Determination of Subcellular Localization of Brambleberry Homolog in *Tetrahymena thermophila.*

By

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ABSTRACT

During early embryonic development in the *Danio rerio*, maternal-effect factor Brambleberry *(bmb)* was shown to assist in pronuclear fusion, is localized on the nuclear membrane. The process of the fusion of chromatin-containing structures known as karyomeres has also been shown to be facilitated by Brambleberry, and is essential in the formation of a single mononucleus following fertilization. Questions concerning the conservation of the function of Brambleberry arose, and an *in-silico* Basic Local Alignment Sequence Tool search unveiled a homolog of *bmb* in the ciliate *Tetrahymena thermophila*. Depending on nutrient levels in the environment, *T. thermophila* is capable of performing either sexual or asexual reproduction. The sexual reproductive process resembles that of higher vertebrates, and involves the exchange of haploid nuclei, of which there are a migratory and residential pair. The migratory nuclei is exchanged with the mating pair, and the migratory and residential pairs then fuse, forming a diploid nucleus with genetic material from both mating pairs. It is this fusion event, and the formation of the diploid nucleus that will be studied through molecular techniques. Through the use of Polymerase Chain Reaction (PCR), a construct will be created containing upstream and downstream homology arms, along with a fluorescent marker, and a selectivity marker. This approach involves observation of the localization of Brambleberry in real time. The function of Brambleberry in *T. thermophila* will be compared to the function of Brambleberry in *Danio rerio.*

INTRODUCTION

Fertilization in vertebrates is a highly regulated set of processes involving multiple protein factors with varying roles. Mutations in these protein factors or malfunctions in the regulatory pathways may lead to early or late stage disruption in development, with either a failure of fertilization entirely, malformation, or the development of a disorder in the eventual fetus. In cases where successful fertilization does not occur after multiple attempts, considerations about the fertility of the parent are considered. In humans, the reason for infertility is complex and there is no single environmental factor or gene responsible. Environmental factors that have been shown to impact the ability of humans to conceive include cigarette smoking or exposure to heavy metals (Hruska et al. 2000). Genetically, the presence of abnormal inherited or mutated genes, including chromosomal abnormalities, or the malfunction of certain vital proteins such as the steroidogenic acute regulatory protein (StAR) (Venkatesh, 2014).

The study of the fertilization process has been extensive and ongoing, through the use of both human gametic cells directly, and model organisms - non-human species which are used in place of more complex organisms to study biological processes. *Saccharomyces cerevisiae*, or baker's yeast, is a common model for human processes as it was one of the first eukaryotic genomes to be sequenced (Bostein *et al.* 1997), and has contributed significantly to the understanding of human genes (Mohammadi *et al.* 2015). An additional model organism that is closely related to humans is *Danio rerio,* commonly known as Zebrafish, which are used as a model for human developmental processes and disorders (Dooley and Zon, 2000).

In yeast and zebrafish, there is a commonality to the fertilization process: membrane fusion events. In the general structure of a cell, a plasma membrane encloses a cytoplasm which

contains a nucleus, housing all genetic information. The nucleus is separated from the cytoplasm through a highly regulated membrane barrier, the nuclear envelope. The envelope has two membranes, the outer and the inner membrane, and for genetic exchange between two gametes, both membranes must fuse (Hetzer, 2010). The fusion event between gametic nuclei, also known as pronuclei, is a highly regulated process during which any disruption may lead to a pause or termination in further development. In vertebrates such as zebrafish, and *Xenopus laevis*, an additional fusion event occurs between, nuclear envelope enclosed chromatin structures known as karyomeres, which function to facilitate the division of large cells present following zygote formation (Abrams *et al.* 2012; Lemaitre *et al.* 1998). Following *in vitro* fertilization, clinical studies have demonstrated that multiple micronuclei in embryos are potentially linked to failed pregnancies (Balakier, 1997). This suggests a possibility that embryonic micronuclei may be formed through conserved processes involving the packaging of chromosomes into nuclear envelopes (Chaves *et al*. 2012).

After fertilization, cell proliferation begins in a highly regulated set of processes, controlled by multiple gene factors. In zebrafish, the first ten division cycles in a developing embryo are controlled by maternal-effect genes and promote cell cleavage in the zygote (Abrams *et al.*. 2012). After the Mid Blastula Transition (MBT), the division and cell movement are controlled by zygotic gene factors. Interruption or mutation in any of the genes involved in fertilization or embryogenesis can result in incomplete development of the embryo, or a halt in embryogenesis.

Initially discovered in zebrafish, the maternal-effect gene Brambleberry (*bmb*) has been shown to be an essential component in pronuclear and karyomere fusion (Abrams *et al*. 2012). The absence of *bmb* results in developmental arrest at the MBT. After anaphase, and going into

telophase, the karyomeres fuse with the assistance of Bmb (Abrams *et al.* 2012). The absence of Bmb inhibits fusion of individual chromatin bodies, resulting in the formation of multi-micronuclei in the blastomeres of the early embryo. Consequently, there is no further development or cell movement associated with the ensuing processes of epiboly and gastrulation.

While the role of Bmb in zebrafish has been determined and well-studied, additional proteins that interact with Bmb have not yet been fully investigated. Examining the function of Bmb in single-celled organisms allows for simpler isolation of the protein and observation of potential interactions through biochemical means. A Basic Local Alignment Search Tool (BLAST) analysis through a mutagenesis screen revealed a homolog of *bmb* in the genome of a single-celled, ciliated protist, *Tetrahymena thermophila*, and the highly conserved Brambleberry Homology Domain (BHD) was also found within the genome (Abrams *et al.* 2012). As *T. thermophila* undergoes nuclear membrane fusion and contains a *bmb* homolog (*Tet-bmb)*, the study of the mechanism of nuclear membrane fusion and potential interacting protein factors can give insight into the full mechanism of nuclear membrane fusion in zebrafish, and by discovering novel protein interactions, there is potential for further understanding of the mechanisms in the analogous process of karyomere fusion in humans.

