Circadian Dysregulation is Associated with Alterations in Tumor Suppressor Activity in Murine Mammary Tissue

by
Hiruni M. Aponso

A THESIS

submitted to

Oregon State University

Honors College

in partial fulfillment of
the requirements for the
degree of

Honors Baccalaureate of Science in Biochemistry and Molecular Biology
(Honors Scholar)

Presented May 29, 2020
Commencement June 2020

AN ABSTRACT OF THE THESIS OF

Hiruni M. Aponso for the degree of Honors Baccalaureate of Science in Biochemistry and Molecular Biology presented on May 29, 2020. Title: Circadian Dysregulation is Associated with Alterations in Tumor Suppressor Activity in Murine Mammary Tissue.

Abstract approved:_____

Patrick Chappell

Breast cancer is the second most common cancer among women in the US, however, only a small fraction of cases are attributable to heritable genetic mutations; the bulk arises from various behavioral and environmental factors. Loss of functional p53 is observed in over 50% of cases, and its activity relies on post-translational acetylation or deacetylation by various cellular proteins. SIRT1 is a class III HDAC that can remove the activating acetyl moiety. In a normal cell, stress activates *p53*, which then transcribes *Hic1*. HIC1 represses transcription of *Sirt1* to prevent it from deactivating P53 by removing its acetyl group. *Hic1* is epigenetically regulated, and hypermethylation of the *Hic1* promoter can result in silencing of its expression. Previous work in our lab demonstrated increased methylation in the promoter region of *Hic1* in mammary tissue derived from mice exposed to extended light at night (LAN) Due to an established link between SIRT1 and the regulation of the circadian rhythm, the present study explores the abundance of P53, SIRT1, and phosphorylated SIRT1 (p-SIRT1) in response to circadian dysregulation. Our results indicate a unique abundance pattern of SIRT1 and P53 in normal murine mammary tissue as well as alterations in P53, SIRT1, and p-SIRT1 abundance after a three-week period of exposure to LAN.

Key Words: Circadian clock, SIRT1, P53, breast cancer, epigenetics

Corresponding e-mail address: aponsoh@oregonstate.edu

Circadian Dysregulation is Associated with Alterations in Tumor Suppressor Activity in Murine Mammary Tissue

by
Hiruni M. Aponso

A THESIS

submitted to

Oregon State University

Honors College

in partial fulfillment of
the requirements for the
degree of

Honors Baccalaureate of Science in Biochemistry and Molecular Biology
(Honors Scholar)

Presented May 2020, 2020
Commencement June 2020

Honors Baccalaureate of Science in Biochemistry and Molecular Biology project of Hiruni M. Aponso presented on May 29, 2020.

APPROVED:

_____

Patrick Chappell, Mentor, representing Biomedical Sciences

_____

Maude David, Committee Member, representing Microbiology

_____

Kate Shay, Committee Member, representing Biochemistry and Biophysics

_____

Toni Doolen, Dean, Oregon State University Honors College

I understand that my project will become part of the permanent collection of Oregon State University, Honors College. My signature below authorizes release of my project to any reader upon request.

_____

Hiruni M. Aponso, Author

Introduction

Many industries and services rely on around-the-clock operations that require night-shift work.[1,2] Shift work is associated with a number of health problems including sleep disturbances, cardiovascular dysfunction, metabolic syndrome, oxidative stress, and breast cancer.[1-4] Under normal conditions, body functions of the respiratory, digestive, and cardiovascular systems rhythmically fluctuate throughout the day in time with the sleep/wake cycle.[2,5] Many essential biological processes occur during sleep that are critical for overall health. When the normal sleep/wake cycle is inverted, our bodies are forced to perform activities during the dark phase, causing a shift in the body's daily rhythmicity.[2,5] The continuous stress of long work hours and nighttime light exposure eventually misaligns the internal clock with the sleep/wake cycle, leading to negative biological effects.[1,2,5]

The circadian clock is a light-entrainable timing system present in most cells that regulates and coordinates physiological processes by temporally inducing expression of target genes.[6-9] In mammals, the master circadian clock is found in the suprachiasmatic nucleus (SCN) of the anterior hypothalamus.[7,9] The clock oscillates with a 24 h periodicity which can be modulated by intercellular signals and light input.[6-10] Functionally, oscillation relies on positive and negative feedback loops generated from the transcription factors Brain and Muscle ARNT-like protein-1 (BMAL1) and Circadian Locomotor Output Cycles Kaput (CLOCK).[6-10] CLOCK and BMAL1 form an active heterodimer which transcribes multiple genes, including the core clock genes *Cryptochrome* (*Cry1* and *Cry2*) and *Period* (*Per1*, *Per2*, and *Per3*).[6-10] Once CRY and PER proteins have accumulated, they form a heterodimer that interacts with CLOCK:-BMAL1 to suppress its activity, in turn repressing their own transcription (Figure 1).[6-10] This positive-

negative feedback loop has a cyclic nature in which the CLOCK:-BMAL1 complex is the most active during the day, and CRY:-PER inhibition peaks during the night.[6-10]

