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THE EFFECTS OF THE PRNP GENE ON CIRCADIAN RHYTHMS

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ABSTRACT

Fatal Familial Insomnia is a rare genetic disorder affecting about 27 families worldwide, caused by mutations in the prion protein gene *PRNP*. Symptoms include loss of sleep and disrupted circadian rhythms. The function of *PNRP* in circadian rhythms is not well understood. The goal of this project was to create a cellular PRNP gene knockout and test the effects on circadian proteins using novel circadian luciferase reporter assays. The results indicate that one CRISPR-treated WI-38 cell line contains a disrupted coding region for *PRNP*, so this cell line can be used in future experiments to observe potential alterations in the expression of several cellular circadian rhythm proteins in the absence of *PRNP*. The results also show that luciferase plasmid hPer-2 works well to monitor circadian rhythm in U-2 OS cells, which validates the use of these novel reporters for studying circadian rhythm.

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ACKNOWLEDGEMENTS

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BACKGROUND

Prion Diseases

Prion diseases are a set of progressive fatal neurodegenerative illnesses that belong to the Transmissible Spongiform Encephalopathies (TSE) and manifest themselves in vertebrates (Prusiner and Dearmond, 1994). They are distinguished by long incubation periods, multi-focal spongiform pathology in the brain associated with neuronal loss, and a failure to induce an inflammatory response. In humans, these diseases can be either sporadic or familial (inherited), and include Creutzfeldt-Jakob disease (CJD), Fatal Familial Insomnia (FFI), Gerstmann-Straussler-Schienker (GSS) disease, and Kuru (Montagna, 2005). Symptoms vary widely, but can include rapidly progressive dementia, visual abnormalities, and cerebellar dysfunction, including muscle incoordination, and gait and speech abnormalities. During the course of the disease, many patients develop pyramidal and extrapyramidal dysfunction including abnormal reflexes, spasticity, tremors, and rigidity. Some patients may also show behavioral changes such as agitation, depression, or confusion. These symptoms often deteriorate into an akinetic mutism in the final fatal stage.

Prion diseases are a type of protein misfolding disease caused by the conversion of prion protein cellular (Prp^{C}) to infectious prion protein scrapie (PrP^{Sc}), the latter is a misfolded version of PrP^{C} characterized by high β-sheet content (Prusiner and Dearmond, 1994). Accumulations of Prp^{Sc} in the brain have been found to induce neural degeneration related to disease symptoms and onset, however, the exact mechanisms of pathogen propagation are not well understood (Montagna, 2011). This MQP project focused on the prion protein and its gene PRNP in relation to FFI and circadian rhythms.

Fatal Familial Insomnia

FFI is a rare, autosomal dominant, prion disease that has been reported in about 27 kindreds worldwide (Montagna, 2011). It is caused by a mutation in PRNP at codon 178 that results in a D178N substitution, and a polymorphism at codon 129 (Montagna *et al.*, 2003). FFI onset can range from 36 to 62 years, with a mean onset age of 51 years, and can have either a short disease duration of less than 11 months, or a prolonged disease duration of greater than 11 months (Montagna, 2011). Initial symptoms typically include the inability to nap or fall asleep, visual fatigue, and autonomic hyperactivity. As the disease progresses, symptoms include complete loss of sleep ability, oneiric stupors, motor impairment, and mental confusion, with later symptoms of akinetic mutism and emaciation, eventually resulting in death (Montagna, 2005). Studies have found that FFI symptoms are most likely caused by the accumulation of PrP^{Sc} in the thalamus and brain, which causes neuronal loss and spongiosis (Montagna *et al.*, 2003).

As is typical of the other spongiform diseases, the mechanism of FFI is not well understood. Many of the symptoms associated with FFI are related to sleep and circadian rhythm, suggesting a possible role for PrP^{C} in sleep regulation and circadian rhythm (Montagna, 2005). However, the prion protein gene PRNP and PrP in both cellular and pathogenic forms have not been extensively studied, hindering our understanding of FFI and potential therapies.

PrP^{C} and PrP^{Sc} Structure

PrP^{C} is composed of approximately 45% α-helix and lacks β-sheet structures (**Figure-1, right panel**). The converted form PrP^{Sc} (**Figure-1, left panel**) contains approximately 30% α-helix and 45% β-sheet (Huang *et al.*, 1996). The two forms differ in that PrP^{C} is soluble and

susceptible to degradation by proteases, while PrP^{Sc} is insoluble and protease resistant. The actual method of PrP^{C} conversion to PrP^{Sc} is not well understood.

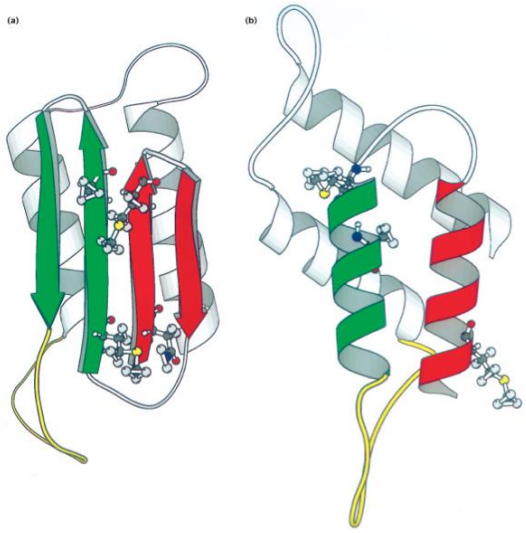

Figure-1: Theoretical 3-Dimensional Structures of Proteins PrP^{Sc} and PrP^{C}. PrP^{Sc} is shown on the **left panel**, and PrP^{C} in the **right panel**. The α-helical domains are shown as gray spirals, while the β-sheets are shown as flat arrows. The green and red arrows in the left panel represent β-sheets, which were converted from the corresponding colored helix in the right panel of PrP^{C}. Note that the right structure for PrP^{C} lacks β-sheets. (Huang e*t al.*, 1996)

Circadian Rhythm

Since many of the FFI symptoms are related to sleep and circadian rhythm, PrP^{C} may itself affect these biological processes. Daily cycles and changes in the physiological and behavioral characteristics of organisms constitute their circadian rhythm. These cycles entrain organisms to a specific, endogenous pattern aligned with day and night cycles, synchronizing their internal and external environments (Vitaterna *et al.*, 2001). Circadian rhythm is controlled primarily by the circadian system, or circadian clock, with localized clocks also found throughout the body in peripheral cells, tissues, and organs (Hida *et al.*, 2012). In mammals, the circadian clock is ultimately regulated by the suprachiasmatic nucleus (SCN), located in the hypothalamus, which serves as a central oscillator, that synchronizes all localized clocks to one

circadian rhythm based on environmental cues (Buhr and Takahashi, 2013). Through synchronization of internal clocks with external environmental cues, the circadian clock system is able to regulate daily physiological rhythms, such as the sleep-wake cycle, body temperature, hormone secretion, and other physiological processes.

