Cell Cycle Determines Critical Temperature in Plasma Vesicles

Honors Thesis in Biophysics

Erin Gray

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Advisor: Dr. Sarah Veatch, University of Michigan

Abstract

Giant plasma membrane vesicles (GPMVs) isolated from RBL-2H3 cells appear uniform at physiological temperatures, contain coexisting liquid-ordered and liquid-disordered phases at low temperatures, and experience micron-sized critical fluctuations close to their critical temperature. We observe a broad distribution of critical temperatures in GPMVs isolated from a dish of cells even though individual vesicles have a well-defined critical temperature. We hypothesized that critical temperatures are linked to cell cycle and tested this hypothesis by isolating GPMVs from cells at various stages. Populations of cells were synchronized at S, G2, M, and G1 stages using a double Thymidine block that arrests cells at the border between G1 and S phases. Critical temperatures were elevated in GPMVs from cells in cell cycle phases that immediately precede cell division (G2 and M) compared to the other stages (G1 and S). In a separate experiment, we plated cells in low serum media to produce a population of cells with arrested growth, and found low critical temperatures in GPMVs isolated from these cells (~10°C). Critical temperatures recovered to typical values (~20°C) after incubating the growth-arrested cells in serum rich media for 24h. We also find that densely plated cells yield GPMVs with lower average critical temperatures than GPMVs from less densely plated cells. Finally, cells were treated with TRAIL, a drug that induces apoptosis, and again critical temperatures were low (~14°C). These results suggest that the magnitude of plasma membrane heterogeneity may be dependent on the cell cycle.

Chapter 1: Introduction

1.1 Membrane structure

Biological membranes are structured according to the 'fluid mosaic model,' with a two dimensional lipid bilayer separating the inner and extracellular environments and interspersed globular proteins and oligosaccharides (Singer, 1972). Membrane lipids are amphipathic molecules characterized by hydrophobic groups or fatty acid chains within and a hydrophobic head group on the surface. Lipids that can be found in biological membranes include phospholipids, glycolipids, and cholesterol. Phospholipids are the primary lipid in the membrane and contain two hydrophobic fatty acid chains. Cholesterol plays many important roles: it alters membrane fluidity, inhibits the formation of gel or solid phases, and is required for the formation of the liquid ordered membrane phase described below (Veatch, 2005). Non-covalent hydrophobic interactions hold the lipids together; however, the structure is still quite fluid, and phospholipids and proteins are free to diffuse laterally across the surface. Additionally, the membrane is coupled to cortical cytoskeleton, which impacts the diffusion rates and localization of lipids and integrated proteins (Kusumi, 2012).

1.2 Lipid Domains and Phase Separation

The lipid bilayer is a mixture of lipids and is present in many different phases, both in cell membranes and synthesized bilayers from purified components. One such phase

is the gel phase, where lipids are packed tightly and can no freely diffuse laterally. In the liquid ordered phase, lipids experience rapid lateral and rotational diffusion while being moderately well packed, which is facilitated by the presence of cholesterol. Finally, the liquid disordered phase is characterized by more loosely packed lipids, which frequently have higher degrees of unsaturation, and therefore more 'kinks' that prevent tight packing (Simons, 1997). Membranes can experience a miscibility transition, where the membrane passes from a uniform single phase at higher temperature to two distinct phases at lower temperatures, either gel and liquid, or liquid-ordered and liquid-disordered.

Plasma membranes from mammalian cells isolated from cortical cytoskeleton have a liquid-liquid miscibility transition below growth temperatures (ref Baumgart). Remarkably, they additionally have critical compositions, meaning they pass through a special critical point at their miscibility transition temperature or critical temperature (T_c) (Veatch et al. , 2008). When above the T_c, the membrane exists as a single, yet heterogeneous liquid phase. Within a few degrees of T_c, micron-sized fluctuations are observed when isolated plasma membrane vesicles are imaged using fluorescence microscopy. Super-critical fluctuations increase in size and magnitude as the temperature is lowered. Right below the critical temperature, two distinct phases are visible on the vesicle surface, but fluctuations are observed at domain boundaries. At low temperatures, vesicles completely separate into two phases, with roughly half the vesicle in each phase. Figure 1.1 shows a vesicle passing through this

transition temperature. As temperature decreases, the domains (fluorescently labeled bright and dark phases) become larger.

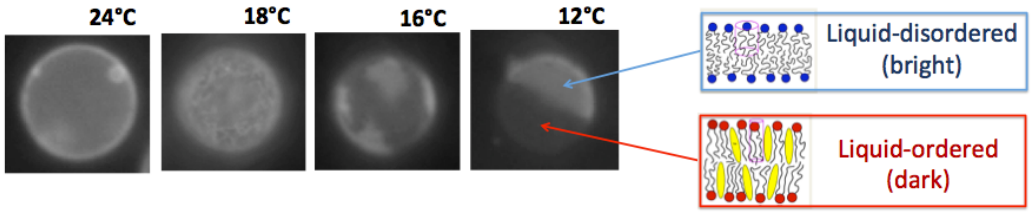

Figure 1.1: A fluorescently labeled giant plasma membrane vesicle as it passes through a phase transition temperature. The liquid-disordered phase is fluorescently labeled while the liquid-ordered phase is not. At 24°C the membrane is one uniform phase, but as the temperature decreases towards 16°C the domains are clearly visible. At 12°C the membrane has completely phase separated in two distinct hemispheres.

