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Identifying the role of cholesterol and the cholesterol transfer protein, YSP1-2, in cell-cell fusion in Tetrahymena thermophila

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Identifying the role of cholesterol and the cholesterol transfer protein, YSP1-2, in cell-cell fusion in *Tetrahymena thermophila*

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Abstract

A cell's shape directly affects how it behaves as one's structure directly correlates to its function. The shape of cells and organelles is driven in part by the lipid bilayer that makes up their membranes. The process by which cells systematically adjust the types of lipids in membranes to facilitate changes in membrane shape remain uncertain. In our research, we aimed to address this issue by studying the role of lipid transfer proteins in the ciliate, *Tetrahymena thermophilia*.

Specifically, we focus on the process of cell-cell fusion during mating in *Tetrahymena* as this requires a drastic change in membrane shape at a specific time and place along the cell membrane. Using methyl-β-cyclodextrin (MBCD), we showed that the curvature accommodating lipid, cholesterol, is required for mating in *Tetrahymena*. Through bioinformatics analysis, we were able to identify a potential cholesterol transfer protein, TTHERM_ 00129660 (YSP1-2), that is expressed during the fusion process and that contains domains consistent with membrane binding and deformation as well as lipid transfer. We were able to clone this gene and its key functional domains with a yellow fluorescent protein with the goal of determining their function and localization.

Future experiments involve determining the localization and function of domains within YSP1-2, as well as testing the effects of cholesterol sequestration on cell-cell fusion specifically. Importantly, we think that because of the conservation and importance of cell-cell fusion in higher eukaryotes, we believe that the information that we glean from this research can be applicable to the broader understanding of cell-cell fusion.

Introduction

Cell-Cell Fusion

Almost all cellular functions are driven by the complex and dynamic interactions of proteins and lipids within cellular membranes (Adhyapak and Kapoor, 2019). These interactions can produce a variety of shape changes to cellular membranes (Adhyapak and Kapoor, 2019). The resulting shape changes support important cell biological process such as cell migration, polarization, and the fusion of cells that underlie the formation of tissues in multicellular organisms (Adhyapak and Kapoor, 2019). Cell-cell fusion involves the direct interaction between two lipid bilayers, accompanied by extensive shape changes within and mixing of each leaflet of

the bilayer subsequently the reformation into a single, fused membrane (Chernomordik and Kozlov, 2008) (Hernández and Podbilewicz, 2017). This interaction begins with a local rupture of continuity of each of the lipid bilayers and their rejoining, facilitated by a strong bending of one or both membrane bilayers, bringing the bilayers into immediate contact. Fusion processes vary greatly in terms of fusing membrane composition, biological environment, and regulatory mechanism (NG et al., 2019). In some fusions, the proteins that mediate fusion, also known as fusogens, are present on either one or both membranes for fusion to occur, resulting in unilateral or bilateral fusion, respectively (NG et al., 2019). These fusogens are used to drive the translation from the pre-fusion to the post-fusion state by bringing lipid bilayers into direct contact, promoting the synthesis of energy-intensive fusion intermediates, and creating fusion pores which allows for the mixing of cytoplasmic contents (NG et al., 2019).

Although fusogen proteins have been a topic of interest, recent work has focused on understanding the potential role of lipid content in the regulation of cell-cell fusion (Biswas et al., 2008) (Sanders et al., 2020). However, it remains unclear exactly how lipid composition is regulated at the sites of fusion.

Lipids Membrane Curvature and Fusion

Plasma membranes consists of lipid bilayers, which are composed of amphiphilic phospholipids that are made of a hydrophilic phosphate head and a hydrophobic tail containing two fatty acid chains (Casares et al., 2019). The shape of a membrane is determined by which lipids are present and their spatial distribution (PA and PK, 2006). Phosphatidylcholine (PC) and sphingomyelin, for example, are cylindrical shaped lipid molecules that tend to form flat bilayer structures (PA and PK, 2006). Inverted conical shaped lipid molecules, such as lysophospholipids

and polyphosphoinositides, tend to form structures with membrane protrusions (referred to as positive membrane curvature or moving away from the cytoplasm) (PA and PK, 2006). Conical shaped lipid molecules, such as diacylglycerol, phosphatidylethanolamine **(**PE), and cholesterol, tend to form structures with a negative curvature (moving towards the cytoplasm), such as membrane invaginations (PA and PK, 2006).

During cell-cell fusion, there is a direct interaction between both plasma membranes that is thought to occur through positive curvature, and this curvature is also seen later at the membrane adjacent to the fusion pore (Hernández and Podbilewicz, 2017). Though these curvatures have been observed during fusion, it is unclear how this curvature is achieved. Importantly, a study done by Kurczy et al observed a significant decrease in PC, a lipid associated with flat membranes, followed by an increase in PE, a lipid that is associated with high curvature membranes, during cell-cell fusion in *Tetrahymena* (Kurczy et al., 2010) and (Figure 1). This change in lipid content occurred when fusion pores, areas of high membrane curvature, are forming (Kurczy et al., 2010). This suggested that lipid content changes as membranes become more curved but does not address whether the change in lipids facilitates the change in curvature. Recent work has begun to address this issue. Specifically, a study from Biswas et al. demonstrated that the addition of exogenous cholesterol, a conical shaped lipid, could facilitate fusion pore opening in an *in-vitro* model of cell-cell fusion (S et al., 2008). However, this study did not address whether a similar alteration of lipid content occurs in cells as part of their normal physiology.