In ciliates such as *Tetrahymena*, the somatic and germline genomes are separated into two distinct nuclei. In *T.thermophila*, the larger somatic macronucleus (MAC) is the primary source of gene transcripts in the cell (Ruehle *et al.* 2016), and directly determines the phenotype of the cell. The smaller germline micronucleus (MIC), is transcriptionally silent in vegetative cells, and stores the genetic information for the sexual progeny (Orias *et al.* 2011).

To ensure genetic diversity in *Tetrahymena* populations, *T.thermophila* are capable of undergoing both sexual and asexual reproduction, influenced by the availability of nutrients in

the surrounding environment. In nutrient-rich environments, the protist enters a vegetative state and reproduces asexually through binary fission (Orias *et al.* 2011). In nutrient-poor environments, the organism enters a conjugative state and undergoes sexual reproduction through conjugation, exchanging nuclei between two cells. This highly conserved eukaryotic process involves meiosis, gametogenesis, and gamete nucleus fusion to form a zygote.

During conjugation, the haploid MIC nuclei of two *T.thermophila* are exchanged, and then fused, forming a diploid nucleus (Orias *et al.* 2011). The factors involved in the pronuclear fusion event and the formation of the diploid nucleus are unknown. As *bmb* has a role in pronuclear fusion in zebrafish, *Tet-bmb*, which is expressed in both vegetative and conjugation stages, may be found to have a similar function during conjugation. The conjugation cycle is shown in **Figure 1**.

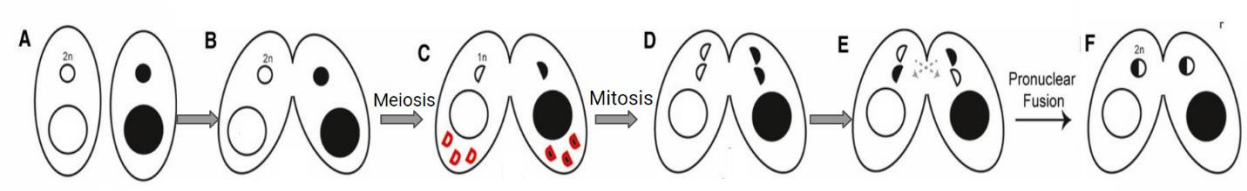

Figure 1: Conjugation cycle of *Tetrahymena thermophila*. Adapted from Ruehle *et al*. 2016. During sexual conjugation, *T.thermophila* undergoes fusion at the plasma membrane, followed by replication of the MIC nuclei through Meiosis, then Mitosis, where a residential and migratory pair are created. The migratory MIC nucleus is exchanged through a pore formed between the two mating types, and the migratory MIC nuclei fuses with the mating pair residential MIC nucleus.

There are seven different mating types that can undergo conjugation, and each mating type can conjugate with another mating, but not itself. The mating types are distinguished by specific membrane-bound proteins localized at contact regions throughout the cell membrane

The two mating types pair with the assistance of surface proteins such as HAP2, which has been shown to have a role in the production of a membrane pore allowing for the MIC nuclei exchange (Cole *et al.* 2014). It is this interaction between these membrane proteins which facilitate the transport and exchange of MIC nuclei between two mating types (Cole *et al.* 2014). While these interactions are understood, the exact mechanism behind the pronuclear fusion of the MIC nuclei are yet to be determined, and the focus of this paper.

Control over the conjugation process of *T.thermophila*, as well as the short doubling time of two to three hours (Ruehle *et al.* 2016). makes this organism an excellent model for this study. The genomes of both the MAC and MIC nuclei have been sequenced, allowing insertion of selective markers, epitope tags, into the MIC genome (Ruehle *et al.* 2016). Furthermore, the conserved eukaryotic processes involved in conjugation allow for comparisons to higher vertebrates.

The hypothesis of this study is that *Tet-bmb* has a role in pronuclear fusion during conjugation in *T. thermophila*. There are multiple molecular methods to determine the functionality of Tet-bmb. Fluorescent tagging is the insertion of a defined amino acid sequence for a specific fluorescent protein on a target protein. These proteins can be viewed under a fluorescent microscope. *T.thermophila* integrates transgenes through homologous recombination, allowing the efficient introduction of exogenous DNA into the chromosome (Kataoka *et al.*. 2012). Since the sequences for both the MAC and MIC nuclei of *T.thermophila* are known, an fluorescent tag can be designed and inserted into the genomic DNA. Foreign DNA can be introduced to organisms through a vector. Plasmids, small circular DNA units found in prokaryotes, serve as fundamental tools for insertion of novel DNA, as they are easily manipulated and linearized by restriction enzymes for placement into eukaryotic organisms. The

approach through fluorescent tagging allows for observation of protein localization in real time. The methods for plasmid-based, as well as PCR-based C-terminal epitope tagging, were first outlined in Kataoka *et al.*. 2012. To ensure proper integration of the vector DNA, selective markers are often introduced, such as neomycin resistance (Mochizuki, 2008). The methods outlined in Kataoka *et al.*. 2012 will be the basis for this study. Insertion of novel sequences into protein-encoding genomic DNA can disrupt the function of the protein. In gene knockout procedures, where the goal is to eliminate the function of the protein, the novel DNA can be inserted at many points along the DNA sequence (Mochizuki, 2008). However, fluorescent tagging is performed in order to examine the localization of a protein, so insertion of the tag at the C-terminus of the protein will be best to conserve function, and prevent misfolding of the protein, or disruption of membrane integration. Electroporation, the introduction of a difference in change across the *Tetrahymena thermophila* cell membranes disrupts the permeability of the of the cell and nuclear membranes, allowing for larger molecules to enter the cell and the nucleus (Ruehle *et al.* 2016). will be used to introduce exogenous DNA into the *Tetrahymena* MAC nucleus genome through homologous recombination to observe the localization.