We use giant plasma membrane vesicles (GMPVs) as a model membrane. GPMVs are vesicles formed from a cell membrane that is no longer coupled to the actin cytoskeleton. They maintain the lipid and protein composition of the original cell and therefore act as a very practical and accurate model of phase behavior in membranes. Phase transition temperatures in mammalian derived GPMVs are typically observed to be from 15 to 25°C. The two distinct regions that form can be imaged using fluorescence microscopy and a fluorescent dye that partitions specifically into one particular phase. Figure 1.2 shows fluorescently labeled GPMVs still attached to cells. While the GPMV can phase separate into large domains, adjacent regions of intact cell remain unstructured on the micro-scale even though they presumably have the same plasma membrane composition.

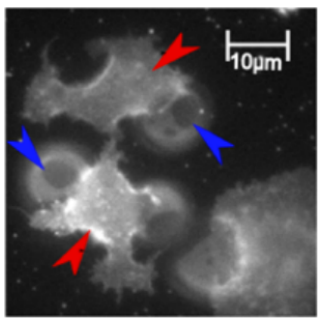

Figure 1.2 Blebs attached to rat basophilic leukemia cells. Blebs are marked with blue arrows and cells with red arrows. The membranes are labeled with DiI-C12 Figure produced from: Machta BB, Papanikolaou S, Sethna JP, Veatch SL (2011). *Biophys J*. **100**: 1668-1677.

1.3 The Cell Cycle

Cell cultures will grow and divide when space and nutrients are provided (Chow, 2010). Cells will progress through the cell cycle and continue to divide until a lack of resources or chemical or mechanical signals indicates that they should stop (Chow, 2010). The major stages of the cell cycle are G1, S, G2, and M (as shown in figure 1.3). G1 phase is the first gap phase. Cells increase in size and must pass the G1 checkpoint, which is a control mechanism that ensures that everything is ready for DNA synthesis. S phase is also known as synthesis, when DNA replication occurs. G2 phase is the second gap phase. During this gap between DNA synthesis and mitosis, the cell continues to grow. The G2 checkpoint control mechanism ensures that everything is ready to enter the M phase and divide. Finally, the M phase, or mitosis, is where cell growth stops. At this stage, cellular energy is focused on the orderly division into two daughter cells. A checkpoint in the middle of mitosis ensures that the cell is ready to complete cell division. There is also the G0 phase, which is the resting state a cell enters when if it stops dividing because of a lack of growth factors or nutrients.

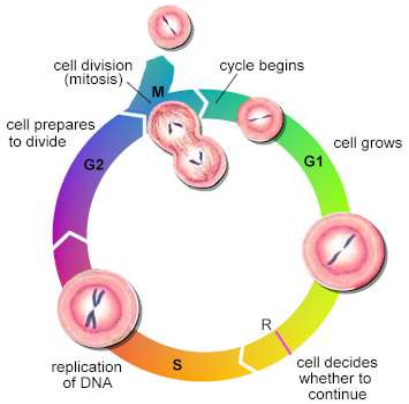

Figure 1.3 Cells progress through the cell cycle G1→S→G2→M. *The Encyclopidia of Science (June 2012)*

1.4 Lipid Changes during the Cell Cycle

Beginning soon after mitosis, cellular phospholipids double during each cell cycle, leaving the bulk of phospholipid accumulation to occur in the S phase (Jackowski, 1994). Beyond the requirements of needing adequate lipids to maintain the integrity of cellular and intracellular compartments, lipids and sterols are known to play important roles in the maintenance and regulation of signaling pathways involved in the cell cycle. For example, several studies have indicated that the tyrosine kinase activity of growth factor receptors is influenced by their local lipid environment (Coskun, 2011; Ringerike, 2002). Also, commonly mutated proteins downstream of growth factor receptors, such as RAS, are regulated in part through plasma membrane lipids (Schubbert, 2007) and alteration of plasma membrane lipids recruits the tumor suppressor PTEN to the plasma membrane, which is vital for its roles in maintaining control of cell growth (Goswami, 2005). Most of past work in this area has focused on the important roles of lipids as second messengers, although some studies have noted changes in the mobility of plasma membrane lipids and proteins as a function of cell cycle position suggesting that membrane physical properties are also affected (de Laat, 1980; Jaconson, 1984).