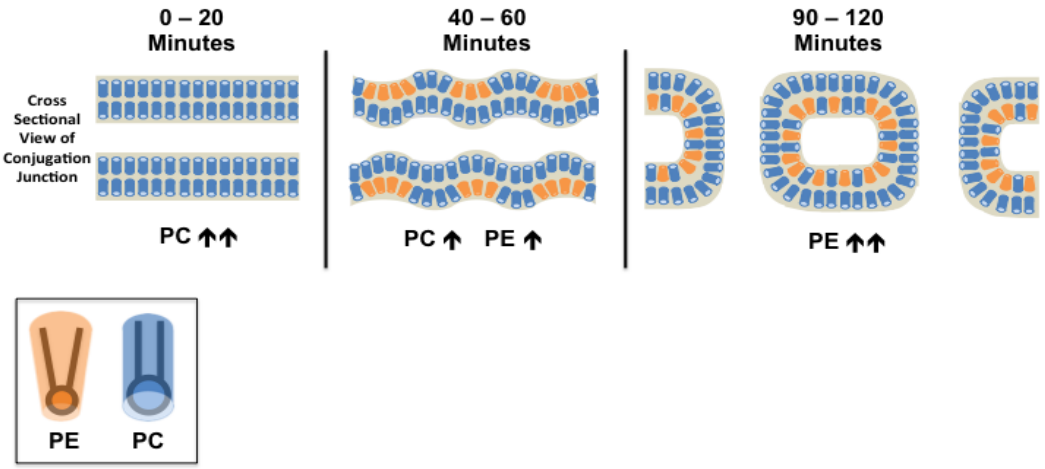

Figure 1. Summary of the observations by Kurczy et al as they relate to membrane lipid composition and fusion in *Tetrahymena*. During *Tetrahymena* fusion, the levels of phosphatidylcholine in the cell membrane decreases while the levels of phosphatidylethanolamine increases. Orange lipids are the conical lipid, phosphatidylethanolamine, and blue lipids are the cylindrical lipid, phosphatidylcholine.

Lipid Transport Proteins: Regulators of Membrane Lipid Content

One physiological mechanism for regulating lipid content during fusion may be lipid transporters. Lipid transfer proteins represent an emerging group of molecules that may regulate the lipid composition of membranes (Lev, 2010). These mechanisms are not well understood; however, it is believed that it may involve transfer of lipids across the cytoplasm (Lev, 2010). A specific type of protein, known as lipid transport proteins (LTPs), have been shown to assist in lipid transport between membrane bilayers at membrane contact sites (Lahiri et al., 2015). LTPs are highly conserved lipid carriers which bind lipids in a hydrophobic pocket, transferring them between the donor and acceptor membranes through an aqueous phase (Lev, 2010).

Recently, a new class of LTPs containing GRAM domains in the pleckstrin homology superfamily (PHg), steroidogenic acute regulatory transfer (StART) proteins, and transmembrane domains have emerged. YSP is among a family of six proteins in yeast that contain StART binding domains, allowing for the binding of sterols (Gatta et al., 2015). Furthermore, GRAM proteins are

among a family of three proteins in humans that contain StART-like domains, mediating inter-membrane sterol transport and regulating sterol homeostasis (Besprozvannaya et al., 2018). These StART-like proteins have been shown to localize to membrane contact sites where they facilitate transfer of sterols, increasing the rate of transfer, from endoplasmic reticulum to the plasma membrane (Gatta et al., 2015). As mentioned before, Biswas et al. has established that sterols are critically important lipids, synthesized in the endoplasmic reticulum, and trafficked via LTPs in non-vesicular organisms (Gatta et al., 2015). Given the emerging role of cholesterol in cell-cell fusion, it would be interesting to test if LTPs such as YSP play any role in this process.

Tetrahymena thermophila **as model for understanding the role of lipids in cell-cell fusion**

In the past several years, work in the Guerrier laboratory has aimed to understand membrane dynamics during cell-cell fusion in *Tetrahymena*. First, in collaboration with the Cole laboratory at St. Olaf College, we were able to observe contacts between the plasma membrane and a membrane that resembles the ER during cell-cell fusion (Cole et al., 2015). In addition, we have shown that reticulon, a protein known to localize to the ER in most eukaryotes, labels the site of fusion in immunofluorescence experiments in *Tetrahymena* (data not shown). These observations are consistent with the formation of ER-plasma membrane contact sites that would be required for lipid transfer. In addition, we know that lipid content at the site of fusion changes in a manner consistent with changes in curvature making plausible to think that lipid content is regulated event during cell-cell fusion in *Tetrahymena*.

Cell-cell fusion in *Tetrahymena* is easily induced and observed in the laboratory and the *Tetrahymena* Gene Expression Database (TGED), facilitates the identification of genes that are expressed during any developmental stage in the *Tetrahymena* life cycle, including cell-cell fusion.

This methodology was used to identify the fusogen, Hap2 (Cole et al., 2014) and reticulon, both of which are highly conserved, demonstrating the usefulness of *Tetrahymena* in the characterization of conserved genes including human orthologs (Eisen et al., 2006). Finally, important advances in methodology facilitate the generation of *Tetrahymena* gene knockouts or that overexpress specific genes of interest (Hai and Gorovsky, 1997). Using these tools, our broad goal is to determine how lipid content is regulated during cell-cell fusion in *Tetrahymena*.

