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Understanding Phase Separation in Mitotic Proteins

Personal Statement: The Importance of Communicating Science

Before I explore phase separation and the conditions which can affect droplet formation in proteins, I want you to understand why I am communicating any of this information. I have always enjoyed the scientific disciplines for their logical methodologies, yet I did not realize I wanted to become a scientist until I joined the Stukenberg Lab at the University of Virginia. Working with Dr. Stukenberg and my mentors have instilled a passion for mitotic biology and critical thinking that I can wish to share with you. Can you imagine a world without science? I can't. It is necessary for a progressive society to have gifted scientists, but also to maintain a public understanding of science (Rull). Science is valuable because we improve living standards via scientific discovery. Without science, we wouldn't understand the shape of our planet. Without science, we are only able to watch darkness as the sun sets. Without science, we cannot defeat COVID-19.

During the Spring semester, I conducted a short survey with UVA students to determine how college students perceive cancer research; the results showed me it was crucial for scientists to share their discoveries. ¾ of respondents indicated they had relationships with individuals diagnosed with some form of cancer. Half of those surveyed incorrectly assumed that cellular mutations cause cancers, while the other half correctly recognized cancers develop from uncontrolled cell growth. ⅔ of participants also suggested the majority of cancer research

involves experimenting with human tissue cultures (Vemuri, ENWR). These are alarming findings even for a survey with a sample size of less than 20 individuals. Even most educated populations appear misinformed of the causes of cancers or the methods in which scientists are investigating potential cures. Science will one day find a sustainable method to eliminate cancers, but until then we must educate the public and inspire the next generation of scientists.

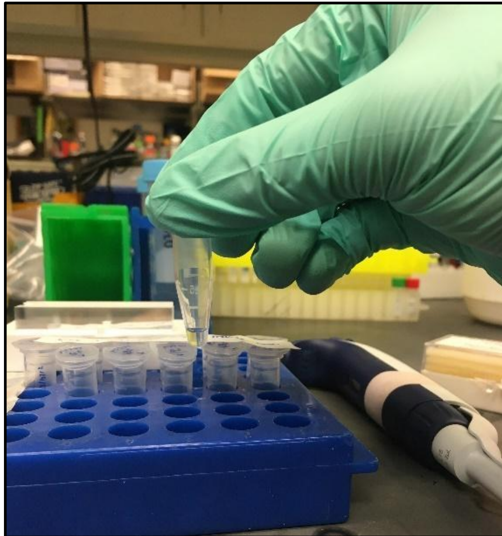

Figure 1: Image of Protein Assay Preparation. The microliter centrifuge tube contains a solution of protein, water, NaCl, DNA, and pH buffer. Less than 3 “drops” of liquid are contained in the test tube, illustrating the potential scale of experiments related to cancer. Solution components are measured using a microliter pipette (bottom right) and stock volumes are stored in environments below 0 ℃ (Vemuri, Photo).

After reading the survey responses, I noticed many students believed cancer research had to interact with cancerous cells. This assumption is inaccurate and produces daunting implications for students with potential in the oncological sciences. My own research with mitotic proteins demonstrates the simplicity of both cancer research and scientific investigation. In Fig. 1, an in vitro protein assay sits within a microliter centrifuge tube (Vemuri, Photo). An in vitro protein assay is a mixture of proteins, water, salts, and target molecules. The microliter pipette resting in the right corner measures all components of the assay precisely. Microscopes analyze the mixtures, providing insights as to how molecules may interact with each other. Notice how little liquid there is in the tube; the experiment is not complex or requiring extensive equipment (Vemuri, Photo). Contrary to the expectations of my survey participants, researching

causes and cures of cancers can be accessible. An action as simple as looking at liquid under a microscope can bring medicine closer to beating cancer.

Our societies must reach a greater public understanding of science. The first step our communities need to take is acknowledging every individual's potential to contribute to science, regardless of how complex the discipline is (Rull). The second step, which I hope to fulfill through my essay, is to educate individuals about the work of scientists.

