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Elucidating the Roles of Membrane Microdomains in Cellular Resilience

A thesis submitted in partial satisfaction of the requirements
for the degree Master of Science

in

Biology

by

Melody Nazarbegian

Committee in charge:

Professor Hemal Patel, Chair
Professor Brenda Bloodgood, Co-Chair
Professor Gulcin Pekkurnaz

2020

The thesis of Melody Nazarbegian is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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University of California San Diego

2020

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ABSTRACT OF THE THESIS

Elucidating the Roles of Membrane Microdomains in Cellular Resilience

by

Melody Nazarbegian

Master of Science in Biology

University of California San Diego 2020

Professor Hemal Patel, Chair
Professor Brenda Bloodgood, Co-Chair

Plasma membrane compartments known as functional microdomains, such as membrane lipid rafts (MLRs) and caveolae, are critical in maintaining membrane structure and function which ultimately influences cellular survival. Numerous studies demonstrate that membrane microdomains are necessary for vital cellular functions like intracellular transport and cell signaling; but it remains unclear how plasma membrane organization is determined, how membrane microdomains regulate membrane repair, and how they work with intracellular organelles to enhance function. To define the role of membrane microdomains in facilitating cellular resilience, we focus on three specific areas: 1) circadian control of membrane dynamics,

2) membrane microdomain regulation of membrane repair, and 3) membrane microdomain regulation of intracellular function. Our findings suggest that membrane structural dynamics follow a daily oscillatory pattern, and that membrane composition and organization may be partially determined by Bmal1 dependent clock genes. Moreover, we found that over-expression of caveolin, a structural protein critical to caveolae, enhances the efficiency of membrane repair. Lastly, we expect that over-expression of caveolin will improve mitochondrial function in upcoming studies. These studies suggest that caveolae improve cellular resilience by enhancing membrane durability and organelle function. Thus, these studies establish a holistic understanding of the plasma membrane and its microdomains, laying the groundwork for further research on oscillatory rhythms at the membrane level, quantification and enhancement of membrane repair kinetics, and optimization of mitochondrial and membrane function via manipulations of membrane composition.

1. INTRODUCTION

The plasma membrane is a vital aspect of cellular structure and function. Cellular homeostasis is strongly influenced by the membrane's facilitation of cellular transport, signaling mechanisms, and structural integrity (Lodish et al., 2000; Krapf, 2018; Blazek et al., 2015). As a semi- permeable barrier between extracellular and intracellular regions, the plasma membrane determines how the cell is affected by and responds to the surrounding environment (Lodish et al., 2000). Hence, the integrity of the plasma membrane is crucial in promoting cell survival, and given the continuous potential for mechanical, osmotic, and pathogen-induced damage in any extracellular environment, cellular membrane repair is necessary for sustaining normal function (Blazek et al., 2015; Demonbreun and Mcnally, 2016). Meanwhile, membrane dysfunction can result in a cascade of downstream issues in the cell, such as unregulated ion influx, protein degradation, and increased susceptibility to damage by harmful physical stressors (Demonbreun and Mcnally, 2016). Hence, functional failures at the plasma membrane give rise to a spectrum of diseases (Demonbreun and Mcnally, 2016). Some of the most extensively studied diseases associated with the plasma membrane include muscular dystrophy, acute kidney injury, and cardiomyopathy, all of which result in cell damage and impaired tissue function in skeletal muscle, kidneys, and cardiac muscle, respectively (Cai et al., 2009; Duann et al., 2015; Woodman et al., 2002). Therefore, tissue and organism-level studies of pathology can be elucidated by investigations into the sub-cellular function of plasma membrane regulation (Schilling and Patel, 2016; Blazek et al., 2015).

The interacting components of the plasma membrane allow it to carry out its essential functions. The cell membrane is a bi-layered, heterogeneous structure composed of phospholipids, glycolipids, sphingolipids, cholesterol, and versatile proteins (Lodish et al., 2000). These

components aggregate and assemble into functional microdomains (Cohen et al., 2004), which are a variety of nanoscale membrane lipid and protein clusters (Laude and Prior, 2004). The study of membrane microdomains has received considerable attention, as the formation of these compartments within the plasma membrane allows the membrane to perform many of its vital functions, like signaling and transport (Krapf, 2018; Laude and Prior, 2004). Evidence that membrane microdomains are distinct compartments in the plasma membrane consists of a large body of electron and fluorescent microscopic images of structurally ordered aggregations, as well as tests utilizing detergents to identify insoluble membrane lipid clusters (Laude and Prior, 2004; Pike, 2003). The most well documented, prominent, and structurally organized membrane microdomains are cholesterol-rich membrane lipid rafts (MLRs) and caveolae (Laude and Prior, 2004).

Membrane lipid rafts are tightly-packed membrane microdomains, characterized by a high concentration of cholesterol and glycosphingolipids (Pike, 2003). Accordingly, membrane lipid rafts are much more orderly than their surroundings in the fluid plasma membrane (Pike, 2003). In addition, lipid rafts often contain proteins involved in signal transduction and are strongly believed to be involved in facilitating signaling mechanisms (Pike, 2003).

Meanwhile, caveolae are a type of membrane lipid raft but are considered a separate microdomain due to their unique protein composition (Pike, 2003). Caveolae are characterized by proteins known as caveolins (Pike, 2003). Caveolins are membrane proteins with three existing isoforms: Caveolin-1, -2, and -3 (Williams and Lisanti, 2004). Caveolin-3, or cav-3, is specifically expressed in muscle tissues while caveolin-1 and -2 are found in most other tissue types (Williams and Lisanti, 2004). The caveolin proteins are scaffolding proteins, key players in cell-signaling pathways, as well as essential structural elements which enable the formation of caveolae (Cohen

et al., 2004). When caveolin proteins oligomerize, the Golgi apparatus adds cholesterol and other small polymers to the complex, which is then transported to the plasma membrane, where proteins called cavins complete the creation of caveolae (Busija et al., 2017). Once formed, caveolae are flask-like membrane invaginations, enriched in sphingolipids and cholesterol (Corrotte et al., 2014). The unique structure of caveolae blurs the distinction between endocytic vesicles and membrane lipid rafts, allowing for a great degree of functional versatility (Cohen et al., 2004; Busija et al. 2017). Caveolins and caveolae have roles in signaling, endocytosis, lipid raft regulation, and lipid trafficking (Schilling and Patel, 2016; Laude and Prior, 2004).

Moreover, numerous studies have shown via genetically modified mouse models, that mutant or knocked-out caveolin genes, and therefore impaired production of caveolae, nearly always result in poor physiological outcomes at the whole-organism level (Stary et al., 2012; Cheng and Nichols, 2016). These outcomes include cardiac, neurological, and muscular dysfunctions such as cardiomyopathy and muscular dystrophy (Stary et al., 2012; Cheng and Nichols, 2016). Meanwhile, cellular models and mouse models, particularly in the case of caveolin over-expression, have produced much evidence that functional caveolin has protective effects, such as cardioprotection, neuroprotection, and mechanoprotection of plasma membranes (Cheng and Nichols, 2016). For example, neurons in caveolin-1 over-expressing mice activate pro-survival signal transduction pathways, while caveolin-3 over-expressing mice demonstrate reduced cardiac hypertrophy, or tissue enlargement (Stary et al., 2012; Horikawa et al., 2011). On the other hand, caveolin-3 knockout mice demonstrate hyperactivated stress responses, via the MAPK signal transduction cascade (Woodman et al., 2002). These protective functions indicate enhanced resilience at the cellular level, meaning more optimal stress responses, more efficient repair

processes, and reduced susceptibility to harmful environmental phenomena (Schilling and Patel, 2016).

Properly functioning plasma membrane microdomains ultimately influence cellular wellbeing. It is therefore imperative to study the factors affecting the ability of the plasma membrane to support cellular mechanisms and structure, as these factors form a network of relationships that collectively determine cellular resilience. Specifically, the structure of the plasma membrane influences its microdomains, and its microdomains go on to protect the membrane itself, as well as interact with intracellular organelles to maintain homeostasis (Schilling and Patel, 2016; Loizides-Mangold et al., 2017). In particular, it has been suggested that membrane composition and fluidity are in constant fluctuation (Loizides-Mangold et al., 2017), and specific changes in these membrane characteristics could optimize the function of membrane microdomains (Casares et al., 2019). In turn, plasma membrane microdomains, like caveolae and lipid rafts, function to protect the cell by playing key roles in membrane repair mechanisms (Andrews and Corrotte, 2014). At the same time, signaling and trafficking between these membrane microdomains and intracellular organelles, like mitochondria, may also be essential in maintaining cellular structure and function (Schilling and Patel, 2016). Thus, the plasma membrane's capacity to perform its essential functions is dependent upon multiple intertwined factors. In the following three sub-chapters, we will review the current foundation of knowledge and gaps in knowledge on the role of plasma membrane functional microdomains in: 1) membrane structural dynamics, 2) membrane repair, and 3) mitochondrial function, all of which help determine overall cellular resilience.

1.1 Dynamic Membrane Composition, Fluidity, and Organization

Plasma membrane structure is characterized by its dynamic nature, as membrane fluidity and lipid composition are not stagnant (Cohen et al., 2004; Casares et al., 2019). These constant changes in the plasma membrane composition, however, do not occur at random. Accordingly, the factors that control membrane lipid dynamics and membrane fluidity are key in the investigation of cellular resilience, because varied membrane structure undoubtedly promises varied membrane function.

For example, cholesterol is known to be an important regulator of membrane fluidity/rigidity ratios (Cooper, 1978). Higher cholesterol/phospholipid ratios result in a more rigid and less permeable plasma membrane, which creates downstream protective effects for the cell, making it less susceptible to harm (Cooper 1978). Cholesterol also increases the organization of the plasma membrane lipid bilayer, while its removal from the membrane results in an increase in membrane tension (Hissa et al., 2013). This influences the success of plasma membrane repair mechanisms (Iaea and Maxfield, 2017; Chabanon et al., 2020), as decreased membrane tension is associated with effective membrane repair outcomes (Togo et al., 2000). Hence, cholesterol is an important determinant of plasma membrane structure. Therefore, cholesterol-rich lipid rafts and caveolae also comprise key aspects of membrane structure that influence its capacity to respond to environmental stressors.

Aside from cholesterol, lipids form the second major component of the plasma membrane. There is evidence that the plasma membrane exhibits periodic oscillations of lipid composition in muscle tissue (Loizides-Mangold et al., 2017), thus altering the cholesterol/phospholipid ratios that affect membrane fluidity (Cooper, 1978). Based on this existing data, as well as current studies in our lab demonstrating periodic fluctuations in the membrane organization of neuronal plasma membranes (J. Zuniga-Hertz, personal communication, December 2019), we have proposed a

novel hypothesis that membrane fluidity and/or composition expresses a periodic oscillatory pattern, partially dictated by a circadian or ultradian rhythm (J. Zuniga-Hertz, personal communication, December 2019). Circadian rhythms are defined as oscillations completing one cycle in the span of 24 hours, while ultradian rhythms complete shorter repeated cycles (Toh, 2008; Goh et al., 2019). These biological rhythms are controlled by clock genes and their major transcription factors, such as Bmal1 (Menet et al. 2014). Meanwhile, disruption of clock gene expression effectively disrupts the periodic oscillations under their control (Menet et al. 2014).