The study of cell-cell fusion can require the use of advanced microscopy and specialized equipment, however cell-cell fusion occurs as part of the mating process in *Tetrahymena* that is characterized by physical and morphological changes that can be observed using common equipment. For example, during the mating process mating *Tetrahymena* first adhere, making weak connections that involve the interaction of proteins on their surface (0-30 minutes Figure 2) and (Cole, 2013). Then over time the membranes between mating *Tetrahymena* begin to mix forming fusion pores (30-60 minutes Figure 2) and (Cole, 2013). Finally, as the number of fusion pores increases and expands the strength of the interactions between the mating *Tetrahymena* increases (60-120 minutes Figure 2) and (Cole, 2013). This means that *Tetrahymena* go from swimming single cells to pairs of weakly interacting cells to pairs of strongly interacting cells when the fully fuse. This transition is easily observable under light microscopy and can be evaluated by coupling physical disruption with light microscopy.

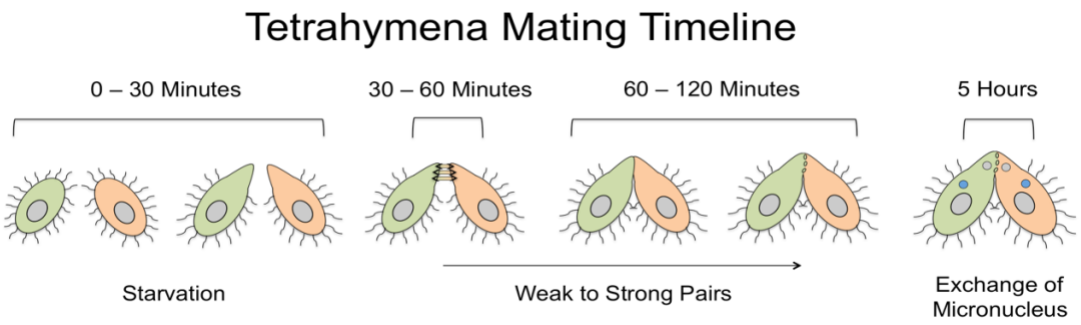

Figure 2. Schematic of the events associated with *Tetrahymena* cell-cell fusion. Weak pairs between the mating partners begin to form roughly 30 minutes after mixing. At around 120 minutes, strong pairs form between the mating partners and fusion pores are created allowing for the exchange of cytoplasmic material. After 5 hours, the nuclei are exchanged between the cells. The cells in green represent cells of one mating type while the cells in orange represent cells of another mating type.

Using *Tetrahymena* Gene Expression Database and structural homology databases (Phyre2 and HHPRED) our lab was able to identify a series of lipid transfer proteins. For this proposal, we chose to focus on the putative lipid transfer protein, TTHERM_00129660, that is highly expressed during cell-cell fusion in *Tetrahymena*. We refer to this protein as YSP1-2, based on homology, to 2 yeast proteins (YSP1 and YSP2). Protein structure prediction programs predict that YSP1-2 contains a F-BAR, START, GRAM and a transmembrane domain. The F-Bar domain is a part of the Bin/Amphiphysin/Rvs (BAR) domain superfamily (Ahmed et al., 2010; Gatta et al., 2015). Its primary function is to bind to the cell membrane and deform it (Ahmed et al., 2010). StART proteins are known to transport cholesterol (Gatta et al., 2015). The GRAM domain has been shown to have functions as a constitutive tether, marking ER-PM contact sites; as well as mediating inter-membrane sterol transport, allowing it to play a role in sterol homeostasis (Besprozvannaya et al., 2018). Finally, using PHYRE2 we were also able to identify a predicted transmembrane domain in the extreme C-terminus of the protein. The length of this transmembrane domain suggests that it would result in localization to the ER. Taken together, this YSP1-2 contains lipid transfer domains that are flanked by a membrane binding/bending domain on its N-terminus and a transmembrane domain on its C-terminus.

The yeast protein has been shown to transport cholesterol between the ER and plasma membrane and as discussed previously, exogenous cholesterol has been shown to increase fusion pore formation (Biswas et al., 2008). Research has shown the interaction of cholesterol with other lipids to be essential for membrane function and fusion (Yèagle, 1989). Within the cell, cholesterol is synthesized in the endoplasmic reticulum and is transported through the Golgi to the plasma membrane, which contains the highest cholesterol concentration of any cellular compartment (Yang et al., 2016). This extreme concentration of cholesterol induces changes in the fluidity, thickness, and compressibility within the bilayer, due to its rigid hydrocarbon rings, reducing lipid bilayer dynamics and fluidity (Yang et al., 2016). A study done by Biswas showed that cholesterol has a role in membrane fusion at two stages: before pore opening and during fusion pore growth (S et al., 2008). They found that cholesterol stimulates the creation and development of contact sites between membranes that allow lipid mixing, and helps to widen the fusion pore, therefore improving fusion efficiency (S et al., 2008). Furthermore, it has been shown that cholesterol has a profound influence on the behavior of membrane proteins present in the lipid bilayer (Epand, 2008). These effects can be attributed to a reduction of free volume in bilayers, stabilization of M2 proton channels, and specialized binding motifs within the membrane (Yang et al., 2016). Despite the extensive research on membrane fusion and the effect of cholesterol on membrane dynamics it remains unclear exactly how cholesterol membrane concentrations are regulated. Although, a role for cholesterol has not been established in *Tetrahymena* cell-cell fusion, several sterol synthesis genes are upregulated during *Tetrahymena* fusion, highlighting the potential role of sterols in this process.

