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Generation of Mutant SNAP-23 to Arrest Mast Cell Degranulation at Trans-SNARE Complex Formation

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Generation of Mutant SNAP-23 to Arrest Mast Cell Degranulation at Trans-SNARE Complex Formation

By

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Abstract

Regulated secretion of pro-inflammatory molecules (e.g, histamines, proteases) from mast cells plays critical roles in immunity, allergic reactions, cardiovascular disease and cancer. These molecules are stored in secretory granules inside the cell and are rapidly released into the extracellular environment when mast cells are activated. It is known that mast cell degranulation depends upon membrane anchored SNAREs (soluble N-ethylmaleidimide-sensitive factor attachment protein receptors) and accessory proteins that form the trans-SNARE complex, a 4 helical bundle central to exocytic fusion. There are three SNARE proteins that contribute to the 4-helical bundle during exocytosis; Syntaxin and VAMP proteins each provide one helix each, SNAP-23 provides two helices. However, the biochemical properties of mast cell exocytosis are unclear because under physiological conditions the granules quickly proceed to fuse to the membrane. To investigate the properties of the degranulation related trans-SNARE complex, we decided to create a C-terminal truncation mutant of SNAP-23, which would arrest membrane fusion after the formation of the 4-helical bundle. Using PCR, SNAP-23 cDNA was amplified and inserted into pMBP-parallel expression vector; the ligation mixture was then transformed into E. coli cells and confirmed by sequencing. The wild type SNAP-23 was used as a template with custom made primers, amplified and inserted to yield a pMBP-parallel expression vector containing a mutant SNAP-23 cDNA lacking the C-terminal portion of the SNARE motif. The SNARE motif was sub-cloned and an IPTG induction process was used to induce to expression in Rosetta 2 *E. coli* cells. In this experiment, we were able to achieve successful cloning and expression of the mutant SNAP-23 omitting the c-terminus.

Key Words: Mast Cell, Degranulation, SNARE, SNAP-23, Cloning, Exocytosis

Dedication

“There is no god but Allah; Muhammad is the Messenger of Allah.”

To my father, their will never be enough for me to say or do to repay you for the life you have provided me. I work to make you proud in everything I do. I love you.

To my step-mother, you have been everything and more than I could have asked for in a mother. I love you to the moon and back.

To my life partner Deondrey Russell, thank you for keeping me sane- so much is meaningless without you. I love you.

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Chapter 1: Statement of Research

The regulatory processes of the human body are under continual development. Advances in human disease and response are continually contributing to the expansion of understanding cell communication, specifically, endo- and exo- cytic pathways. Located in connective and mucosal tissues are major contributors to the human defense towards pathogens, mast cells. Regulated secretion of pro-inflammatory molecules (e.g., histamines, proteases, proteoglycans) from mast cells play critical roles in immunity, allergic reactions, cardiovascular diseases and cancer (Wernersson and Pejler, 2014). These molecules are stored in specialized secretory granules inside the cell, and are rapidly released into the extracellular environment when mast cells are activated, a process known as mast cell degranulation. Mast cells are capable of storing an abundance of these secretory granules in their cytoplasm upon which they can be immediately released via degranulation (Blank, 2004). It has been confirmed that the secretory granules located within mast cells release their contents through the interaction of SNAREs (soluble N-ethylmaleidimide-sensitive factor attachment protein receptors).

The biochemical properties of the trans-SNARE complex are not clear because under physiological conditions they quickly proceed to fuse the membrane. To investigate the properties of the degranulation related trans-SNARE complex we decided to create a C-terminal truncation mutant of SNAP-23, which we hypothesized would arrest membrane fusion after the formation of the 4-helical bundle. Based on published studies of SNAP-23 homologs in neurotransmission and vacuole fusion this goal will be accomplished by using PCR, SNAP-23 cDNA will be amplified and inserted into pMBP-parallel expression vector; the ligation mixture will then be transformed into Nova Blue *E. coli* cells and confirmed by sequencing. The wild type SNAP-23 will be used for custom made primer design and expressed for use as a template

strand in PCR to yield a pMBP-parallel expression vector containing a mutant SNAP-23 cDNA lacking the C-terminal portion of the SNARE motif. The mutant SNAP-23 will be generated using PCR and subcloned into the pMBP-parallel vector for expression in Rosetta 2 E. coli cells.

Chapter 2: Literature Review

Mast cells are leukocytes derived from hematopoietic progenitor cells that have been commonly associated with their role in allergies and inflammatory disease. Mast cells participate in blood circulation and are found in tissues associated with the external environment: skin, airways, intestine, etc. (Urb and Sheppard, 2012). Research in mice has exposed mast cells as having negative responses in various situations. This development contributes to urgency of the research at hand, demonstrating the importance of unraveling the specifics within the process of mast cell degranulation. Upon stimulation mast cells have the ability to release a variety of inflammatory signals stored inside of secretory granules located in the cell in a single stimulator event; this property has given the cells innate and adaptive immunity properties (Blank, 2004). Previous research has revealed that proper loading and releasing of these inflammatory molecules from their granules are highly dependent on the expression of proper proteoglycans. In allergic reactions, sensitized mast cells are activated upon stimulation of IgE surface receptors, allowing the pro-inflammatory molecules to be released via exocytosis (Figure 1) (Blank, 2004). Exocytosis follows a four-step pathway, 1. Targeting 2. Docking 3. Priming and 4. Fusion (Figure 2). There are several mediators bound to the proteoglycan matrix at the plasma membrane fusion site that are required for the proper functioning mast cell degranulation (Blank, 2004). New functional discoveries have sparked investigation in understanding the specific mechanism of secretory degranulation.