Abstract

Although the general public is well acquainted with the detrimental effects of cancer diagnoses, the majority of individuals are unaware of the scientific discoveries that will help medicine defeat cancer. In this paper, I inspect the works of various scientists and myself, which detail the phenomena of phase separation in mitotic proteins. Specifically, analyzing the importance of phase separation in the proteins Aurora B and RBMX provides exciting implications for cancer diagnoses. I recognize that phase separating Aurora B is crucial for the regulation of chromatid segregation during mitosis. The ability of proteins to form membraneless organelles is crucial for preventing genetic anomalies and malignant tumors. This paper is primarily bibliographical, reviewing prior phase separation experiments to connect complex concepts and educate the reader. A detailed empirical study, at the end of the paper, demonstrates the simplicity of phase separation and accessibility of scientific discovery. After reading this paper, readers should have a stronger understanding of how mitotic errors can result in cancer, in addition to an appreciation of phase separating mitotic proteins as membraneless organelles regulating cellular division.

keywords: mitosis; phase separation; Aurora B; RBMX; chromatid segregation; cancer

(177 words)

Introduction: What is Phase Separation?

Eukaryotic cells contain compartmentalized structures known as organelles. Membrane-bound organelles, such as the nucleus, specialize in their function, metabolic processes, and transport of molecules (Carmena). For example, lysosomes digest macromolecules using degradative enzymes contained within their membranes. Eukaryotic cells also contain membraneless organelles composed of proteins and or nucleic acid structures. Recent discoveries in the formation and function of these membraneless organelles propose exciting developments in cell biology. Membraneless organelles form via phase separation (Carmena).

Phase separation is the process in which a solution of proteins separates into a dense phase made of proteins and a dilute phase composed of the supernatant, the solution minus protein components (Klosin). This results in a gel-like droplet made of proteins separated from the surrounding liquid. This spontaneous aggregation occurs as a result of electrostatic

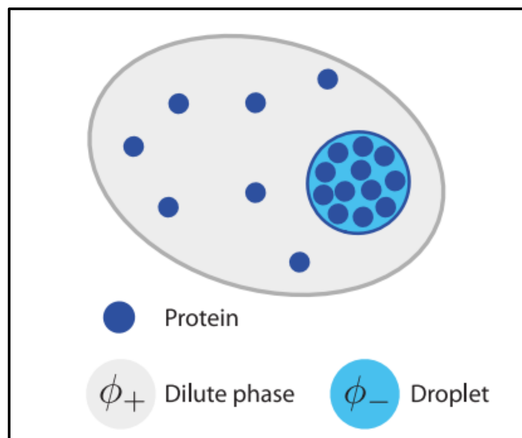

Figure 2: Phase Separation Model. The illustration contains phase separated protein inside a cell; it is drawn from the perspective of a microscope lens looking down at the cell. The dark blue spots represent protein molecules, which aggregate into a droplet in the bottom left corner of this cell. The different phases are indicated by the gray color, identifying the dilute phase, and the turquoise color, identifying the dense phase/droplet (Klosin).

interactions between the proteins, similar to the attraction of magnets. Oppositely charged regions of multiple protein molecules attract each other, resulting in droplet formation. Fig. 2 illustrates the hypothetical phase separation of a dark blue protein in a cell. The majority of the

protein mass has clumped together, forming a droplet structure isolated from the rest of cell components colored gray (Klosin).

Cancer is the malignant, uncontrolled growth of cells in eukaryotes. In the search for a cure, cell biologists are examining the factors which result in uncontrolled cell growth. Recent developments suggest errors with proteins regulating cell growth, mitotic proteins, are the primary culprits of cancerous growth. The phase separation of mitotic proteins permits their accessibility to the vital biochemical reactions which regulate cell growth. Therefore, a lack of phase separated mitotic proteins may correlate with genome instability, replication stress, and organ specific cancers. Investigating the properties of phase separated mitotic proteins develops knowledge of the mechanisms which may lead to cancer or the lack thereof. Phase separation is an exciting topic in biological research, understanding this wonder is essential to the progression of oncology.