Furthermore, existing studies have sought to investigate therapeutic manipulations of plasma membrane structure to optimize functionality (Casares et al., 2019). Changing the concentrations and ratios of components of the plasma membrane ultimately changes its capacity to fulfill various functions. If plasma membrane cholesterol and lipid oscillations follow a regular diurnal pattern, this would strongly suggest that there are daily times of optimal and sub-optimal cell function, especially because cholesterol and lipids are the key components of functional microdomains, such as membrane lipid rafts and caveolae (Casares et al., 2019). All organisms have such intrinsic timekeeping mechanisms which determine a variety of daily and seasonal patterns of physiology; however, the plasma membrane has not been studied in this regard, and its oscillatory patterns have not yet been elucidated.

1.2 The Role of Membrane Microdomains in Plasma Membrane Repair

As stated previously, the dynamic nature of the plasma membrane and membrane microdomains influences the cell's ability to conduct effective repair mechanisms. Membrane microdomains, especially caveolae, have been widely identified as significant components of membrane repair mechanisms, and loss of caveolin protein, critical in caveolae formation, has been associated with cellular damage (Cai et al., 2009). Many investigators of plasma membrane

repair have focused on muscle cell models, as muscle tissue is often subject to a considerable amount of physical stress. In these models, cell injury is often induced via mechanical stress, pore-forming toxins, or osmotic stress (Corrotte et al., 2013) followed by fluorescent probes used to visualize the process of membrane repair (Corrotte et al., 2013). One repair mechanism identified by these studies suggests that caveolae gather in great quantities at the site of membrane injury, fuse together, and ultimately endocytose in order to remove membrane lesions (Corrotte et al., 2013). Meanwhile, there is also a consensus that the caveolin-3 protein works with two other proteins to facilitate membrane repair: mitsugumin-53 (MG53) and dysferlin (Cai et al., 2009; Corrotte et al., 2013). Dysferlin, MG53, and caveolin all independently promote membrane repair, but their synergistic cooperation produces the most successful repair outcomes, thus enhancing cellular resilience (Cai et al., 2009; Chabanon et al., 2020). Furthermore, caveolin over-expression increases interactions between these three essential membrane repair proteins, suggesting that it mediates the colocalization of these proteins, and facilitates plasma membrane repair (Chabanon et al., 2020). Accordingly, the caveolin proteins are believed to be responsible for keeping dysferlin at the cell membrane, as mutant caveolin often results in internalization of dysferlin, preventing it from acting in repair mechanisms at the cell surface (Hernández-Deviez et al., 2007). Our lab has generated evidence suggesting that the colocalization of caveolin, MG53, and dysferlin allows for the formation of a repair protein complex, which facilitates an active resealing mechanism in the plasma membrane that brings a membrane patch to the site of damage via exocytosis (Chabanon et al., 2020).

However, many existing studies fail to consider that injured cells signal and trigger uninjured neighboring cells to potentiate membrane repair (Togo, 2017), suggesting that each cell comprising a tissue does not have an equal capacity to repair its membrane via the aforementioned

mechanisms. Additionally, although the caveolin proteins and caveolae have been accepted as impactful mediators of membrane repair mechanisms, the precise process by which these proteins and membrane microdomains enhance repair is unclear.

1.3 The Influence of Membrane Microdomains on Mitochondrial Function

Beyond the biochemical interactions at the site of the plasma membrane, membrane microdomains promote cellular resilience by engaging with internal organelles (Schilling and Patel, 2016). One of the key organelles that the caveolin proteins and cholesterol-rich caveolae interact with are mitochondria (Schilling and Patel, 2016). Caveolae-mitochondrial interactions modulate mitochondrial structure and function, which has been confirmed by their close proximity in electron microscopic images (Schilling and Patel, 2016; Fridolfsson et al., 2012). It is of interest to study the associations and cross-talk between membrane microdomains and mitochondria, because mitochondrial dysfunction is a key factor in propagating many pathologies, including heart failure and Parkinson's disease (Fridolfsson et al., 2012; Cooper 1978). A large body of research has shown, via mouse models, that caveolin overexpression results in enhanced mitochondrial respiratory function, plasma membrane rigidity, and reduced stress responses, such as decreased production of reactive oxygen species (ROS) that would otherwise damage cellular structures like the plasma membrane (Fridolfsson et al., 2012; Cooper 1978). On the other hand, knocking-out the caveolin genes results in dysfunctional mitochondria and self-harming responses to stress, hence decreasing the resilience of cells and tissues (Fridolfsson et al., 2012; Cooper 1978). Caveolae-mitochondrial interactions are therefore considered important aspects of the cell's ability to adapt to environmental stress, and therefore enhance survival (Fridolfsson et al., 2012; Schilling and Patel, 2016). Thus, membrane microdomains, like caveolae, influence structures beyond the membrane, and these intracellular reactions have significant roles in determining

cellular resilience (Schiling and Patel, 2016). However, despite existing observations, there is still a limited understanding of these interactions, and the precise connection between caveolae-mitochondrial interactions and cellular resilience remains ambiguous.

1.4 Aims

Therefore, the current basis of knowledge on the role of membrane microdomains in the fields of 1) plasma membrane structural dynamics, 2) membrane repair, and 3) mitochondrial function have established that membranes are capable of repairing injuries with the assistance of caveolins and caveolae, that membrane structure and composition is dynamic, and that caveolins are associated with mitochondrial function (Casares et al., 2019; Cai et al., 2009; Schilling and Patel, 2016). These subjects of interest play key roles in the maintenance of cellular homeostasis and resilience in response to environmental stress, and their impact at the cellular level has implications for wellbeing at the tissue and whole-organism level. Elucidating the role of membrane microdomains and the factors which influence cellular resilience will provide a foundation of knowledge for further study of therapies and the cellular basis of disease. The following questions remain: First, do fluctuations in membrane composition and structure follow predictable patterns, and are they in-part dictated by biological clocks? Next, what are the precise roles of caveolins and caveolae in facilitating membrane repair, and how are these roles carried out? Lastly, how do membrane microdomains like caveolae support mitochondrial function? Thus, more work in this field is urgently needed, as the answers to these questions will bring us closer to understanding how cellular resilience can be optimized with respect to functional membrane microdomains.

To uncover this information, this thesis will seek to elucidate the factors influencing cellular resilience via studies which model 1) membrane structural dynamics and 2) plasma

membrane repair, as well as establish a foundation for modeling 3) the relationship between caveolae and mitochondrial function. Our studies therefore have three aims. The first aim is to determine whether the structure and composition of the plasma membrane is partially influenced by intrinsic biological rhythms, which ultimately influences our understanding of the cholesterol-rich microdomains, membrane lipid rafts and caveolae. To investigate this, we utilize a fluorescent membrane lipid raft stain, derived from cholera toxin subunit B (AlexaFluor 488) to probe for patches of cholesterol-rich MLRs in the plasma membranes of murine neurons. We track their dynamics over time via fluorescent microscopy. Then, we employ shRNA (small hairpin RNA) to disrupt production of the clock gene transcription factor Bmal1. We demonstrate that fluctuations in fluorescence intensity follow uniform periodic cycles during spans of 12-48 hours in control cells, and that these patterns are attenuated in cells disrupted with shRNA. Furthermore, the second aim is to elucidate how membrane microdomains function in cellular membrane repair mechanisms. In this study, we seek to identify the precise role of caveolin-3 and caveolae in facilitating plasma membrane repair in muscle cells. To do so, we induce expression of caveolin-3 and MG53, the classic membrane repair protein, in murine myoblasts and cardiomyocytes. We then conduct assays in which we induce laser-damage to single cells, or introduce osmotic stress and pore forming toxins to a large set of cells. Then, we utilize fluorescent probes like FM1-43 and propidium iodide, which stain plasma membrane lesions and nuclear DNA in injured cells, respectively, to track membrane repair over time. This allows us to directly compare the effect of caveolin-3, and therefore, caveolae, on cellular resilience in response to a range of injury methods and conditions. We propose that caveolin-3 is the most influential mediator of membrane repair and affirm its synergistic interactions with MG53. Finally, the third aim will be to ultimately investigate the effects of membrane caveolae on intracellular mitochondrial function, with respect

to maintaining cellular viability. This will be accomplished via high resolution respirometry (HRR). HRR quantifies mitochondrial activity in response to substrates linked to complex I and II of the Electron Transport Chain (ETC) by measuring tissue oxygen consumption and oxidative phosphorylation (Pesta and Gnaiger, 2011). For this study, we will utilize skeletal muscle, heart, hippocampus, and liver tissue samples in caveolin-3 knockout and overexpressing mice. We hypothesize that caveolin-3 overexpressing tissues will have the most efficient mitochondrial function, while knockout mouse tissues will demonstrate compromised mitochondrial function. By quantifying cellular and membrane characteristics over-time, we will therefore create a holistic understanding of the plasma membrane, its microdomains, and cellular resilience. The results of our studies uphold the vital advantages provided by membrane microdomains like caveolae and membrane lipid rafts, uncover novel observations of membrane structural dynamics, and ultimately create a better understanding of how cellular resilience can be optimized by future therapeutic developments.

2. MATERIALS AND METHODS

2.1 Membrane Dynamics: Membrane Lipid Raft Staining and Live Cell Time-Lapse Imaging

To uncover novel observations of membrane dynamics, we developed 48-hour time-lapse assays to track plasma membrane structural components over-time:

Control cells: We cultured murine cortical neurons in a glass-bottom dish. Prior to the experiment, we incubated the cells in 50% FBS media for synchronization. We prepared a 1ug/mL solution of the Alexa Fluor 488 Cholera Toxin Subunit B (CT-B) Conjugate reagent in 2mL of serum-supplemented cell culture media. We incubated the cells in this solution for 10 minutes at 4°C. This cholera toxin derived reagent binds to the ganglioside G_{M1}, which is specifically localized in plasma membrane lipid rafts (Molecular Probes, Inc., 2003). Thus, CT-B positive domains can be considered patches of MLRs or MLR-like structures. We then prepared a rabbit-derived CT-B antibody at a 1:200 dilution in 2mL of serum-supplemented cell culture media. All reagents were kept on ice. After the 10 minute incubation, we washed the cells with 1X PBS, and added the antibody solution. We then incubated the cells for 15 minutes at 4°C. The antibody cross-links to the CT-B. Then, we washed the cells with 1X PBS again, and replenished the plate with serum-supplemented cell culture media. We began time-lapse imaging immediately, with green-fluorescence detection at 40X magnification. We collected images every 10 minutes for 48 hours.

Clock-compromised cells: We cultured murine cortical neurons on a glass-bottom dish and co-incubated them with adenovirus small hairpin RNA (shRNA) targeting the Bmal1 clock gene. The remaining incubation, staining, and imaging protocol was conducted identically to the control cells.

Using the BZ-X Keyence Microscope analysis software, individual neurons in each condition were analyzed by manually selecting each cell, then tracking green fluorescence brightness at each timepoint. We also collected the fluorescence reading for the field background at each timepoint.

2.2 Plasma Membrane Repair: Single-Cell Repair Assays and Live Cell Time-Lapse Imaging

Former researchers in our Cardiac and Neuroprotection Laboratories conducted analyses of membrane repair kinetics in individual cells (Chabanon et al, 2020). They incubated caveolin-3 overexpressing murine cardiomyocytes in FM1-43 fluorescent dye, which fluoresces more brightly when it penetrates the lipid bilayer at an injured site (Corrotte et al., 2015). Then, they utilized a targeted laser to induce injury at a single site on each cell, and tracked fluorescence via live-cell confocal imaging for 450 seconds.

All of our time-lapse imaging assays were conducted in an incubation chamber equipped with a humidifier, CO_2, and temperature control, set to 37°C.