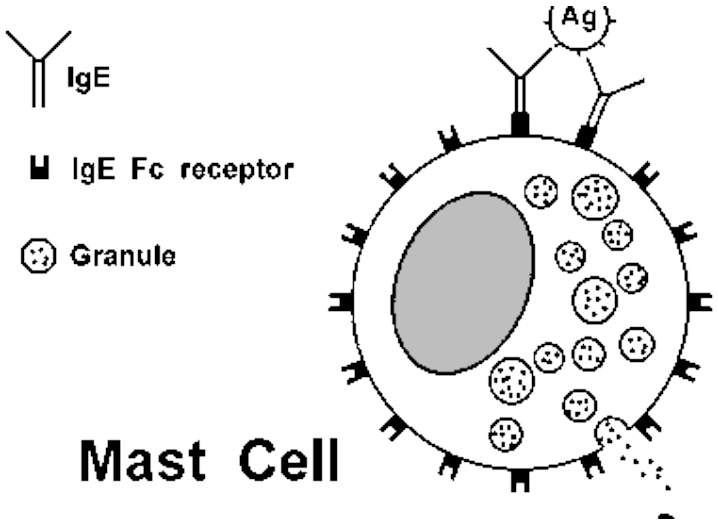

Figure 1. Diagrammed process of mast cell degranulation when IgE becomes bound to mast cells in places such as respiratory tract mucosa. Mast cell degranulation leads to the release of primary mediators (such as histamine, serotonin) and secondary mediators (such as leukotrienes, prostaglandin).

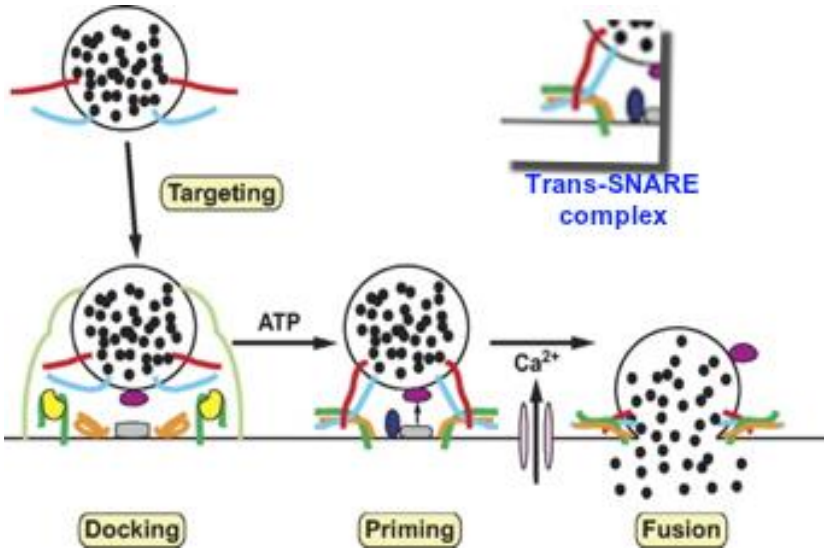

Figure 2. Model of regulated exocytosis. Key components involved in docking, priming, and fusion steps are indicated (Seino, 2005).

Currently, research has revealed that intracellular membrane fusion (Figure 3) involving mast cell exocytosis is dependent upon membrane anchored SNAREs (soluble N-ethylmaleidimide-sensitive factor attachment protein receptors) and accessory proteins for their final fusion with the plasma membrane (Xu et al. 2015). SNARE proteins, which were first identified in the early 1990s, promote fusion by forming a four helical bundle referred to as the trans-SNARE complex, a central step in the fusion process (Marsden et al., 2011). The characterization of SNARE proteins has identified them as crucial components in membrane fusion. The key to understanding the function of SNAREs in membrane fusion rests on two discoveries; the different sets of SNAREs and the disassociation complex that recycles SNAREs caused by *the N-ethylmaleimide* sensitive fusion (NSF) proteins. The two classes of SNAREs involve the v-SNARE (vesicle-membrane SNARE) and the t-SNARE (target-membrane SNARE). Although there are several proteins associated with the SNARE complex, its formation, and its function, these two classes in play a particular role in the fusion in mast cells (Jahn et al., 2006).

In resting cells, Q-SNAREs are bound together in a cis-SNARE complex, which, on binding to secretory vesicle or granule R-SNAREs (VAMP), is transformed into a four-helix bundle called the trans-SNARE complex. Formation of the trans-SNARE complex leads to fusion of the granule and plasma membrane, followed by extrusion of granule contents (Jagath et al., 2001). There are 3 SNARE proteins that contribute to the 4-helical bundle in exocytosis; while syntaxin and VAMP proteins each provide one helix, SNAP-23 protein provides two helices (figure 4). Previous research has identified syntaxin and VAMP homologs that are all capable of functioning in the trans-SNARE complex. However, no other homologs of SNAP-23 have been identified in mast cell exocytosis, making SNAP-23 imperative to the function of the

exocytic trans-SNARE (Marsden et al., 2011). SNARE proteins have since been characterized as key elements involved in mediating membrane fusion of many secretory pathways, as in the secretory granules within mast cells.