Where Cancer Begins: Mitosis & Chromatid Structures

As organisms grow in size and develop in complexity, the number of cells in tissues must increase. Mitosis is the process of cell division in the somatic cells of eukaryotic organisms. During interphase, the first step of mitosis, the parent cell replicates its DNA for subsequent division amongst two daughter cells. The other steps of mitosis arrange DNA along the metaphase plate, the cell’s equator, and equally distribute DNA among two daughter cells (Krenn). Cells organize replicated DNA into pairs of chromosomes which pulled apart by microtubules. The rope-like microtubules attach to the kinetochore areas of chromatids before anaphase (Fig. 3); tension on the kinetochores separates the sister chromatids from each other.

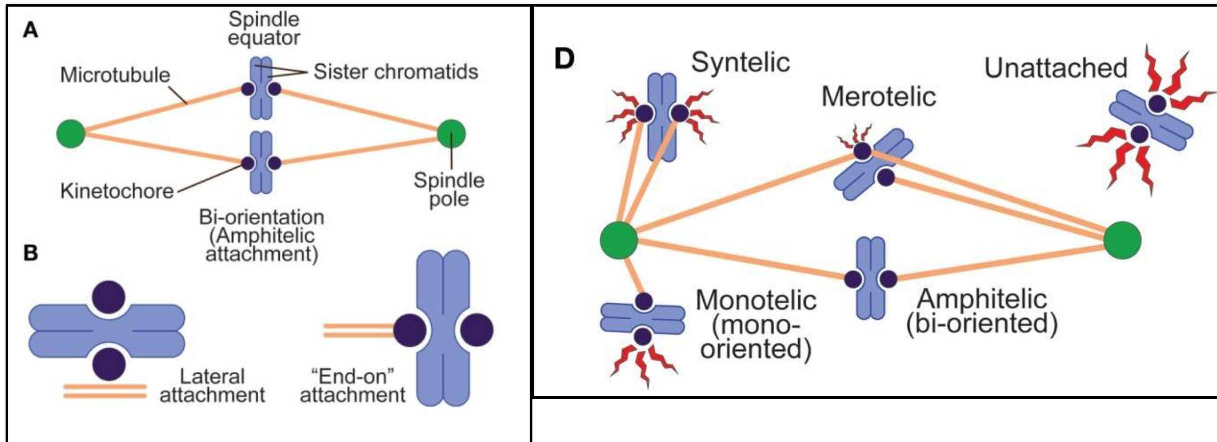

Figure 3: Kinetochore Microtubule Attachments Model. The illustration contains phase separated protein inside a cell; it is drawn from the perspective of a microscope lens looking down at the cell. The dark blue spots represent protein molecules, which aggregate into a droplet in the bottom left corner of this cell. The different phases are indicated by the gray color, identifying the dilute phase, and the turquoise color, identifying the dense phase/droplet.

The connection between microtubule-kinetochore (KC-MT) attachment and uncontrolled cell growth is aneuploidy (Regulation of Mitosis). Aneuploidy is the presence of an abnormal number of chromosomes in a cell. The probability of aneuploid daughter cells increases in the presence of merotelic KC-MT orientations, multiple KC-MT pull a sister chromatid in opposite directions, compared to amphitelic KC-MT orientations, KC-MT pull sister chromatids towards opposite poles (Fig. 3).At the end of the division process, a cell may have too much or too little DNA. Chromosomal instability characterizes these aneuploid cells, which can result in uncontrolled cell division. However, errors like this occur numerous times within our lifespans and our cells are able to correct these mistakes. Why is it that sometimes these errors persist? What is it that attaches microtubules properly? Scientists at the UVA School of Medicine believe that the chromosomal passenger complex (CPC), a protein complex, is the key to answering these questions.