Circadian Rhythm at the Molecular Level

The circadian clock system functions through the expression of clock genes and their transcription factors, which together form positive and negative transcriptional feedback loops **(Figure-2).** In the nucleus, clock genes brain and muscle ARNT-like protein 1 (*BMAL1)* and circadian locomotor output cycles kaput (*CLOCK)* form a positive auto regulatory loop by forming a heterodimer that binds to E-box CACGTG enhancers, activating the transcription of genes period (*PER1, PER2)* and cryptochrome *(CRY1, CRY2),* which form the negative regulatory loop of the clock mechanism. Once *PER* and *CRY* genes are activated, PER and CRY RNA molecules begin to accumulate in the cytoplasm, forming PER-CRY dimers. The PER-CRY dimers then translocate into the nucleus, where they bind to the BMAL1-CLOCK heterodimer, thus repressing the activation of *PER* and *CRY* genes (Nagoshi, *et al*.). In order for a new transcription cycle to begin, PER and CRY must be regulated for degradation. 5' adenosine monophosphate-activated protein kinase (AMPK) and casein kinase 1 (CK1ε/δ) phosphorylate PER and CRY, targeting them for ubiquitination and degradation through the 26S proteasome, allowing for *BMAL1* and *CLOCK* to be activated.

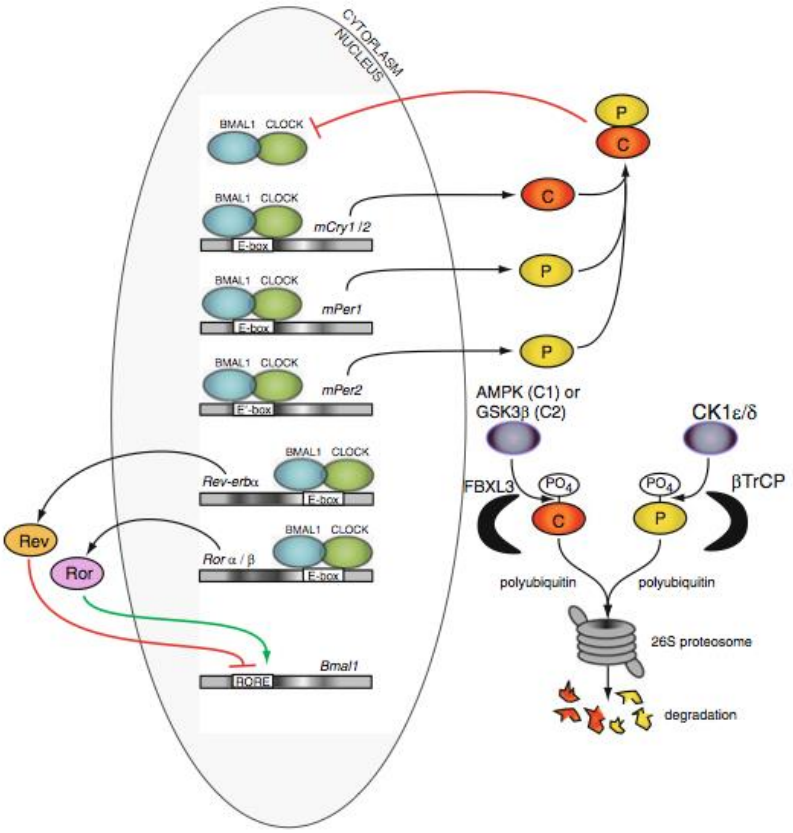

Figure-2: Schematic of the Mammalian Circadian Clock. The blue and green ovals represent BMAL1 and CLOCK heterodimers, which initiate transcription of clock genes *PER* and *CRY*, represented by the yellow and orange ovals, respectively. PER and CRY form a heterodimer that inhibits transcription by the BMAL1-CLOCK heterodimer. PER and CRY are then degraded by AMPK, CK1, and polyubiquitins. (Buhr and Takahashi, 2013)

In addition to this regulatory mechanism, binding of the BMAL1-CLOCK heterodimer also activates transcription of nuclear receptor Rev-erb-α and nuclear receptor retinoid-related orphan receptor (ROR-α), which regulate *BMAL1* through stimulation by ROR-α and repression by Rev-erb-α. Accumulation of Rev-erb-α represses *BMAL1*, with the overall stimulation and repression of *BMAL1* forming a 12 hour peak in RNA levels that are out of phase with *PER* and *CRY* transcription products (Reppert and Weaver, 2002). Altogether, the repression and activation of the clock genes constitute a 24-hour mammalian circadian cycle.

Disruptions in the Circadian Rhythm

Circadian rhythm is intimately linked with different physiological processes, such as, but not limited to, the sleep-wake cycle, metabolism, body temperature, and hormone secretion. Disruptions in circadian rhythm caused by the misalignment of endogenous internal circadian clocks relative to the external environment have been shown to affect cognitive function and sleep, and may result in circadian rhythm sleep disorders (CRSD) (Dodson and Zee, 2010). Such disorders include advanced sleep phase disorder, delayed sleep phase disorder, non-24 hour sleep-wake disorder, and others caused by the disruption of certain clock genes. Delayed sleep phase disorder has the phenotype of late sleep times between 2 AM and 6 AM, and delayed wake times, typically late morning to early afternoon, and although the mechanism is not exactly known, it is believed that polymorphisms in the genes *CLOCK* and *PER* cause the disorder. Similarly, advanced sleep phase disorder manifests as a sleep time between 7 PM to 9PM, and a wake time typically before 5 AM (Jones *et al*., 1999). The exact cause of advanced sleep phase syndrome is also not known, but studies of the disorder have found two families with gene mutations of *PER2* and casein kinase I delta (*CSNK1D*), respectively, demonstrating a genetic basis to the disorder (Dodzon and Zee, 2010). Additional studies in mice have also begun to show evidence for altered neuronal behavior at the cellular level caused by circadian rhythm disruptions due to gene mutations in *Per*, demonstrating the possibility that altered circadian rhythm may play a role in cognitive decline or impairment (Wang *et al.*, 2009).