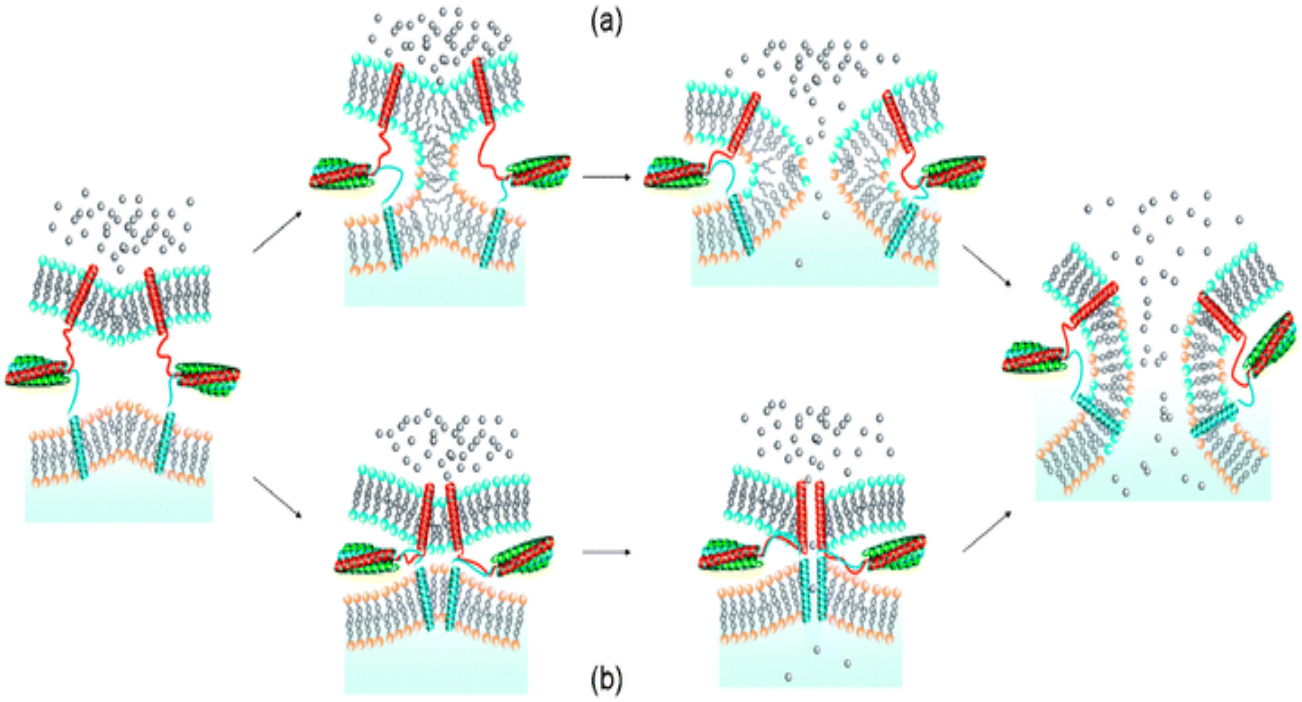

Figure 3. Models for intracellular membrane fusion. Two general models for membrane fusion are depicted, using SNAREs to illustrate the roles of proteins. (a) The "proximity" model shows a fusion pore lined by lipids. Proteins draw two lipid bilayers together, driving hemifusion and then full fusion. (b) The "protein-pore" model shows a fusion pore formed from transmembrane domains (TMD) of proteins. When the fusion pore dilates, the TMDs separate laterally, lipids are incorporated into the pore, and the bilayers merge. While merged the two vesicles become one allowing for the exchange of contents (Stanley and Lacy, 2010).

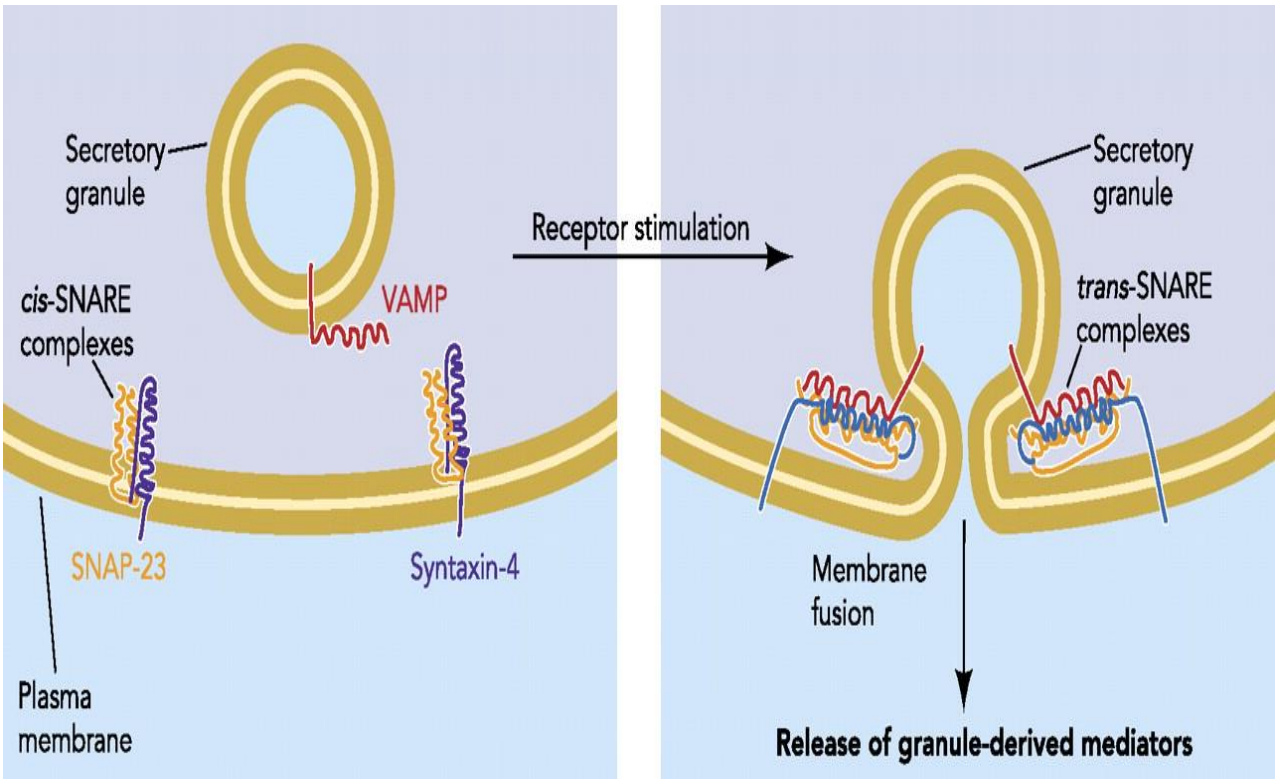

Figure 4. SNARE-mediated vesicle or granule fusion in exocytosis.

Due to the difficulty in mimicking the specificity of in vivo conditions, many mechanisms of the trans-SNARE complex remain unclear. We hypothesize that the creation of a C-terminal truncation mutant of SNAP-23 could arrest membrane fusion of the granule to the plasma membrane. Our hypothesis was tested by using a cloning protocol to yield a mutant SNAP-23 cDNA lacking the final 63bp, C-terminal, portion of the SNARE motif. Upon completion, the research will contribute to future understanding of the regulatory metabolic pathways involved in immune response. Furthering the development in structural function of these pathways will provide pioneers with opportunities for mechanism manipulation in the area of disease treatment and prevention.