Since the MAC nucleus is derived from the MIC nucleus, the localization of bmb in the MAC nucleus should be indicative of the localization of bmb in the MIC nucleus. The use of a ballistic gene gun will then introduce the bmb construct into the MIC nucleus (Gotesman, 2016).

If the *Tet-bmb* is shown to have a similar function to the homolog in zebrafish, it will be possible to then explore potential protein interactions that *Tet-bmb* has within the *Tetrahymena* genome. If there are interactions with other proteins or complexes are formed, these novel proteins can then be sequenced and a sequence or structural homolog can be searched for in *Danio rerio* or other organisms. With the presence of novel protein interactions in zebrafish,

there will be an increased understanding of the mechanisms behind early development and fertilization in higher vertebrates, and potentially a further understanding of the potential analogous process of karyomere fusion in humans.

METHODS

Testing Quality of Genomic DNA

The *T.thermophila* genomic DNA from strain SB210-E was obtained from the Tetrahymena Stock Center at Cornell University. 180µL at 334ng/µL were obtained. This was diluted to a working 50ng/µL for PCR. The quality of the genomic DNA was tested through the amplification Actin, an essential component of the cytoskeleton, as a positive control. The Actin (ACT-1) sequence and the flanking regions were obtained from the *Tetrahymena Genome Database* at *ciliate.org.* Primers for the amplification of Actin were taken from Williams *et al*. 2006.

Primer Design

The sequence of the genomic DNA of TTHERM_00188369 including the 3' and 5' flanks were obtained from the *Tetrahymena Genome Database* at *ciliate.org*. Polymerase Chain Reactions (PCR) are used to amplify specific sequences of DNA. For the insertion of a fluorescent tag, it is essential to have a pure DNA template. To prevent non-specific binding of primers within the genome, smaller sequences of the genome, containing the entire Brambleberry sequence, were amplified to be used as a local PCR genomic DNA template. Approximately 1500bp sequences were amplified to be used as a template for 700bp regions of the C-terminal end of *bmb*, preserving the sequence of the final exon to prevent disruption of the open reading frame, and therefore overall function of the protein. This 700bp sequence will serve as the Upstream Homology Arm (UHA). 800bp sequences were made as a template for a 700bp region for the 3' flanking region of *bmb*. This 700bp sequence will serve as the Downstream Homology

Arm (DHA). It is essential that these homology arms are between 500 and 1000 base pairs to minimize non-specific binding throughout the genome (Kataoka *et al* 2012). Additionally, it is essential that the distance from the stop codon to the beginning of the 4700bp total construct is large, to ensure there is no extra pressure exerted on the construct, disrupting proper insertion.

Placement of the Genomic Editing Primers was adapted from Kataoka *et al.*. 2012. *Integrative DNA Technologies' Oligo Analyzer tool* was used in an effort to minimize complications, and assure melting temperatures were similar. Primers were mapped onto the sequence using the software SerialCloner. Sequences for the Genomic Editing Primers are available in **Table 1**, and **Table 2** adapted after Kataoka *et al.*. 2012.

Primer Set	Primer Name	Sequence
Macro Primers		
Set 1	Tet-gDNA-F2	GCTATGCTAATGTCGAACAAGTT
	Tet-gDNA-R2	TCAGGACGATATTCTTAGTGCCA
Set 2	Bmb-DHA-F1	GAATTGTTTGCTAATCCGCTCT
	Bmb-DHA-1R	TTCACCATTCCACCTCCCTA
Set 3	Bmb-DHA-F1	GAATTGTTTGCTAATCCGCTCT
	Bmb-DHA-2R	AGGTGGAAGAAGGGAGCATT
Set 4	Bmb-DHA-F1	GAATTGTTTGCTAATCCGCTCT
	Bmb-DHA-4R	GAGAATCCCATCAAACCATCA
Set 5	Bmb-DHA-F1	GAATTGTTTGCTAATCCGCTCT
	Bmb-DHA-7R	TTTATTTGCTTTTTCACAACCTAA
Set 6	Tet-gDNA-2F	AAAGACAGATGGATGCGCTTA
	Bmb-DHA-1R	TTCACCATTCCACCTCCCTA
Set 7	Tet-gDNA-2F	AAAGACAGATGGATGCGCTTA
	Bmb-DHA-2R	AGGTGGAAGAAGGGAGCATT
Set 8	Tet-gDNA-2F	AAAGACAGATGGATGCGCTTA
	Bmb-DHA-4R	GAGAATCCCATCAAACCATCA
Set 9	Tet-gDNA-2F	AAAGACAGATGGATGCGCTTA
	Bmb-DHA-6R	CAAACCTGGATCATCAAATTCA
Set 10	Tet-gDNA-2F	AAAGACAGATGGATGCGCTTA
	Bmb-DHA-7R	TTTATTTGCTTTTTCACAACCTAA

Table 1: Primers needed for Local PCR Template Reactions.

Sequenced designed through the use of SerialCloner and Primer3.