1.5 Overview

We aim to explore the changes in the temperature at which the membrane experiences critical fluctuations during the cell cycle. In this study, a broad distribution of critical temperatures was observed in GMPVs isolated from a dish of

cells even though individual vesicles have a well-defined critical temperature. Populations of cells were then synchronized at the different stages of the cell cycle, using a double Thymidine block that arrests cells. Critical temperatures were elevated in GPMVs from cells in cell cycle phases that immediately precede cell division (G2 and M) compared to the other stages (G1 and S). In a separate experiment, cells were plated in low serum media to produce a population of cells with arrested growth, and we found low critical temperatures in GPMVs isolated from these cells (~10°C). We find that densely plated cells, also growth arrested, yield GPMVs with similar lower average critical temperatures than GPMVs from less densely plated cells. Using giant plasma membrane vesicles and fluorescence microscopy, we can begin to characterize the impact cell cycle phase has on determining membrane behavior.

Chapter 2: Methodology

2.1 Experimental Set-Up

2.1.1 Cell Culture

In a 25cm^2 cell culture flask, Rat Basophilic Leukemia (RBL) – 2H3 mast cells were incubated at 37°C in 5% CO2 and were passaged using 0.25% Trypsin/EDTA and RBL media 24 hours prior to the GPMV preparation process. RBL media was composed of 800 mL MEM 1X with L-glutamine and earle salts, 200mL fetal bovine serum, and 1 mL gentamicin. Trypsin/EDTA and RBL media were warmed to 37°C. The cells were washed twice with 0.25% Trypsin/EDTA and then incubated for 5 min with 2 mL Trypsin/EDTA. After the cells no longer adhered to the flask, they were centrifuged at 500

rev/min for five minutes with 4 mL media and resuspended in an appropriate amount of media for a final concentration of 10^6 cells/mL. The RBL cells were plated in new flasks and doubled in concentration overnight.

2.1.2 Preparation of Labeling Buffer

To prepare the labeling buffer, 2 mL DiI-C12 (Invitrogen) at a concentration of 10 mg/mL in methanol (Sigma Aldrich) was added to 100 mL methanol. 100 µL of the solution was then added to 10 mL bleb buffer. DiI-C12 was a fluorescent dye that integrates into the liquid disordered region of the membrane, had an extinction coefficient of 150,00 $M^{-1}cm^{-1}$, and was excited by 532 nm light. When viewed under the microscope, this allowed for the imaging of a distinct light phase and dark phase when vesicles were phase separated.

2.1.3 Preparation of Active Buffer

To prepare the active buffer, 15 mg dithiothreitol (DTT) (Sigma Aldrich) was dissolved in 100 µL of 36% formaldehyde (Fisher Scientific). This was vortexed for a few seconds until the DTT was completely dissolved in formaldehyde. 20 µL of this solution was added to 10 mL bleb buffer for a final concentration of 0.3 mg/mL DTT.

2.1.4 GMPV Preparation

The process of preparing GMPVs involved chemically interfering with cell functioning in a manner such that parts of the cell membrane detached from the cytoskeleton and pinched off to form vesicles containing cytoplasm (Holowka and Baird 1984). Bleb buffer was prepared as a 1 L stock solution with water and 150 mM NaCl, 2 mM $CaCl_2$, and 20 mM

Hepes. The solution was set to a pH of 7.4. RBL cells were washed twice with bleb buffer and then 1 mL of the labeling buffer was added. The cells were left to shake at 100 rev/min for five minutes at 37°C. After the cells had labeled, they were washed twice with bleb buffer, once with active buffer, and then 1 mL of active buffer was added to the flask. The cells were left to shake at 100 rev/min for one hour. After one hour, the blebs were floating in the buffer and extracted from the flask with a pipette, without disturbing attached cells. 20μL of this volume was pipetted onto a cover slip, and another cover slip was placed on top using vacuum grease. For a higher density of blebs, the vacuum grease can be applied thicker so as to allow a greater number of blebs per unit area after the blebs sink to a single plane of view.

2.1.5 Synchronizing Cells

Cells were cultured similarly to that described in 2.1.1. When the RBL cell culture was 25%-30% confluent, they were washed twice with 1X sterile PBS and then 2mL of RBL media and 2mM Thymidine (Signma Aldrich) was added. Excess thymidine in a mitotic cell generates negative feedback on the production of deoxycytidine triphosphate from cytidine-5'-phosphate. Excess quantities of deoxyadenosine and deoxyguanosine also block progression through S-phase. However, as a reagent for the control of replication timing, thymidine has been found to work best as its blocking activity can be applied and reversed more consistently.

This cell culture was incubated for 18 hours (first block). Afterwards, the thymidine was removed by washing with 1X PBS. The flask must then be split into multiple flasks so that

a sample is available for each desired time point. If 1 sample per phase of the cell cycle was needed, the cell culture was split into 4 separate flasks by following the protocol in 2.1.1. If 1 sample was needed per hour, the cell culture was split into 12 separate flasks. After this process, fresh RBL media was added for 9 hours to release cells so they would continue to progress through the cell cycle. Next, 2mL of RBL media and 2mM thymidine was added for 17 hours (2^{nd} block). Finally, this thymidine was removed by washing with 1X PBS. Cells were released by adding fresh RBL media. All cells then began progressing through the cell cycle beginning in the S phase.