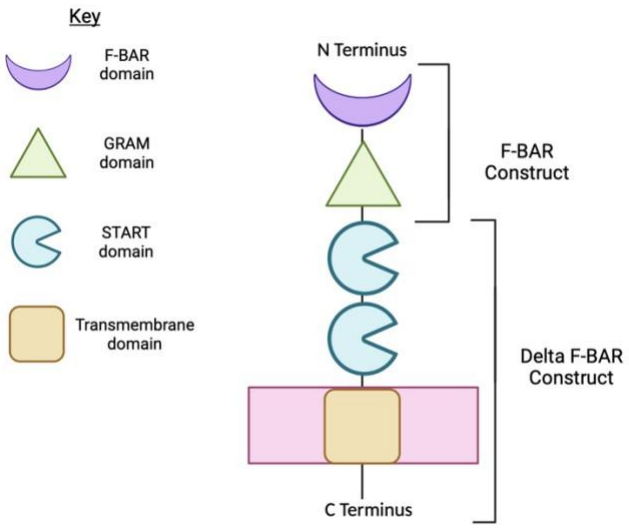

Figure 3. Using the *Tetrahymena* gene expression database a candidate lipid transfer protein, TTHERM_00129660 (YSP1-2) was identified. By combining the protein sequence from this database and aligning it to other homologs that are known to play a role in cell-cell fusion using PHYRE2 and HHPRED we were able to predict that YSP1-2 contains a F-BAR domain, a GRAM domain, a START domain, and a transmembrane domain. The F-BAR domain is 466 amino acids long, the GRAM domain is 115 amino acids long, and the start domain is 586 amino acids long.

Ultimately, our goal is to test the hypothesis that alteration of composition of lipids at the conjugation junction is required for cell-cell fusion. Specifically, that the transfer of cholesterol from the endoplasmic reticulum (ER) to the plasma membrane, via the lipid transfer protein YSP12, is required for the formation and/or expansion of fusion pores during cell-cell fusion in *Tetrahymena*. To this end we aimed to 1) test if cholesterol was required for fusion in mating *Tetrahymena* by observing the effects of cholesterol sequestration on *Tetrahymena* mating, 2) determine the cellular localization of the lipid transfer protein, YSP1-2 be expressing a visualizing fluorescently tagged YSP1-2 and 3) to express truncated forms of YSP1-2 (Delta-F-BAR and F-BAR constructs) in order to determine their localization and contribution to YSP1-2 function (Figure 3).

We predict that cholesterol plays an essential role in cell-cell fusion as several sterol synthesizing genes are upregulated during *Tetrahymena* conjugation. To test this hypothesis, we

will assess what effect cholesterol sequestration by methyl-β-cyclodextrin (MBCD) has on mating in *Tetrahymena*. Future experiments will continue to test at which point during the mating process cholesterol is required.

Based on our analysis of the domain organization of YSP1-2, we expect at least partial localization of YSP1-2 to the ER. PHYRE2 analysis predicts that the C-terminus of YSP1-2 contains a putative transmembrane domain whose length is consistent with localization to the ER membrane (Watson and Pessin, 2001). Since our analysis also predicts an F-BAR domain on the N-terminus of the protein, we feel strongly that this will connect YSP1-2 to another membrane (Liu et al., 2015). We expect *Tetrahymena* YSP1-2 to act similarly at the ER-PM interface and we expect that Delta-F-BAR will localize to the ER and the F-BAR domain to localize to the plasma membrane.

Materials and Methods

Cholesterol Sequestration by MBCD on Tetrahymena thermophilia Mating

Tetrahymena thermophila 428 and B2086 cells were counted using a coulter counter. Cells were then seeded and grown overnight at a concentration to yield 150,000 cells/mL. Cells were then resuspended in 10 mM tris and starved overnight. Cells were then counted using the coulter counter and mixed at equal concentrations. Cells were allowed to mate in different concentrations of 150mg/ml MBCD (1:10 dilution, 1:20 dilution, and 1:50 dilution) in 10 mM Tris at 30°C for 4 hours. A control of 10mM Tris was used. Cells were removed and pairs were counted via light microscopy.

Addition of MBCD at Varying Times During Tetrahymena Mating

Tetrahymena thermophila 428 and B2086 cells were counted using a coulter counter. Cells were then seeded and grown overnight at a concentration to yield 130,000 cells/mL and 100,000 cells/mL. Cells were then resuspended in 10 mM tris and starved overnight. Cells were then counted using the coulter counter, mixed at equal concentrations, and allowed to mate in at 30°C for 4 hours. A 1:20 dilution of 150mg/ml MBCD in 10 mM Tris was added to the cells every 30 minutes for 150 minutes. A control of 10mM Tris was added at the same time as each drug addition. Cells were removed and pairs were counted via light microscopy.

Trituration of MBCD Treated Tetrahymena thermophila Mating cells

Tetrahymena thermophila 428 and B2086 cells were counted using a coulter counter. Cells were then seeded and grown overnight at a concentration to yield 115,000 cells/mL and 193,000 cells/mL. Cells were then resuspended in 10 mM tris and starved overnight. Cells were then counted using the coulter counter, mixed at equal concentrations, and allowed to mate in at 30°C for 4 hours. A 1:20 dilution of 150mg/ml MBCD in 10 mM Tris was added to two wells and a 1:20 dilution of 10mM tris was added to 2 wells. After 2.5 h and 4.5 h 800 µL of cell suspension was removed from the mating mix, transferred to an empty well on the 12 well plate and pipetted up and down 5 times before being returned to the well. The triturated and undisturbed mating mixtures were then treated with 100 µL of 2.4 percent glutaraldehyde in 10 mM Tris. Cells were removed and pairs were counted via light microscopy.

Cloning into entry vector

DNA fragment corresponding to full length YSP1-2, the F-BAR domain of YSP1-2, and the delta F-BAR domain of YSP1-2 were amplified from genomic *Tetrahymena thermophila* DNA

using polymerase chain reaction (PCR). PCR fragments were subjected to agarose gel electrophoresis and then fragments were extracted and purified from agarose gels. Fragments were cloned in pENTR-D plasmid using Topo cloning technology (Thermofisher). Topo reactions were then be transformed into competent *E. coli* and selected based on resistance to Kanamycin. After colonies were picked, liquid cultures, and plasmid DNA was isolated, cloning of the region of interest was verified using restriction enzyme digest. Positive clones were then sent out for DNA sequencing.