CLOCK also possesses histone acetyltransferase (HAT) activity, which facilitates chromatin remodeling events.[11,12] In addition, CLOCK is able to acetylate non-histone proteins, including its own binding partner BMAL1 (Figure 1).[11,12] In parallel with the suppression of clock-controlled gene transcription, CLOCK acetylates BMAL1 at a conserved Lys537 residue. This acetylation directs CRY to the CLOCK:-BMAL1 complex and instigates the repression of transcriptional activity (Figure 1).[11,12,13] To counterbalance BMAL1 acetylation, histone deacetylase (HDAC) SIRT1 removes the acetyl group from Lys537 allowing the CLOCK:-BMAL1 complex to resume transcriptional activity (Figure 1).[11,12]

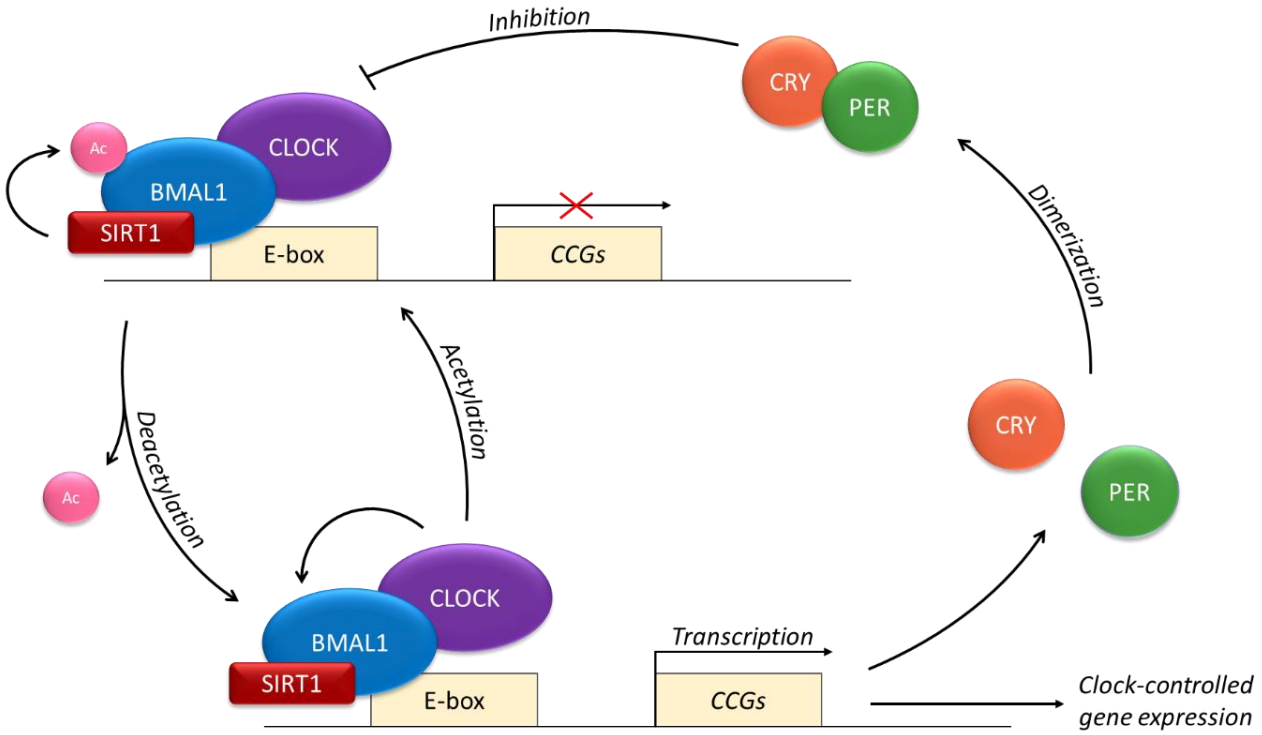

Figure 1. The regulatory mechanism of the circadian clock. SIRT1 deacetylates BMAL1 at Lys537 allowing the CLOCK:-BMAL1 heterodimer to bind to E-box elements in the promoter regions of clock-controlled genes (CCGs) including *cry* and *per*. CRY and PER proteins accumulate and form a dimer. The HAT activity of CLOCK acetylates BMAL1 at Lys537 and thereby directs the CRY:-PER dimer to the CLOCK:-BMAL1 complex. The CRY:-PER dimer inhibits CLOCK-BMAL1 activity and restarts the transcriptional cycle.