Regulators of Mitosis: Chromosome Passenger Complex (CPC) & Aurora B Kinase

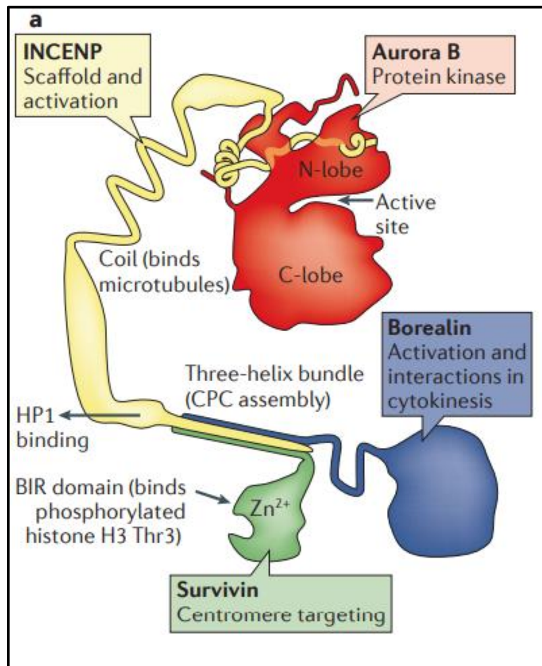

Figure 4: The Components of the Chromosome Passenger Complex (CPC). This illustration depicts the four domains of CPC: INCENP, Aurora B, Borealin, and Survivin. INCENP, Borealin, and Survivin recognize and bind to chromosomes. After the targeting domain binds to chromosomes, the kinase domain, Aurora B, can add phosphate groups to alter the structure and function of proteins. Specifically, Aurora B corrects chromosome-microtubule errors, regulates the mechanisms driving the contractions of cytokinesis, and activates the spindle assembly checkpoint (Carmena).

CPC is the master control of cell division in eukaryotic cells. CPC is a protein complex composed of four different proteins: Aurora B kinase, INCENP, Borealin, and Survivin. The localization module of CPC, ISB (INCENP, Survivin, Borealin), recognizes and binds to chromosomes (Carmena). The kinase module of CPC, Aurora B and a small subunit of INCEP, corrects chromosome-microtubule errors, regulates the mechanisms driving the contractions of cytokinesis, and activates the spindle assembly checkpoint. The Stukenberg Lab is interested in the regulation of mitotic errors via the kinase module of CPC. They previously discovered high concentrations of CPC near the inner centromere of sister chromatids performing a vital operation; kinase modules correct errors in microtubule attachment (Regulation of Mitosis).

The methods controlling the process of KC-MT attachment are known as error correction (EC) and spindle assembly checkpoint (SAC). EC is dependent on the cell's ability to detect the amount of tension on orientations of microtubules. A lack of tension in microtubules (monotelic attachment Fig. 3) will result in addition of microtubules to create tension-bearing structures. An

excess of tension (merotelic attachment Fig. 3) will result in the deconstruction of abnormal KC-MT attachments. Aurora B interacts with the surface proteins of microtubules, playing a vital role in EC. An excess of Aurora B will cause a disruption of KC-MT attachments, while the inhibition of Aurora B can promote the assembly of KC-MT attachments (Krenn).

SAC is a more ubiquitous pathway, which halts mitosis if there is even a single unattached microtubule. Cells containing mutant SAC will be able to continue mitosis in the presence of incorrectly attached chromosomes and will be susceptible to mis-segregation. Aurora B appears to be an indirect participant in SAC by producing unattached kinetochores, the only structures activating SAC (Krenn). Thus, Aurora B corrects KC-MT attachments by producing microtubules, destabilizing KC-MT attachments, and producing unattached kinetochores. Research investigating Aurora B activity indicates the protein produces the outermost regions of kinetochores --necessary for attachment-- and regulates the polymerization of tubulin subunits --forming microtubules-- (Trivedi, Regulation). These findings suggest Aurora B is a necessary agent in preventing the formation of cancerous cells by correcting KC-MT attachments, which would otherwise result in aneuploid cells (Krenn).

The CPC is a part of the centromere signaling network, a network of reactions controlling the timing of cell division. The Stukenberg Lab at UVA has also recognized that all CIN breast tumors have an overexpression of proteins within the centromere signaling network, suggesting that an excess of mitotic regulators contributes to CIN (Regulation of Mitosis). These data agree with the cellular mechanisms which govern KC-MT regulation; an excess of CPC would result in the destabilization of healthy KC-MT attachments, due to high concentrations of Aurora B. CPC is also found in low concentrations of 10 μM near the inner centromere in healthy cells, further supporting the hypothesis that an excess of CPC leads to the lack of improper KC-MT

correction. But how is it that CPC is able to localize near the inner centromere? What conditions result in an excess of CPC localizing? Investigating the phase separation of CPC may provide answers to these questions.