Primer Set	Primer Name	Sequence
3'DHA Primers		
	Bmb-DHA-7F	CAAAGAGAAGCTTGCATTGAGA
	Bmb-DNA-7R	TTAGGTTGTGAAAAAGCAAATAAA
bmb Editing Primers*		
	Tet_F-Cterm	**GCTGATGGCGATGAATGAACACTG**AAGAAGAGGCAAACAGATCT
	Tet_R-3Flank	**GCGAGCACAGAATTAATACGACT**GTGTCATTCTAATTTAAGCAAGC
	Tet_Neo4_F.2	**CCCGGGGGATCTGAATTCGATATCAAGCTT**CAAAGAGAAGCTTGCATTGAGA
	Tet-pur4_F.2	**GATATCAAGCTTATCGATACCGTCGACCT**CAAAGAGAAGCTTGCATTGAGA
	Tet_mEGFP_R-Cterm	**AAGTTCTTCACCCTTAGAAACCATGGATCC**TTTTGGAAAATCAGAGAAACTTC
	Tet_mCherry_R-Cterm	**ATCTTCTTCTCCTTTTGAAACCATGGATCC**TTTTGGAAAATCAGAGAAACTTC
mEGFP-Neo4 Primers		
	BamH1-EGFP_Fw	GGATCCATGGTTTCTAAGGGTGAAGAACTTTTCACTGGTGTTGTTC
	HindIII-Neo4_Rv.2	AAGCTTGATATCGAATTCAGATCCCCCGGGCTGCA
mCherry-pur4 Primers		
	BamH1-mCherry_Fw	GGATCCATGGTTTCAAAAGGAGAAGAAGATAACATGGC
	HindIII-pur4_Rv	CGAGGTCGACGGTATCGATAAGCTTGATATC
Casette Primers		
	5'RACE-Outer	GCTGATGCGATGATGAACACTG
	3'RACE-Outer	GCGAGACAGAATTAAACGACT
Actin Primers		
	ACT-1Fwd	CCTCACGCCATCTTGAGAAT
	ACT-1Rev	AACTTCTCTTCCACTGCCGA

Table 2: Primers Needed for Gene Editing PCR Reactions

Sequence in bold and underlined indicates a single-stranded primer tail, that will anneal to additional sequences in the design.

TA Cloning

Once the local PCR genomic DNA templates have been successfully amplified, it is necessary to clone them further for use in future reactions. Following initial amplification using PCR, the local genomic DNA templates are inserted into a plasmid vector, and bacterial colonies are transformed. *Thermus aquaticus*, or *Taq* polymerase, a thermostable polymerase commonly used in PCR reactions, has terminal transferase activity which adds an adenosine to the 3' ends double stranded DNA. The majority of products amplified using *Taq* polymerase have a 3' adenosine overhang. Using a T-vector, which is engineered to have a 3' tyrosine overhang,

allows ligation of the PCR product into the T-vector without the need for a restriction digest. (Zhou, 2000). Additionally, Topoisomerase I from *Vaccinia* virus binds to the duplex DNA at specific sites, cleaving the phosphodiester backbone one one strand (Shuman, 1991). The energy from breaking this bond provides the energy needed to form a new phospho-tyrosol covalent bond between the tyrosyl residue of topoisomerase I and the 3' phosphate of the newly cleaved strand. The phosphor-tyrosol bond can then be reversed and the topoisomerase released, creating a new bond between the PCR product and the T-vector (Shuman, 1991). This allows the bacterial colonies to efficiently clone the PCR product in a convenient and labor-saving method (Zhou, 2000).

The T-vector in this experiment is the pCR-4-TOPO plasmid provided by Invitrogen™ TOPO® TA Cloning® Kit for Sequencing, (Invitrogen, 2014). The plasmid, shown in **Figure 2** contains a selectivity marker for Kanamycin and Ampicillin allowing for growth of *Escherichia coli* colonies on selectivity plates to select for colonies that have undergone transformation. These colonies are then grown in liquid media for isolation of plasmid DNA through the use of MiniPrep protocol.

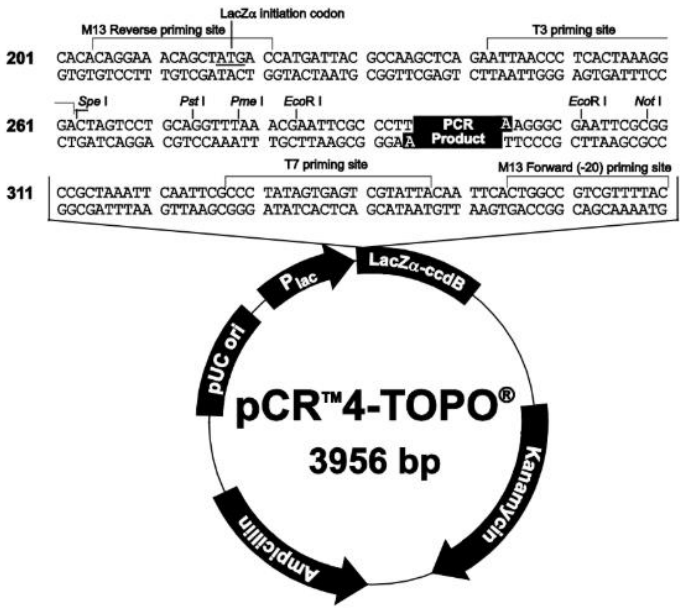

Figure 2: pCR ™ 4- TOPO ® plasmid from Invitrogen™ TOPO® TA Cloning® Kit for Sequencing

Plasmid used for cloning of the PCR products for the local genomic DNA template amplification through transformation of *E.coli* and growth of cultures.

Plasmid DNA Isolation

To use the sequence amplified through TA cloning, the pCR-4 TOPO plasmid is then isolated from the *Escherichia Coli* bacteria through GenElute™ MiniPrep. This method involves the use of DNA binding columns and centrifugations to isolate plasmid DNA. The bacterial cultures are harvested from the liquid media, then spun down into pellets. These pellets are then resuspended with an RNAse A buffer, then the cells are lysed using a lysis buffer. The lysis reaction is neutralized, and the columns are prepared, then the lysate is run over the columns following multiple washes to remove undesired compounds. The plasmid DNA is eluted from

the column using an elution solution, and the end result is purified plasmid DNA that can be used as a local genomic DNA template (Invitrogen, 2011).