SIRT1 is the mammalian homolog of yeast *Sir2* and is a NAD^+-dependent deacetylase involved in metabolic and physiological processes.[7,10-12,14] The SIRT1 enzymatic reaction involves hydrolysis of NAD^+ and transfer of the target protein's acetyl group to the 2'-OH position of ADP-ribose, producing nicotinamide and *O*-acetyl-ADP-ribose.[7,14] Since SIRT1 exclusively utilizes NAD^+ as a co-substrate, its activity is dependent on the availability of NAD^+.[7,11,12,14] The rate-limiting step of the NAD+ salvage pathway is performed by nicotinamide phosphoribosyltransferase (NAMPT), and SIRT1 activity correlates with NAMPT abundance.[11,12,14] CLOCK:-BMAL1 regulate induction of *Nampt* transcription, resulting in oscillatory abundance of NAMPT.[11,12] Thus, because the NAD+ salvage pathway is circadian-regulated by the clock, the activity of SIRT1 is modulated in a circadian manner in phase with CLOCK:-BMAL1 activity.[7,11,12]

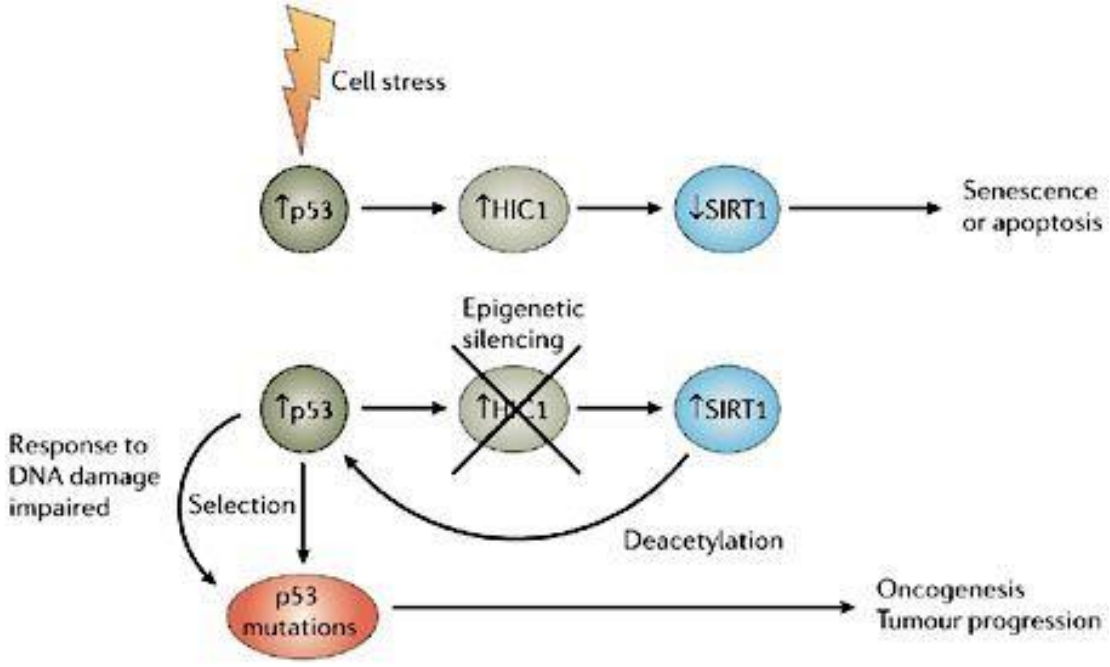

Figure 2. Effects of epigenetic silencing of HIC1 on the DNA-damage response. Silencing of HIC1 allows the transcription of SIRT1. SIRT1 HDAC activity can inhibit P53 activity. Baylin, S. B., & Ohm, J. E., Nature Reviews Cancer, 2006.[19]

SIRT1 is a class III HDAC with the ability to deacetylate both histone and non-histone proteins, including the key tumor suppressor P53.[12,15] Under normal cell conditions, P53 remained inactive at low concentrations.[16] In response to DNA damage, P53 is rabidly induced and post-translationally modified to the active acetylated form which can direct transcription of proapoptotic genes. (Figure 2)[6,12] *Hic1* (*Hypermethylated in Cancer 1*), a P53 target gene, is transcriptional repressor which inhibits the transcription of SIRT1, preventing SIRT1-mediated deacetylation of Ac-P53 (Figure 2).[15] Mutations or epigenetic silencing that disrupts P53 activity is often found in cancer patients, suppressing the ability to undergo cell cycle arrest, which then results in malignant cell growth.

Preliminary studies conducted at the Chappell lab has indicated changes in transcript levels of *Hic1* and *Sirt1* as well as increases in methylation at the *Hic1* loci after prolonged exposure to light-at-night (LAN) (Figure 3). Exposure to 3wks of LAN caused an increase in promoter methylation of the *Hic1* locus (Figure 3a), and differential expression of *Hic1* transcripts assessed by qPCR (Figure 3b). These observations were accompanied by insignificantly changed transcription of *Sirt1* at all Zeitgeber times (ZT), with ZT times being time of day cues, after 3wks LAN (Figure 3c), but significant upregulation of *Sirt1* transcription in mice exposed for 6wks LAN (Figure 3d). We believe extended exposure to LAN will cause circadian dysregulation and thereby induce epigenetic silencing of *Hic1* and alter expression and activity of SIRT1 and P53, which may lead to abnormal cell growth.