2.3 Plasma Membrane Repair: Global Repair Assays and Live Cell Time-Lapse Imaging

For our current membrane repair studies, we developed and optimized protocols for global membrane repair assays, in which we tracked membrane repair kinetics in multiple cells simultaneously. In preparation for these assays, we seeded 20,000 C2C12 murine myoblasts per well in a standard 48-well tissue culture plate. These assays may also be done with L6 murine myoblasts. We then added 0.5U, 1.0U, 1.5U, or 2.0U of each virus to the appropriate wells. Each well either received no virus, MG53 virus, cav-3 virus, or both MG53 and cav-3 viruses. Each condition was prepared in triplicates. The cells were allowed to incubate with the viruses for 24 hours prior to replenishing the wells with fresh culture media. The cells were then incubated for

another 24 hours. We prepared solutions of 50U/uL streptolysin-O (SLO) toxin and 4uM FM1-43 fluorescent dye in calcium-free HBSS. On ice, to each well we add 100uL of the diluted SLO solution and 100uL of the diluted FM1-43 solution, for a total volume of 200uL per well. An example of this assay setup is demonstrated in Table 1.

We captured images of the FM1-43 red fluorescence, magnified at 20X every 5 minutes for 50 minutes. To analyze the total area of fluorescence at each timepoint, we utilized ImageJ's "Analyze Particles" function.

Table 1. Example of Membrane Repair Assay Setup for a Viral Dose (*X*).

	Contents of Wells in 48-Well Plate:		
Wild-Type Triplicates:	Wild-Type Myoblasts	Wild-Type Myoblasts	Wild-Type Myoblasts
Cav-3 Triplicates at Dose *X:*	*X Dose of* Caveolin-3	*X Dose of* Caveolin-3	*X Dose of* Caveolin-3
MG53 Triplicates at Dose *X:*	*X Dose of* MG53	*X Dose of* MG53	*X Dose of* MG53
Cav-3 + MG53 Triplicates at Dose *X:*	*X Dose of* Caveolin-3 + *X Dose of* MG53	*X Dose of* Caveolin-3 + *X Dose of* MG53	*X Dose of* Caveolin-3 + *X Dose of* MG53

Furthermore, in order to elucidate membrane repair kinetics immediately after injury, we conducted a modified version of the aforementioned global assays. In preparation for these assays, we seeded 20,000 C2C12 myoblasts per well in a standard 48-well tissue culture plate. These assays may also be done with L6 myoblasts. We then added 0.125U, 0.25U, 0.5U, or 1.0U of each virus to the appropriate wells, in an effort to reduce the likelihood of viral toxicity at higher doses. Each well either received no virus, MG53 virus, cav-3 virus, or both MG53 and cav-3 viruses. Each condition was prepared in triplicates. The cells were allowed to incubate with the viruses for 24 hours prior to replenishing the wells with serum-free culture media. The

cells were then incubated for another 24 hours. On the day of the assay, we prepared 1:10 dilutions of DMEM (10% DMEM) in sterile water, in order to induce hypo-osmotic shock (Barzilai-Tutsch et al., 2018). We exposed each condition to 10% DMEM. We also included a 100% DMEM control with wild-type cells. To track cell injury, we utilized propidium iodide (PI), which stains nuclear DNA; PI can only reach DNA if the integrity of the plasma membrane has been disrupted (Molecular Probes, Inc., 2006). We used 2uL of 15 mg/ml per 100uL of reaction (Barzilai-Tutsch et al., 2018). We captured images of the PI red fluorescence, magnified at 20X every 20 seconds for 160 seconds. To analyze the total area of fluorescence at each timepoint, we utilized ImageJ's "Analyze Particles" function.

All of our time-lapse imaging assays were conducted in an incubation chamber equipped with a humidifier, CO_2, and temperature control, set to 37°C.

2.4 Mitochondrial Function: High Resolution Respirometry

To assess the relationship between mitochondrial function and the membrane microdomains caveolae, we will analyze live tissue from caveolin-3 over-expressing and knockout mice using the Oroboros O2k High Resolution Respirometry (HRR) apparatus. We will test skeletal muscle, heart, hippocampus, and liver tissue samples. We will be following a substrate-uncoupler-inhibitor-titration protocol (Pesta and Gnaiger, 2011), using the substrates glutamate, malate, ADP, pyruvate, succinate, the uncoupler FCCP, the inhibitor rotenone, and the inhibitor antimycin A. This will allow us to measure capacities for tissue oxygen consumption and oxidative phosphorylation, and draw connections to the effects of caveolin-3 and caveolae.

3. RESULTS

3.1 Plasma Membrane Structural Dynamics May Be Partially Controlled by Bmal1 Dependent Clock Genes

Membrane structural dynamics play important roles in determining membrane functionality and cellular viability. In order to elucidate the factors governing plasma membrane dynamics, we sought to test our novel hypothesis that circadian control is at partially play, as well as identify if membrane dynamics behave in a regular, cyclical manner. Because membrane lipid rafts (MLRs) are prevalent microdomains in the plasma membrane, tracking their organization and distribution sheds light upon potential patterns of plasma membrane dynamics. To study trends in membrane dynamics over-time, we stained patches of membrane lipid rafts in mouse cortical neurons with a fluorescent probe derived from the cholera B toxin (Alexa Fluor CT-B 488). Via fluorescence microscopy, the brightness of fluorescence was measured every 10 minutes over a 48-hour span in six individual cells. Oscillations of average brightness at each timepoint were observed. There was a cycle of increasing and decreasing fluorescence brightness, where there was a rise to a peak, followed by return to a trough, with consistent amplitudes, approximately every 12 hours (Figure 1A). Meanwhile, the variance of fluorescence brightness, quantified by standard deviation, demonstrated a notable distinction from the background fluorescence, as the entire range of fluorescence values measured in the mouse cortical neurons was notably higher than the background. The background fluorescence in the microscopic field maintained steadily and did not demonstrate periodic oscillations. This suggests that the observed trend in mouse cortical neuron membranes is not artificial. The prominent, periodic oscillations of brightness emitted by fluorescently stained MLR patches, or CT-B positive domains, suggest that an ultradian rhythm, possibly spanning cycles of 12 hours,

is at play in controlling plasma membrane composition and organization. Meanwhile, in murine cortical neurons infected with adenovirus small hairpin RNA (shRNA) specifically designed to disrupt expression of Bmal1, a prominent clock gene transcription factor, and subjected to the aforementioned MLR staining protocol, the same trend is not observed (Figure 1B). There is no prominent oscillatory pattern of membrane organization in the clock-compromised condition (Figure 1B). Moreover, the control cell MLR patches visibly redistribute and reorganize over-time, where areas of the membrane become more and less concentrated in fluorescence, while shRNA treated cells show no notable changes (Figure 1C). These results uncover novel information on patterns of the plasma membrane's daily behavior, suggesting that Bmal1 dependent clock genes play a partial role in determining membrane dynamics, and that there are regular temporospatial fluctuations in membrane structural components.

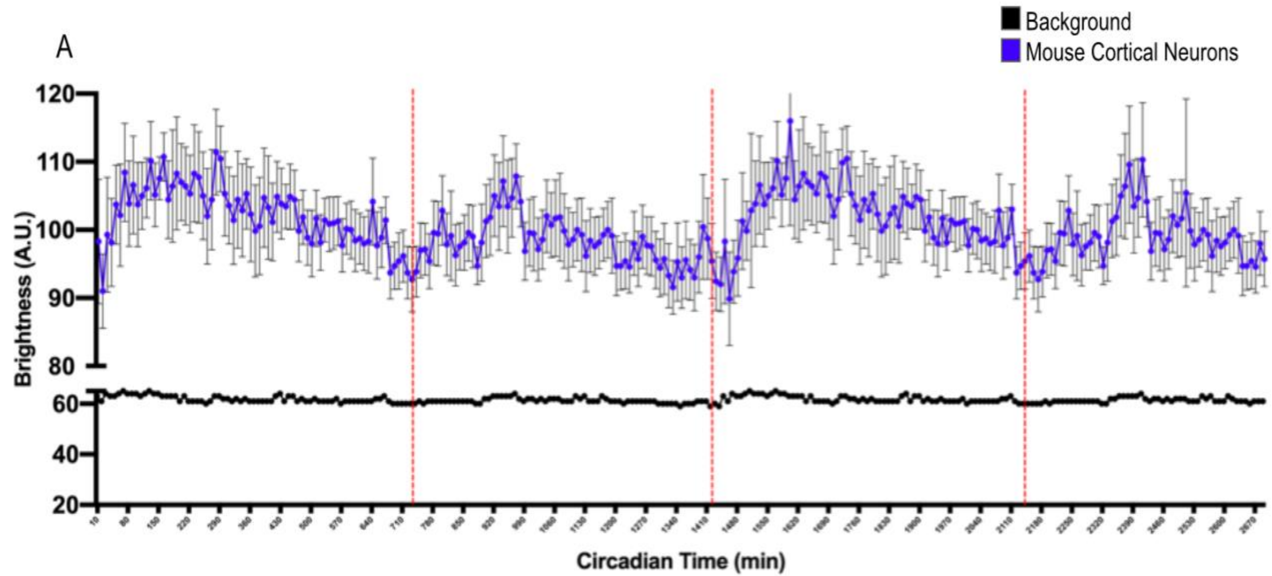

Figure 1A. Plasma Membrane Organization in Murine Cortical Neurons Oscillates. A fluorescent probe derived from the cholera B toxin (Alexa Fluor CT-B 488) was used to stain patches of membrane lipid rafts (MLRs) in murine cortical neurons. The brightness of fluorescence, detected by fluorescence microscopy, was measured every 10 minutes over a span of 48 hours in six individual cells. Average fluorescence brightness ± standard deviation is presented by the blue line. The black line represents the background fluorescence detected at each timepoint. Fluorescence values were measured in arbitrary units (A.U.). The oscillatory trend suggests a partial influence by a biological clbck, and the red dotted lines indicate the proposed boundaries of the ultradian cycles, spanning 12 hours each.

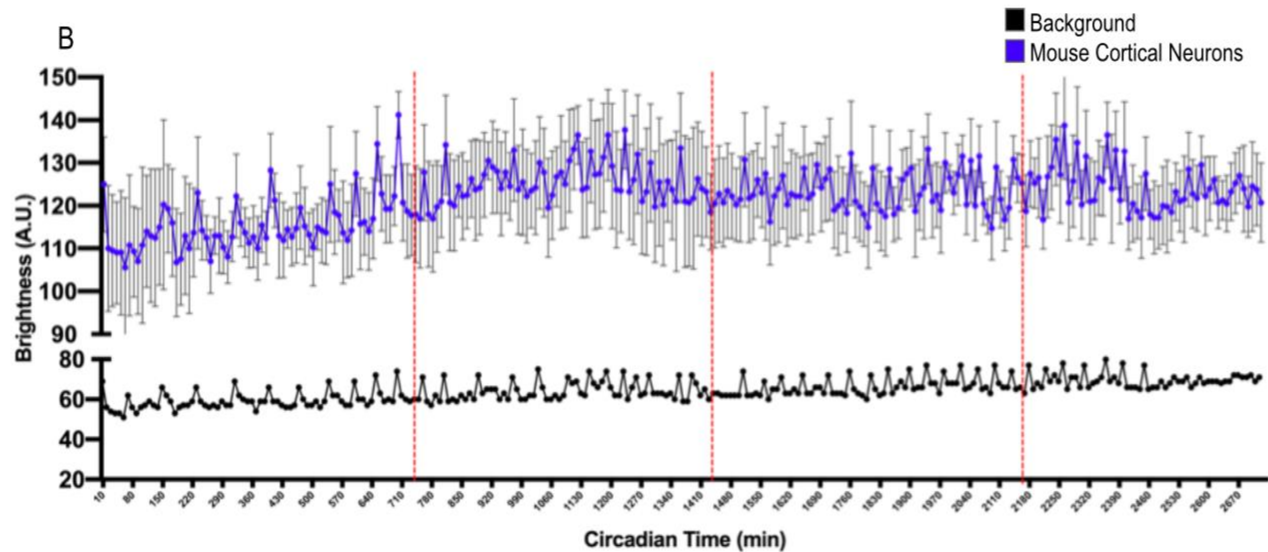

Figure 1B. RNA Interference (RNAi) of the Clock Gene Transcription Factor Bmal1 Disrupts Ultradian Patterns. Murine cortical neurons were infected with adenovirus small hairpin RNA (shRNA) targeting Bmal1, a common clock gene transcription factor, via a 24 hour co-incubation. MLRs were stained with fluorescent CT-B. The brightness of fluorescence, detected by fluorescence microscopy, was measured every 10 minutes over a span of 48 hours in six individual cells. Average fluorescence brightness ± standard deviation is presented by the blue line. The black line represents the background fluorescence detected at each timepoint. Fluorescence values were measured in arbitrary units (A.U.). The trend does not resemble the cyclic behavior seen in control cells.