PRNP and Circadian Rhythm

Studies have found that sleep loss or disrupted circadian rhythms may be caused by mutations in *PRNP*, demonstrating that PrP^{C} may play an important role in circadian rhythm pathways. In one study, 24-hour recordings of core-body temperatures, heart rate, mean arterial

pressure, norepinephrine, and cortisol and melatonin concentrations in FFI patients showed differences between the patients and controls (**Figure-3**) (Montagna *et al*., 2003). In the healthy control, melatonin, which plays a role in the sleep-wake cycle, rises sharply during the dark period. In comparison, the FFI patient has consistently low melatonin levels throughout day and night, demonstrating how a change in circadian rhythm may also affect sleep, with progressive sleep loss being one of FFI's main symptoms.

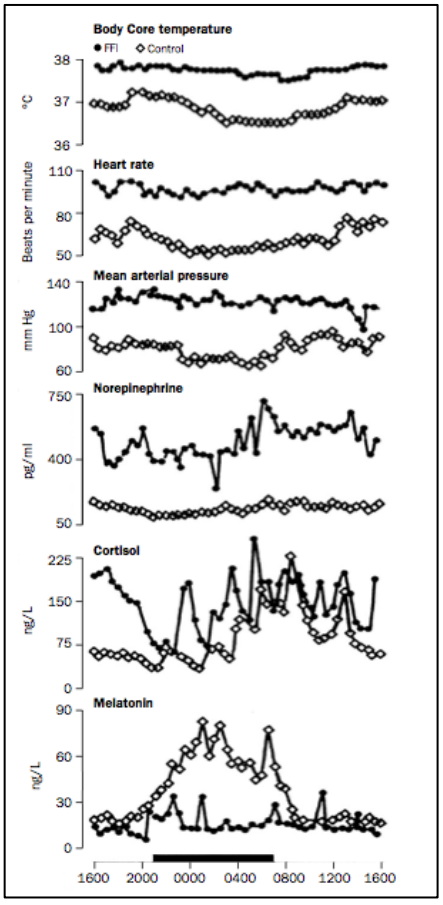

Figure-3: Twenty Four Hour Study of FFI Patients vs. Healthy Controls. FFI patients are represented as filled black circles and healthy controls are represented as non-filled diamonds. 24-hour recordings began at time 16:00, with the black bar representing dark periods. (Montagna *et al*., 2003)

In another study, *PRNP* knockout mice were raised and observed for differences in development and neurophysiology (Tobler et al., 1996). Mice lacking *PRNP* developed and

reproduced normally, showing no deficiencies or impairments. Circadian rhythm was observed through the use of running wheels to gage motor activity rhythms under light dark conditions. It was found that *PRNP*-deficient mice had altered, longer, period lengths when compared to wild type mice, suggesting that PrP^C may have a direct role in normal circadian rhythm functions (Tobler *et al.*, 1996).

Although the exact function of *PRNP* in relation to circadian rhythm is unknown, learning how *PRNP* affects circadian rhythm physiologically and molecularly is a key to understanding the cause and related symptoms of prion diseases, such as fatal familial insomnia.

CRISPR Genome Editing

The creation of a *PRNP* knockout in human cell lines for the purpose of identifying changes in cellular circadian rhythm is an integral part of this MQP project. Genome editing is the process of making targeted DNA mutations using tools to cut and then incorporate mutations of interest through homologous or non-homologous DNA recombination. These tools include zinc finger nucleases (ZFNs), transcription activator like effector nucleases (TALENs), and more recently, clustered regularly interspersed short palindromic repeats (CRISPR) (**Figure-4**). Of the three methodologies mentioned, CRISPRs are the newest. We chose to use the CRISPR system for performing the knockout in WI-38 cell lines because of its ease of design and high efficiency. The CRISPR and CRISPR associated (Cas) is a natural defense system found in bacteria and archae, where the CRISPR is used to recognize invading phage DNA and plasmid DNA by incorporating sequences from the invading phage in a small RNA-based repertoire, that is then used for further recognition and destruction of the foreign genetic elements through DNA cleavage (Horvath and Barrangou, 2010; Karginov and Hannon, 2010; Wiedenheft *et al.*, 2012). As a genome-editing tool, the CRISPR/Cas system functions in a similar manner to that of the

bacterial defense system. The CRISPR/Cas system requires a Cas nuclease, such as cas9 (green in the diagram), CRISPR RNA (crRNA) (orange in the diagram), and a guide RNA (blue in the diagram). The crRNA guides the Cas9 nuclease to a specific DNA sequence and a double-stranded break is induced. For gene removal, two guide RNAs can be designed to target the Cas nuclease to sites flanking the gene targeted for removal, and then the DNA ends are rejoined in vivo by DNA ligase.