Dr. Trivdei's Biochemistry: Evidence for Phase Separated CPC

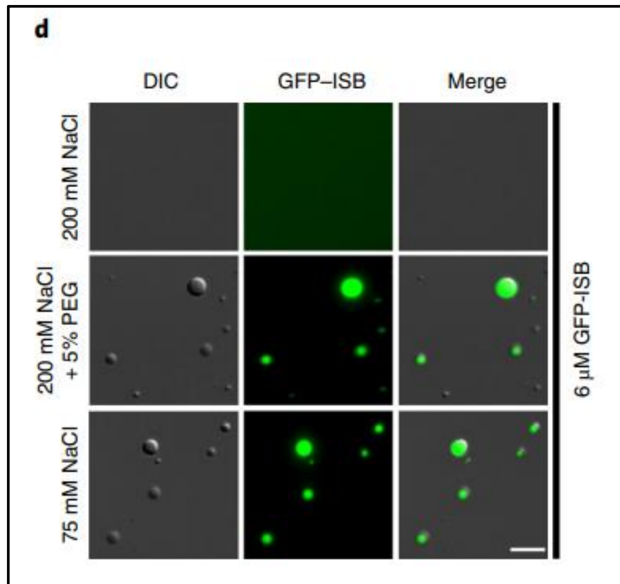

Figure 5: GFP Tagged ISB Droplets. These images depict the behavior of fluorescently tagged phase separating ISB proteins. The three different assays (rows) indicate the effects of increasing salt concentration and adding polyethylene glycol (PEG) on droplet formation. Increasing salt concentration appears to decrease droplet size. Adding PEG appears to increase the propensity of ISB to form droplets (Trivedi, The inner).

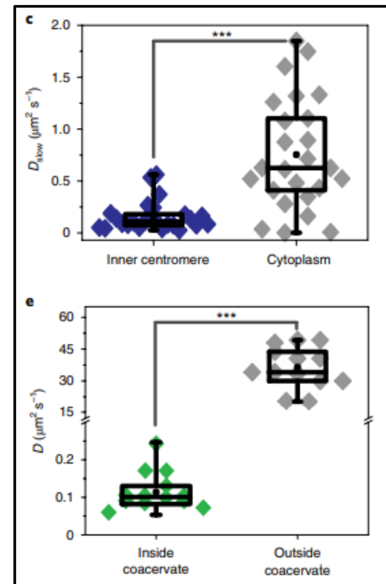

Figure 6: Droplet Diffusion Box Plots. The blue data represents the diffusion of molecules in suspected phase separated CPC. The green data represents the diffusion of molecules within in vitro phase separated ISB. The diffusion rates inside the protein complexes are much lower than outside the protein complexes. This suggests in vitro phase separated CPC has properties similar to CPC found in human cells (Trivedi, The inner).

Phase separation is an emergent property of proteins present at a high local concentration, resulting in membraneless organelles. The Stukenberg Lab has helped identify a new membraneless organelle composed of CPC, whose function is to correct mitotic errors via EC.

Former UVA student Dr. Prasad Trivedi presented evidence for the phase separation of CPC and the necessity of this process for our cells in his paper published by *Nature Cell Biology* last year.

Fig. 5 demonstrates the phase separation of fluorescently tagged ISB, demonstrating that the droplets are composed of CPC proteins. These droplets increased in size as the concentration of protein increased and as the concentration of salt decreased (Trivedi, The inner). Testing the physical characteristics of the droplets revealed gel-like properties. Fig. 6 presents the diffusion rates of fluorescently tagged Borealin within a cell (blue data) and fluorescently tagged ISB within a cell (green data). Diffusion rates outside the centromere and coacervate are both much higher than within the suspected phase separated droplets. The diffusion rates within the suspected phase separated droplets are also very similar to each other. These data suggest CPC exists in a phase separated state near the inner centromere (Trivedi, The inner).