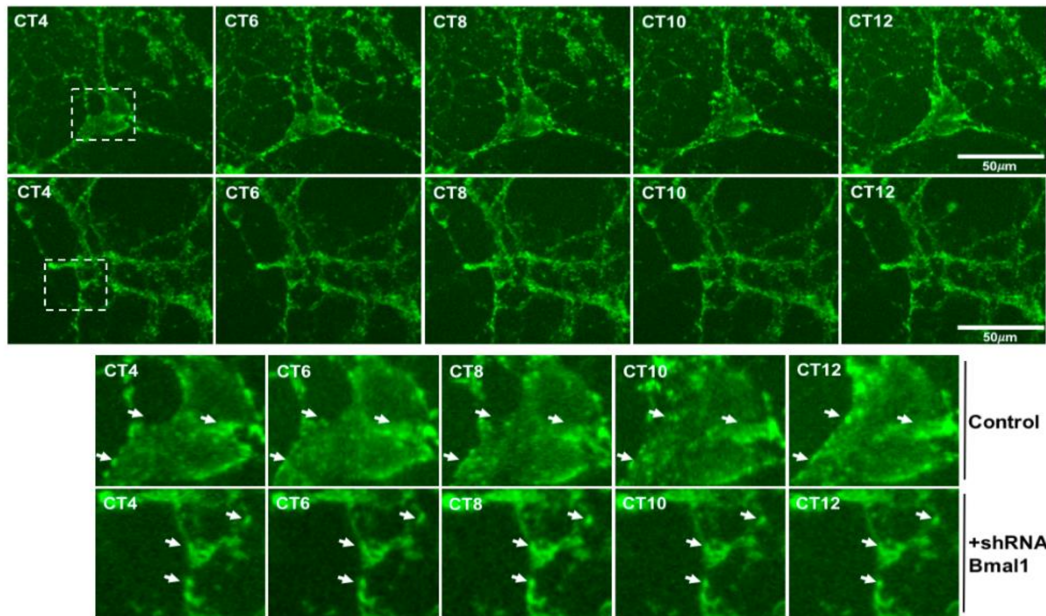

Figure 1C. Organization of Membrane Lipid Rafts in Mouse Cortical Neurons Visibly Changes Over-Time. Tracking areas of a mouse cortical neuron plasma membrane, indicated by the white arrows, in a control cell over-time shows that fluorescently stained MLR patches (CT-B positive domains) constantly redistribute and demonstrate dynamic characteristics in the membrane. Meanwhile, in mouse cortical neurons infected with adenovirus shRNA targeting Bmal1, there are not many prominent changes in membrane organization. This suggests that Bmal1 dependent clock genes partially determine plasma membrane dynamics.

3.2 Caveolin-3 Enhances Plasma Membrane Repair

Furthermore, in order to withstand damage-inducing stress, the plasma membrane relies on repair mechanisms, which involve membrane microdomains such as caveolae, to reseal membrane wounds and maintain cellular structure (Corrotte et al., 2013). Accordingly, we conducted multiple investigations to demonstrate how cellular resilience is enabled by membrane microdomains:

To uncover the role of caveolin, the unique structural protein in caveolae, in plasma membrane repair, we transfected murine myoblasts with cav-3, MG53, or both cav-3 and MG53 expressing viruses, then induced damage by introducing them to the pore forming toxin streptolysin-O (SLO). MG53 is a classic membrane repair protein that is thought to interact with

cav-3 to facilitate membrane resealing (Cai et al., 2009). We tested four viral doses (Figure 2): A) 0.5U, B) 1.0U, C) 1.5U, and D) 2.0U. Then we used the fluorescent dye FM1-43, which fluoresces more brightly when it penetrates the lipid bilayer (Corrotte et al., 2015). The dye can only be incorporated between the bilayer if the plasma membrane has been injured. We tracked the injured cells over-time for 50 minutes. All of the cav-3, MG53, and cav-3 and MG53 transfected myoblast fields exhibited lower average FM1-43 fluorescence over-time, than wild-type myoblast fields (Figure 2). This data presented a high degree of variation, quantified by standard error of the mean (SEM). However, general trends in the average change in field fluorescence were still observed. At the lowest viral dose (Figure 2A), we found that cav-3 and MG53 transfected cells incorporated less FM1-43 dye than wild-type myoblasts, while co-expression of cav-3 and MG53 produced a much more notable decrease in fluorescence, suggesting a tendency for cellular resilience over-time when these essential membrane repair proteins interact. Interestingly, as the viral doses increased, the effects of each transfection condition became less distinguishable from one another; however, with each increasing viral dose, there was a clear trend of decreasing FM1-43 fluorescence, suggesting that cav-3 and MG53 enabled cellular resilience over-time in the presence of the pore forming SLO toxin. This distinction is clear in comparison to the wild-type myoblasts, which exhibited a continual increase in FM-143 fluorescence over-time (Figure 2B-D). This potentially suggests that over the span of 50 minutes, the cav-3 and MG53 transfected cells in the presence of deleterious SLO were able to demonstrate cellular resilience by re-establishing or maintaining membrane integrity with more efficient membrane repair than wild-type cells.

In Figure 2, between the first and second time-points, we see prevalent changes in FM1-43 fluorescence in a span of just five minutes. However, the plasma membrane is known to

repair itself within minutes after injury (Terasaki et al., 1997). To elucidate the kinetics of membrane repair, our laboratory conducted assays focused on the minutes immediately following the membrane injury. To do so, cav-3 over-expressing murine cardiac myocytes were incubated with the fluorescent dye FM1-43. Individual cardiomyocytes were penetrated at a single site via laser-induced injury. Live cell confocal imaging was employed to track fluorescence over-time for 450 seconds. If membrane repair mechanisms are efficient, the membrane reseals faster than FM1-43 is incorporated. During the 450 second span, wild-type cells demonstrated a more rapid increase in FM1-43 fluorescence than cav-3 overexpressing cells. The trends in fluorescence over-time suggest, as expected, that cav-3 overexpressing cardiomyocytes repaired the membrane wound more efficiently than wild-type cells, at about double the rate (Figure 3). Similarly, in murine cardiomyocytes transfected with caveolin-3, MG53, or both caveolin-3 and MG53 expressing viruses at a range of viral doses, the aforementioned method of single-cell injury and fluorescent probing was utilized to observe dose-dependent effects of membrane repair machinery. At a given dose, caveolin-3 transfected cardiomyocytes tended to increase the rate of repair more effectively than MG53 transfected cardiomyocytes. Meanwhile, co-expression of both proteins produced the most substantial rate increase. This suggests that caveolin-3, and subsequently caveolae, play a critical role in mediating membrane repair kinetics (Chabanon et al., 2020).

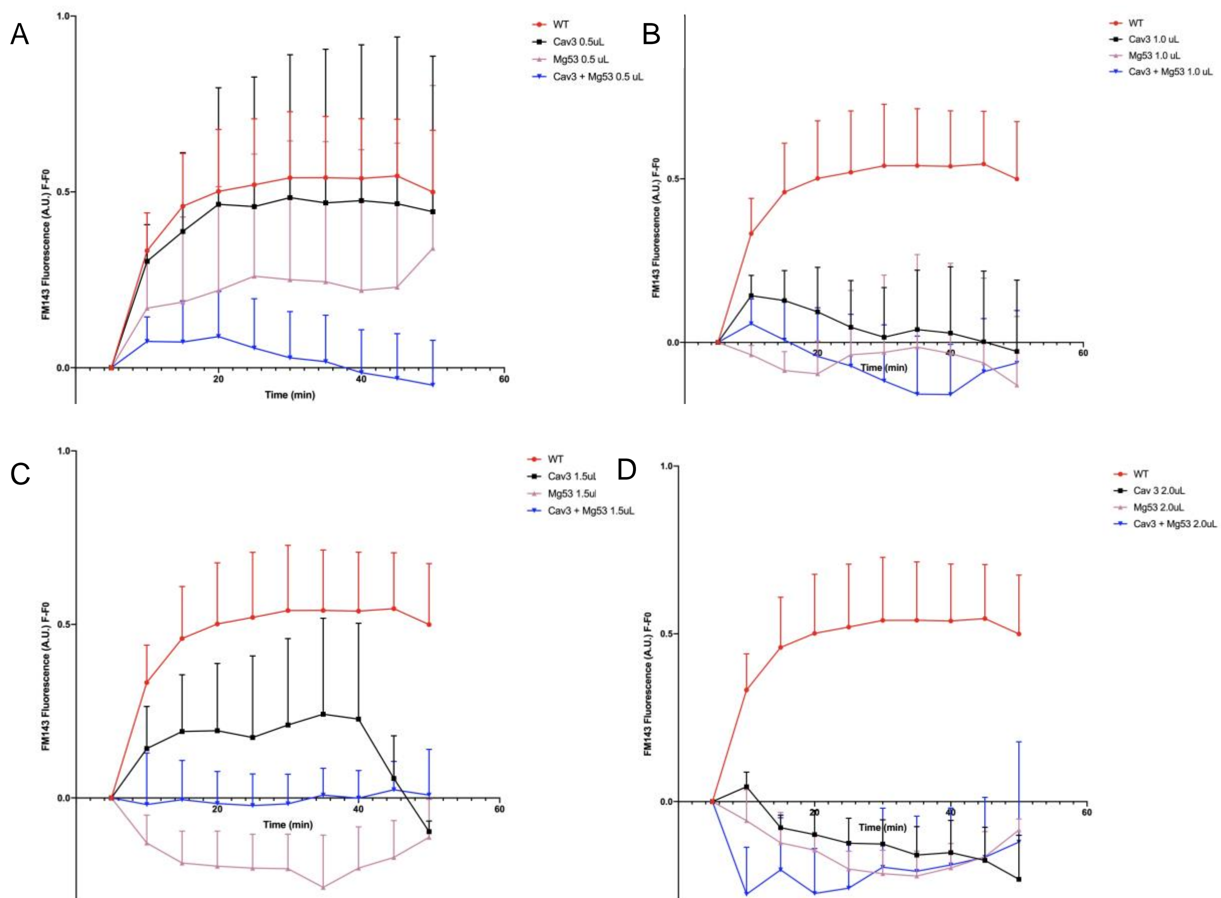

Figure 2. MG53 and Cav-3 Transfected Cells Incorpo e Less FM1-43 Dye Over Time in the Presence of Pore Forming SLO Toxin. Murine myoblasts were transfected with Cav-3, MG53, or both Cav-3 and MG53 expressing viruses 48-hours prior to the assay. The viral dose range was as follows: A) 0.5U, B) 1.0U, C) 1.5U, and D) 2.0U. Each set of cells was then injured with pore-forming SLO Toxin. The fluorescent probe FM1-43, which fluoresces at membrane pores, was added simultaneously. A fluorescence microscope was used to capture fluorescence in a selected field every 5 minutes for 50 minutes. Average change in whole-field fluorescence ± SEM suggests that MG53 and Cav-3 tend to facilitate in a decrease in membrane injury, and that the transfected cells may be more resilient than wild-type cells. (n=4)

