On Stentor growth, regeneration timing, vaults, and proteins

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Abstract

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Cells need to be able to regenerate their parts to recover from external perturbations. The unicellular ciliate *Stentor coeruleus* is an excellent model organism to study wound healing and subsequent cell regeneration. The *Stentor* genome became available recently, along with modern molecular biology methods, such as RNAi. These tools make it possible to study single-cell regeneration at the molecular level. The first section of the protocol covers establishing *Stentor* cell cultures from single cells or cell fragments, along with general guidelines for maintaining *Stentor* cultures. Culturing *Stentor* in large quantities allows for the use of valuable tools like biochemistry, sequencing, and mass spectrometry. Subsequent sections of the protocol cover different approaches to inducing regeneration in *Stentor*. Manually cutting cells with a glass needle allows studying the regeneration of large cell parts, while treating cells with either sucrose or urea allows studying the regeneration of specific structures located at the anterior end of the cell. A method for imaging individual regenerating cells is provided, along with a rubric for staging and analyzing the dynamics of regeneration. The entire process of regeneration is divided in three stages. By visualizing the dynamics of the progression of a population of cells through the stages, the heterogeneity in regeneration timing is demonstrated. The molecular mechanism for how *Stentor* regenerates is a complete mystery, however, the process of regeneration shows striking similarities to the process of cell division. On a morphological level, the process of creating a second mouth in division or a new oral apparatus in regeneration have the same steps and occur in the same order. On the transcriptional level, genes encoding elements of the cell division and cell cycle regulatory machinery, including Aurora kinases, are differentially expressed during regeneration. This suggests that there may be some common regulatory mechanisms involved in both regeneration and cell

division. If the cell cycle machinery really does play a role in regeneration, then inhibition of proteins that regulate the timing of cell division may also affect the timing of regeneration in *Stentor*. Here we show that two well-characterized Aurora kinase A+B inhibitors that affect the timing of regeneration. ZM447439 slows down regeneration by at least one hour. PF03814735 completely suppresses regeneration until the drug is removed. Here we provide the first direct experimental evidence that *Stentor* may harness the cell division machinery to regulate the sequential process of regeneration. Despite its conservation through many species, Vault's function as a ribonucleic protein remains unknown. Many theories including signaling, immunity, and drug resistance have been questioned. Here we explored their role in *Stentor.* Knockdown of vaults have no effect on regeneration in *Stentor*. Knockdown of vaults did not increase sensitivity to holospora, a ciliate parasite. *Stentor coeruleus* is a useful model organism to study single-cell regeneration. They have a distinctive cell shape and large size. They can stretch to 1mm in length. At their anterior end, they have a membranellar band consisting of tightly woven layers of long cilia that beat in synchrony. They have an oral apparatus where they phagocytose unfortunate microorganisms caught in their flow. On the posterior end, they have a holdfast to attach themselves to underwater surfaces (Figure one). And, when cut in half, they can regenerate. The process of regeneration takes eight hours and their transcriptome during regeneration has been characterized. The next step to understanding their regeneration is to have an account of proteins so that we can see the building blocks of *Stentor*. Because of the size of Stentor, we are able to dissect them into parts and look for enrichment and depletion of proteins in each part. We analyzed over 4000 proteins, providing an inventory of proteins that Stentor will use for regeneration, as well as the proteins required to make a new membranellar band.

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Chapter 1 - Introduction

The power to regenerate is a useful survival mechanism in the single-celled world. Cells face constant external perturbations and must adapt quickly in order to continue propagating. The ciliate *Stentor coeruleus* is an excellent model organism to study wound healing and subsequent cell regeneration. They live in freshwater ponds, a constant war zone where other animals such as small fish and amoebas try to eat them. Other than *Stentor*, regeneration has not been thoroughly studied in ciliates. Small laser ablations to the gullet of Paramecium can sometimes be regenerated[1]. Few cells are capable of putting themselves back together like *Stentor*. Imagine if a cell was a bag of enzymes, then if it ruptures, all the enzymes would mix with adjacent media and the cell would be lost. However, cells are much more capable than a bag. *Stentor* are able to pull back in their enzymes, seal the wound, and regenerate missing parts. This dissertation is on the topic of regenerating missing parts. On the other hand, multicellular regeneration is based solely on cell division of remaining cell. Neoblasts in planarian flatworm Schmidtea mediterranea are capable of dividing into whole animals[2]. However for single-cells, cell division is their entire development cycle. A whole new organism is born when they divide. Therefore for single cells to survive, regeneration is a key skill to have.

When *Stentor* cells divide, the anterior daughter cell inherits the oral apparatus from the mother cell. A second OA forms de novo in the mother cell just prior to cytokinesis and is inherited by the posterior daughter cell (Figure 1.1). There are several striking similarities between *Stentor* regeneration and division. First, regeneration of the OA in *Stentor* proceeds through a series of distinct morphological stages, which are identical to the sequence of stages seen in dividing cells. Second, when *Stentor* regenerates its OA, the macronucleus, which normally takes the form of nodes on a string, becomes compacted into an oblong shape. Interestingly, these same nuclear shape changes take place during cell division at precisely the moment when the cell is building a new oral apparatus. These similarities led

us to hypothesize that some common regulatory mechanisms may be involved in both regeneration and cell division.