2.1.6 Serum Deprivation

A confluent RBL cell culture was used to make 3 flasks with differing amounts of nutrients. One was a control, with the normal 20% serum in the RBL media. The other two were 0.5% serum and 0% serum. The slowed growth rate must be taken into account when deciding how many cells to plate in each flask so that a similar density of cells is obtained at the time of GPMV preparation. To begin, the cells were washed twice with 0.25% Trypsin/EDTA at 37°C and then incubated for 5 min with 2 mL Trypsin/EDTA.

After the cells no longer adhered to the flask by adding Trypsin, a tube of cells and media was made for each serum condition. In the 20% tube, 0.5mL of cells/Trypsin was added to 4mL of 20% serum RBL media. In the 0.5% tube, 0.75mL of cells/Trypsin was added to 4mL of 0.5% serum RBL media. In the 0% tube, 0.75mL of cells/Trypsin was added to 4mL of 0% serum RBL media. (See 2.1.1 for 20% serum media composition.) To make 0.5% serum RBL media, 250µL of 20% serum and 10µL Gentamicin were added to

9.74 mL of MEM. To make 0% serum RBL media, 10μL Gentamicin was added to 8mL of MEM. Each of these samples of cells were then centrifuged at 500 rev/min for five minutes and next resuspended in an appropriate amount of media (either 20%, 0.5%, or 0% serum for a final concentration of 10^6 cells/mL. The RBL cells were plated in new flasks and left overnight overnight.

2.1.7.1 Adding Back Serum

Following the procedure outlined in 2.1.6, one 20% serum flask and four 0% serum flasks were created. The breakdown of the cells/Trypsin solution was 0.3mL cells/Trypsin into 20% serum tube with 4mL 20% serum RBL media and 1.7mL cells/Trypsin into 0% serum tube with 4mL 0% serum RBL media. After centrifuging, resuspending, and letting sit overnight, one 20% serum flask and one 0% serum flask were processed to create GMPVs. 20% serum RBL media was added back into the other three 0% serum flasks. One of these flasks is processed to make GMPVs after one hour, the next is processed after 4 hours, and the final one is processed the following day.

2.1.8 Apoptosis

A flask of confluent RBL cells was split into two flasks and left to sit overnight. The next day, one flask was drained and .1μg of TRAIL and 1mL of media was added. This was shaken at 37 °C for half an hour at 80 rev/min. GPMV preparation followed.

2.2 Data Acquisition

2.2.1 Microscope

An IX81 microscope, equipped with a mercury lamp (OLYMPUS) and SCMOS camera (ANDOR) was used to image the GPMV samples. Light was from an Olympus U-RFL-T power source, which passes through a filter (selects for 535±25nm excitation and 610±33nm emission wavelengths) and a 40X objective. Temperature of the sample was controlled using a temperature stage from Oven Industries, Inc. Cover slips were adhered to the stage using Arctic Alumina Premium Ceramic Polysynthetic Thermal Compound and Arctic Silver 5 High-Density Polysynthetic Silver Thermal Compound.

2.2.2 Imaging

Images samples are viewed with an Andor Neo Camera using Andor SOLIS software. The display was set to min/max mode and acquisition time between .01 and 0.15 seconds. Blebs were imaged at temperatures ranging from 25°C to 10°C in increments of 2 or 3°C. Near the phase transition temperature data was acquired in smaller temperature intervals. At each temperature, about 200 to 500 blebs were imaged (about 10 frames as shown in figure 2.1) in which the plane of focus was on the membrane surface for the majority of blebs. It was also confirmed for each frame that the majority of GPMVs could be distinguished as being ‘phase separated’ (two-phase) or ‘non-phase separated’ (one-phase). Images were saved in individual files for each temperature.