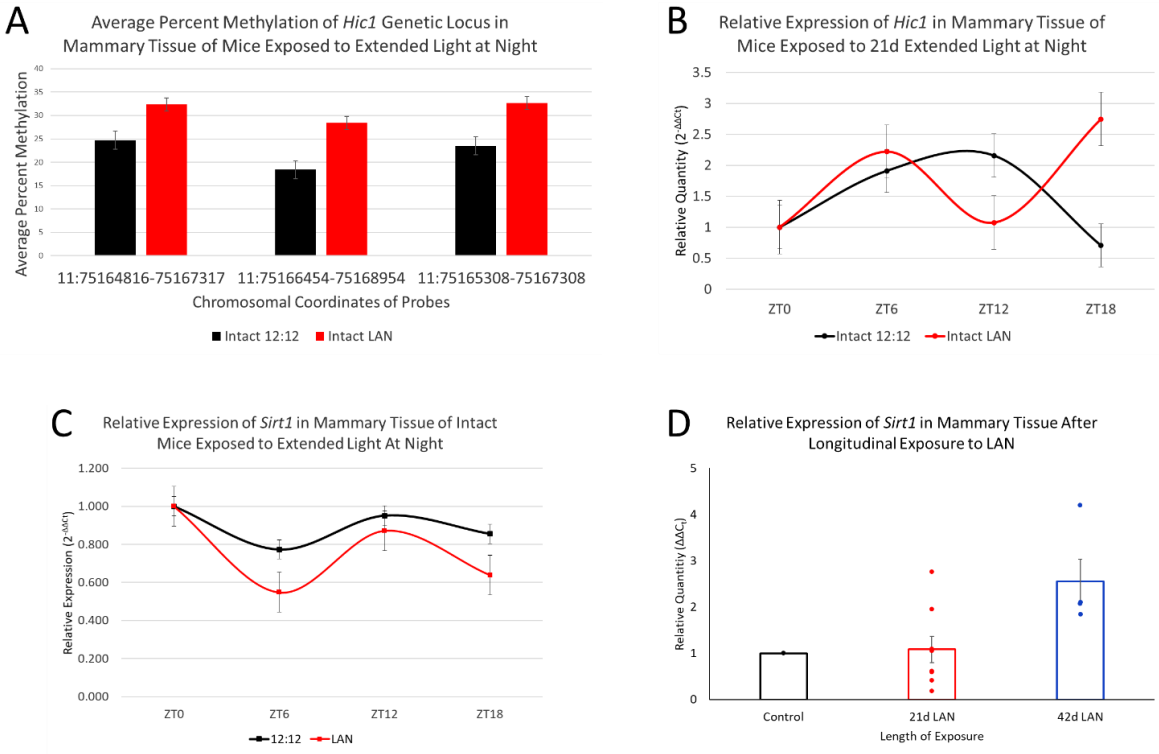

Figure 3. Methylation of *Hic1* and transcript levels of *Hic1* and *Sirt1* in mammary tissue. (a) Methylation at the Hic1 genetic locus a normal light cycle (black) and three weeks of light at night (red). (b) Transcript levels of Hic1 after a normal light cycle (black) and exposure to LAN (red). (c) Transcript levels of Sirt1 after a normal light cycle (black) and LAN (red). (d) Relative Sirt1 expression after a normal light cycle (black), 3wk LAN (red), and 6wk LAN (blue).

We chose to measure the abundance of SIRT1 and P53 in their active and inactive states to determine whether subjecting mice to LAN alters the DNA damage response activity of P53. We utilized immunoblotting techniques to quantify protein abundance in tissue samples collected from the mammary glands of LAN exposed mice at various ZT times. This allowed us to determine specific temporal windows in which the target proteins are most abundant/active, and whether exposure to LAN causes a shift or disruption in the normal abundance/activity levels.

Materials and Methods

Animal treatments and tissue harvesting. Wild type female C57BL6 mice were unaltered and were housed in either a control 12 h light/12 h dark (12:12LD) cycle or an extended light-at-

night (LAN, 18:6LD) cycle which was an 18 h light/6 h dark cycle. Mice were exposed to their respective light cycles for 3wks before mammary tissue samples were harvested at Zeitgeber times (ZT) ZT00, ZT06, ZT12, and ZT18. Tissue samples were flash frozen in liquid nitrogen immediately after collection and stored at -80°C.

Protein extraction from tissue samples. Mammary tissue samples were homogenized in Cell Signaling Technology RIPA buffer containing 1.0% protease inhibitor, 2.0% phosphatase inhibitors, and 0.5% PMSF, then rocked at 4°C for 2 h. Samples were then centrifuged at 12,000 rpm for 20 min and the supernatant was collected and stored at -80°C.

Dot blotting for primary antibody and protein concentration optimization. Protein extracts were directly deposited onto a nitrocellulose membrane with varying total protein concentration (10 – 50 µg) per dot and left to dry. Membrane was blocked with 5% milk (for anti-p53 and anti-sirt1 antibodies) or 5% BSA (for anti-P-sirt1 and anti-Ac-p53 antibodies) block in TBS-T for 1 h at RT. Block was discarded before incubation in varying dilutions (1:250 – 1:2000) of primary antibody for 1 h at RT. Membrane was washed with TBS-T and incubated in secondary antibody for 30 min at RT. After washing with TBS-T, membrane was visualized via chemiluminescence at 425 nm.

