionality of the cell (Davis & Maher, 1994; Morimoto & Koshland, 1990). In addition to this finding, I also found that the transfection may be improved by increasing the amount of DNA added to the cells. I also incorporated the use of antibiotics to improve the transfection.

I repeated the transfection using three different conditions: (1) Increase the amount of DNA from 1.5 μg to 1.9 μg, (2) increase the amount of DNA to 2.3 μg, and (3) use 1.5 μg of DNA with the addition of the antibiotics puromycin and zeocin. I used Benchling to identify which antibiotics Cas9 and the sgRNAs are resistant to. Cas9 is antibiotic resistant to puromycin and the sgRNAs are antibiotic resistant to zeocin. The utilization of antibiotics would ensure the DNA collected was successfully transfected with Cas9 and the sgRNA, and if the cell did not contain the DNA, it would die.

I completed the transfection under these three different conditions and successfully extracted the DNA, following the same protocol. I then proceeded to complete the cutting efficiency experiment using these samples. The conditions of the left-sided samples were successfully obtained. The conditions for the transfection are shown in **Figure 5** below. The concentration of the obtained DNA and purity levels are seen in **Table 7** below.

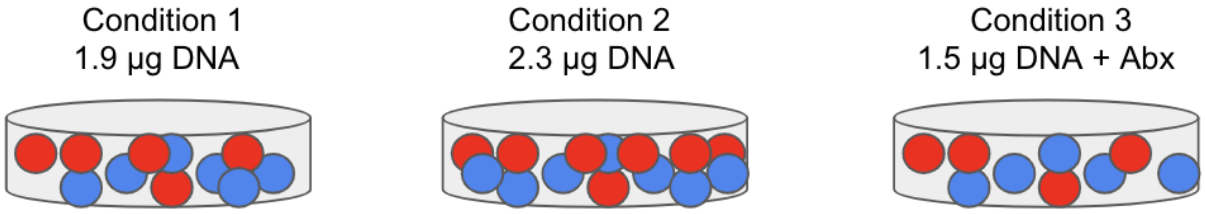


**Figure 5 Transfection with varying conditions**

**Table 7 Left-sided samples cutting efficiency under varying conditions**

| Sample | Cutting Efficiency | sgRNA |
|---|---|---|
| 1A | 0.50% | L1 |
| 1B | 0.43% | L2 |
| 2A | 0.57% | L1 |
| 2B | 0.74% | L2 |
| 3A | 0.85% | L1 |
| 3B | 0.31% | L2 |

I was unable to repeat this step of the experiment in time for the completion of the thesis, however, I believe that this step of the project was unsuccessful because the enzyme was denatured due to being a room temperature for too long. I plan to repeat this experiment using the DNA from the antibiotic condition and I expect the cutting efficiency to be much stronger.

Broadly, the next step in this project will be to insert the designed DNA sequence into the target region of the gene. The enzyme, Cas9, will inflict a DSB at the desired location on *Per1*. The DSB will be repaired through the precise method of HDR which utilizes a template that contains the desired edit to make specific changes to the region of interest. This template will contain the desired edits with sequences homologous to the endogenous DNA flanking the edit, which are referred to as homology arms. The larger the edited sequence, the larger the homology arms must be. Since this is a large 5.5kb region, these homology arms will have to be larger, which makes this process more challenging to successfully complete. Finally, the completed strand is ligated together, and the resultant strand now has the desired DNA sequence (Hida et al., 2000; Ran et al., 2013).

Future work will finish designing the donor plasmid to insert this desired sequence into the *Per1* gene. Once two homologous strands are established this can be put into mice. It should be put into mice and a behavioral experiment should be conducted to evaluate the effect of the mutated CRE and E-box sites influence on learning across the day/night cycle. The behavioral experiment should be set follow protocols of previous work by Bellfy and colleagues (2022) to compare the behaviors of mice with wild-type DNA to these mutated sites. Contrasts between these experiments will reveal how these sites are affecting learning and memory across the circadian day.

In conclusion, understanding the mechanisms behind how the circadian day influences learning and memory is a crucial area of research. Many studies have supported *Per1*'s influence over many of our bodies' mechanisms and it is important to uncover the other processes it is affecting. The findings from my thesis will be utilized in future work to reveal the mechanisms behind this gene and its influence on learning and memory formation.

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# ACADEMIC VITA

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## EDUCATION

**Pennsylvania State University | Schreyer Honors College** — University Park, PA
Bachelor of Science in Biology, with a Minor in Neuroscience — Expected in 05/2023

## PROFESSIONAL EXPERIENCE

**Kwapis Lab, Pennsylvania State University** — February 2020 to Present
*Research Assistant*

- Conduct mice behavioral experiments to evaluate memory formation, storage, and updating in the young and aging
- Perform brain surgery, brain extractions, slicing, and staining of mice brains to analyze learning
- Utilize CRISPR technology as an independent project to identify specific roles of target proteins in memory formation
- Plan experiments and write protocols for an independent project while collaborating with the principal investigator and graduate researchers in the lab

**Boas Vision Associates, Exton, PA** — December 2018 to August 2019
*Intern*

- Aided in administrative duties, obtained patients' medical insurance, and organized medical records
- Assisted clinicians throughout the practice with visual field tests and retina tests
- Attended and participated in meetings and brainstorming sessions with team members

## PUBLICATIONS (*in progress)*

Bellfy, L., Smies, C. W., Bernhardt, A. R., Bodinayake, K. K., Sebastian, A., Stuart, E. M., Wright, D. S., Lo, C.-Y., Murakami, S., Boyd, H. M., Von Abo, M. J., Albert, I., & Kwapis, J. L. (2022). The clock gene Per1 expression may exert diurnal control over hippocampal memory consolidation. *BioRxiv*. https://doi.org/10.1101/2022.10.11.511798

## CERTIFICATIONS

**Emergency Medical Technician (EMT)** — December 2018 to Present
**Centre LifeLink EMS, State College, PA** — February 2021 to Present

- Volunteer as an EMT
- Attentively aid patients during basic life support calls and assist paramedics with advanced life support (ALS) calls
- Communicate efficiently and effectively with paramedics, EMTs, and the emergency department

## COLLEGE INVOLVEMENT

**Epsilon Sigma Alpha** — September 2019 to Present
*President* — January 2021 to January 2022

- Managed a $15,000 budget through payment of dues and distribution across events and fundraisers
- Ensured our organization reached the set goal for fundraising contributions with THON and St. Jude
- Developed and maintained strong relationships with national senior members, alumni, and PSU officials
- Conducted biweekly chapter meetings with 200+ members, and assisted with events and new member recruitment

**Remote Area Medical** — September 2019 to Present

- Assist clinicians at clinics to provide medical, dental, and vision care to underserved and uninsured individuals

*Grant Team* — October 2020 to October 2021

- Wrote grant proposals to earn funding for the first RAM clinic in PA, which will be held in March of 2023

## SHADOWING

**Hospital of the University of Pennsylvania** — Philadelphia, PA
*Radiology* - Dr. Ali Nabavizadeh (MD)

- 24 hours, shadowed a Neuro-radiologist and his team

***Penn State* Health *Family and Community Medicine*** — State College, PA
*Family and Community Medicine* - Dr. Ryan Kipp (MD)

- 40 hours, shadowed the Chief of Residency through the out-patient practice