Chapter 3: Materials and Methods

PCR

The template DNA sample HXP_O34, 10uM primer, and 10X pfu buffer were put on ice to thaw. A PCR mix containing: 40uL sterile milipore water, 5uL 10x pfu buffer (G Biosciences Lot No: 123105), 1uL of template DNA sample, 1uL of 10uM NcoI forward specific primer, 1uL of 10uM EcorI reverse specific primer, 10mM dNTPs (New England Biolabs Lot No: 0711209), and 1uL pfu DNA polymerase (G Biosciences); were placed in a sterile PCR tubes. Using a thermocyler a PCR was run using the following program: Initial denaturation at 94°C x 5 min, denaturation at 94°C x 30 sec-annealing at 55°C x 30 sec-extension at 72°C x 1 min for 5 cycles; 94°C x 30 sec- 60°C x 30 sec- 72°C x 2 min for 25 cycles; and 72° x 10min and held at 4°C. Following completion

of the PCR, the PCR product was run on agarose gel for 1 hour at 80V to verify presence of gene of interest. To prepare the 0.7% agarose, 0.35g agarose powder was weighed out, 50mlL of 5x TBE buffer was added and the mixture was microwaved for 1min (or until completely dissolved). The dissolved mixture was poured into gel plate, well combs were added and the mixture was allowed to solidify for 1 hour. 10uL of the PCR product was mixed with 2uL of 6x loading dye and loaded in the well; 10ul of lamda DNA standard was added to a separate well to aid I product verification. The remaining 40uL of PCR product was stored in -20° freezer for late use. Following completion of the gel run, the gel was placed under UV light in biohazard area for image capture of the presence verification.

To purify the PCR product containing the mutant SNAP-23 omitting the c-terminus, the remaining 41uL of PCR product was used with a QIAquick PCR purification kit (Cat. No: 28104). 41uL of PCR product was transferred to a sterile 1.5mL centrifuge tube. 200uL (5x

volume of PCR product) PB buffer was added to the tube as well as 10uL sodium acetate which caused the solution to turn yellow. The mixture was transferred to a sterile QIAquick purple spin column and spun down using a centrifuge set at parameters 13krpm x 1 min to bind the DNA. The flow through was duped and 750uL of PE buffer was added to wash, the solution was then spun again at 13krpm x 1 min 3 times. The column was transferred to a sterile 1.5mL centrifuge tube and 50uL of sterile milipore water was added to column to sit at room temperature x 1 min. The solution was eluted by spinning down at 13krpm x 1 min. The purified product taken to the Nanodrop to determine the concentration of DNA in ng/uL and then stored at -20°C.

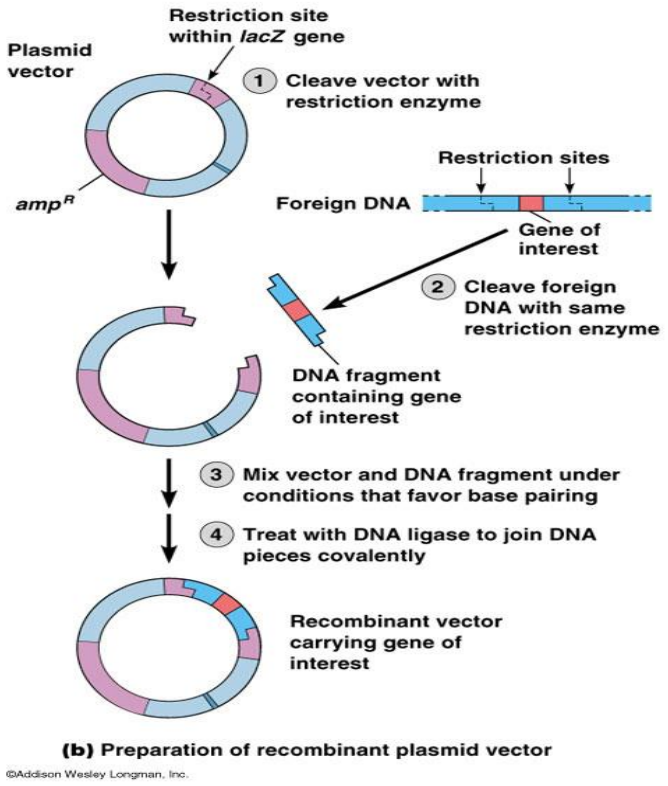

Figure 5: The cloning process involves using restriction enzymes to cut the insert and vector in preparation for ligation to create a recombinant plasmid vector containing the gene of interest.

Plasmid Isolation of Vector

In preparation for restriction digestion of the gene of interest the vector pMBP-parallel (HXB_OC21) was inoculated for plasmid isolation. The lab bench was sterilized using ethanol and near a Bunsen burner 100mg/mL ampicillin was obtained form -20°C freezer and allowed to thaw and 1000x dilution to 100ug/mL was performed by adding 6uL of ampicillin to 6mL. A sample of the bacterial vector was obtained using a sterile wooden applicator were mixed in a a sterile beaker and incubated at 220rpm at 37°C overnight. The culture was retrieved after overnight incubation and a QIAprep Spin Miniprep kit (Cat. No: 27106) was used for plasmid isolation. The overnight culture was gently vortexed and 1.5mL of the culture was transferred to a sterile 1.5mL centrifuge tube. The tube was centrifuged at 13krpm x 1 min at 4°C, the supernatant was dumbed and the process was repeated to obtain an acceptable pellet. The tube was vortexed to re-suspend the pellet and 250ul P1 buffer, 250uL p2 buffer was added to the tube then the sample was inverted 10x for mixing. 350uL of N3 buffer was added and the tube was inverted and then placed on ice for 5 minutes. The tube was then spun at 13krpm x 10 min at 4°C and the supernatant was transferred to a sterile blue QIAprep spin column. The column was centrifuged at 13krpm x 1 min at 4°C, then followed by the addition of PB buffer and centrifuged at the previous parameters. 750uL of PE buffer was added to the tube and sat at room temperature for 1 min and centrifuge at the previous parameters again, followed by the discard of flow through. The spin column was transferred to a sterile centrifuge tube and 50uL of EB buffer was used for elution. The isolated plasmid was taken to the Nano drop to obtain the DNA concentration and then stored at -20°C.

Restriction, Digestion, & Ligation

To perform a restriction-digestion and ligation of the mutant SNAP-23 and vector pMBP-parallel a reaction was set up using two separate sterile PCR tubes. 41uL of purified PCR product was added to one tube and 41uL of pMBP-parallel vector was added to the other tube; in addition, 5uL 10x NEB Buffer (Cutsmart), 2uL EcorI restriction enzyme, and 2uL NcoI was also added to each tube. The two tubes were placed in a 37°C water bath for 3 hours, after 3 hours, the tubes were put on ice for 2 minutes, and then heat inactivated at 65°C for 20 minutes. The vector was treated with 1uL alkaline phosphatase (rSAP Shrimp alkaline phosphatase) and added back to the water bath for an additional hour while the tube containing the insert DNA was stored at -20°C freezer for later use. After an hour, the vector containing tube was heat inactivated at 65°C and stored in the -20°C freezer as well. The products were thawed, each tube was mixed with 10uL 18X gel red loading dye and ran on a 1% agarose gel at 80V for 1 hour.