Immunoblotting. A total of 20 µg of protein per sample was prepared in 2.5% 4X Bolt LDS Sample Buffer and 1% 10X Bolt Reducing agent and heated at 70°C for 10 min. Prepared protein samples were loaded into a Bolt 4-12% Bis-Tris Plus precast gel and run at 100V for 50-60 min in MOPS running buffer. Samples were transferred onto a 0.2 µm nitrocellulose

membrane at 10V for 1 h in 1X Novex Transfer Buffer. Membranes were blocked with 5% milk (for anti-p53 and anti-sirt1 antibodies) or 5% BSA (for anti-P-sirt1 and anti-Ac-p53 antibodies) block in TBS-T milk block in TBS-T followed by overnight incubation in either anti-p53, anti-Ac-p53, anti-sirt1, anti-P-sirt1, or anti-β-actin primary antibodies in a 1:500 dilution in 5% block. Membranes were washed with TBS-T and incubated with anti-rabbit or anti-mouse secondary antibodies for 1.5 h before visualization via chemiluminescence.

Results and Discussion

Light-at-night alters SIRT1 abundance in murine mammary tissue. Immunoblot analysis was conducted for 3wk control and LAN-exposed mammary tissue samples to assess the effects of LAN on SIRT1 abundance (Figure 4a). In the 3wk 12:12LD control group, normalized SIRT1 expression revealed an unexpected rhythmic abundance of SIRT1 peaking at ZT06 and ZT12; although SIRT1 activity oscillates, its abundance is believed to remain static. The underlying cause of the observed fluctuation in SIRT1 abundance is unknown, however, it is possible that this observation is mammary tissue-specific (Figure 4b). There appears to be a temporal shift in peak SIRT1 abundance to ZT18 after exposure to LAN, which occurs concomitantly with overall diminished abundance and loss of circadian oscillatory accumulation. (Figure 4b). The observed depletion of SIRT1 in LAN-exposed mice may be attributable to disruptions in *Hic1* transcription (Figure 3b). It is possible that HIC1 abundance is altered, thus repression of *Sirt1* expression is affected after LAN.

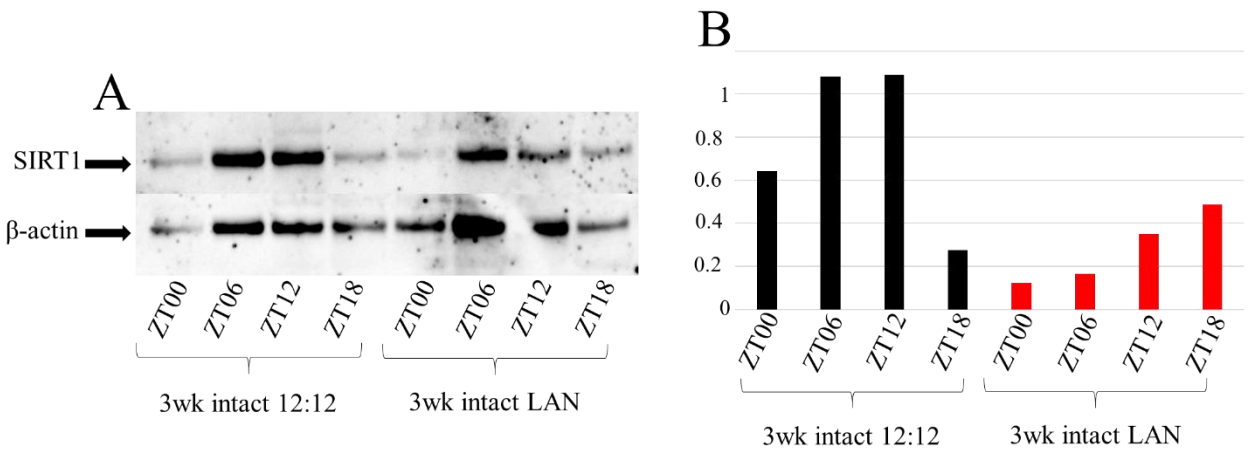

Figure 4. SIRT1 protein expression levels in mammary tissue. (a) Immunoblot of samples taken from mice exposed to 3wk 12:12 and 3wk LAN treatment conditions. (b) SIRT1 levels in mice exposed to 3wk 12:12 and 3wk LAN normalized to β-actin expression.

SIRT1 activity is constitutively low in mouse mammary and nearly undetectable after exposure to LAN. Immunoblot analysis of p-SIRT1 abundance demonstrates the expected oscillatory pattern in 3wk 12:12 mice but almost no expression in LAN exposed mice (Figure 5a). Figure 5b shows normalized p-SIRT1 modification in 3wk 12:12 and 3wk LAN. Although the control group exhibits expected circadian patterns of increased SIRT1 in tandem with the activity of the CLOCK- BMAL1 heterodimers, the low observed abundance of p-SIRT1 suggests that these findings are insignificant (Figure 5b). Surprisingly, p-SIRT1 was nearly undetectable in LAN-exposed mice (Figure 5b). There is seemingly a decline in SIRT1 activity with extended LAN exposure possibly due to a disruption or downregulation of a kinase responsible for phosphorylation and activation of SIRT1.