Restriction Diagnostics

Restriction Digests are then used to determine the presence of an insert, as well as the sequence of the insert within the plasmid. Specifically, EcoRI is present on either side of the insertion within the pCR-4 TOPO plasmid, and can be used to determine the size of the insert. The sequence cut by SpeI is present within the amplicon itself, and digest products can be used to determine whether the desired amplicon was inserted, as well as the orientation of the insert thorough running on a DNA gel. Once it is confirmed that the inserted sequence is correct, the plasmid can then be diluted dramatically and be used as a template for the homology arms and for further PCR reactions.

Tagging Constructs

The tagging strategy was adapted from Kataoka *et al* 2012, in which a series of PCR reactions is used to synthesize a designed construct. To design the procedure to accommodate the lab equipment available, plasmids mEGFP-neo4 and mCherry-pur4 were used. This process is shown in **Figure 3**. Primers Bmb-cont-1F and Bmb-cont-4R were used to amplify 1500bp, containing sequences from both the C-terminal and 3'flanking regions. Once amplified, cloned and isolated, the B1F/B4R local genomic DNA template will be used to amplify roughly 800 base pairs of the C-terminal region of *bmb* using primers Tet-F-Cterm and Tet_mEGFP_R-3Flank (Tet-mCherry_R-3Flank for mCherry approach). Primers Bmb-DHA-7F and Bmb-DHA-7R are then used to amplify roughly 800 base pairs of the 3' flanking region of the amplicon.

The 7F/7R local genomic DNA template will then be used to amplify 800 base pairs of the 3' Flanking region, using primers Tet-Neo4_F.2 (Tet-pur4_F.2 for mCherry approach) and Tet-Cterm-R. The sequences in **Table 2** that are underlined and in bold are showing single stranded primer tails. These primer tails allow for annealing to the fluorescence and selectivity marker amplicons, as well as serving as primers for amplification of the entire construct.

Amplification of the 5' end of the mEGFP region of the pmEGFP-neo4 construct was done by primers BamH1-mEGFP-F. The 3' end of the neo4 region of the mEGFP-neo4 construct was done by primers HindIII-Neo4-R. Amplification of the 5' end of the mCherry region of the mCherry-pur4 construct was done by primers BamH1-mCherry-F. The 3' end of the pur4 region of the mCherry-pur4 construct was done by primers HindIII-pur4-R. These sequences can be found in **Table 2**. A depiction of the approach can be seen in **Figure 3**, adapted and modified from Kataoka *et al* 2012.

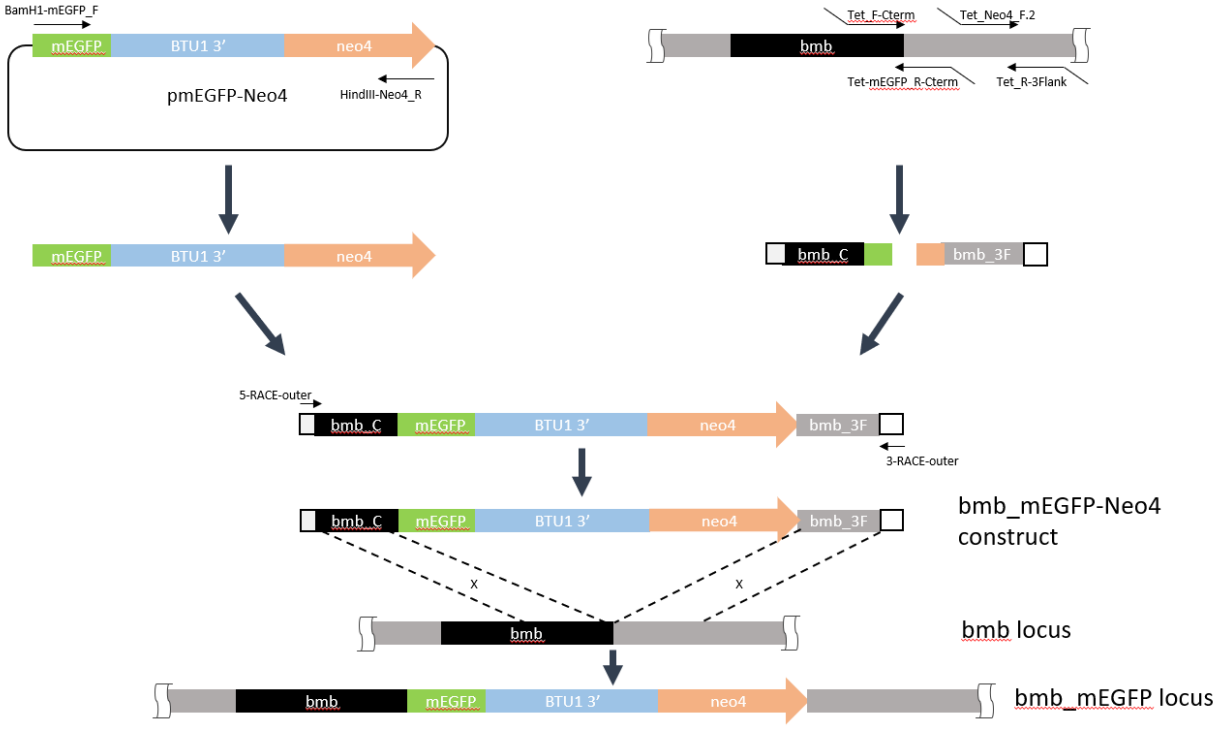

Figure 3: PCR Genomic Editing Strategy Schematic. Modelled after Kataoka *et al.*. 2012).