Upon completion of the gel run, the digested products in the gel were extracted and purified using a QIAquick gel extraction kit (Cat. No: 28704). The gel band was excised with a sterile scalpel and weighed (0.40g-insert & 0.26g-vector), placed in a sterile purple QIAquick spin column. Three times the volume of buffer: 0.40g x 3= 1200uL insert digestion product and 0.26g x 3= 780uL vector digestion product, was added to the appropriate tubes and incubated at 50°C for 10 min, or until gel is completely dissolved. One gel volume of isopropanol was added to the sample and the column was centrifuged at 13krpm for 1 min. The flow through was discarded and 750uL PE buffer was added and centrifuged again at the previous parameters. The column was placed in sterile centrifuge tube and eluted with 50uL sterile milipore water. The elution was taken to the Nano drop to obtain the DNA concentration.

Ligation of the mutant SNAP-23 and pMBP-parallel to create a recombinant plasmid was achieved by setting up two reaction mixtures in PCR tubes. One uL of 10x T4 ligase buffer (#BO2025 10mM New England BioLabs), 1uL T4 ligase was added to each tube; 6uL vector and 2uL insert was added to one tube and 6uL vector and 2uL milipore water was added to the other tube. The amount of vector and insert that needed to be added was determined by using 2 formulas: Insert ng = 5[BP insert/BP vector] x vector ng, and ng of insert + ng of vector = 100ng.The cocktails were incubated at 17 overnight in the PCR thermocycler. After the PCR thermocycler cycle finished the samples were stored then stored at 4°C for transformation.

Transformation of Novablue

The ligated products were transformed into competent *Escherichia coli* Novablue cells (Novagen). Competent Novablue cells were obtained from the -70°C freeze and allowed to thaw on ice. In a sterile centrifuge tubes 15uL of cells were mixed with 1.5uL ligation products and mixed by gently flicking. The tubes were put on ice for 30 minutes, followed by a heat shock at 42°C for 45 seconds and immediately returned to ice for an additional 10 minutes. 75uL of SOC medium (Novagen Lot No: 3163c400) was added to each tube and the tubes were incubated at 37°C for 1 hour. Following the 1-hour incubation period, the mixture was transferred to 100ug/mL Ampicillin and 34ug/mL Chloramphenicol resistant selective agar plates and spread by rotating 5-7 sterile glass beads horizontally on the lab bench. The glass beads were removed and the plates were incubated at 37°C for 20 minutes then inverted and incubated at 37°C overnight.

Plasmid Isolation and Sequencing

The plates were removed from the incubator after the overnight incubation period and the colonies were recorded and picked for use in plasmid isolation. A colony from each

transformation was plucked using a sterile wooden applicator and transferred to sterile test tubes containing 5mL sterile LB/Amp, 100ug/mL/Cam, 34ug/mL broth and incubated overnight at 220rpm at 37°C overnight. The overnight culture was removed from the incubator and the the plasmid isolation was done using a QIAprep Spin Kit (Cat. No: 27106), according to the same protocol used in plasmid isolation of vector. The elutions were taken to the Nanodrop to obtain the DNA concentration and the plasmids were logged and stored in -20°C.

Samples of the mutant SNAP-23 omitting the C-terminus were prepared and sent out for sequencing for confirmation. The two plasmids with highest recorded concentration from the Nanodrop were prepared by thawing both the primers and plasmids on ice. The sequencing solution was composed of 5uL DNA, 3uL of sterile milipore water, and 4uL of each primer for a total volume of 12uL. The sequencing results were analyzed using nucleotide BLAST to determine any errors in the cloned sequences before transformation into expression *E. coli* Rosetta 2 (DE3) competent cells (Novagen).

Transformation into Rosetta 2

The mutant SNAP-23 was transformed into competent Rosetta 2 (DE3) cells to prepare for expression. Competent Rosetta 2 cells were obtained from the -70°C freezer and thawed out on ice. In a sterile 1.5mL centrifuge tube on ice 10uL of competent cells were mixed with 1uL of each plasmid by gently flicking. The tubes were iced for 30 minutes, followed by heat shock at 42°C x 30 sec and immediately placed on ice for 10 minutes. 90uL of sterile LB brother were added to each tube and the tubes were incubated at 37°C for 1 hour. 50yL of each mixture were plated onto Amp, Amp100/Cam34 resistant plates. The plates were incubated at 37°C for 20 minutes then inverted and incubated at 37°C overnight.

Small-Scale Induction

The overnight cultures were removed from the incubator and a single colony was inoculated in 5mL Amp100/Cam34 LB at 220rpm at 37°C overnight. The OD_{600} of the overnight bacterial cultures was taken and the overnight cultures were transferred to 5mL LB, Amp100, and Cam34 into two separate test tubes labeled +IPTG and –IPTG. The tubes were placed back into the rotating shaker at 220rpm at 37°C for 2 hours (or until OD_{600} reaches 0.4-0.8). After 2-hour incubation, or OD_{600}, period 2.5uL 1M IPTG was added to the +IPTG tube and the tube was returned to the rotating shaker. The tubes were incubated for an additional 4 hours at 37°C/220rpm. The OD_{600} was taken at the end of the 4-hour incubation period and 1mL of each sample was transferred to 1.5mL sterile centrifuge tube. +IPTG and –IPTG tubes were centrifuged at 13krpm for 1 min and the supernatant was discarded. The pellet was re-suspended in 100uL 2x SDS Page loading buffer containing 1mM fresh PMSF. Small glass beads were added to each tube, the tubes were vortexed for 1 minute and then boiled at 95°C x 5 minutes and stored at -20°C.