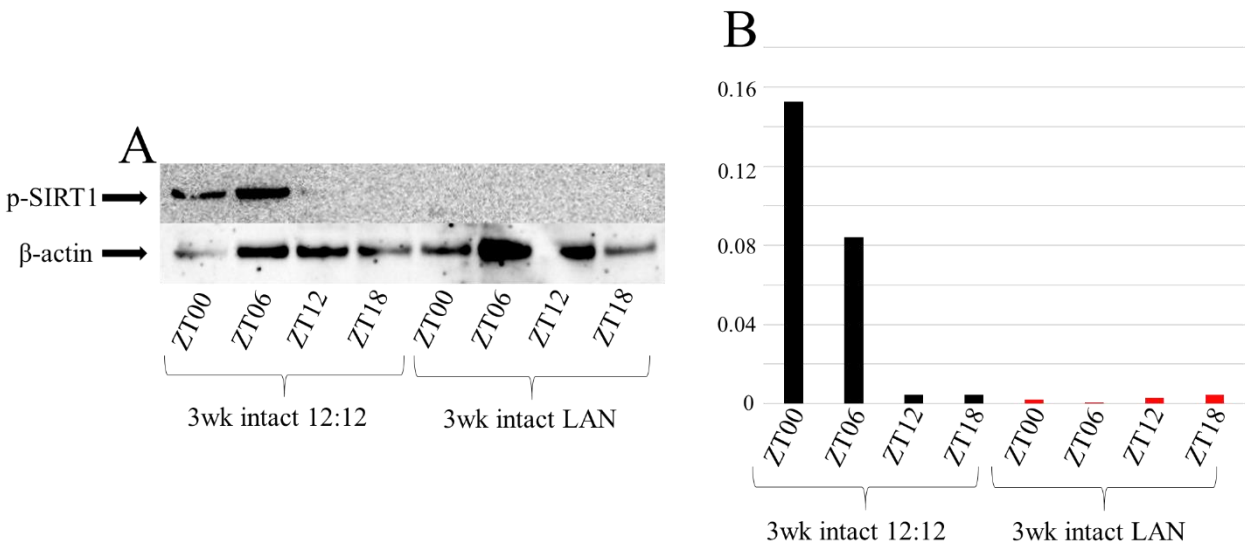

Figure 5. p-SIRT1 protein expression levels in mammary tissue. (a) Immunoblot of samples taken from mice exposed to 3wk 12:12 and 3wk LAN treatment conditions. (b) p-SIRT1 levels in mice exposed to 3wk 12:12 and 3wk LAN normalized to β-actin expression.

P53 abundance fluctuate over the circadian period in mouse mammary. Immunoblot analysis of P53 abundance in 3wk 12:12 and 3wk LAN mice indicates strong peaks at ZT06 and ZT18 in both groups (Figure 6). An immunoblot of β-actin for the same conditions further emphasizes a cyclic abundance of P53 over a 24 h cycle (Figure 6). Since P53 is not a clock-controlled gene, rhythmic expression of P53 is unexpected and has not yet been reported. P53 was expected to be constitutively abundant throughout the day. However, exposure to LAN appears to diminish P53 abundance as indicated by the comparatively lighter-weight bands in the LAN group (Figure 6). This data, however, is not indicative of P53 activity. Although we did attempt to probe for Ac-P53 to determine the relative activity of P53 in these samples, the antibody at hand did not yield any signal for either control or LAN samples. Nonetheless, the observed alternating abundances of P53 may provide insight into timepoints in which cells are more susceptible to DNA damage.

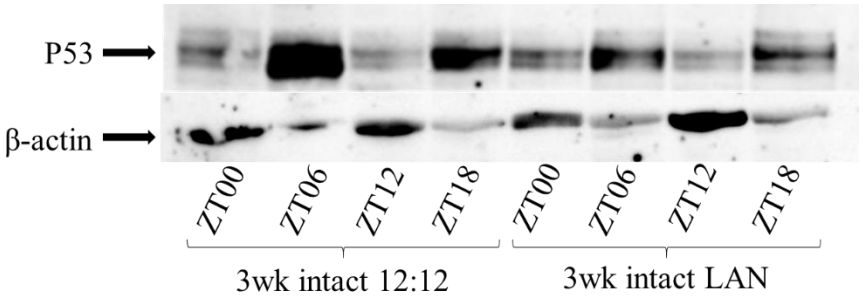

Figure 6. Immunoblot of mammary tissue samples probed for P53 and β-actin. Samples were taken from mice exposed to 3wk 12:12 and 3wk LAN treatment conditions.