Approach modelled after Kataoka *et al*. 2012. For the mCherry approach, primers BamHI-mCherry_F will be used in place of BamHI-mEGFP_F, and HindIII-pur4_R will be used in place of HindIII-Neo4_R. Additionally, Tet-pur4_F.2 will be used in place of Tet_Neo4_F.2, and Tet-mCherry_R-Cterm will be used in place of Tet-mEGFP_R-Cterm. Sequences for these primers can be found in **Table 2**.

RESULTS

Local Genomic DNA Template Amplification:

In order to begin the design of the construct, a smaller, more manageable sequence must be amplified for use in the TA cloning reaction, and will act as a local genomic DNA template. The results of the PCR reaction are shown in **Figure 4** and **Figure 5**. The lanes shown in green were the proper sizes.

Restriction Diagnostic

In order to confirm the identity of the sequence incorporated into the pCR™ TOPO® plasmid, a restriction diagnostic must be done using EcoRI and SpeI. The results of these digests are shown in **Figure 6**. The lanes with the asterisk were the proper sizes.

Homology Arm Amplification

With the identity of the PCR product confirmed, the local genomic DNA templates can now be used to amplify the upstream and downstream homology arms. The results of this are shown in **Figure 7**.

Fluorescent and Selectivity Marker Amplification.

To confirm the integrity of the fluorescent and selectivity markers, and plasmids, a control test was done alongside the EcoRI digests following TA Cloning of the mCherry-pur4 and mEGFP-neo4 plasmids. The results of this are shown in **Figure 8.** The mEGFP-neo4 undigested control was 6,125 base pairs in total, and the digest drop-out was expected to be

1,697 base pairs. The remaining linearized plasmid was expected to be 4,428 base pairs. The mCherry-pur4 undigested control was 5,882 base pairs in total, and the digest drop-out was expected to be 1,796 base pairs. The remaining linearized plasmid 4,086 base pairs. As evident in **Figure 8**, these results were not obtained.

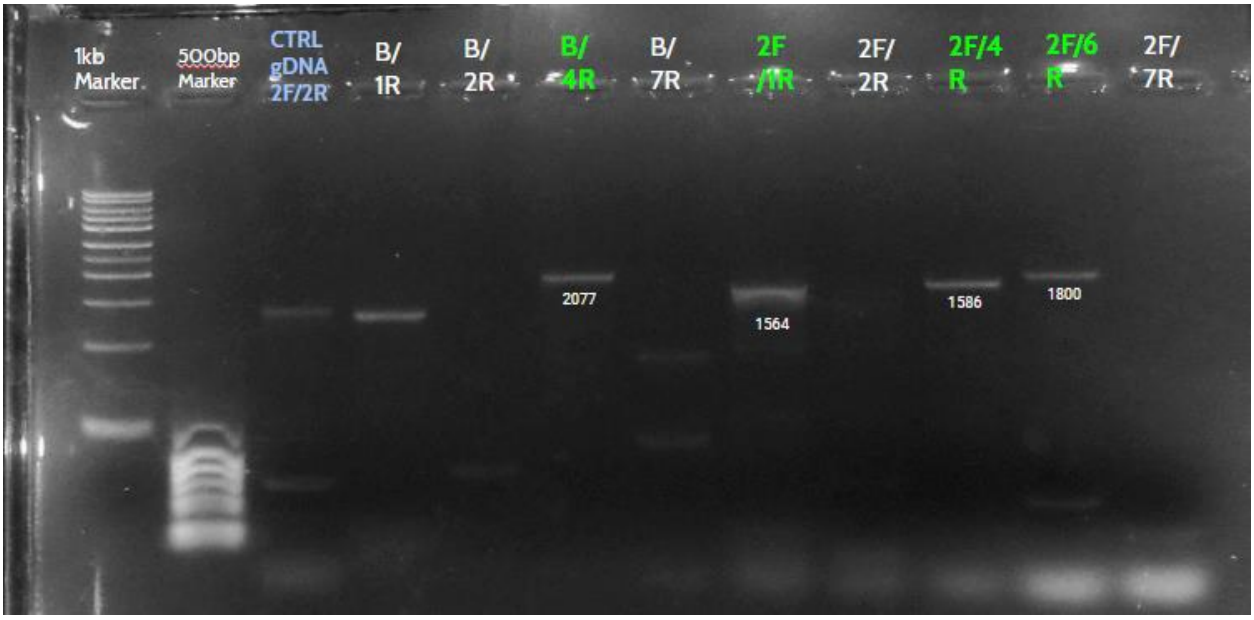

Figure 4: Local Genomic DNA Template amplification.

Lanes in green (B/4R, 2F/1R, 2F/4R. and 2F/6R) indicate successful amplification of the Local Genomic DNA Template.

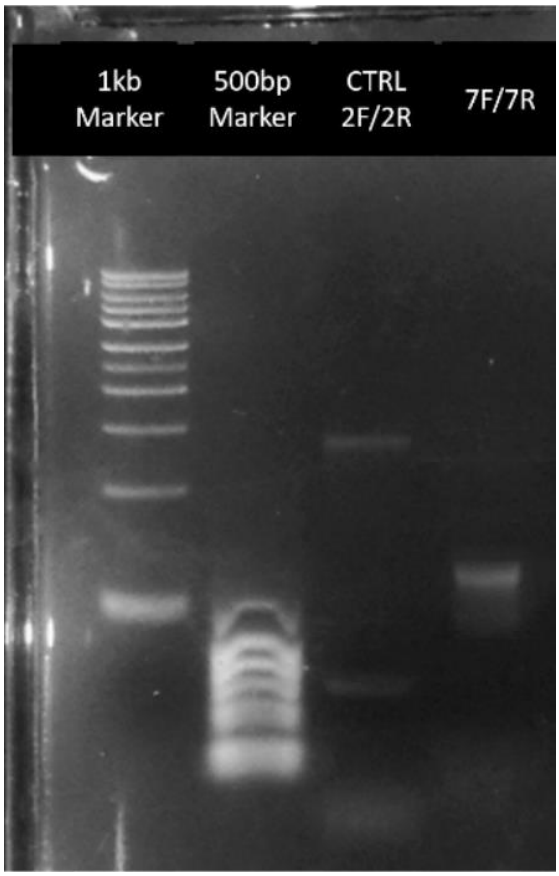

Figure 5: Local Genomic DNA Template Amplification, DHA.