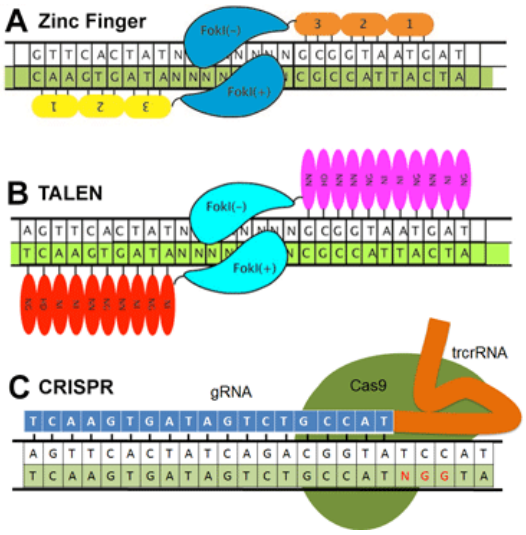

Figure-4: Diagram of the Three Main Methodologies for Genome Editing. Shown are the three main techniques currently used for genomic editing, including zinc finger nucleases (**Panel-A**), Talens (**Panel-B**), and CRISPRs (**Panel-C**). In the case of the CRISPR system, a guide RNA (blue in the diagram) is used to target the Cas-9 nuclease (green) to induce a double-stranded cut. Two cuts can be designed to excise a specific gene, and then the DNA ends are resealed in vivo by DNA ligase. (Addgene's Guide to CRISPR Technologies, 2013)

PROJECT PURPOSE

Fatal familial insomnia (FFI) is a rare prion disorder caused by mutations in the PRNP gene, and affects approximately 27 families worldwide. Of the various symptoms associated with FFI, a loss of circadian rhythm has been found to be detrimental to patients. Although it is known that mutations in PRNP can result in FFI, it is not well understood whether normal WT PRNP plays a role in circadian rhythm, and whether the mutations associated with FFI affect circadian rhythm, causing further disorder progression. The purpose of this project was to develop and utilize novel cellular assays to determine whether a PRNP gene knockout disrupts circadian rhythm, implicating a role for PRNP in circadian rhythm. The CRISPR system will be used to create PRNP knockouts in WI-38 cell lines, and the effects on various circadian proteins will be assayed using luciferase reporters with circadian protein promoters.

METHODS

Exome Variant Server FFI Assessment

In order to find the prevalence of the FFI mutations in a general population, *PRNP* was queried for variants using the Exome Variant Server (EVS). Under Data Browser in the EVS, *PRNP* was queried using gene information from the National Center for Biotechnology Information (NCBI). Data sets for both African American and European American populations were selected and downloaded as text files. Text files were converted to spread sheet format and analyzed for *PRNP* mutations related to FFI.

Creating a CRISPR

In order to create a CRISPR that targets and creates a gene knockout (KO) in *PRNP*, a target guide RNA as well as sense and antisense oligos with vector specific overhangs were designed and ordered from Invitrogen. Oligos were annealed using a PCR program that heats samples to a high temperature, and then it slowly lowers the temperature to allow for natural formation of double stranded DNA. The samples were treated with polynucleotide kinase (PNK) after being annealed in order to add a phosphate to the 5' end. The annealed oligos were ligated into backbone vector px330, a vector consisting of a chimeric guide RNA and Cas9 nuclease. The resulting plasmid was then transformed into *E. coli* bacteria in preparation for sequencing. Chosen colonies were prepared using the Qiagen Mini Prep Kit, and sequenced using the U6 promoter forward primer in order to confirm the desired crRNA. A total of three PRNP CRISPR plasmids were made, identified as PRNP CRISPR 1, 2, and 3, and were transfected into WI-38 cells.

Cell Culture and Maintenance

The WI-38 (ATCC® CCL-75™) cell line is derived from human fetal lung tissue. We used it as a transfection host for the PRNP KO CRISPR due to its genomic stability. WI-38 cells were grown in Eagle's Minimum Essential Media (EMEM), supplemented with 2 mM L-glutamine and a final concentration of 1% non-essential amino acids (NEAA) and 15% fetal bovine serum (FBS). WI-38 cells were plated from a frozen stock, and passaged 1:2 once a high confluence had been reached. Cells were transfected 4-5 days later using the Optifect WI-38 transfection kit (Life Technologies) once 80% or greater confluence had been achieved.

Optifect WI-38 Cell Transfection

WI-38 cells were pre-plated onto a 6 well plate with a cell volume of 250,000 cells per well. Plasmid DNAs for GFP (control) and PRNP KO's were mixed with a lipid-based transfection reagent and incubated at room temperature for 15 minutes before being added drop-wise to the pre-plated cells. Once the DNA and reagent were added to the wells, the volume was raised to a total volume of 1.0 mL using Optimem media. For maintenance, cells were cultured after the transfection with puromycin and EMEM in order to place selective pressure on the cells.

CRISPR Efficacy Testing

In order to test the efficacy of the designed CRISPRs, CRISPRs were transfected into WI-38 cells. Cells were grown after transfection for approximately 48 hours, after which genomic DNA was extracted using the Qiagen DNeasy kit for blood and animal cells. Once the DNA was extracted, genomic regions expected to contain the CRISPR-induced mutations were amplified using PCR and run on a gel. The amplicon was excised from the gel and purified using

the Qiagen Gel Extraction kit. Amplicons were subsequently denatured and reannealed using the Surveyor Assay (described below).

Surveyor Assay

Genomic DNA (gDNA) was isolated from WI-38 cells transfected with plasmids PRNP CRISPR 1, 2, or 3, using the DNeasy WI-38 DNA extraction kit for blood and animal cells. The gDNA CRISPR insertion site was amplified using PCR. The PCR product was then annealed and processed using the Surveyor Mutation Detection Kit. If the CRISPR plasmid successfully induced a mutation at its target site, the isolated gDNA will contain both WT and mutant type amplicons. If WT DNA anneals with mutant DNA, a DNA mismatch will occur, and the amplicon will be sensitive to digestion with a single-strand-specific Surveyor nuclease. The final surveyor products were run on a 2% agarose TBE gel alongside the original WT amplicon to determine whether any of the CRISPR plasmids had induced any PRNP mutations. The original amplicon is approximately 600 bp in length and a possible mutation site occurs at position of 200 bp. If a mismatch is present, this will result in a 200 bp fragment plus a 400 bp fragment in the sample lane.

Gel Mutation Assay

A second assay for identifying potential mutations in the PRNP CRISPR transfected WI-38 cells is a Gel Mutation Assay. This was performed using gDNA isolated from from WI-38 cells treated with PRNP CRISPR 1, 2, or 3. Using PCR, a 100 bp region near the CRISPR insertion site was amplified from the gDNA. The PCR product was then run on a 4% agarose TBE gel and analyzed for possible insertions or deletions, which would present as smaller or larger bands in the sample lanes.