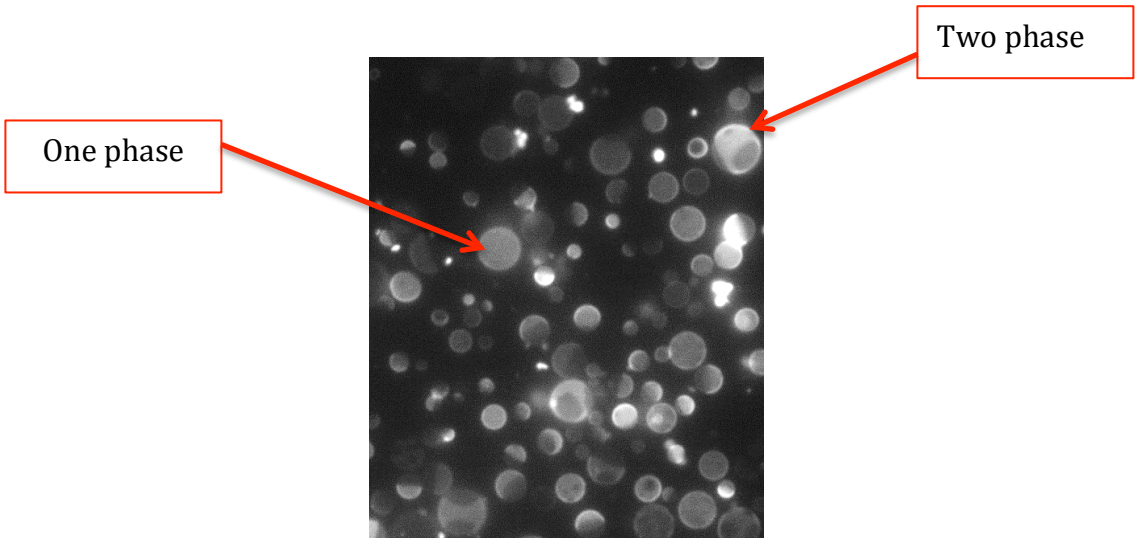

Figure 2.1: Image of a field of blebs in which almost all GPMVs can be unambiguously labeled as one phase or two phase.

2.3 Data Analysis

Data was analyzed using the MatLab program 'count_vesicles'. In this program, at each given temperature of a sample the program runs through all frames and the user right-clicks for non-phase separated vesicles and left-clicks for phase separated vesicles. Phase separation is defined as the presence of a distinct light and dark phase in a single vesicle. Right-clicked vesicles are tagged with a yellow marker and lift-clicked with a red marker to prevent the double counting of vesicles. The program creates a histogram plot for these two states at each temperature. After this procedure is followed the program 'plot_countdata' is used to fit the data to a sigmoidal curve (equation 2.1) showing the percentage of phase-separated vesicles versus temperature. T_M, the phase transition temperature, is determined to be the point at which fifty percent of vesicles are phase separated.

$$\%Separated = 100 \times (1 - \frac{1}{1 + e^{(T - T_M)/B}}) \qquad (2.1)$$

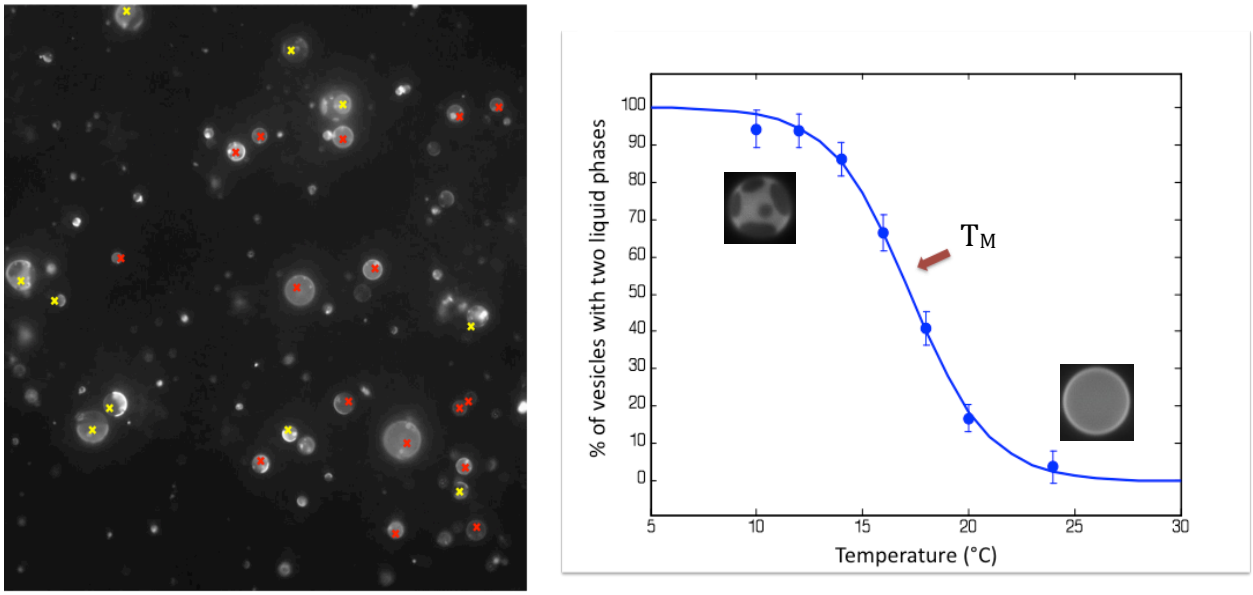

Figure 2.2: (a) Field of blebs in which phase separated blebs are labeled with a red marker and non-phase separated blebs are labeled with a yellow marker. (b) Sigmoidal curve fit showing the percentage of phase separated vesicles over a range of temperatures. The transition temperature, T_M, is marked at the point where 50% of vesicles are phase separated.

Chapter 3: Results

3.1 Variation in Transition Temperature During the Cell Cycle

After cells were synchronized, a sample of GMPVs was taken from each phase of the cell cycle, and plates were imaged, the changes in transition temperature were analyzed. A sigmoidal curve was created for each of the four major phases in the cycle (Figure 3.1a). Error bars were defined as 100/sqrt(N-1) where N is the number of vesicles counted for each temperature. Each of the four flasks of cells were blebbed to create GMPVs, imaged, and analyzed to calculate the temperature at which half of the GMPVs transitioned to displaying two phases. In each trial, there was a

distinct difference between the phase transition temperatures of the first two stages versus the final phases of the division.