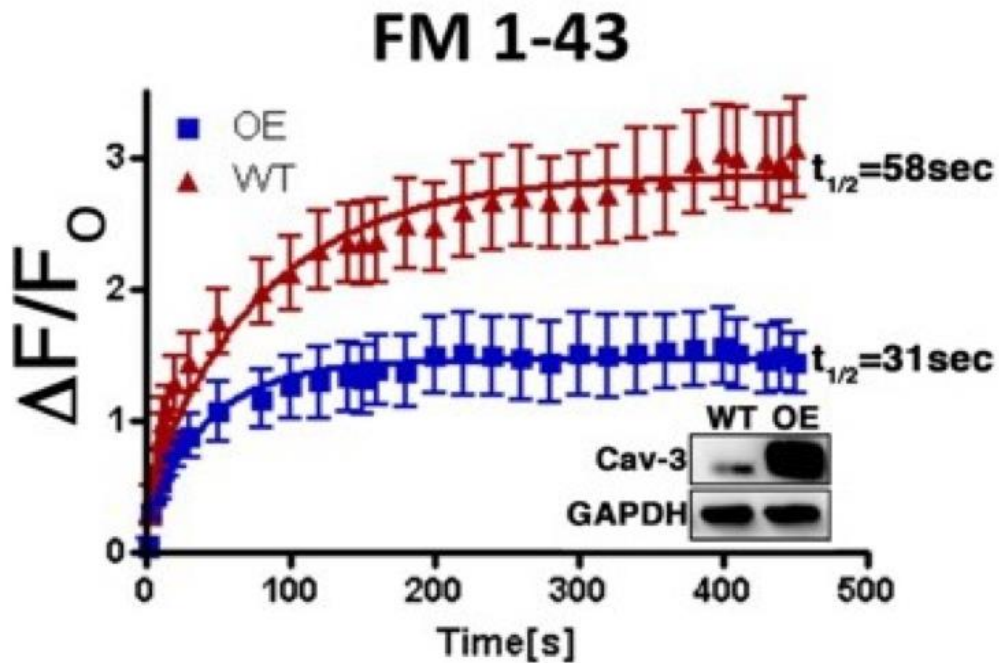

Figure 3. Cav-3 Overexpression Improves Membrane Repair Kinetics. Overexpression of cav-3 in murine cardiomyocytes, verified by immunoblotting results, was induced. The cells were incubated with the fluorescent probe FM1-43, which fluoresces at membrane pores. The plasma membrane was wounded via laser-induced injury at a single site. Live cell confocal imaging was used to detect fluorescence (F) over-time for 450 seconds. Greater fluorescence corresponds to more FM1-43 dye incorporation, while low fluorescence corresponds to less FM1-43 dye incorporation. Efficient repair mechanisms result in less dye incorporation. Based on the fractional change in fluorescence over-time, cav-3 overexpressing cardiomyocytes repaired the site of injury approximately twice as fast as wild-type cells. (n=8) (Chabanon et al., 2020)

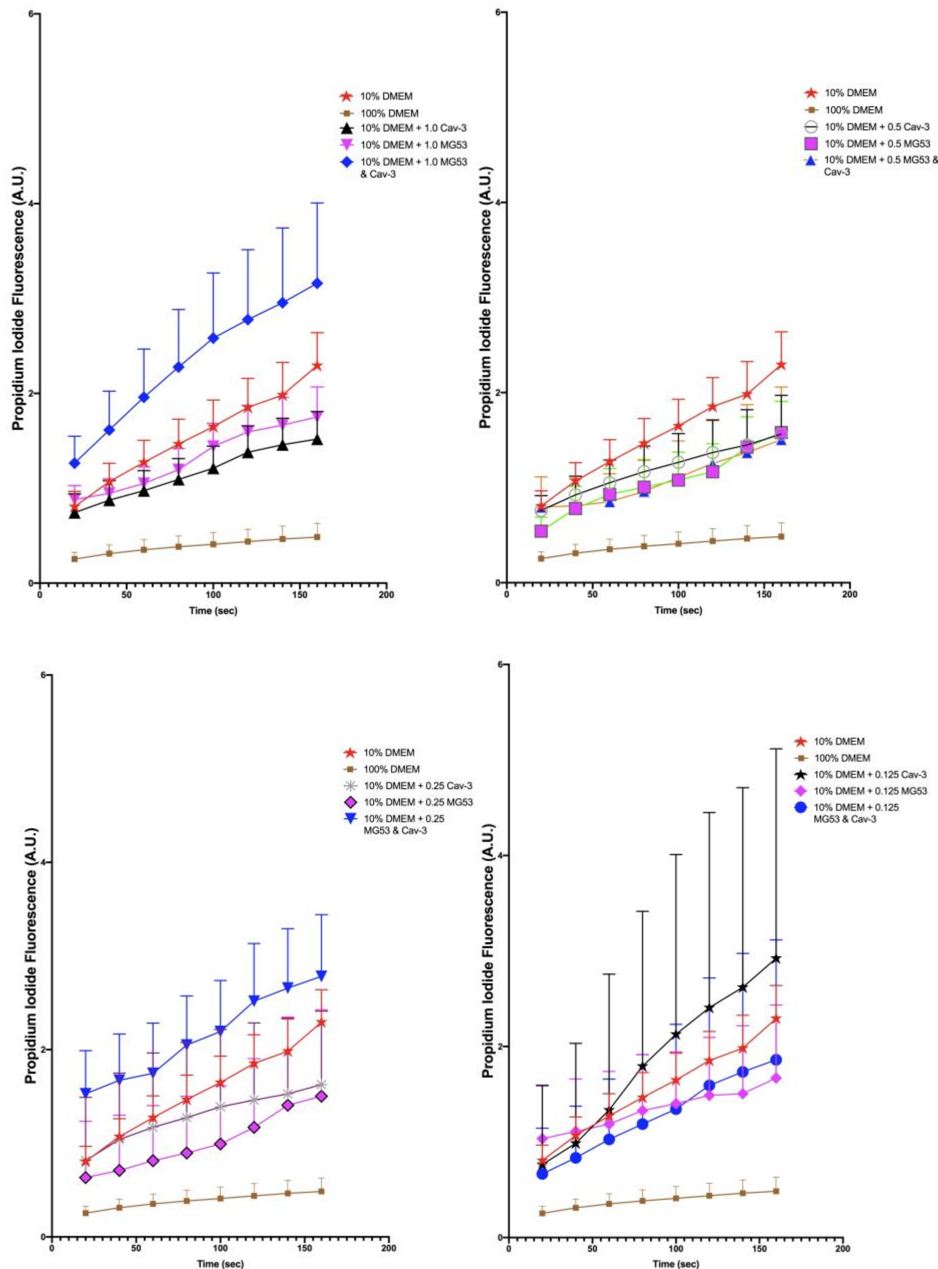

Figure 4. MG53 and Cav-3 Transfected Cells May Incorporate Propidium Iodide Dye More Slowly than Wild-Type Cells in Hypo-Osmotic Conditions. Murine myoblasts were transfected with Cav-3, MG53, or Cav-3 and MG53 expressing viruses 48-hours prior to the assay. The viral dose range was as follows: A) 1.0U, B) 0.5U, C) 0.25U, and D) 0.125U. Each set of cells was then injured via exposure to hypo-osmotic culture media (1:10 dilution of DMEM, or 10% DMEM), and the fluorescent probe propidium iodide (PI), which fluoresces in contact with DNA, was added simultaneously. We assume PI can only access DNA if the integrity of the outer plasma membrane is compromised. A fluorescence microscope was used to capture fluorescence in a selected field every 20 seconds for 160 seconds. Average whole-field fluorescence ± SEM demonstrates that MG53 and cav-3 may slow the progress of membrane injury at certain doses. (n=3)

In the aforementioned study (Figure 3), the laboratory was limited to analysis of single-cells. When assessing individual cells, factors like cell-cell signaling to potentiate repair (Togo, 2017) are not accounted for. Additionally, the single-cell experimental model cannot feasibly analyze a large number of replicates. Hence, it is of interest to develop a global assay which accounts for multiple cells subjected to injury. To explore membrane repair kinetics in larger sets of cells simultaneously, we transfected murine myoblasts with A) 1.0U, B) 0.5U, C) 0.25U, and D) 0.125U of cav-3, MG53, or both cav-3 and MG53 expressing viruses. We then induced membrane damage by introducing hypo-osmotic culture media, and probed for membrane damage with the fluorescent dye propidium iodide (PI). Propidium iodide stains DNA, which it can only access if the plasma membrane has been injured. We tracked field fluorescence over-time for 160 seconds. Due to a high degree of variance (SEM), there was not a distinct viral dose-dependent effect on membrane repair under these experimental conditions. However, the trends in average fluorescence suggest that cav-3 and MG53, when expressed separately at viral doses of 0.25U, 0.5U, and 1.0U, may have enabled slower PI incorporation than untreated cells subjected to the same hypo-osmotic media (Figure 4A-C). Surprisingly, co-expression of MG53 and cav-3 did not consistently demonstrate the synergistic, protective effect expected to result from the interaction of key repair proteins (Figure 4A, 4C), potentially due to viral toxicity. On the other hand, the field of cells that received the 0.125U viral dose of cav-3 (Figure 4D) demonstrated an increase in fluorescence that surpassed wild-type cells in hypo-osmotic conditions, which we expected to sustain the most unrepaired damage; the inconsistencies in Figure 4D may be because the viral dose was too low to take effect.

However, these data overall suggest that caveolin, and its microdomain successor caveolae, increase the efficiency of membrane kinetics, likely by enabling the production of

caveolae and working with essential repair proteins, thus enabling cellular resilience (Figures 2-4).

Chapter 3.1 includes unpublished material coauthored with Zuniga-Hertz, Juan.

Chapter 3.2, in part, includes the following material from our laboratory being prepared for submission for publication: Chabanon, Morgan; Ray, Supriyo; Schilling, Jan; Dhanani, Mehul; Rangamani, Padmini; Patel, Hemal. “Phenomenological Model of Cav-3-mediated Plasma Membrane Repair”.

4. DISCUSSION

Through investigations of plasma membrane structure, function, and microdomains, our research seeks to elucidate a holistic understanding of how membrane-level activity has downstream implications for cellular survival and responses to stress. To establish this basis of knowledge, we studied membrane structural dynamics and membrane repair mechanisms, with respect to membrane microdomains. We hope to not only build upon these areas of interest, but also extend our studies to the intracellular implications of membrane microdomains, like mitochondrial function, as all of these aspects ultimately determine a cell's capacity to function optimally and resiliently.

Seeking to elucidate specific temporospatial patterns of membrane dynamics, we speculated that the plasma membrane is partially subject to control by a biological clock, as all organisms ubiquitously exhibit daily circadian and ultradian rhythms. We found that in wild type mouse cortical neurons, membrane lipid raft (MLR) distribution in the plasma membrane fluctuates, following an oscillatory pattern with repeating cycles every 12 hours (Figure 1A and 1C). However, in the cortical neurons that underwent RNA interference (RNAi), targeting the clock gene transcription factor Bmal1, this prominent oscillation was diminished (Figure 1B and 1C). Hence, when the cell's intrinsic biological clock was compromised, membrane organization became irregular and lost its clear pattern of behavior.