Circadian Luciferase Reporter Assay

Four plasmids received from the Weitz lab at Harvard Medical School were transfected into a U-2 OS (ATCC® HTB-96™) cell line in order to test synchronization using different conditions, as well as to evaluate the ability of the circadian reporter plasmids. The four plasmids consisted of an hPer2 promoter short fragment, the first two kb of the hBmal1b-promoter, the first two kb of the mPer1 promoter, and the first two kb of the mPer2 promoter, upstream from the luciferase reporter gene. Transfected cells were pre-plated in a 96 well plate at a cell density of approximately 3×10^6 cells per well, under four different synchronization methods, no synchronization media, Forskolin, Dexamethasone, and serum shock, and then incubated. After incubation, the medium was aspirated from each cell and replaced with phenol red-free medium containing 0.1 μM luciferin. The plate was then sealed using a plastic plate cover and vacuum grease to prevent the media from evaporating during the assay. Cells were placed in a Tecan Infinite 200 Pro plate reader at 37° Celsius, with luciferase readings taken every 8 minutes for 5 days for a total of 960 time points.

RESULTS

FFI is a rare genetic disorder caused by mutations in the *PRNP* gene. Of the many symptoms associated with FFI, loss of circadian rhythm and sleep are most detrimental to patients. In order to better understand the role of *PRNP* in circadian rhythm, we created *PRNP* KO's using the CRISPR/Cas system, and tested novel circadian rhythm assays to assess whether circadian rhythm behaviors of cells could be measured quantitatively. These assays represent the preliminary stages of a future project that will test whether circadian proteins are altered in cells containing PRNP mutations or in cells completely lacking PRNP.

Transfection Optimization

To introduce genetic mutations into the genome of WI-38 cells, we first need to introduce CRISPR plasmids using lipid-mediated transfection. We first optimized our transfection protocol using WI-38 cells. A cell optimization test was performed to find the optimal number of cells needed for transfection and the optimal amount of reagent required for the highest transfection rate. Conditions were tested using three different cell densities, either pre-plated prior to lipid addition, or plated simultaneously with lipid addition, using two different lipid concentrations, and using two different plasmid masses (**Figure-5**). A green fluorescent protein (GFP) plasmid was used for this test transfection, allowing transfected cells to easily be observed under a microscope. GFP-positive cell counts performed for the different reactions showed that reaction four, which contained 1.0 μg of DNA and 1.0 μl of transfection reagent, for both pre-plating prior to lipid addition and plating with simultaneous lipid addition under all cell seed numbers, had the highest transfection rates when compared to the other three reactions under similar plating and cell seed number conditions.

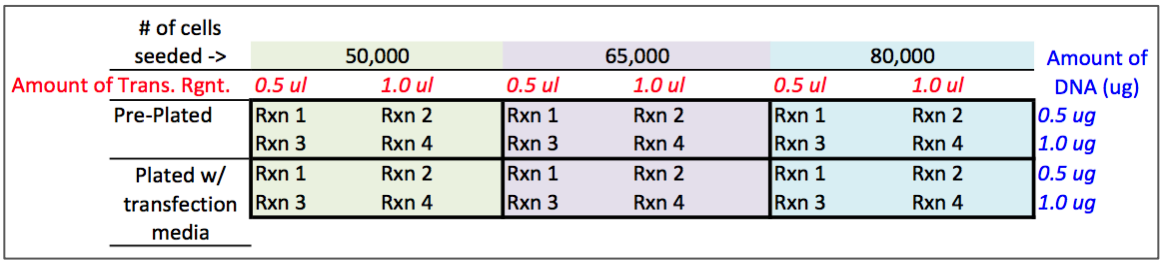

Figure-5: Experimental Design of WI-38 Transfection Optimization Conditions. Shown above are the conditions used to optimize the transfection conditions for WI-38 cells. The location of each reaction in the table specifies the constituents of the reaction. For example, Reaction 1 under the light green shading on the left (50,000 cells seeded) in the pre-plated box contains 0.5 µl of transfection reagent and 0.5 µg of DNA.

In order to assess whether pre-plating the cells prior to lipid addition or adding the lipid simultaneous to plating is best, three cell counts were taken of random fields of the transfected cells. This data confirmed that pre-plating the cells prior to lipid addition generally produced higher levels of transfection (**Table-1**). Cells pre-plated at a cell density of 65,000 cells produced an average transfection percentage of 22.7% with the smallest standard deviation (best reproducibility) compared to other transfection conditions that may have had a higher transfection percentage. Combining the results of Figure-5 and Table-I, it was determined that WI-38 cells transfect optimally using 65,000 cells pre-plated prior to lipid addition, and a total of 1.0 µg of DNA and 1.0 µl of transfection reagent. These optimized transfection conditions were used later on the WI-38 cells when transfecting the PRNP KO CRISPR plasmids.

	Pre-Plated Reaction 4				Mixed with Media Reaction 4		
Seed #	50,000	65,000	80,000	*Seed #*	50,000	65,000	80,000
Field Count 1	23	13	18	*Field Count 1*	6	17	50
Field Count 2	15	16	15	*Field Count 2*	15	10	19
Field Count 3	20	17	8	*Field Count 3*	13	15	9
Average	19.33	15.33	13.67	*Average*	11.33	14.00	26.00
Avg. Transfection Percentage	37.20	22.69	16.43	*Avg. Transfection Percentage*	21.81	20.72	31.27
Standard Dev.	4.04	2.08	5.13	*Standard Dev.*	4.73	3.61	21.38

Table-1. Transfected Cell Field Counts of Reaction Four. GFP-positive cell counts from cells plated using Reaction 4 of the optimized transfection conditions were conducted on random fields directly comparing pre-plated cells versus simultaneous addition of the lipid at different cell densities.