Five trials were performed and the results were averaged to see the change in transition temperature at each of the four stages of the cell cycle. The transition temperature of the GMPVs created from the cells in the G1 phase was used as a base line that the GMPVs from the other phases were compared. The GMPVs from the cells in the S phase were found to be the lowest, approximately 2°C below the G1 GMPVs. On the other hand, GMPVs from the G2 and M phase exhibited transition temperatures greater than G1 temperature by about 2-3°C (Figure 3.1b). Error bars were created by calculating the standard error of the mean for the five trials.

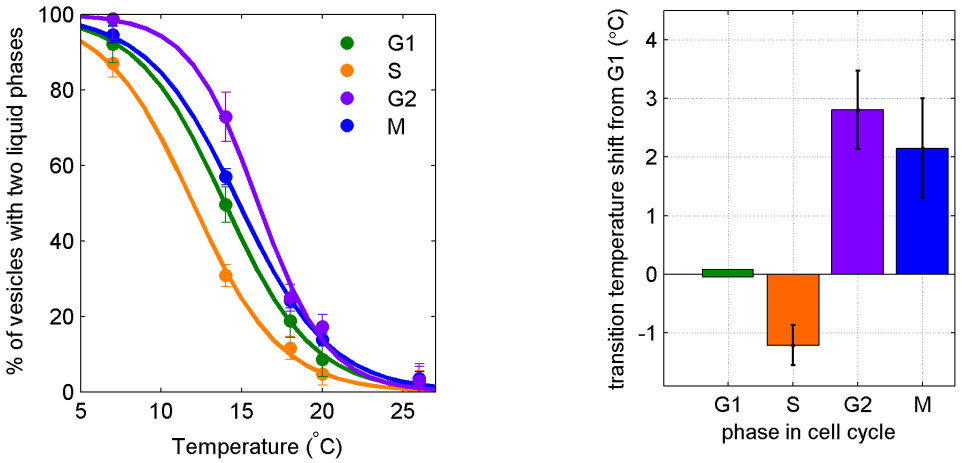

Figure 3.1: (a) Sigmoidal curves fir for a sample of GMPVs from each of the four phases of the cell cycle. (b) Change in transition temperature of a sample of GMPVs as a cell progresses through the cell cycle. Transition temperatures are elevated in G2 and M phase cells.

An experiment was also run to see if the transition temperature gradually changed between phases of the cell cycle, or if the change occurred abruptly. A sample of cells

was taken every hour for 16 hours as the cell progressed through the cell cycle. GPMVs were prepared, imaged, and analyzed to calculate the temperature at which half of the GMPVs transitioned to displaying two phases. Three trials were conducted and the results were averaged to determine the transition temperature throughout the cell cycle. The original data from each trial can be seen as points plotted in gray on the below graph. Error bars were created by calculating the standard error of the mean for the four trials. It was clear that the big changes in transition temperature we saw in out first trial between the S and G2 stage and M and G1 phase were indeed a product of the change cell cycle phase (Figure 3.2). It was found that transition temperatures were significantly elevated when the cell is dividing (G2 and M phase) compared to t

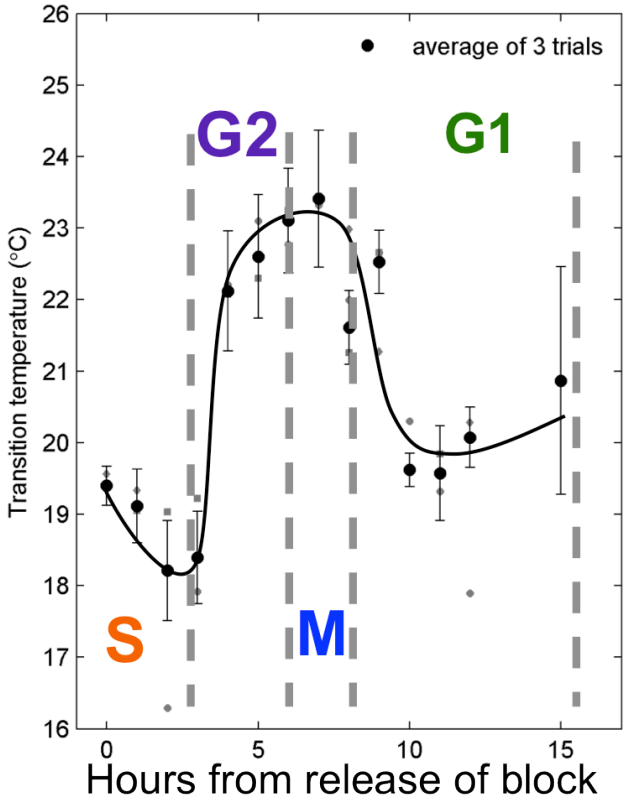

Figure 3.2: Continuous transition temperatures of sample of cells progressing through the cell cycle, taken every hour.