Cloning into YFP expression vector

pENTR-D clones that contained the fragments of interest were mixed with YFP expression vectors. Fragments of interest were recombined into YFP expression vectors using the LR Clonase reaction. After the LR clonase reaction, High Efficiency Cloning *E. Coli* cells were transformed, and positive clones were identified using restriction enzyme analysis. Positive clones were then sent to sequencing.

Results

Cholesterol is required for mating in Tetrahymena and may be required for fusion

Lipids in general have been shown to change during the cell-cell fusion process. Treatment of cells with exogenous cholesterol has been shown to affect viral cell fusion and fusion pore expansion in mammalian cells but it remains unclear if cholesterol is required for fusion. To test this, we used MBCD to determine if cholesterol sequestration would inhibit fusion in mating *Tetrahymena*. To test this, *Tetrahymena* were mated in the presence of 10mM Tris, 15mg/ml, 7.5mg/ml, or 3mg/ml MBCD. We found that *Tetrahymena* that received 7.5 mg/ml and 15 mg/ml

of MBCD showed a statically lower amount of percent pairing than cells that received 10 mM Tris (Figure 4). This suggests that cholesterol is required for *Tetrahymena* mating.

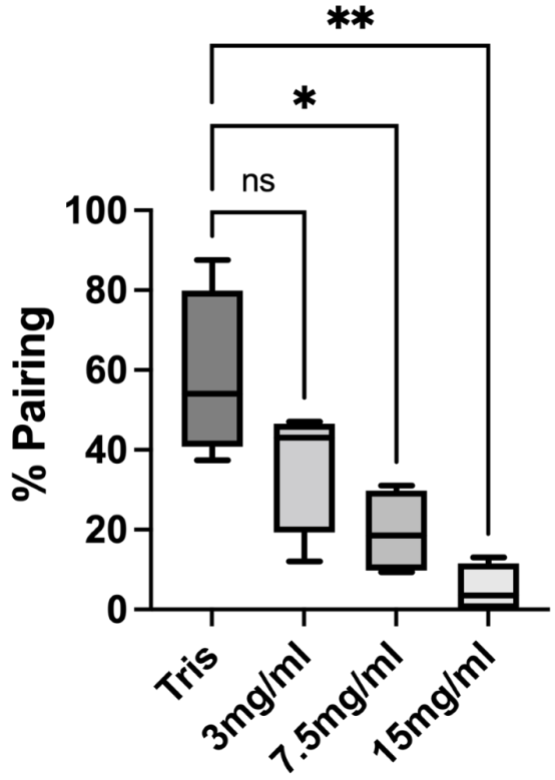

Figure 4. *Tetrahymena thermophila* cells that received 7.5 mg/ml and 15 mg/ml of MBCD showed a statically lower amount of percent pairing than cells that received 10 mM Tris. Mating *Tetrahymena* were treated with Tris (negative control) or 3mg/ml, 7.5mg/ml, or 15mg/ml MBCD. Cells were allowed to mate for 3-4 hours and then percent pairing was calculated by counting 50 total objects using several locations along each slide. Boxes represent the 10^{th} and 90^{th} percentile. Whiskers represent the minimum and maximum percent pairing and line at center of the box represents the mean. A Mann-Whitney test was performed to determined statistical significance where * = $p<.05$ and ** = $p<.01$

We were able to show a dose dependent effect of MBCD on Tetrahymena mating, suggesting that cholesterol was required for some part of the fusion process. To understand if MBCD was impacting fusion specifically, we performed a time course experiment with the hypothesis that *Tetrahymena* would be insensitive to MBCD if it were added after the fusion has occurred. To do this we added 7.5mg/ml MBCD directly after mixing Tetrahymena mating partners, or 30, 60, 90, 120, 180 minutes after mixing. Similarly, Tris was added to adjacent wells

containing *Tetrahymena* as a control. Cells were removed from each well 4 hours after mixing mating partners. The cells were then fixed, and the percent pairing was calculated by counting 100 total objects using several locations along each slide.

We found that *Tetrahymena thermophila* cells that received MBCD before 120 minutes showed a statically lower amount of percent pairing than cells that received 10 mM Tris at the same time (Figure 5). Since it has already been established that *Tetrahymena thermophila* make strong pairs at 120 minutes, and that strong pairs are a result of membrane fusion, we were able to conclude that MBCD may be impairing pairing by inhibiting fusion. This suggests that cholesterol plays a role in fusion during *Tetrahymena* mating.