PCR amplification of the local genomic DNA template Downstream Homology Arm, 7F/7R was successful, with an expected size of 800bp. 2F/2R was previously shown as a successful PCR amplicon, and was used as a control in this gel.

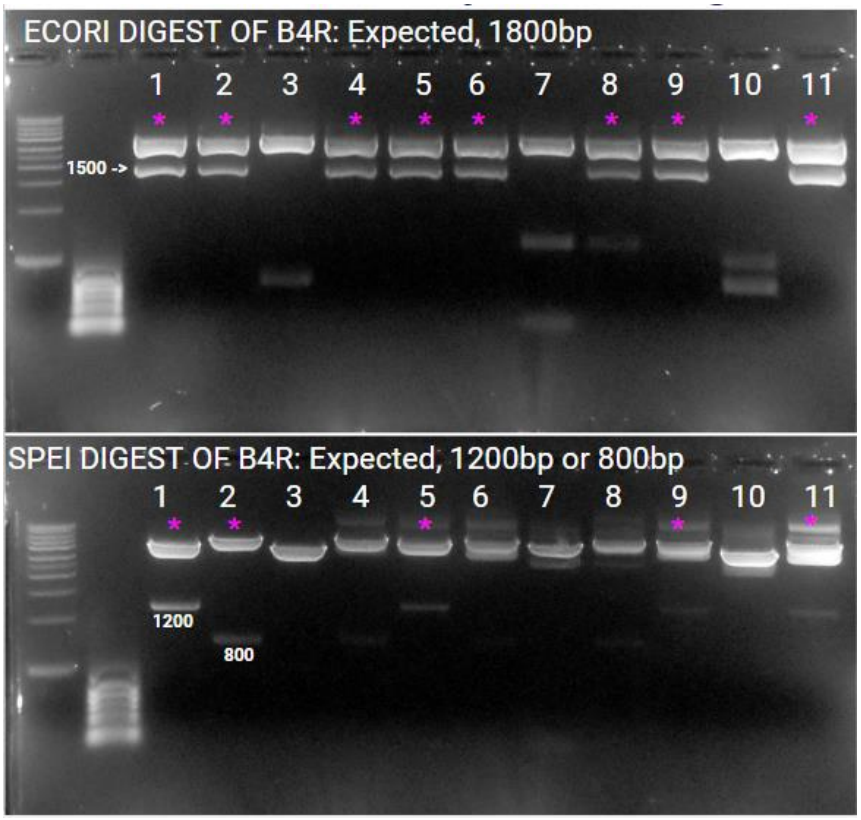

Figure 6: Restriction diagnostic of Bmb-cont-1F/Bmb-cont-4R.

Lanes with asterisk indicate correct sized bands were observed. In the SpeI digest of B4R, both orientations of the insert were seen at 1200bp and 800bp.

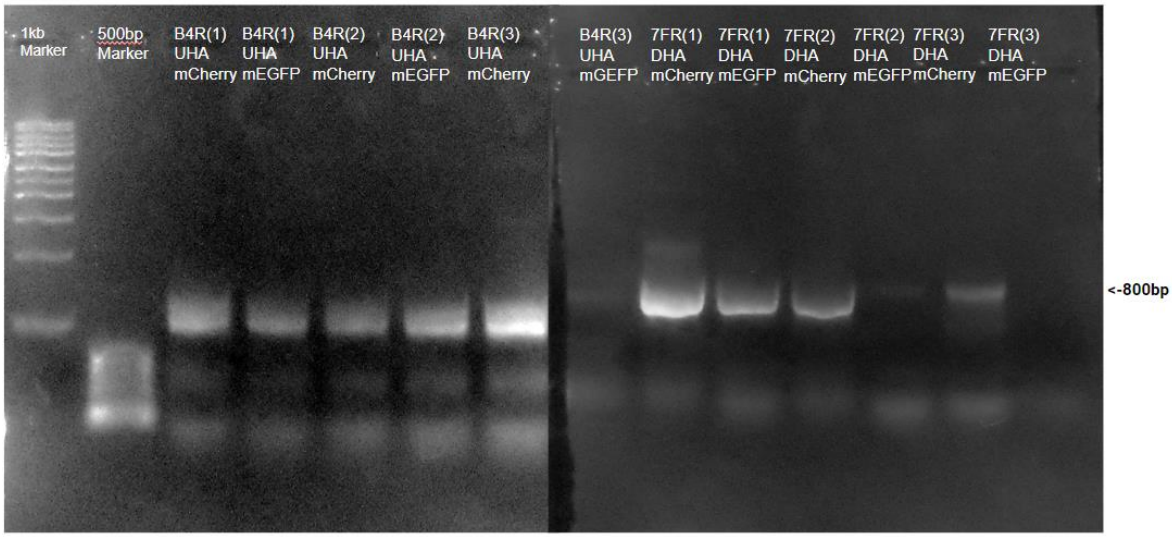

Figure 7: UHA & DHA Amplification

Amplification of the Upstream and Downstream Homology Arms from the local genomic DNA template was successful, each fragment had an expected size of 800bp.