Conclusion

Proper circadian regulation is necessary for the management of numerous biological processes including the sleep/wake cycle, body temperature maintenance, and hormone secretion. Dysregulation of circadian rhythms alters key physiological and metabolic mechanisms resulting in increased vulnerability to diseases such as Alzheimer's disease, bipolar spectrum disorders, and metabolic syndromes.[17,18] This study investigated the implications of circadian dysregulation on tumor susceptibility in the mammary caused by alterations in the abundance and activity of SIRT1 and P53.

Mammary tissue samples were collected from mice exposed to extended light-at-night at various Zeitgeber times to quantify the abundance of SIRT1 and P53 via immunoblotting. Our results suggest a downregulation of SIRT1 and P53 abundance after three-week exposure to LAN. Surprisingly, SIRT1 protein levels exhibited oscillations peaking at ZT06 and ZT12 in mice exposed to a normal light cycle (Figure 7a), whereas peak SIRT1 abundance in LAN-exposed mice was shifted to ZT18 and overall protein levels were decreased (Figure 7b). Both control and LAN groups failed to demonstrate significant SIRT1 activity, as seen in Figures 5a and 5b. Interestingly, P53 abundance fluctuated at different time points; both the control and LAN exposed mice exhibited peak P53 abundance at ZT06 and ZT18 (Figure 7). These results suggest that chronic circadian dysregulation induced by exposure to light-at-night affects the

abundance and activity of SIRT1 and P53 which play a role in DNA damage repair and cell cycle arrest. Changes in the abundance of these proteins in the mammary offers insight into how circadian disruption may initiate breast tumor formation.

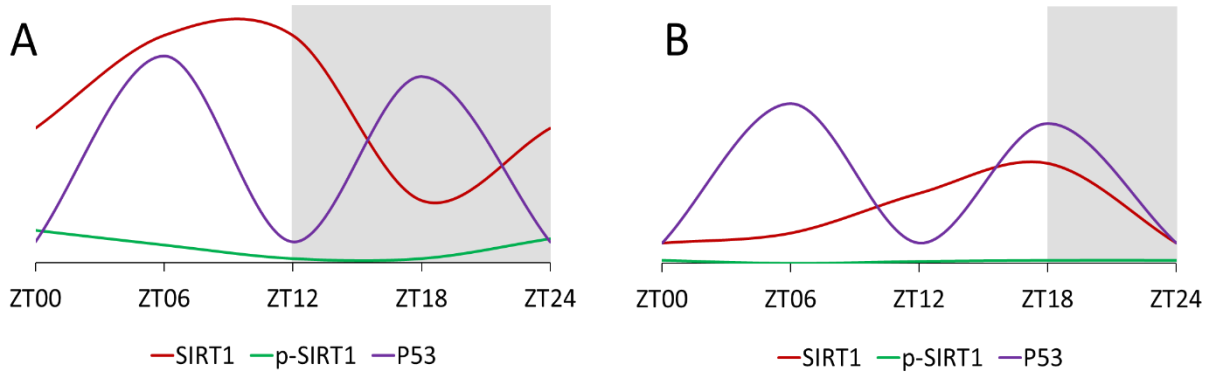

Figure 7. Model illustrating the observed abundance patterns of SIRT1 (red), p-SIRT1 (green), and P53 (purple) with the shaded regions indicating when the mice were in the dark. (a) Abundance patterns in intact mice exposed to a 3wk 12:12 cycle. SIRT1 abundance peaks at ZT06 and ZT18 while p-SIRT1 abundance is relatively low at all points of the day. P53 shows a unique oscillation with peaks at ZT06 and ZT18. (b) Abundance patterns in intact mice exposed to a 3wk LAN cycle. SIRT1 abundance is diminished and reverse peaked while p-SIRT1 is not detectable. P53 slightly diminished but maintains the same oscillation seen in the control group.

References

1. Jin, Y., Hur, T., & Hong, Y. (2017). Circadian Rhythm Disruption and Subsequent Neurological Disorders in Night-Shift Workers. Journal of Lifestyle Medicine, 7(2), 45–50. https://doi.org/10.15280/jlm.2017.7.2.45
2. James, S. M., Honn, K. A., Gaddameedhi, S., & Van Dongen, H. P. A. (2017). Shift Work: Disrupted Circadian Rhythms and Sleep—Implications for Health and Well-Being. Current Sleep Medicine Reports, 3(2), 104–112. https://doi.org/10.1007/s40675-017-0071-6
3. Garbarino, S., Beelke, M., Costa, G., Violani, C., Lucidi, F., Ferrillo, F., & Sannita, W. G. (2002). Brain Function and Effects of Shift Work: Implications for Clinical Neuropharmacology. Neuropsychobiology, 45(1), 50–56. https://doi.org/10.1159/000048674
4. Davis, S., Mirick, D. K., & Stevens, R. G. (2001). Night Shift Work, Light at Night, and Risk of Breast Cancer. JNCI Journal of the National Cancer Institute, 93(20), 1557–1562. https://doi.org/10.1093/jnci/93.20.1557
5. Costa, G. (1996). The impact of shift and night work on health. Applied Ergonomics, 27(1), 9–16. https://doi.org/10.1016/0003-6870(95)00047-X
6. Ramsey, M. R., & Ellisen, L. W. (2011). Circadian function in cancer: Regulating the DNA damage response. *Proceedings of the National Academy of Sciences*, *108*(26), 10379–10380. https://doi.org/10.1073/pnas.1107319108
7. Asher, G., Gatfield, D., Stratmann, M., Reinke, H., Dibner, C., Kreppel, F., Mostoslavsky, R., Alt, F. W., & Schibler, U. (2008). SIRT1 Regulates Circadian Clock Gene Expression through PER2 Deacetylation. *Cell*, *134*(2), 317–328. https://doi.org/10.1016/j.cell.2008.06.050