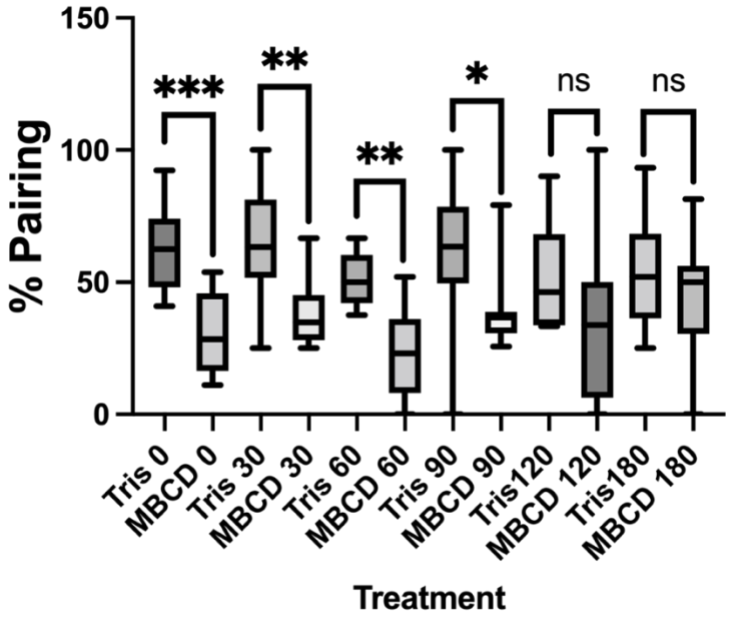

Figure 5. *Tetrahymena thermophila* cells that received 7.5 mg/ml MBCD before 120 minutes showed a statically lower amount of percent pairing than cells that received 10 mM Tris at the same time. Mating Tetrahymena received Tris or MBCD at 0, 30, 60, 90, 120, or 180 minutes after mixing mating partners. Cells were allowed to mate for a total of 4 hours then extracted fixed and then percent pairing was calculated by counting 100 total objects using several locations along each slide. Boxes represent the 10^{th} and 90^{th} percentile. Whiskers represent the minimum and maximum percent pairing and line at center of the box represents the

mean. A Mann-Whitney test was performed to determined statistical significance where * = $p<.05$, ** = $p<.01$, and *** = $p<.005$.

Cloning of candidate cholesterol transfer protein, YSP1-2, in YFP containing vector.

We hypothesized that our candidate protein, TTHERM_ 00129660 (YSP1-2), was a cholesterol transfer protein that aided in the process of cell-cell fusion. We predicted that it had 3 domains: F-BAR domain, START domain, and a transmembrane domain. The F-BAR domain was thought to bind and deform the membrane while the START/GRAM domain was thought to facilitate cholesterol transfer. To begin to characterize this protein, we focused on generating fluorescently labeled parts of YSP1-2. Specifically, we wanted to generate YFP tagged full length YSP1-2 protein, the F-BAR construct of YSP1-2 (contains F-BAR and GRAM domain) and the delta F-BAR construct of YSP1-2 (containing only the two START domains) (Figure 3). The general cloning scheme is described in figure 6. Delta F-BAR domain of YSP1-2 from genomic *Tetrahymena* genomic DNA to a pENTR-D vector, and then to a YFP-tagged pBS-ICY vector (Figure 6).

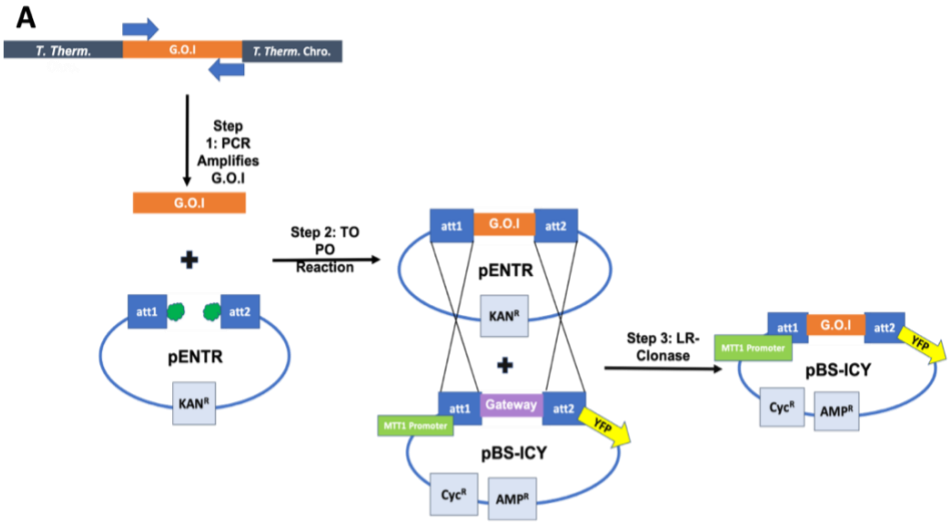

Figure 6. Outline of cloning process for YSP1-2 into YFP vector. Genomic DNA from *Tetrahymena thermophila* DNA was amplified via PCR. The gene of interest was interested into a shuttle vector known as pENTR via a TOPO reaction. The shuttle vector was then interested into a YFP tagged vector know as pBS-ICY using a LR-Clonase reaction.

We began by amplifying the DNA fragment that coded for delta F-BAR of YSP1-2 from the *Tetrahymena* genome using PCR. The amplification of the DNA fragments was confirmed and purified from the agarose gel (Figure 7). A topoisomerase-mediated cloning process was used to integrate the fragment into a kanamycin-resistant pENTR plasmid. The samples were subjected to a diagnostic digest to ensure that the delta F-BAR of YSP1-2 construct was successfully cloned into the vector (Figure 8). pENTR clones for FBAR were generated by a previous research group, prior to my tenure within the lab, forming pENTR-YSP1-2 F-BAR plasmid. Due to difficulty in our ability to PCR the full-length DNA, pENTR clones for full length YSP1-2 were synthesized by Invitrogen. pENTR F-BAR and full-length YSP1-2 constructs were also verified using diagnostic digest (data not shown). Importantly, all constructs were also verified by DNA sequencing.

We used the LR clonase reaction to move Delta F-BAR, F-BAR, and full-length YSP1-2 to YFP containing vector. Our cloning was successful as demonstrated by restriction enzyme analysis (Figures 9 – 11).