Our findings are in agreement with existing studies that have identified oscillatory patterns in membrane lipids, and have also suggested that these oscillations are in-part enforced by clock gene expression (Loizides-Mangold et al., 2017; Aviram et al., 2016). Disruption of Bmal1 has been shown to directly disrupt the regular patterns of biological clocks (Menet et al. 2014), which corresponds to our observed loss of the MLR oscillatory pattern when mouse

cortical neurons underwent RNAi to impede Bmal1 expression (Figures 1A-C). Other recent studies of membrane dynamics present results which align with our findings of circadian oscillation in the membrane (Figure 1A). Lipidomics studies in human skeletal muscle tissue demonstrate daily patterns of oscillation in membrane lipid composition, length of sphingolipid and phospholipid chains, and expression of lipid metabolites; meanwhile, comparison of wild-type to clock-gene compromised conditions upholds that biological clocks may be partially responsible for these patterns (Loizides-Mangold et al., 2017). Similarly, there is a body of evidence suggesting that the temporospatial arrangement and distribution of lipids among intracellular organelles, such as mitochondria, also follow daily patterns (Aviram et al., 2016). Therefore, in the context of the current literature, our results ultimately suggest that Bmal1 influences the organization in the plasma membrane, which indicates that a circadian clock may contribute to membrane dynamics.

These findings have significant implications for the field of membrane biology, as the composition of the plasma membrane directly impacts its capacity to carry out vital signaling, transport, and self-repairing functions (Loizides-Mangold et al., 2017). Meanwhile, circadian control of the plasma membrane dynamics has not been studied in depth. Hence, our novel research expands upon the existing field of knowledge by focusing on functional microdomains of the plasma membrane, such as MLRs, and tracking their daily patterns of behavior. The need to understand MLRs and other membrane microdomains in the context of circadian rhythms also stems from their importance in membrane function. MLRs are cholesterol-rich, tightly packed functional domains that influence membrane characteristics like fluidity, rigidity, and structural integrity (Cooper, 1978). Membrane microdomains are a prevalent, key component of membrane structure, and some studies have even suggested that optimizing the structure and composition of

the plasma membrane will provide the solution to numerous health issues, like metabolic diseases (Casares et al., 2019). For example, in human patients whose biological clock is compromised, studies have identified altered red blood cell membrane composition due to fatty acid (FA) lipid pathologies, leading to deleterious effects on metabolic homeostasis (Garaulet et al., 2009). In another study, increasing the cholesterol content in rat liver cell membranes altered the composition of caveolar microdomains, which changed the cells' capacity for insulin signaling (Hahn-Obercyger et al., 2008; Perona, 2017). There are also a variety of daily, seasonal, or even lifelong patterns in lipid and cholesterol homeostasis (Gooley et al., 2016; Gooley and Chua, 2014; Gordon et al., 1987; Blüher et al., 2001). As major components of the plasma membrane and its microdomains, studying circadian lipid and cholesterol fluctuations may give insight into how membrane composition results in increased or decreased capacities for function, and should be explored as a future therapeutic method. Future studies of optimizing membrane composition may be possible via regulation of gene expression, enzyme function, or even diet (Casares et al., 2019; Barceló et al., 2009). Accordingly, our study of membrane dynamics makes necessary strides toward elucidating the specific temporospatial patterns of membrane structure, and identifying the potential circadian factors that help determine changes in plasma membrane composition over-time. Therefore, our results establish a basis of knowledge which can be used to work toward not only identifying time points at which membrane composition is optimal, but also how the membrane's composition can be therapeutically optimized, especially with respect to microdomain composition, to enhance cellular and organismal resilience.

The observed oscillatory pattern (Figure 1A and 1C), however, may be attributed to multiple biological rhythms which culminate into the phenomena we observed in our study. In

any organism, many biological rhythms likely occur simultaneously, each with their own frequency of periodic gene expression (Veen and Gerkema, 2016). Clock genes enable rhythmic gene expression that governs a multitude of concurrent processes, like lipid metabolism and protein catabolism (Veen and Gerkema, 2016). Our experimental design does not allow us to distinguish between multiple simultaneous rhythms. Although we were able to identify the ultradian fluctuation of membrane organization over-time, where each oscillation spanned 12-hours (Figure 1A), it remains unclear whether there are multiple biological rhythms that contribute to the observed pattern. Hence, in future investigations, it would be of interest to resolve individual rhythms, such as lipid and cholesterol metabolism, contributing to the membrane fluctuations we observed, because some circadian rhythms may mask others (Veen and Gerkema, 2016).

On the other hand, it cannot be assumed that the observed oscillations in Figure 1 are solely due to a biological clock. Our experimental model was limited to testing the effects of Bmal1, however, there are numerous other clock genes, like Per and Cry (Menet et al., 2014), and even factors not involving gene expression, which have not been tested in this regard. Thus, our results exclusively suggest that the membrane is indeed dynamic and that its structure and composition fluctuates in a regular temporospatial pattern that is partially regulated by Bmal1 dependent clock genes.

Furthermore, our experimental model was limited to one cell type, cortical neurons. However, many biological rhythms, such as lipid homeostasis, are regulated with tissue and cell specificity (Gooley, 2016; Gooley and Chua, 2014). Although the mammalian hypothalamic suprachiasmatic nucleus (SCN) orchestrates the major circadian rhythms, including the sleep-wake cycle, cell-specific clock genes and their gene products form molecular-level biological

clocks that are specific to tissue function (Moore, 2001; Gooley, 2016). Circadian patterns of lipid metabolism, for example, in one tissue may not be representative of all tissues (Gooley, 2016; Gooley and Chua, 2014). Subsequently, circadian patterns in membrane composition may not be well represented by one type of cell, as lipids are major structural components in the plasma membrane. Hence, it would be of interest to conduct our membrane dynamics study with a variety of cell types.

Overall, understanding how the structure of the plasma membrane behaves, and the factors which create its daily patterns, ultimately reveals how plasma membrane function may be predisposed to having differing capacities to function throughout the day, which has downstream implications on cellular resilience. One of these implications may be specific to the plasma membrane's capacity to repair itself in response to stress or injury. In order to withstand damage-inducing stress, the plasma membrane relies on repair mechanisms, which involve membrane microdomains like caveolae, to reseal injured membranes to maintain cellular structure.

To investigate the precise role of the caveolae-forming protein, caveolin, in membrane repair mechanisms, our laboratory conducted multiple sets of investigations to demonstrate how cellular resilience is enabled by membrane microdomains. We used the fluorescent dyes FM1-43 and propidium iodide to track membrane injury, such that higher fluorescence corresponds to greater dye incorporation by injured membranes. We found that during spans of 50 minutes, sets of injured myoblasts transfected with caveolin-3, MG53, or both caveolin-3 and MG53 tended to demonstrate a decrease in FM1-43 fluorescence, while wild-type myoblasts showed a continual increase in fluorescence (Figure 2). Our laboratory also found that in the immediate 450 seconds after injury, individual caveolin-3 overexpressing cardiomyocytes repaired laser-induced injury more efficiently than individual wild-type cells, at about double the rate, as the overexpressing

cells incorporated FM1-43 much slower (Figure 3). Our laboratory's other studies also demonstrated dose-dependent protective effects of caveolin-3 expression, MG53 expression, and co-expression of caveolin-3 and MG53 in single cells, where the co-expressing condition is the most protective against laser-induced injury, and caveolin-3 expression is more protective than MG53 expression alone (Chabanon et al., 2020). As expected, these results suggest that caveolin-3, and subsequently caveolae, play a critical role in membrane repair kinetics. Since the key kinetics of membrane repair occur almost immediately after the initial injury is sustained, we also developed a global assay that studied the first three minutes after injury, from which we observed that cav-3 and MG53 transfected myocytes incorporate propidium iodide slightly slower than wild-type cells, on average, but each viral dose did not produce an effect that was distinctly unique from the others (Figure 4).

Our findings are in alignment with current studies in the field, which predominantly study membrane repair in the context of membrane microdomains and the key repair proteins, caveolin-3 and MG53. Corrotte et al. (2013) and Andrews et al. (2014) have demonstrated that caveolin-3, and caveolae, play direct roles in enabling membrane repair mechanisms, and that caveolin overexpression consistently results in enhanced rates of repair. They've also shown that caveolae accumulate and endocytose membrane lesions when pore forming toxins have induced membrane injury (Corrotte et a., 2013). Our laboratory's previous studies have also observed a synergistic relationship between caveolin-3 and MG53 during membrane repair, whereby caveolin-3 likely plays the most influential role in enabling the formation of a repair protein complex with MG53, and other key proteins like dysferlin (Chabanon et al. 2020). Our studies, which utilized lasers, hypo-osmotic culture media, and the pore forming toxin streptolysin-O to induce membrane injury, therefore uphold existing findings in the field and contribute to the

body of data that has identified caveolae as key facilitators of efficient membrane repair, and therefore in cellular resilience.

Furthermore, although biological membranes repair themselves within a few seconds after injury (Andrews et al., 2014), Figure 2 showed an observable trend in membrane fluorescence across a much longer span of time (50 minutes) after injury with SLO. This longer-term effect aligns with other current studies, which employ the pore forming toxin listeriolysin-O to induce damage and propidium iodide fluorescence to track repair, that showed differences in membrane repair efficiency can be reflected across 30-minute timespans (Pathak-Sharma et al., 2017). It is uncertain what the long-term trends in fluorescence represent, as these far exceed the membrane resealing timeline. Han et al. (2012) attributes decreases in FM1-43 fluorescence across 20 minutes in endothelial cells to lysosomal fusion, which is a potential membrane repair mechanism. More specifically, this refers to calcium-triggered exocytosis of lysosomes, which causes lesion removal via endocytosis, possibly mediated by caveolar vesicles (Andrews & Corrotte, 2018; Corrotte et al., 2013). Hence, our results in Figure 2 may suggest that caveolin-3 and MG53 enable a more resilient membrane by promoting lysosomal fusion after membrane injury.

However, the limitations of our studies and experimental design do not allow us to distinguish between different mechanisms or modes of membrane repair. The current body of data in this field concurs that there are multiple mechanisms of membrane repair, where injury to the membrane triggers calcium ion influx, followed by a repair mechanism that corresponds to the mode and size of membrane injury (Andrews & Corrotte, 2018; Jimenez et al., 2014). For instance, budding-off of the plasma membrane has been associated with small lesions, while larger wounds may require the formation of a patch (Andrews & Corrotte, 2018; Jimenez et al.,

2014; Terasaki et al., 1997). These mechanisms differ in rate, can occur simultaneously, and vary by cell type (Terasaki et al., 1997). In our experiments using SLO and hypo-osmotic media to induce membrane injury, we aren't able to control for the size of the lesions created. Also, our models only used cardiac and skeletal muscle cells, thus, the rates of membrane repair are likely not representative of all cell types.

Furthermore, in our laboratory's studies that utilized a targeted laser to produce a single membrane lesion (Figure 3), although the size of the lesion was known, the study was limited to observing one cell at a time. With that in consideration, along with studies that suggest cell-cell signaling causes unwounded cells to potentiate membrane repair when in the vicinity of injured cells (Togo 2017), we sought to demonstrate membrane kinetics in a global assay that ideally would represent how multicellular tissues behave in response to injury. In these global assays, although we observed general trends in membrane repair over-time which suggest that caveolin-3 and MG53 were improving membrane resilience, we also observed a remarkable amount of variability from assay to assay (Figures 4 and 5). The trends observed in our global assays did not match the dose-dependent effects of cav-3 and MG53 seen in our laboratories studies of membrane repair in single cells (Chabanon et al., 2020). The highly variable outcomes of our global assays have been due to the lack of control for lesion size (Jimenez et al., 2014).