8. Kawamura, G., Hattori, M., Takamatsu, K., Tsukada, T., Ninomiya, Y., Benjamin, I., Sassone-Corsi, P., Ozawa, T., & Tamaru, T. (2018). Cooperative interaction among BMAL1, HSF1, and p53 protects mammalian cells from UV stress. *Communications Biology*, *1*(1), 204. https://doi.org/10.1038/s42003-018-0209-1
9. Maiese, K. (2017). Moving to the Rhythm with Clock (Circadian) Genes, Autophagy, mTOR, and SIRT1 in Degenerative Disease and Cancer. *Current Neurovascular Research*, *14*(3). https://doi.org/10.2174/1567202614666170718092010
10. Chang, H.-C., & Guarente, L. (2013). SIRT1 Mediates Central Circadian Control in the SCN by a Mechanism that Decays with Aging. *Cell*, *153*(7), 1448–1460. https://doi.org/10.1016/j.cell.2013.05.027
11. Nakahata, Y., Sahar, S., Astarita, G., Kaluzova, M., & Sassone-Corsi, P. (2009). Circadian Control of the NAD+ Salvage Pathway by CLOCK-SIRT1. *Science*, *324*(5927), 654–657. https://doi.org/10.1126/science.1170803
12. Nakahata, Y., Kaluzova, M., Grimaldi, B., Sahar, S., Hirayama, J., Chen, D., Guarente, L. P., & Sassone-Corsi, P. (2008). The NAD+-Dependent Deacetylase SIRT1 Modulates CLOCK-Mediated Chromatin Remodeling and Circadian Control. *Cell*, *134*(2), 329–340. https://doi.org/10.1016/j.cell.2008.07.002
13. Hirayama, J., Sahar, S., Grimaldi, B., Tamaru, T., Takamatsu, K., Nakahata, Y., & Sassone-Corsi, P. (2007). CLOCK-mediated acetylation of BMAL1 controls circadian function. *Nature*, *450*(7172), 1086–1090. https://doi.org/10.1038/nature06394
14. Revollo, J. R., & Li, X. (2013). The ways and means that fine tune Sirt1 activity. *Trends in Biochemical Sciences*, *38*(3), 160–167. https://doi.org/10.1016/j.tibs.2012.12.004

15. Baylin, S. B., & Ohm, J. E. (2006). Epigenetic gene silencing in cancer – a mechanism for early oncogenic pathway addiction? *Nature Reviews Cancer*, *6*(2), 107–116. https://doi.org/10.1038/nrc1799
16. Lakin, N. D., & Jackson, S. P. (1999). Regulation of p53 in response to DNA damage. *Oncogene*, *18*(53), 7644–7655. https://doi.org/10.1038/sj.onc.1203015
17. Alloy, L. B., Ng, T. H., Titone, M. K., & Boland, E. M. (2017). Circadian Rhythm Dysregulation in Bipolar Spectrum Disorders. *Current Psychiatry Reports*, *19*(4), 21. https://doi.org/10.1007/s11920-017-0772-z
18. Potter, G. D. M., Skene, D. J., Arendt, J., Cade, J. E., Grant, P. J., & Hardie, L. J. (2016). Circadian Rhythm and Sleep Disruption: Causes, Metabolic Consequences, and Countermeasures. *Endocrine Reviews*, *37*(6), 584–608. https://doi.org/10.1210/er.2016-1083
19. Baylin, S. B., & Ohm, J. E. (2006). Epigenetic gene silencing in cancer – a mechanism for early oncogenic pathway addiction? Nature Reviews Cancer, 6(2), 107–116. https://doi.org/10.1038/nrc1799

Acknowledgements

I would like to thank my mentors Dr. Patrick Chappell and Rebecca Veitch for their unwavering support and guidance throughout this process. I appreciate their patience as I learned basic techniques and made numerous mistakes along the way. They pushed me to challenge myself and think critically which has immensely improved my skills as a scientist. This project would not have been possible without them.

I would like to thank my committee members Dr. Maude David and Dr. Kate Shay for providing advice and critical improvements on my thesis.

I would like to thank all the undergraduates of the Chappell lab, Chris Markgraf, Noa Rayzman, Teagan James, and Zack Rogers, for their continuous encouragement and support throughout the years. They kept me motivated after numerous failed experiments and was always willing to lend a helping hand when I felt overwhelmed.

Lastly, I would like to thank my friends and family for their constant support.