After analyzing the Borealin protein, Dr. Trivedi removed specific regions of the protein believed to be inactive during phase separation. In vitro assays of the mutant Borealin resulted in little to no phase separation compared to the wild-type Borealin. Although the removed regions were not active during phase separation, they affected the propensity of Borealin to form phase separated droplets. Examining the properties of the inactive Borealin regions revealed large positively charged amino acid chains. These data suggest inactive regions of Borealin are drivers of phase separation and charge is important for phase separation (Trivedi, The inner).

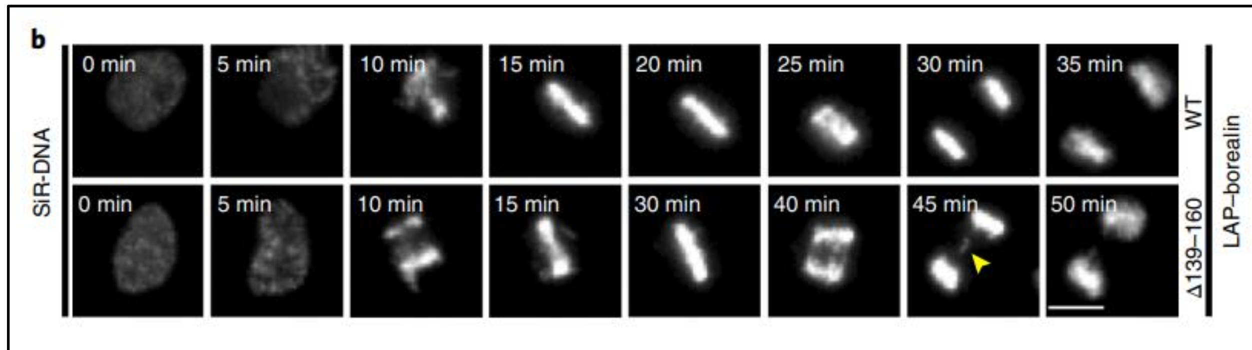

Figure 7: Mitosis Length vs LAP-Borealin. These microscope images depict the different stages of mitosis across two different cell cultures. The top row contains regular human cells and the bottom row contains human cells with mutated Borealin. The muted Borealin does not contain amino acid residues that were observed to be inactive during in vitro phase separation. The mutated cell line has difficulty dividing chromosomes and takes longer to complete mitosis (Trivedi, The inner).

Lastly, Dr. Trivedi compared the mitotic activity of human cells expressed with the mutated Borealin (missing charged regions) with the mitotic activity of regular human cells. Cells containing the mutant proteins did not correct KC-MT errors and took longer to complete mitosis. Notice in Fig. 7 how the cell with mutant Borealin (bottom row) takes an additional 15 minutes to complete the division of DNA compared to the cell with wild-type Borealin (top row). The percentage of lagging chromosomes in anaphase were also higher in the cells containing the mutant Borealin compared to cells containing the wild-type Borealin. Furthermore, additional comparisons between cells revealed removing charged regions of Borealin correlates with a decrease in the amount of Aurora B near the inner. These data support the hypothesis that phase separation is essential for the regulation of mitosis and the localization of CPC to the inner centromere (Trivedi, The inner).

Dr. Trivedi's findings present a strong case for the importance of phase separation in all organisms. Mitosis is one the most important cellular processes across various lineages. Our previous exploration of division has demonstrated the consequences of erroneous mitosis. The ability to correct improper KC-MT attachments prevents the formation of cancerous cells.

Biochemical evidence suggests the phase separation of CPC localizes the protein complex and therefore Aurora B, which degrades KC-MT attachments in excess quantity. Without a doubt, exploring the phase separation of mitotic proteins brings us one step close to defeating cancer.

The Impact of Phase Separation Research: Selecting Appropriate Cancer Therapies

Dr. Trivedi has demonstrated CPC exists in a phase separated state within human cells. Dividing cells use these membraneless organelles to regulate mitosis viva correcting improper KC-MT attachments. What does this mean for oncological procedures? Well, it suggests identifying tumors with poor phase separation of CPC can spare up to 40% of breast cancer patients from ineffective treatments (Barney). In fact, identifying patients with poor phase separation of CPC in their cells will eliminate the inclusion of chemotherapy in their treatment programs. Dr. Stukenberg hopes to "identify the patients where treatments such as [chemotherapy medication] paclitaxel are going to be most effective" (Barney). Removing the possibility of chemotherapy will rid patients of potential detrimental side effects and select for more appropriate treatment options such as immunotherapy or surgery.