Also, in Figure 4 we observed unexpectedly high fluorescence values for the co-expressed cav-3 and MG53 condition, which was expected to result in less dye incorporation since these proteins are thought to work synergistically to promote repair. This may be due to viral dose toxicity, as the addition of both the cav-3 and MG53 lentiviruses doubles the dose at each condition, which may have been too high to be effective without causing harm to the cell (Castellani et al., 2010). In Figure 4, we also observed that at the lowest viral dose (0.125U), the

wild type cells sustained less membrane damage than some of the transfected conditions, which may indicate that this dose was too low to have a notable effect on the cells, especially when considering the substantial overlap in error bars for all tested conditions. In future studies, we can test more viral doses to determine the upper and lower limits where the transfection becomes unsuccessful.

Despite these limitations, the findings from our single-cell and global membrane repair assays (Figures 2-4) still provide valuable insight to the field of membrane biology. Our results further affirm that caveolin-3, and therefore caveolar microdomains, help enhance the rate of membrane repair. Making these strides toward defining the precise roles of membrane microdomains like caveolae in membrane repair will ultimately pave the way for understanding diseases like muscular dystrophy, which is a result of unrepaired membrane damage in skeletal muscle cells that directly relates to caveolin-3 mutations, or caveolinopathies (Hagiwara et al., 2000). Understanding these membrane-level malfunctions or inefficiencies with respect to self-repair mechanisms has significant downstream implications in understanding and treating a wide range of diseases, as caveolins, caveolae, and MG53 have been shown to protect kidney tissue from acute kidney injury (AKI), and even have neuroprotective effects (Duan et al., 2015; Cheng and Nichols, 2016). Furthermore, linking our understanding of membrane repair kinetics with our understanding of membrane structural dynamics, this provides even more potential to pursue therapies that optimize membrane structure and composition. An immediate future step for our studies could be to hybridize our membrane repair and membrane dynamics studies to determine if there are certain times throughout the day at which the plasma membrane has the optimal capacity to perform repair mechanisms. Optimizing membrane composition, and subsequently

caveolar composition, may allow for membrane repair to be enhanced, thus increasing cellular resilience (Casares et al., 2019; Hahn-Obercyger et al., 2008; Perona, 2017).

Moreover, in prospective studies, we will seek to identify the precise role of membrane microdomains, particularly caveolae, in mitochondrial function. Caveolin proteins have been found to not only localize at the site of the membrane, but also in intracellular structures like the mitochondria (Schilling and Patel, 2016). Also, the cross-talk between mitochondria and caveolae is thought to play a role in maintaining mitochondrial structure and function (Fridolfsson et al., 2012). Meanwhile, malfunctioning mitochondria, potentially stemming from caveolinopathies, cause harm to the cell, not only by failing to respire successfully, but also by releasing harmful reactive oxygen species (ROS) (Schilling and Patel, 2016; Fridolfsson et al., 2012). These ROS can then further harm the plasma membrane, creating a magnified effect where there would already be a decreased capacity for membrane repair paired with an added stressor. Therefore, caveolins and caveolae play crucial roles not only in membrane function, but also in intracellular mitochondrial function, while the welfare of the mitochondria may be crucial to maintaining plasma membrane structure (Schilling and Patel, 2016). This intracellular relationship is key in facilitating successful, resilient responses to stress (Schilling and Patel, 2016). Furthermore, considering this in the context of membrane dynamics, it would be of interest to investigate how biological clocks influence mitochondrial membranes, and how this may coordinate with the plasma membrane to optimize cellular function. Overall, expanding our studies to membrane-mitochondrial interactions will define crucial roles for membrane microdomains, and can ultimately pave the way for elucidating the cellular basis of many pathophysiologies.

Hence, our findings present a holistic view of membrane microdomains as they pertain to maintaining cellular structure and function, and ultimately determining the cell's capacity to be resilient in its environment. Our studies lay groundwork for understanding how, when, and why the plasma membrane can function optimally, and helps identify targets for therapies that could optimize membrane function. Elucidating the crucial roles of functional membrane microdomains as essential players in membrane structure, membrane repair, and organelle function not only affirms their versatility, but also their fundamental position as mediators of cellular resilience.

REFERENCES

Andrews, N. W., Almeida, P. E., & Corrotte, M. (2014). Damage control: cellular mechanisms of plasma membrane repair. *Trends in Cell Biology*, *24*(12), 734–742. doi: 10.1016/j.tcb.2014.07.008

Aviram, R., Manella, G., Kopelman, N., Neufeld-Cohen, A., Zwighaft, Z., Elimelech, M., Adamovich, Y., Golik, M., Wang, C., Han, X., Asher, G. (2016). Lipidomics Analyses Reveal Temporal and Spatial Lipid Organization and Uncover Daily Oscillations in Intracellular Organelles. *Molecular Cell*, *62*(4), 636–648. doi: 10.1016/j.molcel.2016.04.002

Barceló Francisca, Perona, J. S., Prades Jesús, Funari Sérgio S., Gomez-Gracia, E., Conde, M., Estruch, R., Ruiz-Gutiérrez Valentina. (2009). Mediterranean-Style Diet Effect on the Structural Properties of the Erythrocyte Cell Membrane of Hypertensive Patients. *Hypertension*, *54*(5), 1143–1150. doi: 10.1161/hypertensionaha.109.137471

Barzilai-Tutsch, H., Dewulf, M., Lamaze, C., Browne, G. B., Pines, M., & Halevy, O. (2018). A promotive effect for halofuginone on membrane repair and synaptotagmin-7 levels in muscle cells of dysferlin-null mice. *Human Molecular Genetics*, *27*(16), 2817–2829. doi: 10.1093/hmg/ddy185

Blazek, A. D., Paleo, B. J., & Weisleder, N. (2015). Plasma Membrane Repair: A Central Process for Maintaining Cellular Homeostasis. *Physiology*, *30*(6), 438–448. doi: 10.1152/physiol.00019.2015

Blüher, M., Hentschel, B., Rassoul, F., & Richter, V. (2001). Influence Of Dietary Intake And Physical Activity On Annual Rhythm Of Human Blood Cholesterol Concentrations. Chronobiology International, 18(3), 541–557. doi: 10.1081/cbi-100103975

Busija, A. R., Patel, H. H., & Insel, P. A. (2017). Caveolins and cavins in the trafficking, maturation, and degradation of caveolae: implications for cell physiology. *American Journal of Physiology-Cell Physiology*, *312*(4). doi: 10.1152/ajpcell.00355.2016

Cai, C., Weisleder, N., Ko, J., Komazaki, S., Sunada, Y., Nishi, M., Takeshima, H., Ma, J. (2009). Membrane Repair Defects in Muscular Dystrophy Are Linked to Altered Interaction between MG53, Caveolin-3, and Dysferlin. *Journal of Biological Chemistry,284*(23), 15894-15902. doi:10.1074/jbc.m109.009589

Casares, D., Escribá, P. V., & Rosselló, C. A. (2019). Membrane Lipid Composition: Effect on Membrane and Organelle Structure, Function and Compartmentalization and Therapeutic Avenues. International Journal of Molecular Sciences, 20(9), 2167. doi: 10.3390/ijms20092167

Castellani, S., Gioia, S. D., Trotta, T., Maffione, A. B., & Conese, M. (2010). Impact of Lentiviral Vector-Mediated Transduction on the Tightness of a Polarized Model of Airway Epithelium and Effect of Cationic Polymer Polyethylenimine. *Journal of Biomedicine and Biotechnology*, *2010*, 1–11. doi: 10.1155/2010/103976

Cazzola, R., Rondanelli, M., Russo-Volpe, S., Ferrari, E., & Cestaro, B. (2004). Decreased membrane fluidity and altered susceptibility to peroxidation and lipid composition in overweight

and obese female erythrocytes. *Journal of Lipid Research*, *45*(10), 1846–1851. doi: 10.1194/jlr.m300509-jlr200

Chabanon, M., Ray, S., Schilling, J., Dhanani, M., Rangamani, P., Patel, H. (2020). Phenomenological Model of Cav-3-mediated Plasma Membrane Repair. Manuscript in preparation.

Cheng, J. P., & Nichols, B. J. (2016). Caveolae: One Function or Many? *Trends in Cell Biology*, *26*(3), 177–189. doi: 10.1016/j.tcb.2015.10.010

Cohen, A. W., Hnasko, R., Schubert, W., & Lisanti, M. P. (2004). Role of Caveolae and Caveolins in Health and Disease. *Physiological Reviews*, *84*(4), 1341–1379. doi: 10.1152/physrev.00046.2003

Cooper, R. A. (1978). Influence of increased membrane cholesterol on membrane fluidity and cell function in human red blood cells. *Journal of Supramolecular Structure*, *8*(4), 413–430. doi: 10.1002/jss.400080404

Corrotte, M., Almeida, P. E., Tam, C., Castro-Gomes, T., Fernandes, M. C., Millis, B. A., Cortez, M., Miller, H., Song, W., Maugel, T. K., Andrews, N. W. (2013). Caveolae internalization repairs wounded cells and muscle fibers. *ELife,2*. doi:10.7554/elife.00926

Corrotte, M., Castro-Gomes, T., Koushik, A., & Andrews, N. (2015). Approaches for plasma membrane wounding and assessment of lysosome-mediated repair responses. *Methods in Cell Biology Lysosomes and Lysosomal Diseases*, 139–158. doi: 10.1016/bs.mcb.2014.11.009

Demonbreun, A. R., & Mcnally, E. M. (2016). Plasma Membrane Repair in Health and Disease. *Current Topics in Membranes Dynamic Plasma Membranes - Portals Between Cells and Physiology*, 67–96. doi: 10.1016/bs.ctm.2015.10.006

Duann, P., Li, H., Lin, P., Tan, T., Wang, Z., Chen, K., Zhou, X., Gumpper, K., Zhu, H., Ludwig, T., Mohler, P. J., Rovin, B., Abraham, W. T., Zeng, C., Ma, J. (2015). MG53-mediated cell membrane repair protects against acute kidney injury. *Science Translational Medicine*, *7*(279). doi: 10.1126/scitranslmed.3010755

Fridolfsson, H. N., Kawaraguchi, Y., Ali, S. S., Panneerselvam, M., Niesman, I. R., Finley, J. C., Kellerhals, S. E., Migita, M. Y., Okada, H., Moreno, A. L., Jennings, M., Kidd, M. W., Bonds, J. A., Balijepalli, R. C., Ross, R. S., Patel, P. M., Miyanohara, A., Chen, Q., Lesnefsky, E. J., Head, B. P., Roth, D. M., Insel, P. A., Patel, H. H. (2012). Mitochondria-localized caveolin in adaptation to cellular stress and injury. *The FASEB Journal*, *26*(11), 4637–4649. doi: 10.1096/fj.12-215798

Garaulet, M., Lee, Y.-C., Shen, J., Parnell, L. D., Arnett, D. K., Tsai, M. Y., Lai, C.-Q., Ordovas, J. M. (2009). CLOCK genetic variation and metabolic syndrome risk: modulation by monounsaturated fatty acids. *The American Journal of Clinical Nutrition*, *90*(6), 1466–1475. doi: 10.3945/ajcn.2009.27536

Goh, G., Maloney, S., Mark, P., & Blache, D. (2019). Episodic Ultradian Events—Ultradian Rhythms. *Biology*, *8*(1), 15. doi: 10.3390/biology8010015