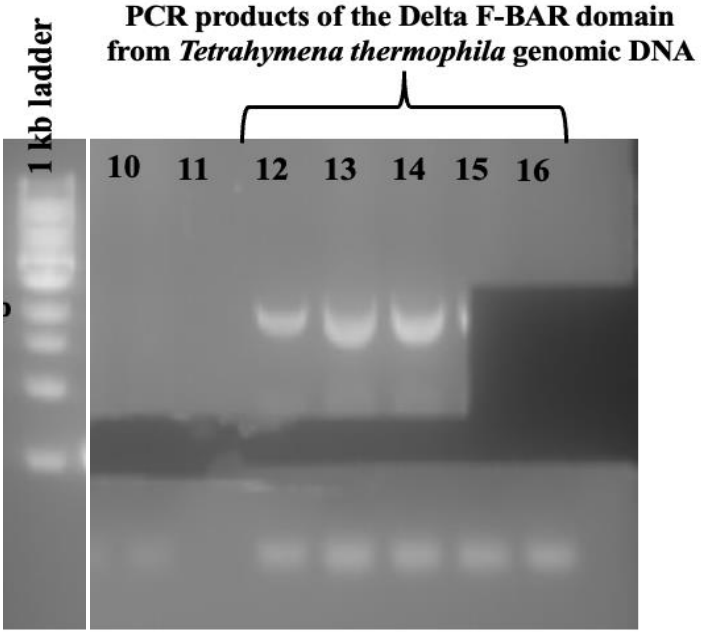

Figure 7. PCR of delta F-BAR of YSP1-2 from *Tetrahymena* genomic DNA. Lane 1 contained the ladder (Neb N3232L). Lanes 12-16 contained the bands of the PCR products of the delta F-BAR domain from *Tetrahymena thermophila* genomic DNA. Positive bands were approximately 1938bp. Bands from lanes 15 and 16 were excised for gel purification.

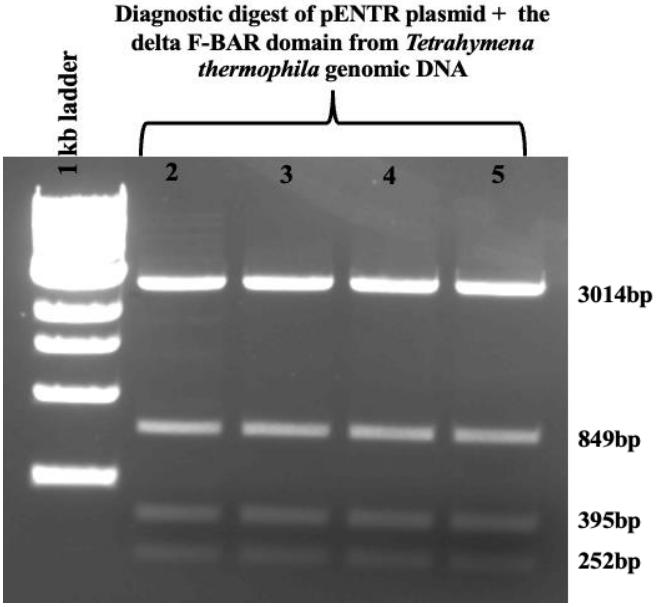

Figure 8. Diagnostic digest of topo Clones of pENTR-YSP1-2 Delta F-Bar. Lane 1 contains a 1 kb Ladder (Neb N3232L). Lanes 2 –5 are a diagnostic digest of the pENTR-D vectors plus the YSP1-2 C-terminus from *Tetrahymena* Genomic DNA insert with enzymes HindIII and EcoRI. Each lane has bands at approximately 3014bp, 849bp, 395bp, and 252bp.

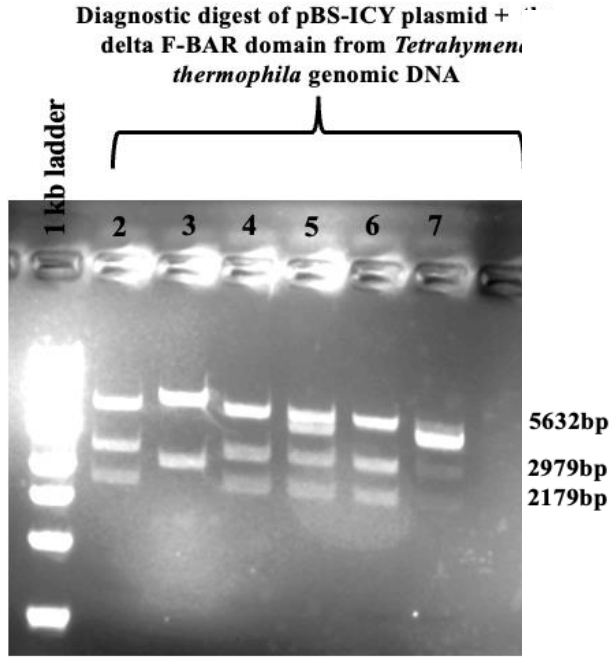

Figure 9. Diagnostic digest of YFP Clones of pBICY-YSP1-2 Delta F-Bar. Lane 1 contains a 1 kb Ladder (Neb N3232L). Lanes 2 –7 are a diagnostic digest of the YFP vectors plus the YSP1-2 C-terminus from *Tetrahymena* Genomic DNA insert with enzymes HindIII. Lanes two and four have bands at approximately 2179bp, 2975bp and 5632bp.