My Personal Project: Potential Phase Separation in RBMX

After learning about what phase separation and the previous accomplishments of the Stukenberg Lab during my initial training, Dr. Stukenberg informed me of his current goal: to investigate if other proteins interacting with CPC can phase separate. Similarities in the properties governing phase separation also hint similarities in proteins' function, creating additional avenues for exploring the pathways which prevent cancer. Recognizing these reactions will help identify which regulatory pathways failed to act in cancer patients. Yukiko Cho, a postdoctoral researcher at Kumamoto University, outlines the interactions of the protein RBMX (RNA-binding motif protein, X-linked) with Satellite I RNA and Aurora B during the mitotic

phase. Her work suggests Aurora B binds to Satellite I RNA to produce a protein complex necessary for chromosome segregation (Cho). Purifying this complex from cell cultures revealed RBMX proteins bound to RNA during mitosis (Cho). Decreasing the concentrations of both RBMX and Satellite I RNA in cells resulted in premature chromosome segregation. After reading this source I had two questions: Does RBMX phase separate to interact with Aurora B and how do nucleic acid structures play a role in activating this protein?

Investigating these questions required me to first determine any possible conditions for the phase separation of RBMX droplets. I conducted an experiment, exploring how salt concentration and DNA concentration affect the propensity of the protein to form phase separated droplets (Vemuri, Understanding).

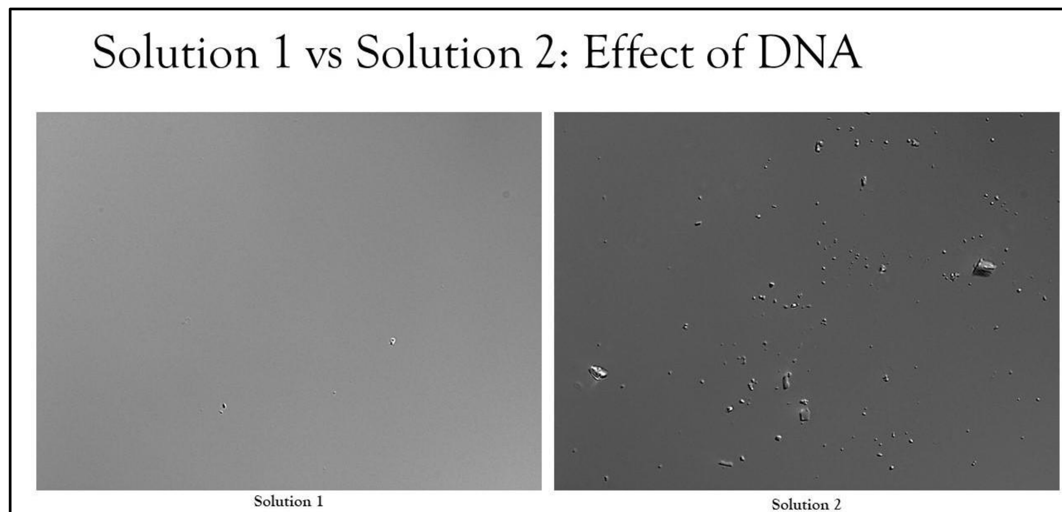

Figure 8: DNA Increases Droplet Size. These images compare the in vitro assays of Solution 1 and Solution 2. Solution 1 contains no DNA; Solution 2 contains 50 μM DNA. Imaging under a microscope reveals phase separated RBMX in Solution 2. Solution 1 contains no phase separated RBMX. These data suggest DNA is vital for the phase separation of RBMX (Vemuri, Understanding).