12% SDS-PAGE

Using a sterile falcon tube: 6.6mL milipore water, 5mL 1.5M Tris, 200uL 10% SDS, 8mL 30% acrylamide mix, 200uL 10% ammonium persulfate and 8uL TEMED were all mixed together. 4.5mL of the solution was then poured into 1mm glass plates and isopropanol was added to each pocket and allowed to solidify. Once solidified, the isopropanol was rinsed off using sterile milipore water. To prepare the 5% stacking layer 5.5mL sterile milipore water, 1mL of 1.0M Tris, 1.3mL 30% acrylamide, 80uL 10% SDS, and 80uL 10% Ammonium Persulfate and 8uL TEMED were mixed to create the 5% stacking layer. The 5% stacker layer was poured onto the 10% SDS-PAGE and the appropriate combs were inserted in the gel to form the

appropriate wells. A protein ladder was prepared by mixing 10uL of sample buffer with 0.5uL SDS Protein Standard, boiled for 5 minutes and loaded into a well. The samples were thawed from -20°C and 10uL of each sample were loaded; the gel was run at 150V for 70 minutes using BioRad Mini-PROTEAN Tetra System. Following completion of the gel run, the gel was removed from the apparatus and a pry tool was used to cut away the stacking gel. The gel was placed in a plastic staining container and covered with Coomassie Blue Staining Solution for 1 hours, then destained overnight with destining solution.

Chapter 4: Data and Results

We used SNAP-23 cDNA obtained from a rat and amplified it using PCR. The amplified DNA was used to perform a restriction, digestion and ligation into pMBP-parallel expression vector. The plasmid was transformed into Nova Blue E. coli cells and the plasmid was re-isolated to be sent for sequencing and transformation in Rosetta 2 (DE3) competent cells for induction using IPTG. The induced products were run on an SDS-PAGE to confirm expression.

Rattus norvegicus

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>gi|117935058:149-781 Rattus norvegicus synaptosomal-associated protein 23
(Snap23), mRNA
ATGGATGATCTATCACCAGAAGAAATTCAGCTTCGGGCTCACCAAGTTACTGATGAGTCTCTGGAAAGCA
CAAGGAGAATCCTGGGTTTAGCCATTGAGTCTCAGGATGCAGGAATCAAGACTATCACTATGCTGGATGA
GCAAGGGGAACAACTAAATCGCATAGAAGAAGGCATGGACCAAATAAATAAAGACATGAGAGAGGCAGAG
AAGACTTTAACAGAACTCAACAAGTGTTGTGGCCTCTGCGTCTGCCCTTGTAATAGGACCAAGAACTTTG
AGTCTGGAAAGAACTATAAGGCAACATGGGGTGATGGTGGAGACAGCTCACCTAGCAATGTGGTATCTAA
GCAACCAAGCCGGATAACAAATGGTCAGCCTCAGCAGACTACAGGAGCGGCCAGCGGTGGATACATTAAA
CGCATAACTAATGATGCCAGAGAAGATGAGATGGAAGAGAACCTGACTCAAGTGGGCAGCATCCTAGGGA
ACCTAAAGAACATGGCTCTGGATATGGGCAATGAAATTGATGCTCAAAACCAGCAAATACAGAAGATCAC
AGAAAAGGCTGACACCAACAAGAATCGCATTGACATTGCCAATACAAGAGCAAAGAAACTCATTGACAGC
TAA
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synaptosomal-associated protein 23 (Snap23) Fasta sequence

Figure 6: Fasta sequence of Rat SNAP-23. Goal will be to delete final 63bp (outlined in red) that are responsible for encoding domain of complete SNARE-complex.

PCR results

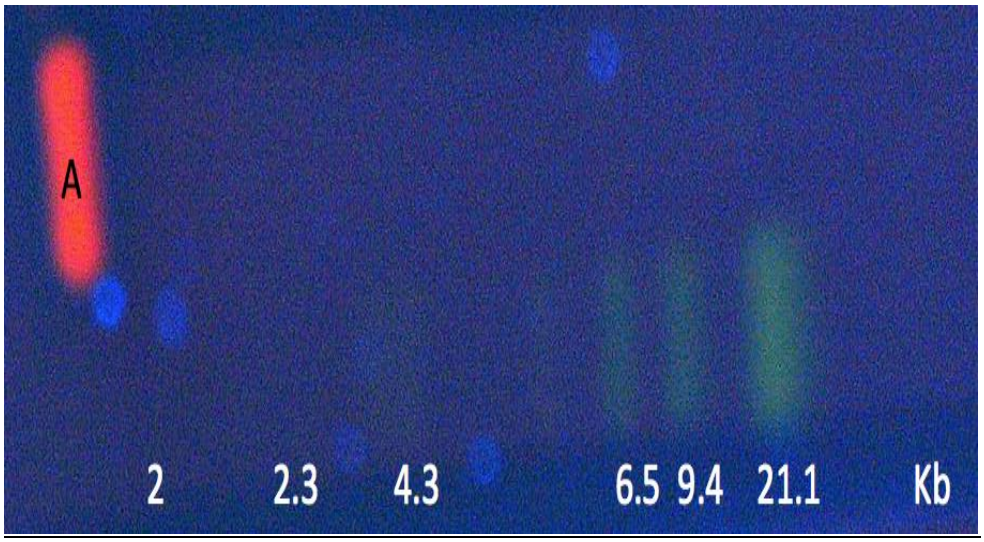

Figure 7: PCR confirmation of mutant SNAP-23 using gel electrophoresis.
The visible band of the truncated SNAP-23 (A) omitting the final 63 bp was measured was measured at 0.57 Kb.

Table 1: Nano-drop readings after PCR purification, vector isolation, gel extraction, and recombinant plasmid isolation

		ng/uL	260/280	260/230
PCR purification	SNAP-23 (mutant)	76.3	1.82	2.10
Vector Isolation	pMBP-parallel	47.5	2.15	2.15
Gel extraction (vector)	pMBP-parallel	32.4	2.99	0.01
Gel extraction (insert)	SNAP-23 (mutant)	20.5	1.54	0.04
Plasmid Isolation of plasmid from Rosetta 2	Sample #1	102.7	1.84	1.54
Plasmid Isolation of plasmid from Rosetta 2	Sample #2	102.1	1.86	1.85
Plasmid Isolation of plasmid from Rosetta 2	Sample #3	90.4	1.88	2.03
Plasmid Isolation of plasmid from Rosetta 2	Sample #4	88.7	1.90	2.04

Table 2: Ligation set-up

	Vector & Insert (pMBP-parallel + SNAP-23 mutant)	Insert (pMBP-parallel/control)
Vector	6uL	6uL
Insert	2uL	0uL
10x T4 Ligase	1uL	1uL
Tu ligase enzyme	1uL	1uL
Milipore water	0uL	2uL