Test of Circadian Rhythm Plasmids

To determine whether cellular circadian rhythm could be measured using the provided reporter plasmids, a luciferase assay was designed that focused on cell synchronization methods and luciferase expression driven by the promoter of core circadian clock genes. The assay was designed according to previously established methods that use human U-2 OS cells to monitor circadian clock rhythms *in vitro* (Vollmers *et al*., 2008). We tested the synchronization methods and reporter plasmids in the U-2 OS cell line which is known to express circadian rhythms. U-2 OS cells transfect efficiently and do not require the optimized transfection protocol described above for the WI-38 cells. U-2 OS cells were plated at a cell density of 250,000 cells per well in a 96-well plate and were transfected with circadian rhythm reporter plasmids for human and mouse circadian proteins. To determine the best method of cell synchronization, four conditions were tested: forskolin, dexamathosone, serum shock, or untreated cell media (no synchronization). Cells were transfected with reporter plasmid, incubated with one of the four conditions, and then assayed for luciferase activity using a plate reader every eight minutes over five days for a total of 960 time points. Cells transfected with plasmid hPer2-luc confirmed that circadian rhythm could be measured (**Figure-6**). At periods where hPer2 was active, luciferase was also activated, as shown by several re-occurring peaks. All other luciferase reporter

plasmids tested did not produce detectable levels of rhythm, and showed no re-occurring peaks in the graphed data. All four cell synchronization methods produced similar results, indicating that any synchronization can be used in future experiments with the PNRP knockout cells.

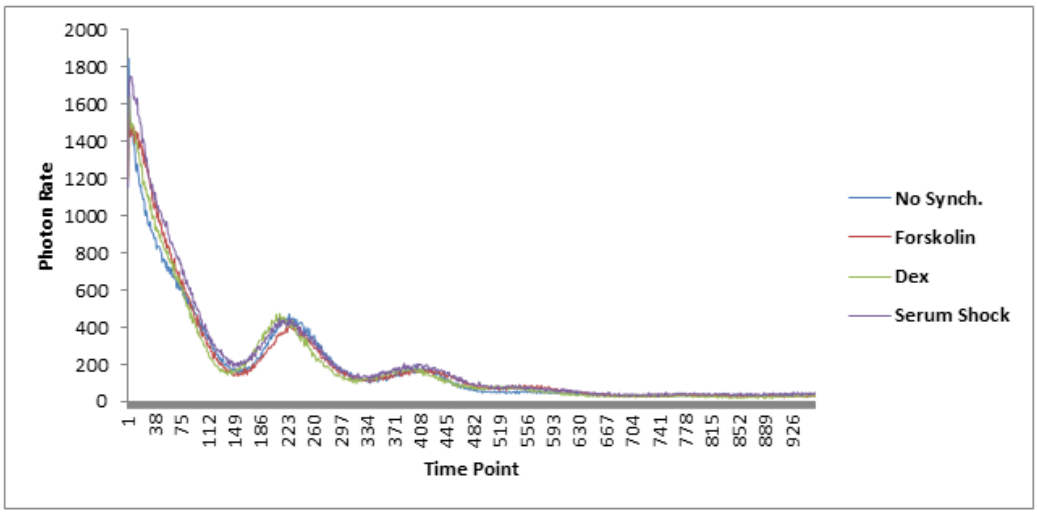

Figure-6: U-2 OS hPER2 Circadian Luciferase Assay Results. Four different cell synchronization methods were compared: blue (no synchronization), red (Forskolin), green (Dexamethasone), and purple (serum shock). The photon rate was determined every eight minutes over five days.

CRISPR Knockout of PRNP

To determine whether the *PRNP* gene can be knocked out in WI-38 cells using our CRISPR methodology, a target RNA was designed against the *PRNP* gene to create a KO. We created three PRNP CRISPR plasmids and focused on exon-2 of the *PRNP* gene as it contains the transcriptional start site. By selecting a CRISPR target sequence before the exon-2 start codon (**Figure-7**), the CRISPR/Cas system will induce a frame shift mutation if successful.

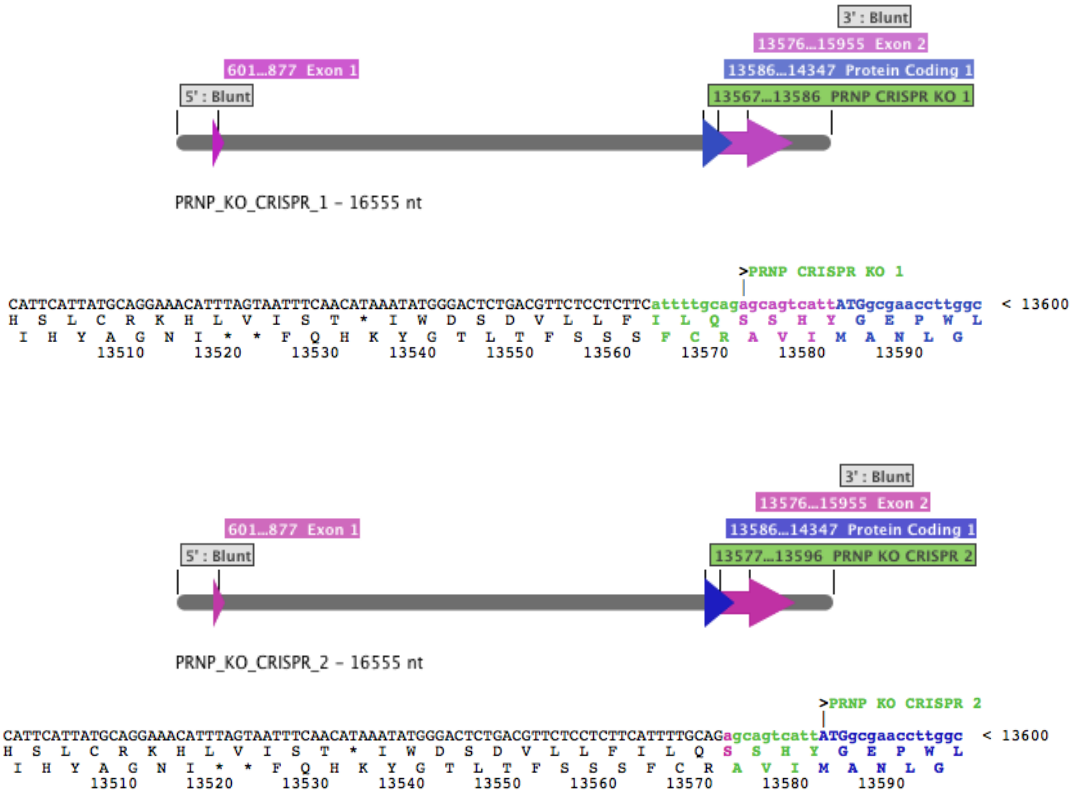

Figure-7: PRNP CRISPR Graphic Map and Insertion Sites. Using molecular biology software, the insertion sites of PRNP CRISPR 1 and 2 are shown. Pink indicates the exon regions of the PRNP gene, blue indicates the protein-coding region, and green indicates the CRISPR.