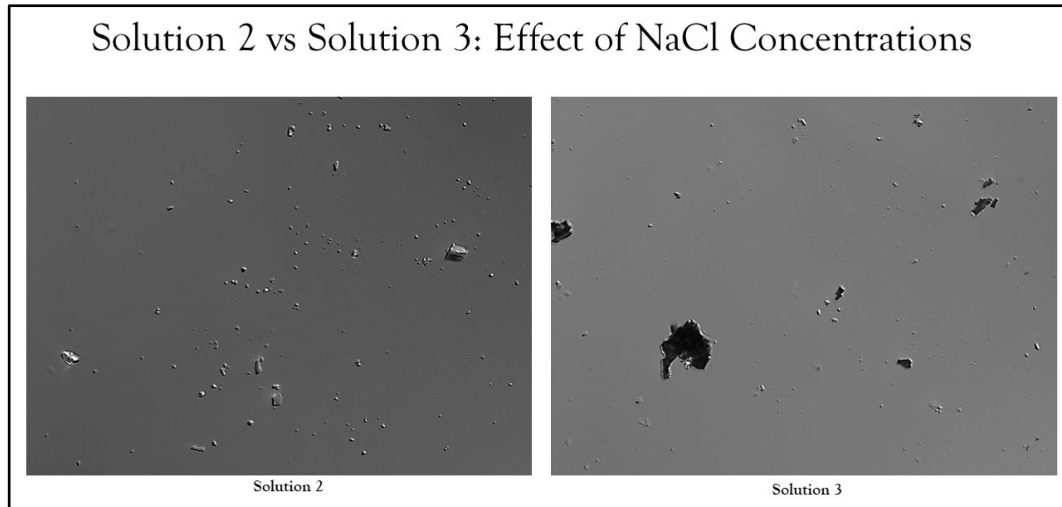

Figure 10: Increasing Salt Concentration Reduces Droplet Formation. These images compare the in vitro assays of Solution 2 and Solution 3. Solution 2 contains 10.9 mM NaCl & 50 μM DNA; Solution contains 150 mM NaCl & 50 μM DNA. Imaging under a microscope reveals phase separated RBMX in Solution 2 and Solution 3. Solution 3 appears to have fewer droplets compared to Solution 2. These data suggest increasing salt concentration reduces the propensity of RBMX to form phase separated droplets (Vemuri, Understanding).

Imaging the solutions revealed important properties of RBMX (Vemuri, Understanding). Comparing Solution 1 and Solution 2 demonstrates that DNA is essential for the formation of phase separated RBMX droplets (Fig. 8). This finding is in agreement with Dr. Trivedi's claim that charge is important for phase separation since the negative charge of DNA can interact with the positive charge on RBMX. Dr. Cho's description of RBMX binding to RNA also supports this claim because DNA and RNA are very similar. Comparing Solution 2 to Solution 3 shows that decreasing salt concentration increases droplet frequency and size (Fig. 9). This trend is similar to the effect of salt on in vitro ISB droplets and further supports the idea that charge is important for phase separation. The positive charges from the Na^+ atoms can result in possible repulsion, explaining the reduction in droplet formation. The findings of this experiment indicate RBMX is able to phase separate. However, there are still many questions left unanswered before we can understand the role of phase separation in RBMX.

Conclusion: Future Implications and the Value of Understanding Phase Separation

Phase separation is a unique property of macromolecules that has long-lasting implications for biology and medical procedures. The phase separation of CPC in eukaryotes explains the ability of our cells to correct the thousands of mitotic mistakes occurring during an organism's lifespan. Phase separated CPC produces a membraneless organelle, permitting the kinase domain, Aurora B, to conduct regulatory biochemical reactions specific to mitosis. Aurora B disassembles improper KC-MT attachments, synthesizes appropriate KC-MT attachments, and produces free kinetochores. These functions prevent the formation of aneuploid cells, reducing the risk of uncontrolled cell growth – cancer--. The Stukenberg at UVA has outlined alternate cancer treatments methods upon carefully study of phase separated CPC. My personal research on the mitotic protein RBMX confirms its ability to phase separate. I hope that I can this information to replicate and understand the biochemical reactions unique to this regulatory protein in the near future. Educating the public and scientific communities on the importance of phase separation has two distinct purposes. One, it demonstrates the accessibility and impact of scientific research simple in nature. Two, it provides an additional tool for scientists to explore the unknown biochemical pathways through which cancer emerges.

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