Sequencing Results

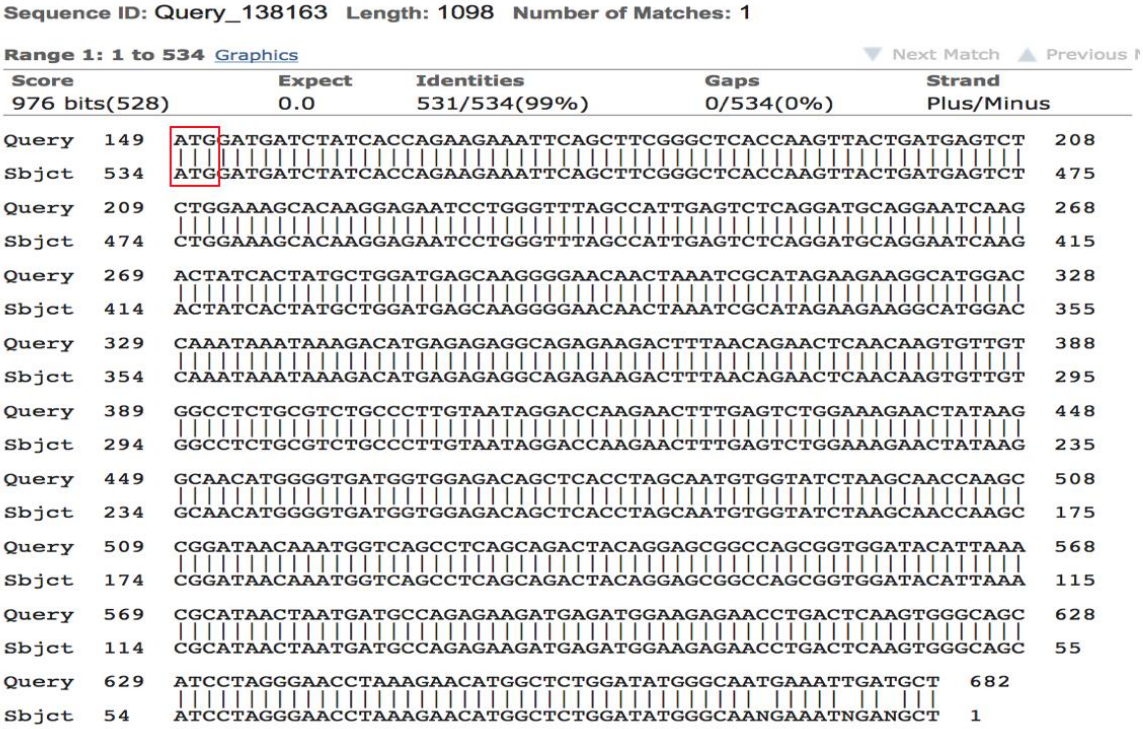

Figure 8: Truncated SNAP-23 forward primer (start codon outlined in red) sequencing results matched with Rat wild-type SNAP-23 using a NCBI Nucleotide BLAST

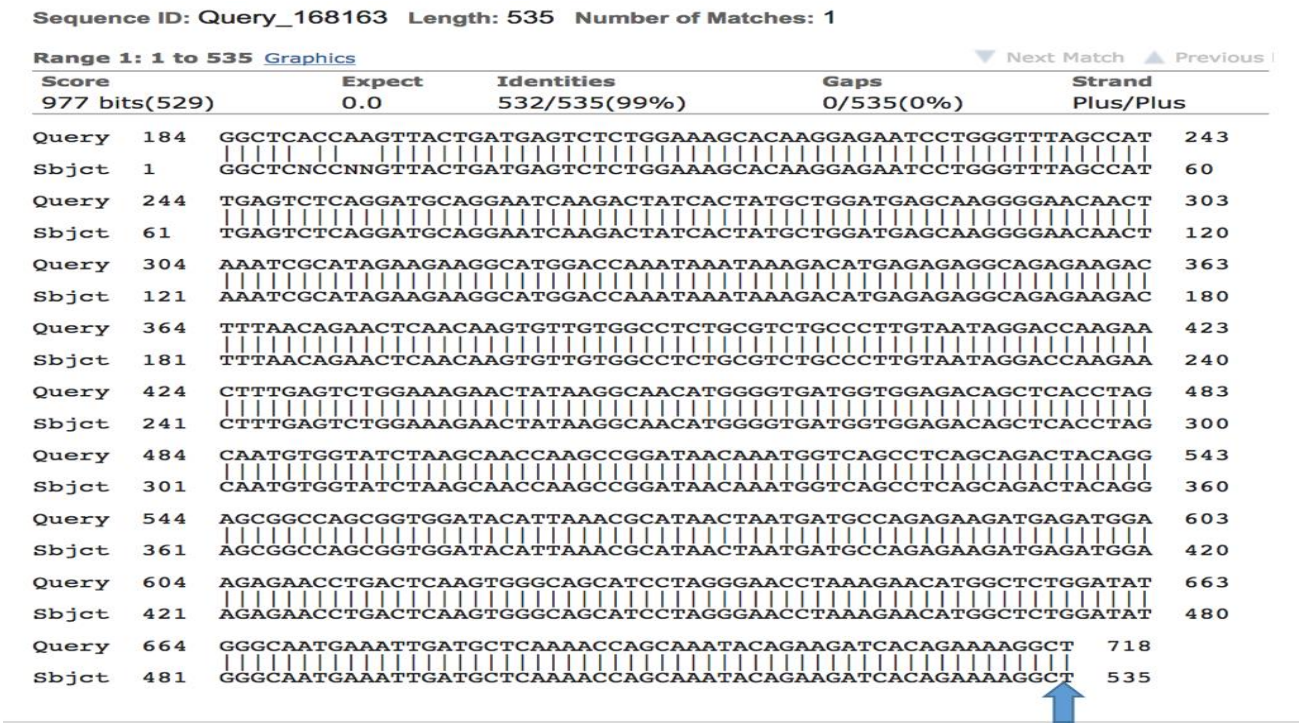

Figure 9: Truncated SNAP-23 reverse primer sequencing results matched with Rat wild-type SNAP-23 using NCBI Nucleotide BLAST. Stop codon TGA followed final nucleotide, designated by the blue arrow, indicting deletion of final 63bp.