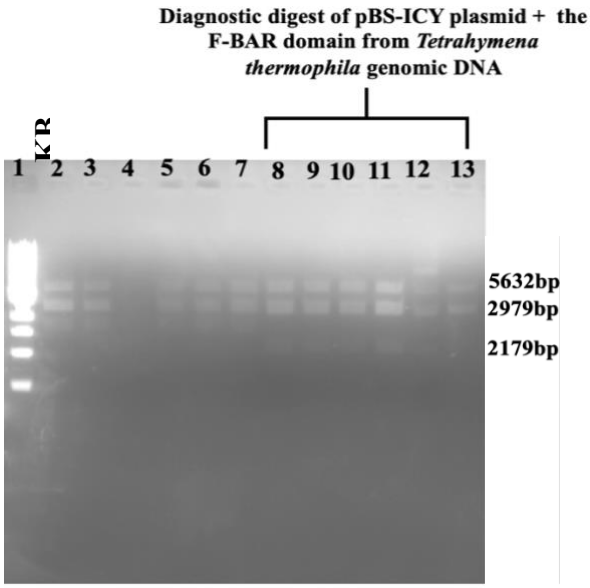

Figure 10. Diagnostic digest of pBS-ICY-YSP1-2 F-BAR. Lane 1 contained a 1 kb Ladder (Neb N3232L). Lanes 8-13 are diagnostic digests of the YFP vectors plus the YSP1-2 F-BAR from *Tetrahymena* Genomic DNA insert with enzyme HindIII. Lanes 8, 9, 10, 11, and 13 have bands at approximately 4739bp, 2981bp, 2975 bp, and 1388bp.

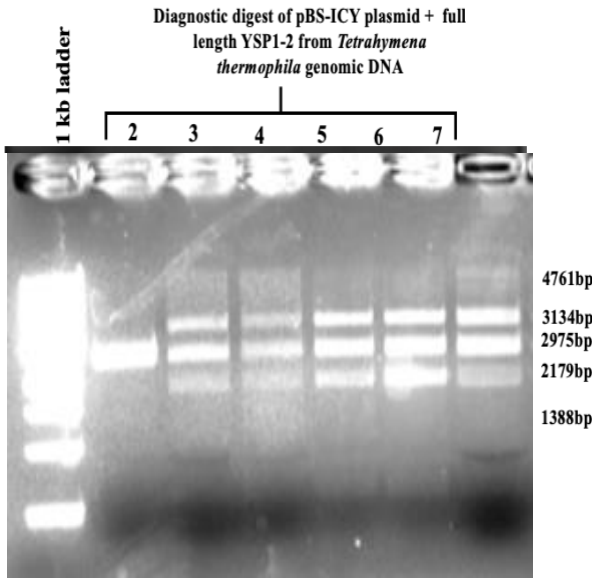

Figure 11. Diagnostic digest of pBS-ICY-YSP1-2. Lane 1 contains a 1 kb Ladder (Neb N3232L). Lane 2-7 is a diagnostic digest of the YFP vectors plus the full length YSP1-2 from *Tetrahymena* Genomic DNA insert with enzyme HindIII. Lanes 3-7 have bands at approximately 4761bp, 3134bp, 2975bp, 2179bp, and 1388bp.

Conclusion

Ultimately, our goal was to test the hypothesis that cholesterol transfer from the endoplasmic reticulum (ER) to the plasma membrane is required for fusion during *Tetrahymena* mating. To test this, we first tested the effects cholesterol sequestration by methyl-β-cyclodextrin (MBCD) on fusion during Tetrahymena mating. We found that MBCD treatment reduces mating in *Tetrahymena* suggesting that cholesterol is required for this process. We were also able to show that cholesterol sequestration by MBCD before 120 minutes significantly reduces mating in *Tetrahymena* suggesting that cholesterol is required during the mating process before strong pairs are made. Importantly, this supports the role of cholesterol in fusion since strong pair formation is predicated on membrane fusion. It will be important to test fusion formally in the future. We have done some preliminary experiments to test this. Specifically, we used a pair disruption assay to determine if MBCD treatment would make pairs more sensitive to mechanical disruption, a sign that fewer strong pairs are forming. However, the results were inconclusive, and the experiment needs to be repeated. In addition, fusion can be measured using dye transfer between mating partners. It will be interesting to measure dye transfer in the presence of MBCD.

We were successful in cloning YSP1-2 as well as truncated forms (Delta-F-BAR and F-BAR constructs). The medium-term goal was to express each of these constructs into *Tetrahymena* and to determine their localization. Based on our analysis of the domain organization of YSP1-2, we expected at least partial localization of YSP1-2 to the ER. PHYRE2 analysis predicted that the C-terminus of YSP1-2 contains a putative transmembrane domain whose length is consistent with localization to the ER membrane (Watson and Pessin, 2001). Since our analysis also predicted an F-BAR domain on the N-terminus of the protein, we felt strongly that this would connect YSP1-2 to another membrane (Liu et al., 2015). Indeed, we called this protein YSP1-2 because this domain

organization is similar to YSP proteins in yeast that act at ER-PM interface (Gatta et al., 2015). We expected *Tetrahymena* YSP1-2 to act similarly at the ER-PM interface and we expected that Delta-F-BAR would localize to the ER and the F-BAR domain to localize to the plasma membrane.

Yeast YSP proteins transfer cholesterol between the ER and the plasma membrane. We expected YSP1-2 to act similarly. We also tested the requirement for YSP1-2 in this regulation by expressing GRAM (cholesterol transfer domain) domain mutants and generating YSP1-2 knockout *Tetrahymena*.

To continue the investigation on the localization and function of YSP1-2 and the deletion mutations we need to isolate sufficient plasmid DNA and transform it into *Tetrahymena thermophila.* We are not successful in isolating a sufficient amount of plasmid DNA however we should be able to isolate sufficient plasmid DNA after generating larger bacterial cultures. We hope to be able to transform tetrahymena with these constructs in the near future.

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