The three *PRNP* CRISPR plasmids, designated PRNP-1, PRNP-2, and PRNP-3, were transfected into WI-38 cells using the optimized transfection protocol described above. The WI-38 cells were placed under selective pressure using puromycin to select for CRISPR-positive cells. To determine whether the PRNP CRISPR plasmids caused the expected mutations in the PRNP gene, the genomic DNA (gDNA) from each population of WI-38 cells was extracted, the target site was amplified by PCR, and the amplicons were analyzed using a gel mutation assay (**Figure-8**). The gel mutation assay was performed to observe whether any mutations such as

deletions or insertions are present in the *PRNP* KO gDNA samples at the target sites. Any mutations greater than about 5 bp will be observed as a shift in band mobility relative to WT. The data indicates that possible mutations occurred using *PRNP* KO CRISPRs 2 and 3, as visualized using Primer set-2 (black arrows, left side of the figure).

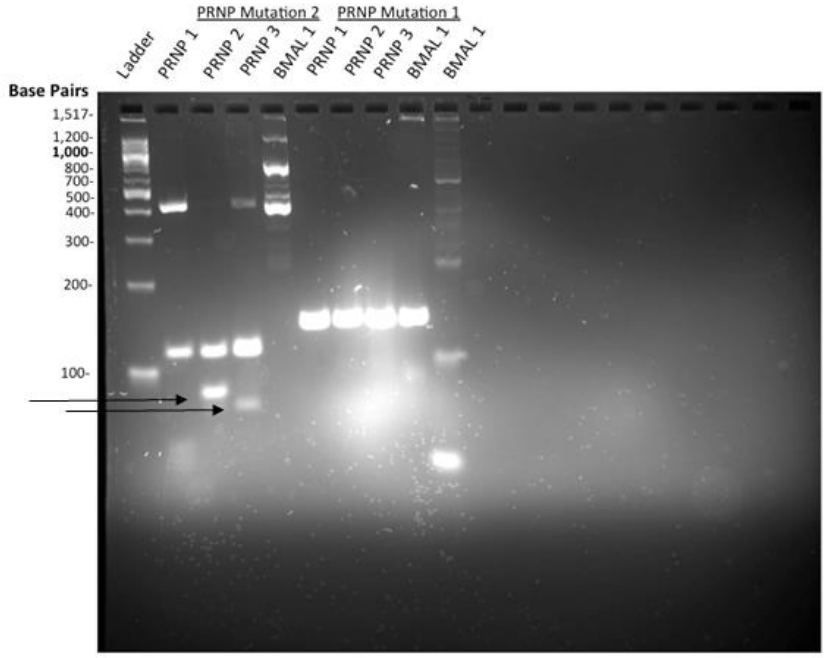

Figure-8: Gel Mutation Assay of PRNP KO's. Three different PRNP KO gDNA's were tested: PRNP-1, PRNP-2, and PRNP-3. Two different primer sets were used, PRNP Mutation 1 (right side of figure) and PRNP Mutation 2 (left side of figure). BMAL 1 gDNA was used as a control in both primer sets and with an additional BMAL 1 primer set. Arrows indicate the smaller bands likely harboring deletion-type mutations.

The surveyor assay (**Figure-9**) was performed as a backup assay for identifying CRISPR-induced mutations. The Surveyor assay identifies mutations in a different manner, and was performed to compare possible mutations found between the Surveyor assay and the gel mutation assay. In the Surveyor assay, a 600 bp PCR amplicon from the WT and potentially mutated target sites are mixed, denatured, reannealed, and cut with a single-strand-specific Surveyor nuclease. If a mutation is present in the CRISPR-treated DNA, a mismatch occurs in

the formed heteroduplex, making the DNA sensitive to digestion with Surveyor nuclease. A potential mutation site exists at 200 bp from the left terminus of the 600 bp amplicon, so if that mutation is present, cleavage by the Surveyor nuclease would result in the formation of a 200 bp band and a 400 bp band in the lane. Unfortunately, the Surveyor assay data was inconclusive. The PCR failed to amplify the *PRNP* region of interest for several different reactions, and the positive control reaction also failed. Thus, this assay needs to be redone.