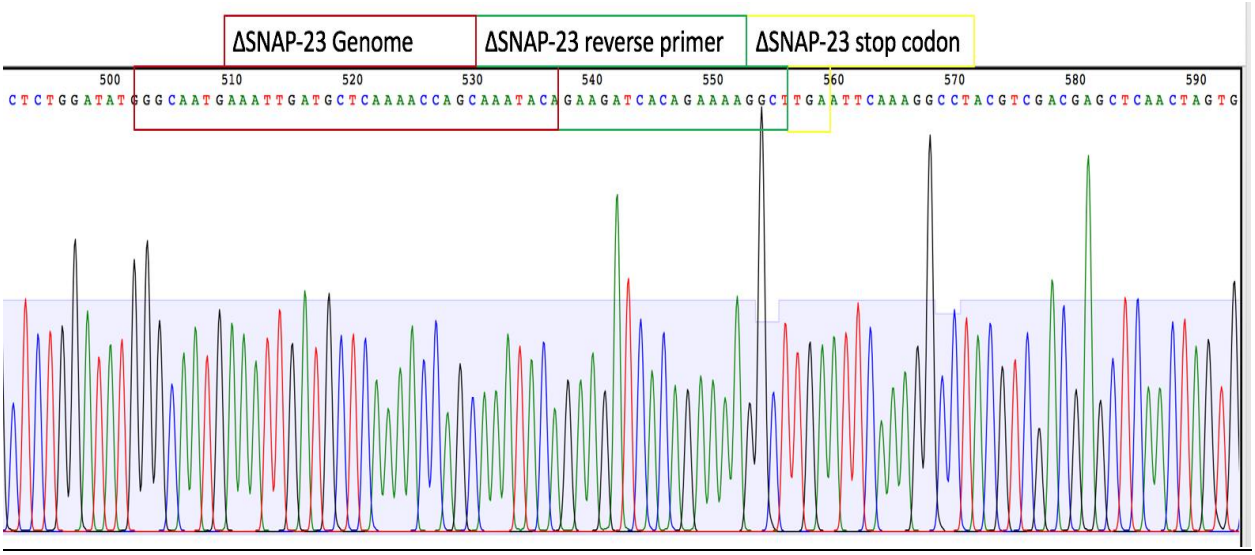

Figure 10: Confirmed sequence for truncated synaptosomal-associated protein 23 [Rattus norvegicus] A sample of the isolation was sent for sequencing and verified using the wild-type sequence against reverse primer omitting the final 63bp.

Six points were identified following the analysis of the BLAST results. The N's in the forward primer sequence in figure 8 were all identified as base T. The N's in the reverse primer sequence in figure 9 were all identified as base A. There was no mutation determined in the sequencing results, and confirmation that the truncated SNAP-we was successfully transformed and isolated without error.

SDS-PAGE results

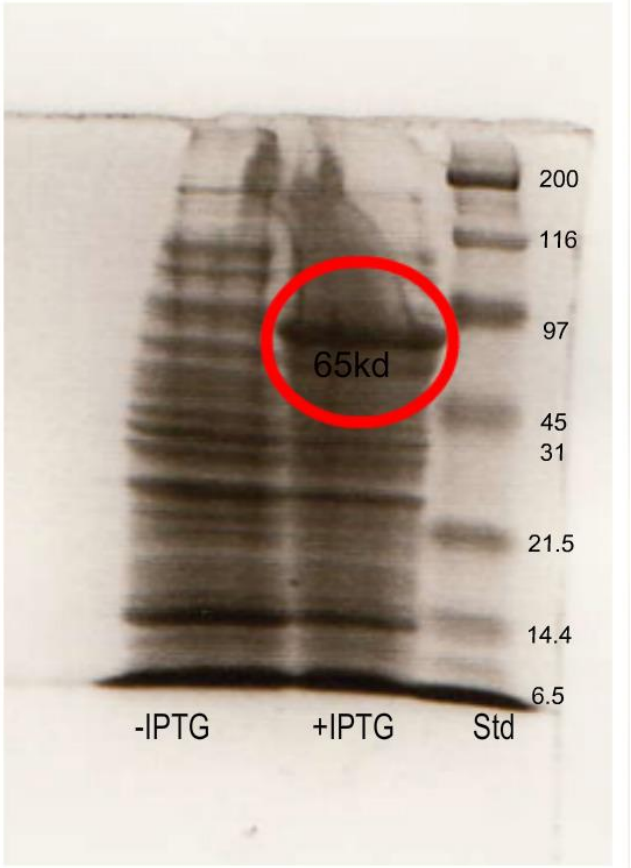

Figure 11: SDS-PAGE results for small scale protein induction of recombinant plasmid. –IPTG had no IPTG added and therefore did not express. + IPTG containing IPTG, was expressed can be seen between bands 3 and 4, corresponding to the calculated 65kd.

Chapter 5: Discussion

To investigate the properties of the degranulation related trans-SNARE complex, we created a C-terminal truncation mutant of SNAP-23. This was done by using a cloning expression vector and transforming into *E. coli* Rosetta 2 (DE3) expression cells to allow for IPTG induction to verify expression. The pMPB-parallel vector contains a maltose binding protein tag to allow for protein isolation from bacterial lysates to be tested in already-established fusion reactions. We proposed the mutant SNAP-23 will halt membrane fusion following the formation of the Trans-SNARE complex and provide fresh insights into the specific properties of the trans-SNARE complex and its dynamics in regulation of mast cell exocytosis.

We successfully amplified SNAP-23 cDNA using PCR as seen in figure 7. Following PCR, the product and pMBP-parallel vector were digested using restriction enzymes EcorI and NcoI in preparation for ligation reaction. The digestion products were ligated using the set up listed in table 2, and the recombinant plasmid was transformed into *E. Coli* Nova Blue cells. The plasmid was re-isolated from the Nova Blue cells and sent off for sequencing to confirm we created the right mutant with the correct c-terminal truncation. A 99% sequence matched confirmed that we created a C-terminal truncation of SNAP-23 mutant omitting the final 63bp in the SNARE motif using NCBI nucleotide BLAST as seen in figure 8 and 9. After confirmation, we ran a small-scale induction using IPTG to induce expression in Rosetta 2 (DE3) cells. Following induction, the products were used to run and SDS-PAGE gel to confirm expression the mutant. A strong visible band can be seen in figure 11 and the mutant was calculated to be located at 65kd as seen in the gel.

In conclusion, after successfully cloning and expressing the mutant isoform future direction include protein isolation and purification, and bacterial lysate testing to determine if the

the truncated mutant can halt exocytosis in mast cells following the formation of the trans-SNARE complex.

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