3.2 Decreased Transition Temperatures in G0 GMPVs

The change in transition temperature when the cell was resting in the G0 phase was also explored. Two methods were used to arrest the cells in this phase, by overgrowing cells in a flask and by depriving cells of serum. It is well established that when the density of a flask is increased, cell growth was limited due to contact inhibition (Klug, 2012). When there was no more space for cells to divide, the cells became growth arrested until more space became available (Stoker, 1967). Previously unpublished work by Sarah Veatch looked at the impact of cell flask density on transition temperature. The temperature measurements were done using the same methods described in this thesis. The number of cells per frame was determined by taking pictures of cells prior to GPMV preparation, then counting the number of cells per area using an automated image-processing algorithm. This experiment was repeated for three separate trials with 4-5 flasks of different number of cells per trial. It was found that transition temperature was inversely proportional to the surface density of the flask from which the GPMVs are extracted (Figure 3.3a). The higher density flasks exhibited lower transition temperatures than the lower density flasks.

The second method of arresting cells was by growing cells in serum-deprived media. GPMVs from cells grown without serum for 24h exhibited lower transition temperatures than GPMVs from cells grown in normal (20%) serum (Figure 3.3b). Transition temperatures were able to recover to control values when serum starved cells were incubated in normal serum for 24 hours prior to GPMV preparation.

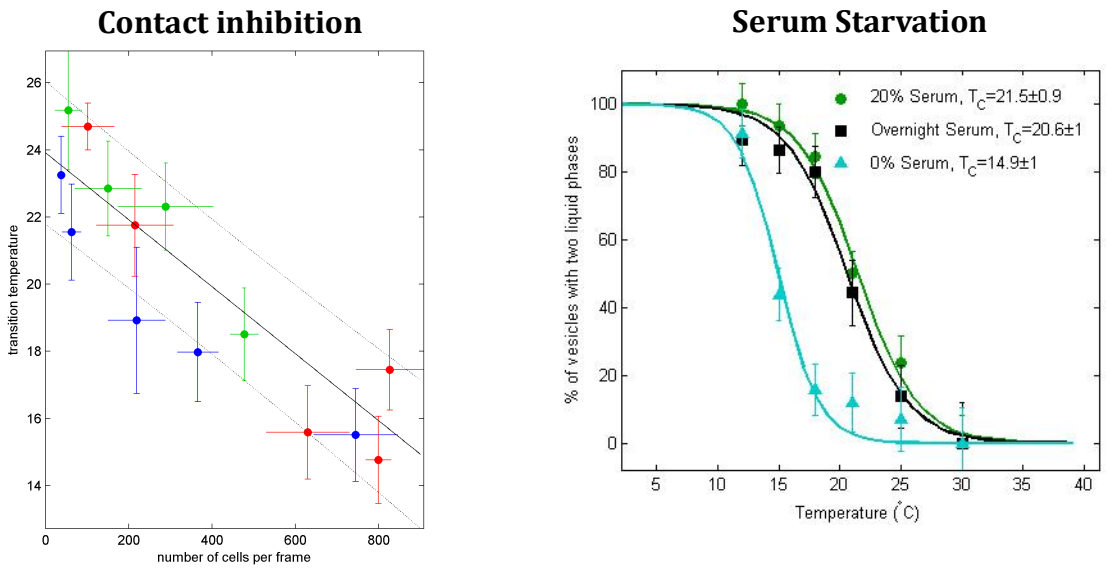

Figure 3.3: (a) Critical temperature is inversely proportional to the surface density of the flask from which the GPMVs are extracted. Crowded cells are contact inhibited. (b) Sigmoidal curves of GPMVs from cells grown without serum for 24h, cells grown with serum, and cells that had serum added after 24 hours. GMPVs exhibited lower critical temperatures when cells were grown without serum.

Overall, the GMPVs that were arrested in the G0 phase by either serum starvation or contact inhibition experienced lower transition temperatures than cells that were able to divide. Four trials were performed and the results were averaged to determine the transition temperature of GMPVs from cells in the G0 phase (Figure 3.4). This followed the previous trend, because GMPVs that were far away from dividing (G0, G1, and S cells) all experienced lower critical temperatures than GMPVs that could actively divide (M and G2 cells).

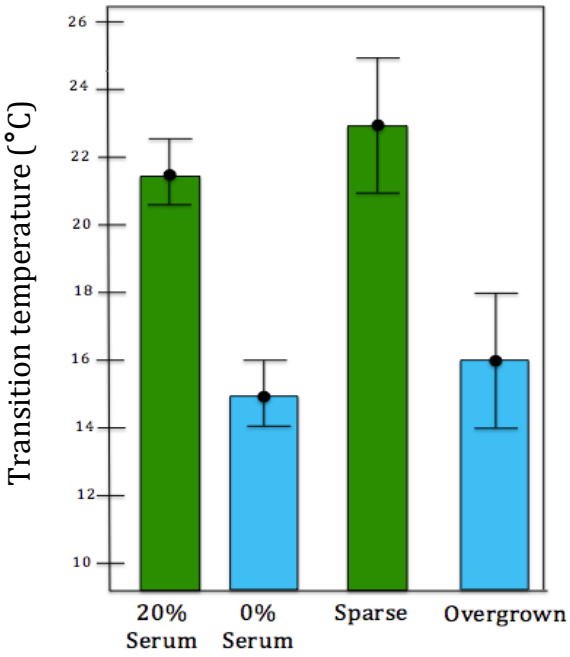

Figure 3.4: Critical temperatures are depressed in serum starved and contact inhibited cells.