Gooley, J. J., & Chua, E. C.-P. (2014). Diurnal Regulation of Lipid Metabolism and Applications of Circadian Lipidomics. *Journal of Genetics and Genomics*, *41*(5), 231–250. doi: 10.1016/j.jgg.2014.04.001

Gooley, J. J. (2016). Circadian regulation of lipid metabolism. *Proceedings of the Nutrition Society*, *75*(4), 440–450. doi: 10.1017/s0029665116000288

Gordon, D. J., Trost, D. C., Hyde, J., Whaley, F. S., Hannan, P. J., Jacobs, D. R., & Ekelund, L. G. (1987). Seasonal cholesterol cycles: the Lipid Research Clinics Coronary Primary Prevention Trial placebo group. *Circulation*, *76*(6), 1224–1231. doi: 10.1161/01.cir.76.6.1224

Hagiwara, Y., Sasaoka, T., Araishi, K., Imamura, M., Yorifuji, H., Nonaka, I., Ozawa, E., Kikuchi, T. (2000). Caveolin-3 deficiency causes muscle degeneration in mice. *Human Molecular Genetics, 9*(20), 3047-3054. doi:10.1093/hmg/9.20.3047

Hahn-Obercyger, M., Graeve, L., & Madar, Z. (2008). A high-cholesterol diet increases the association between caveolae and insulin receptors in rat liver. *Journal of Lipid Research*, *50*(1), 98–107. doi: 10.1194/jlr.m800441-jlr200

Han, W.-Q., Xia, M., Xu, M., Boini, K. M., Ritter, J. K., Li, N.-J., & Li, P.-L. (2012). Lysosome fusion to the cell membrane is mediated by the dysferlin C2A domain in coronary arterial endothelial cells. *Journal of Cell Science*, *125*(5), 1225–1234. doi: 10.1242/jcs.094565

Hissa, B., Pontes, B., Roma, P. M. S., Alves, A. P., Rocha, C. D., Valverde, T. M., Aguiar, P. H. N., Almeda, F. P., Guimaraes, A. J., Guatimosim, C., Silva, A. M., Fernandes, M. C., Andrews, N. W., Viana, N. B., Mesquita, O. N., Agero, U., Andrade, L. O. (2013). Membrane Cholesterol Removal Changes Mechanical Properties of Cells and Induces Secretion of a Specific Pool of Lysosomes. *PLoS ONE*, *8*(12). doi: 10.1371/journal.pone.0082988

Hölttä-Vuori, M., Sezgin, E., Eggeling, C., & Ikonen, E. (2016). Use of BODIPY-Cholesterol (TF-Chol) for Visualizing Lysosomal Cholesterol Accumulation. *Traffic*, *17*(9), 1054–1057. doi: 10.1111/tra.12414

Hernández-Deviez, D. J., Howes, M. T., Laval, S. H., Bushby, K., Hancock, J. F., & Parton, R. G. (2007). Caveolin Regulates Endocytosis of the Muscle Repair Protein, Dysferlin. Journal of Biological Chemistry, 283(10), 6476–6488. doi: 10.1074/jbc.m708776200

Horikawa, Y. T., Panneerselvam, M., Kawaraguchi, Y., Tsutsumi, Y. M., Ali, S. S., Balijepalli, R. C., Murray, F., Head, B. P., Niesman, I. R., Rieg, T., Vallon, V., Insel, P. A., Patel, H. H., Roth, D. M. (2011). Cardiac-Specific Overexpression of Caveolin-3 Attenuates Cardiac Hypertrophy and Increases Natriuretic Peptide Expression and Signaling. *Journal of the American College of Cardiology*, *57*(22), 2273–2283. doi: 10.1016/j.jacc.2010.12.032

Iaea, D. B., & Maxfield, F. R. (2017). Membrane order in the plasma membrane and endocytic recycling compartment. *Plos One*, *12*(11). doi: 10.1371/journal.pone.0188041

Jimenez, A. J., Maiuri, P., Lafaurie-Janvore, J., Divoux, S., Piel, M., & Perez, F. (2014). ESCRT machinery is required for plasma membrane repair. *Science, 343*(6174), 1247136. doi:10.1126/science.1247136

Krapf, D. (2018). Compartmentalization of the plasma membrane. Current Opinion in Cell Biology, 53, 15–21. doi: 10.1016/j.ceb.2018.04.002

Laude, A. J., & Prior, I. A. (2004). Plasma membrane microdomains: Organization, function and trafficking (Review). Molecular Membrane Biology, 21(3), 193–205. doi: 10.1080/09687680410001700517

Lodish, H., Berk, A., Zipursky, S. L., Matsudaira, P., Baltimore, D., & Darnell, J. (2000). Transport Across Cell Membranes. In *Molecular Cell Biology 4th edition.* W H Freeman & Co.

Loizides-Mangold, U., Perrin, L., Vandereycken, B., Betts, J. A., Walhin, J.-P., Templeman, I., Chanon, S., Weger, B. D., Durand, C., Robert, M., Monyoya, J. P., Moniatte, M., Karagounis, L. G., Johnston, J. D., Gachon, F., Lefai, E., Riezman, H., Dibner, C. (2017). Lipidomics reveals diurnal lipid oscillations in human skeletal muscle persisting in cellular myotubes cultured in vitro. *Proceedings of the National Academy of Sciences*, *114*(41). doi: 10.1073/pnas.1705821114

Menet, J. S., Pescatore, S., & Rosbash, M. (2014). CLOCK:BMAL1 is a pioneer-like transcription factor. *Genes & Development*, *28*(1), 8–13. doi: 10.1101/gad.228536.113

Modzel, M., Lund, F. W., & Wüstner, D. (2017). Synthesis and Live-Cell Imaging of Fluorescent Sterols for Analysis of Intracellular Cholesterol Transport. *Methods in Molecular Biology Cholesterol Homeostasis*, 111–140. doi: 10.1007/978-1-4939-6875-6_10

Molecular Probes, Inc., Vybrant Lipid Raft Labeling Kits. (2003, September 4). Retrieved from *https://www.thermofisher.com/document-connect/document-connect.html?url=https://assets.thermofisher.com/TFS-Assets/LSG/manuals/mp34403.pdf&title=VnlicmFudCBMaXBpZCBSYWZ0IExhYmVsaW5nIEtpdHM*

Molecular Probes, Inc., Propidium Iodide Nucleic Acid Stain. (2006). Retrieved from *https://www.thermofisher.com/document-connect/document-connect.html?url=https://assets.thermofisher.com/TFS-Assets/LSG/manuals/mp01304.pdf&title=UHJvcGlkaXVtIElvZGlkZSBOdWNsZWljIEFjaWQgU3RhaW4*

Moore, R. Y. (2001). Suprachiasmatic Nucleus. In *International Encyclopedia of the Social & Behavioral Sciences*(pp. 15290–15294).

Pathak-Sharma, S., Zhang, X., Lam, J. G. T., Weisleder, N., & Seveau, S. M. (2017). High-Throughput Microplate-Based Assay to Monitor Plasma Membrane Wounding and Repair. *Frontiers in Cellular and Infection Microbiology*, *7*. doi: 10.3389/fcimb.2017.00305

Pesta, D., & Gnaiger, E. (2011). High-Resolution Respirometry: OXPHOS Protocols for Human Cells and Permeabilized Fibers from Small Biopsies of Human Muscle. *Mitochondrial Bioenergetics Methods in Molecular Biology*, 25–58. doi: 10.1007/978-1-61779-382-0_3

Perona, J. S. (2017). Membrane lipid alterations in the metabolic syndrome and the role of dietary oils. *Biochimica Et Biophysica Acta (BBA) - Biomembranes*, *1859*(9), 1690–1703. doi: 10.1016/j.bbamem.2017.04.015

Pike, L. J. (2003). Lipid rafts. *Journal of Lipid Research*, *44*(4), 655–667. doi: 10.1194/jlr.r200021-jlr200

Schilling, J. M., & Patel, H. H. (2016). Non-canonical roles for caveolin in regulation of membrane repair and mitochondria: Implications for stress adaptation with age. *The Journal of Physiology,594*(16), 4581- 4589. doi:10.1113/jp270591

Schilling, J. M., Head, B. P., & Patel, H. H. (2018). Caveolins as Regulators of Stress Adaptation. *Molecular Pharmacology*, *93*(4), 277–285. doi: 10.1124/mol.117.111237

Sharma, D. K., Brown, J. C., Choudhury, A., Peterson, T. E., Holicky, E., Marks, D. L., Simari, R., Parton, R. G., Pagano, R. E. (2004). Selective Stimulation of Caveolar Endocytosis by Glycosphingolipids and Cholesterol. *Molecular Biology of the Cell*, *15*(7), 3114–3122. doi: 10.1091/mbc.e04-03-0189

Shvets, E., Bitsikas, V., Howard, G., Hansen, C. G., & Nichols, B. J. (2015). Dynamic caveolae exclude bulk membrane proteins and are required for sorting of excess glycosphingolipids. *Nature Communications*, *6*(1). doi: 10.1038/ncomms7867

Stary, C. M., Tsutsumi, Y. M., Patel, P. M., Head, B. P., Patel, H. H., & Roth, D. M. (2012). Caveolins: targeting pro-survival signaling in the heart and brain. *Frontiers in Physiology*, *3*. doi: 10.3389/fphys.2012.00393

Terasaki, M., Miyake, K., & Mcneil, P. L. (1997). Large Plasma Membrane Disruptions Are Rapidly Resealed by Ca2 -dependent Vesicle–Vesicle Fusion Events. The Journal of Cell Biology, 139(1), 63–74. doi: 10.1083/jcb.139.1.63

Togo, T., Krasieva, T. B., & Steinhardt, R. A. (2000). A Decrease in Membrane Tension Precedes Successful Cell-Membrane Repair. *Molecular Biology of the Cell*, *11*(12), 4339–4346. doi: 10.1091/mbc.11.12.4339

Togo, T. (2017). Cell membrane disruption stimulates cAMP and Ca2 signaling to potentiate cell membrane resealing in neighboring cells. Biology Open, 6(12), 1814–1819. doi: 10.1242/bio.028977

Toh, K. L. (2008). Basic Science Review on Circadian Rhythm Biology and Circadian Sleep Disorders. *Annals Academy of Medicine Singapore*. Retrieved from https://www.ncbi.nlm.nih.gov/pubmed/18797559

Veen, D. R., & Gerkema, M. P. (2016). Unmasking ultradian rhythms in gene expression. *The FASEB Journal*, *31*(2), 743–750. doi: 10.1096/fj.201600872r

Williams, T. M., & Lisanti, M. P. (2004). The Caveolin genes: from cell biology to medicine. Annals of Medicine, 36(8), 584–595. doi: 10.1080/07853890410018899

Woodman, S. E., Park, D. S., Cohen, A. W., Cheung, M. W.-C., Chandra, M., Shirani, J., Tang, B., Jelicks, L. A., Kitsis, R. N., Christ, G. J., Factor, S. M., Tanowitz, H. B., Lisanti, M. P. (2002). Caveolin-3 Knock-out Mice Develop a Progressive Cardiomyopathy and Show Hyperactivation of the p42/44 MAPK Cascade. *Journal of Biological Chemistry*, *277*(41), 38988–38997. doi: 10.1074/jbc.m205511200

Yang, S.-T., Kreutzberger, A. J., Lee, J., Kiessling, V., & Tamm, L. K. (2016). The role of cholesterol in membrane fusion. Chemistry and Physics of Lipids, 199, 136–143. doi: 10.1016/j.chemphyslip.2016.05.003