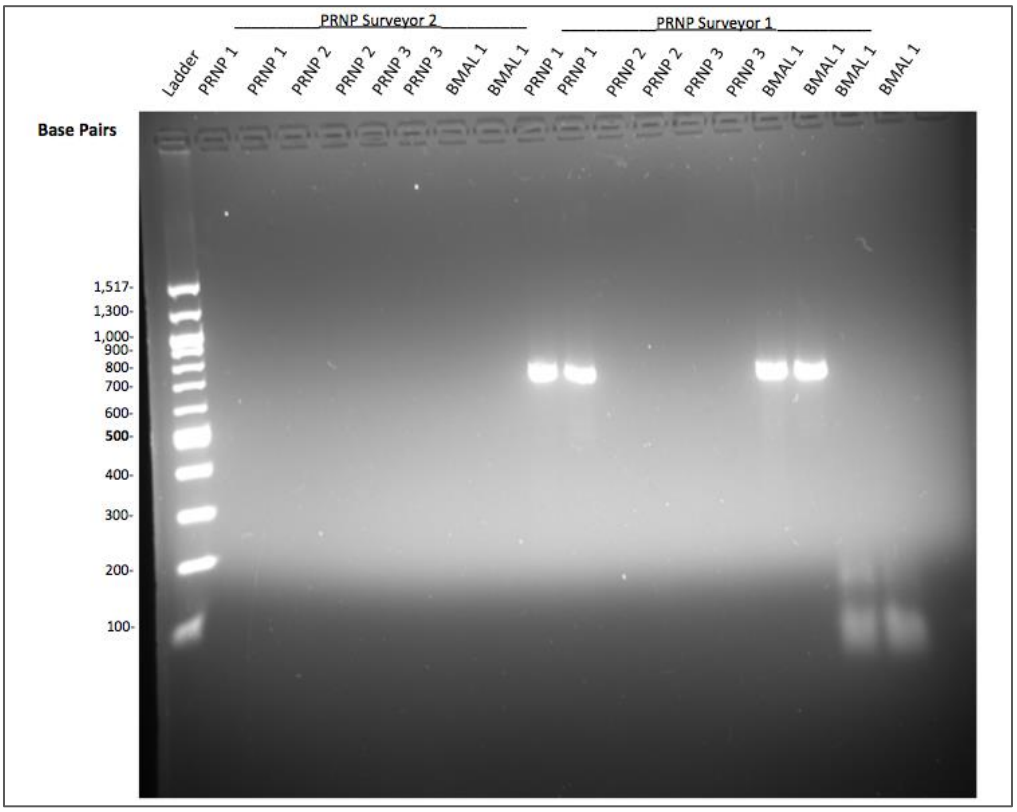

Figure-9: Surveyor Assay Results. Three different CRISPR PRNP KO gDNA's were used in this assay: PRNP-1, PRNP-2, and PRNP-3. Two different primer sets were used, PRNP Surveyor-1 (right side), and PRNP Surveyor-2 (left side). BMAL-1 gDNA was used as a control in both primer sets and with an additional BMAL-1 Surveyor primer set. Surveyor PRNP samples were run on a 2% agarose gel alongside the respective PCR product.

Although the Surveyor assay did not work, the gel mutation assay identified two potential positives created by CRISPR's 2 and 3, so those two potentially mutated amplicons were cloned and sequenced. The *PRNP* KO DNA amplicons were ligated into a plasmid and transformed

into *E. coli*. The plasmid DNA was then extracted from the *E. coli* using the Qiagen MiniPrep Kit and sequenced. The sequencing results were then aligned with the WT *PRNP* coding region to assess whether mutations were present (**Figure-10**). We sequenced 25 colonies and discovered one insertion, indicating that our *PRNP* CRISPRs can yield mutations in the *PRNP* gene of the WI-38 cell line.

Figure-10: Sequence Analysis of Potential PRNP Mutants. The figure shows the alignment of WT *PRNP* DNA (upper row) versus 13 clones of the PCR amplicons from WI-38 gDNA treated with CRISPR's 2 and 3. Genomic DNA was isolated from cells transfected with the *PRNP* CRISPRs targeting exon 2 of the PRNP gene. The PRNP target location was amplified, cloned, and sequenced. Each sequence represents the genome of one WI-38 cell. One cell contains an inserted sequence at the target site (red), which is a candidate for future studies investigating the consequences of loss of functional *PRNP* on circadian characteristics.

DISCUSSION

The goal of this project was to assess the potential effects of the *PRNP* gene on circadian rhythm by creating a *PRNP* knockout in WI-38 cells using the CRISPR/Cas system, and assaying the *PRNP*-KO cells for the levels of various circadian rhythm proteins using luciferase reporter plasmids. Using the CRISPR/Cas system, the data indicate that we created a frame shift mutation in the genome of one WI-38 cell, indicating the designed PRNP CRISPR is capable of editing the *PRNP* gene in the genome of WI-38 cells. The results also show that a reporter plasmid for hPer-2 circadian protein can successfully monitor circadian rhythm in U-2 OS cells, and in the future this assay will hopefully allow for analysis of circadian behavior of WI-38 cells bearing CRISPR-mediated *PRNP* gene KO's.

Fatal familial insomnia (FFI) is a rare autosomal dominant disease that is highly penetrant and fatal in those affected by the disease. FFI is caused by mutations in *PRNP*, which result in the misfolding of prion protein from a normal cellular form to a pathogenic form. Of the associated symptoms, loss of circadian rhythm is apparent (Montagna *et al*., 2003). However, it is unknown whether PRNP loss or mutations associated with FFI affect the cellular levels of various circadian proteins. In order to understand whether *PRNP* plays a role in circadian rhythms, the CRISPR/Cas genome editing system was used to create a KO in the *PRNP* gene in WI-38 cells. Designed PRNP CRISPRs were transfected into WI-38 cells, and subsequent screening identified one WI-38 cell line with the expected insertion in exon-2 of *PRNP*. Previous studies observed robust circadian oscillations in human U-2 OS cells using luciferase reporter plasmids driven by promoters from circadian proteins (Vollmers *et al*., 2008). Using similar procedures, we tested two human circadian promoters, hPER2 and hBMAL1, and two mouse circadian promoters, mPer1 and mPer2. The data indicated that of the four circadian

promoters tested, only the hPer2 promoter successfully demonstrated circadian rhythm in the U-2 OS cells. But at least this shows that cellular circadian rhythm can be successfully measured using the luciferase reporter approach.

Although the overall goal of the project was to assay whether a PRNP-KO affects cellular circadian rhythm, the assays conducted in this project represent vital preliminary steps needed to begin to understand the role that *PRNP* may have in circadian rhythm. The genome of one WI-38 cell was successfully mutated using the designed PRNP CRISPR, showing that this designed plasmid works well. In the future, this KO cell line will be grown to a monoclonal population, and western blots will be performed to verify the PRNP protein has been knocked down. The KO cells will then be transfected with a designed luciferase reporter plasmid and assayed for alterations in circadian protein levels. If WI-38 cells fail to show a circadian rhythm, the PRNP KO will be repeated in other cell lines of interest. Ultimately, various FFI *PRNP* mutations will be created to determine whether they show the same phenotype as the *PRNP* KO.

In general, this project encountered two main challenges. First, the WI-38 cells proved difficult to transfect, and they grew slowly. The transfection assay was optimized for these cells, however, even with optimized conditions, many of the WI-38 cell populations failed to grow or transfect, perhaps due to the primary-like nature of WI-38 cells. Although the circadian luciferase assay worked well for U-2 OS cells, it failed to detect a circadian rhythm in WI-38 cells, so the assay will need to be repeated. This project used novel CRISPR and luciferase circadian protein assays, each of which had to be perfected before we are able to perform the final test of circadian rhythms in PRNP KO cells. In addition, the FFI project is novel to our laboratory. It is also very time consuming to prepare monoclonal populations of WI-38 cells.

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