3.3 Depressed Transition Temperatures in GMPVs from cells undergoing Apoptosis

Cells were treated with TRAIL (TNF-related apoptosis-inducing ligand), a ligand that induces the process of cell death called apoptosis. It is a cytokine that is naturally produced and secreted by most normal tissue cells. An example of the GPMVs created from cells undergoing apoptosis can be seen in Figure 3.5. GMPVs from the cells undergoing apoptosis were expected to have membranes comparable to G0 and G1 cells, as opposed to the ready to divide M and G2 phase cells. This was seen in by the decrease in transition temperature comparable to that of the non-dividing cells (Figure 3.5b).

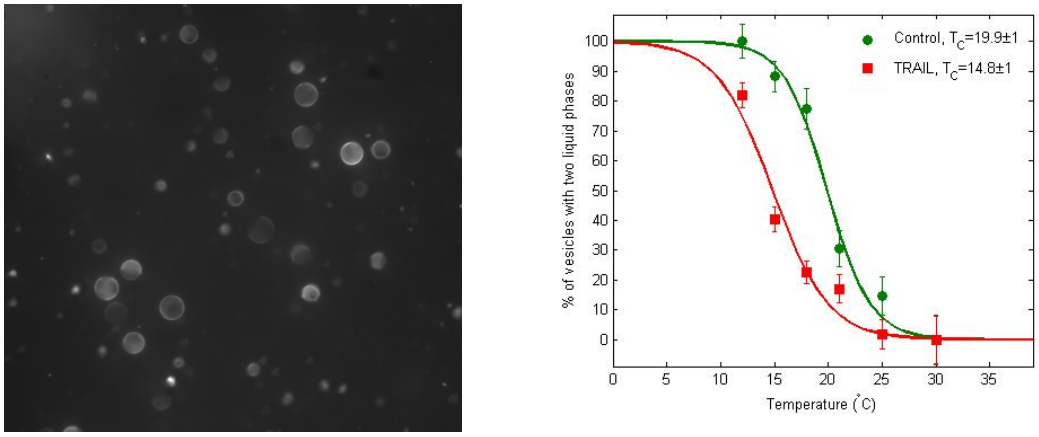

Figure 3.5: (a) GPMVs from cells treated with TRAIL to induce apoptosis. (b) Cells treated for 30 min with TRAIL produced GPMVs with lower critical temperatures.

Chapter 4: Discussion

In this study, we demonstrate that the stage of the cell cycle prior to GMPV preparation impacts the transition temperature by several degrees. During the stages directly prior to division, G2 and M, transition temperatures are elevated while in the other stages (G0, G1, and S) transition temperatures were depressed. Since the transition temperature varies, it can be said with reasonable certainty that cells have a different lipid composition of their membranes during these different stages. In order to test this hypothesis, Lipodomics can be done on a population of cells from each of the five stages.

One specific property that we can study is the formation of lipid domains, sometimes referred to as 'lipid rafts' in cellular membranes. It has been proposed that the

formation of these lipid rafts has a functional role in key biological processes such as immune signaling and endocytosis. I hypothesize that cells about to go through cell division have increased membrane heterogeneity or organization. On the other hand, cells that are in the G0 phase are in a state of rest and do not need well coordinated membrane activities to be occurring. To test this hypothesis, we will explore the potential mean force using 2-color, super resolution microscopy at different stages of the cell cycle. This will shed light on the organization within the plasma membrane at each phase.

The above experiments were not performed in full cell or live cells, but rather using very accurate model membranes that was not synthetically created. We hypothesize that membrane organization is needed for increased efficiency in signaling cascades. During actively dividing stages, cells will be communicating with their environment, and hence be performing more signaling functions. This would not be as crucial for G0 or G1 cells so they will not have increased signaling and membrane organization. This can be tested in live cells by investigating the immune response using a calcium assay. This assay can be done to test lipid dependent cellular functions by monitoring levels of cytoplasmic calcium in the RBL cells.

Using fluorescence microscopy and RBL GMPVs, the phase separation of cell plasma membranes with a critical composition was observed. By viewing this system during different stages of the cell cycle, it was found that in the stages where the cell was actively dividing, transition temperatures were elevated. In the future, lipid composition,

membrane organization, and cellular signaling will be explored in order to make a further connection to transition temperatures. Comparing the results of this cell cycle experiment with future lipodomics, calcium assays and 2-color, super resolution experiments will provide further evidence in support of the effect of cell cycle stage on plasma membrane behavior.

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