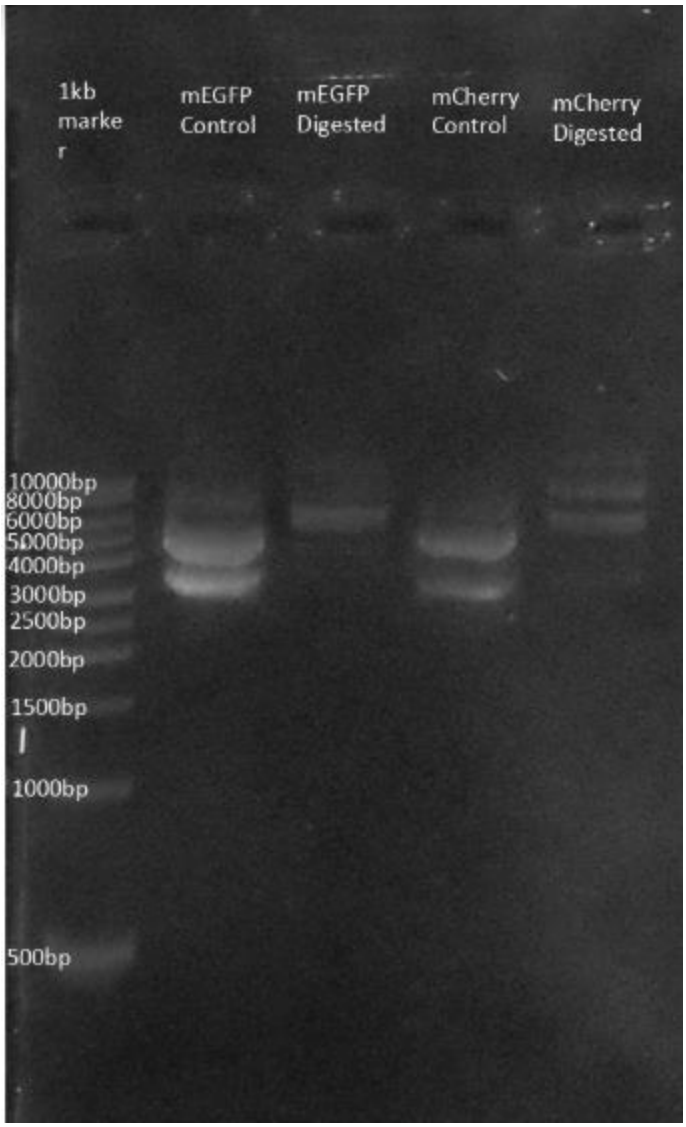

Figure 8: Amplification of mCherry-pur4 and mEGFP-neo4 plasmids.

A control test using the undigested mEGFP-neo4 and mCherry-pur4 plasmids was done, alongside the endonuclease digested plasmids to observe bands. Expected sizes were not seen, indicating a potential issue with the protocol, or polymerase.

DISCUSSION

Local Genomic DNA Template Amplification

The amplification of the local genomic DNA templates Bmb-cont-1F/Bmb-cont-4R and Tet-gDNA-7F/Tet-gDNA-7R through PCR appears to be successful as shown in **Figures 4 and 5.** This indicates proper primer design and PCR protocol.

Restriction Diagnostic

Following TA Cloning of the Bmb-cont-1F/Bmb-cont-4R local genomic DNA template, a restriction diagnostic was done using EcoRI and SpeI. This diagnostic confirmed the identity of the inserted sequences for further use in homology arm amplification.

Homology Arm Amplification

The use of the local genomic DNA template from Bmb-cont-1F/Bmb-cont-4R and Tet-gDNA-7F/Tet-gDNA-7R to create homology arms was successful. Each homology arm had an expected size of 800 base pairs, and each lane shows the correct sizes for the amplicons. Additionally, these homology arms have single stranded primer tails that can then be used to anneal to the fluorescence/selectivity marker to create the overall construct for insertion into *Tetrahymena thermophila*.

Fluorescent and Selectivity Marker Amplification

The failure of the amplification of the mEGFP-neo4 and mCherry-pur4 constructs is likely due to a number of factors. The Platinum™ polymerase used in the experiment has length of 5,000 base pairs (Invitrogen, 2015). The mEGFP-neo4 construct is 6,125 base pairs, which is

above the polymerase tolerance, and the mCherry-pur4 construct is 5,882 base pairs, also above the tolerance of the polymerase. The presence of multiple bands suggests that there was an incomplete digest of the plasmid. Further work and use of a higher length polymerase must be done.

Insertion of Construct into MAC and MIC Nuclei

Once the construct has successfully been made through further work, the construct must be inserted into the nuclei of *Tetrahymena thermophila*. To do this, two methods are used. The first of which, electroporation, will introduce the construct into the MAC nucleus and allow for observation of the localization under a fluorescent microscope. Electroporation introduces a temporary difference in charge across the *T. thermophila* membranes, disrupting the permeability of both the cell and nuclear membranes. This disruption allows for larger molecules to enter the cell and nucleus, and for the construct to be inserted into the genome through homologous recombination (Ruehle *et al.*2016).

As the structure of the MAC and MIC nuclei is the same, they are both contained in a nuclear envelope, the localization of *bmb* in the MAC nucleus should resemble the localization in the MIC nucleus. To confirm this, the construct will be inserted through the use of a biolistics "gene-gun"

Biochemical Approach

Examination of protein-protein interactions in the ciliate *Tetrahymena thermophila* can provide insight into potential novel proteins involved in pronuclear fusion in zebrafish. Through the use of affinity chromatography, highly specific interactions can be found, and can detect

weak interactions between proteins (Srinivasa Rao *et al*. 2014). However, affinity columns can occasionally produce false results.

The discovery of novel protein interactions in *T.thermophila* and examination of these proteins in zebrafish can provide further insight into the mechanisms behind pronuclear fusion in zebrafish, as well as potentially develop our understanding of the analogous process of karyomere fusion in human embryos.

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