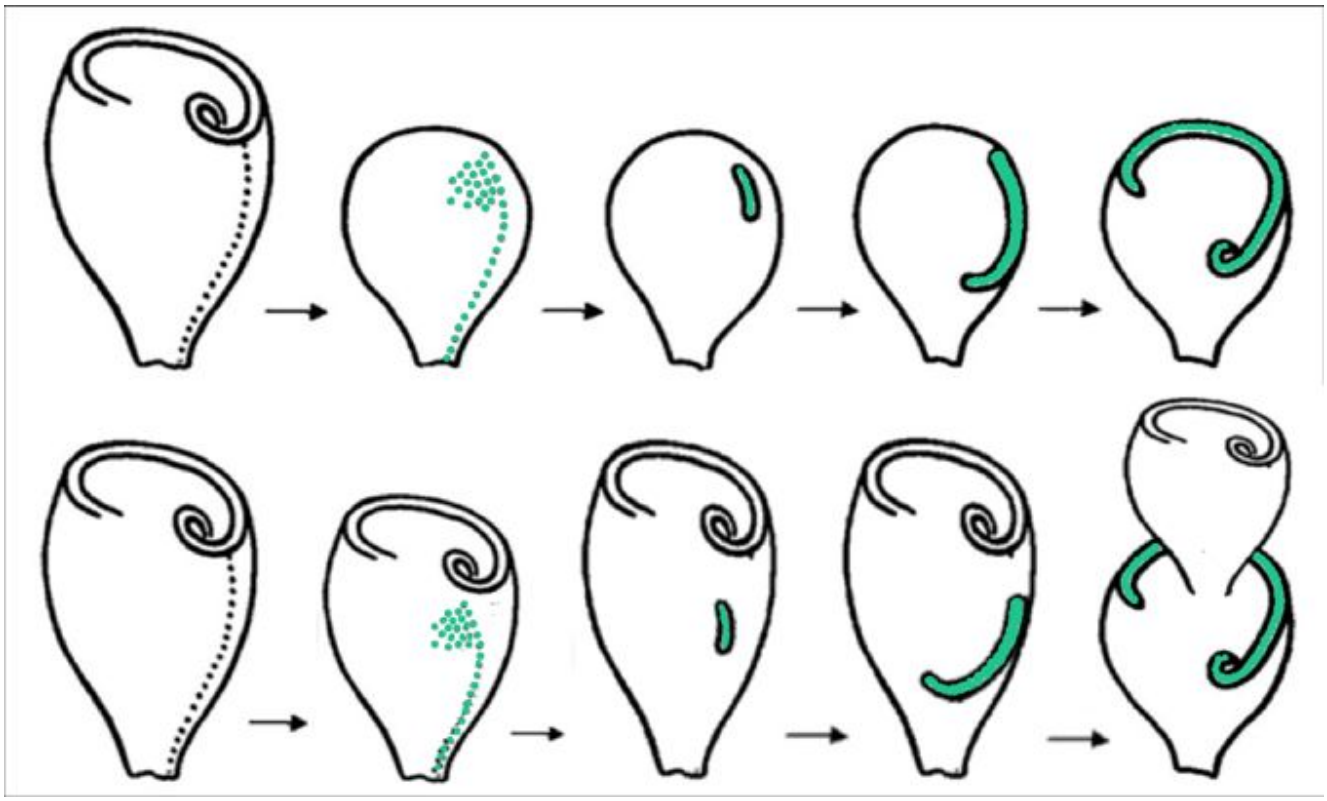

Figure 1.1: Regeneration (top row) and cell division (bottom row) in *Stentor* have similar morphological steps. Modified from De Terra.

The *Stentor* genome became available recently, along with modern molecular biology methods, such as RNAi. These tools make it possible to study single-cell regeneration at the molecular level. Manually cutting cells with a glass needle allows studying the regeneration of large cell parts, while treating cells with either sucrose or urea allows studying the regeneration of specific structures located at the anterior end of the cell. A method for imaging individual regenerating cells is provided, along with a rubric for staging and analyzing the dynamics of regeneration. The entire process of regeneration is divided in three stages. By visualizing the dynamics of the progression of a population of cells through the stages, the heterogeneity in regeneration timing is demonstrated.

Cells are not simple bags of enzymes, but rather highly complex machines whose components are carefully scaled to the correct size and arranged in well-defined positions. The morphogenesis of individual cells represents a key process in cell and developmental biology, but its molecular mechanism is unknown[3,4]. While some cultured cells resemble blobs, unicellular organisms can have extremely complicated architectures, exemplified by the complex cortical patterns seen in ciliates[4,5].

Perhaps the most extreme example of a highly structured cell is *Stentor coeruleus*, a giant heterotrichous ciliate distantly related to *Tetrahymena* and *Paramecium. Stentor* is 1 mm long and is covered with more than 100 longitudinal stripes of blue pigment alternating with rows of cilia organized by parallel stacks of microtubule ribbons that run the length of the entire cell. The cell is trumpet-shaped (Figure 1.2), with a membranellar band and an oral apparatus (OA) at its anterior end, and a holdfast that attaches the cell to the substrate at its posterior end. In addition to the clear anterior-posterior polarity, the cell also shows a distinctive chiral patterning, such that spacing between ciliary rows gradually increases in a clockwise direction. This results in a discontinuity where the narrowest row meets the widest row, and this region of the cell surface, known as the locus of stripe contrast, can induce the formation of the second set of anterior end structures when grafted onto another cell[6], making it formally equivalent to Spemann's Organizer. Thus, all key processes of developmental biology have their analogs in *Stentor*: axiation, pattern formation, and induction. In an embryo, these processes are driven by fate differences between different cells, but in *Stentor*, they must be driven by fate differences between different regions within a single cell. What defines the differences between the regions within *Stentor* is a mystery.

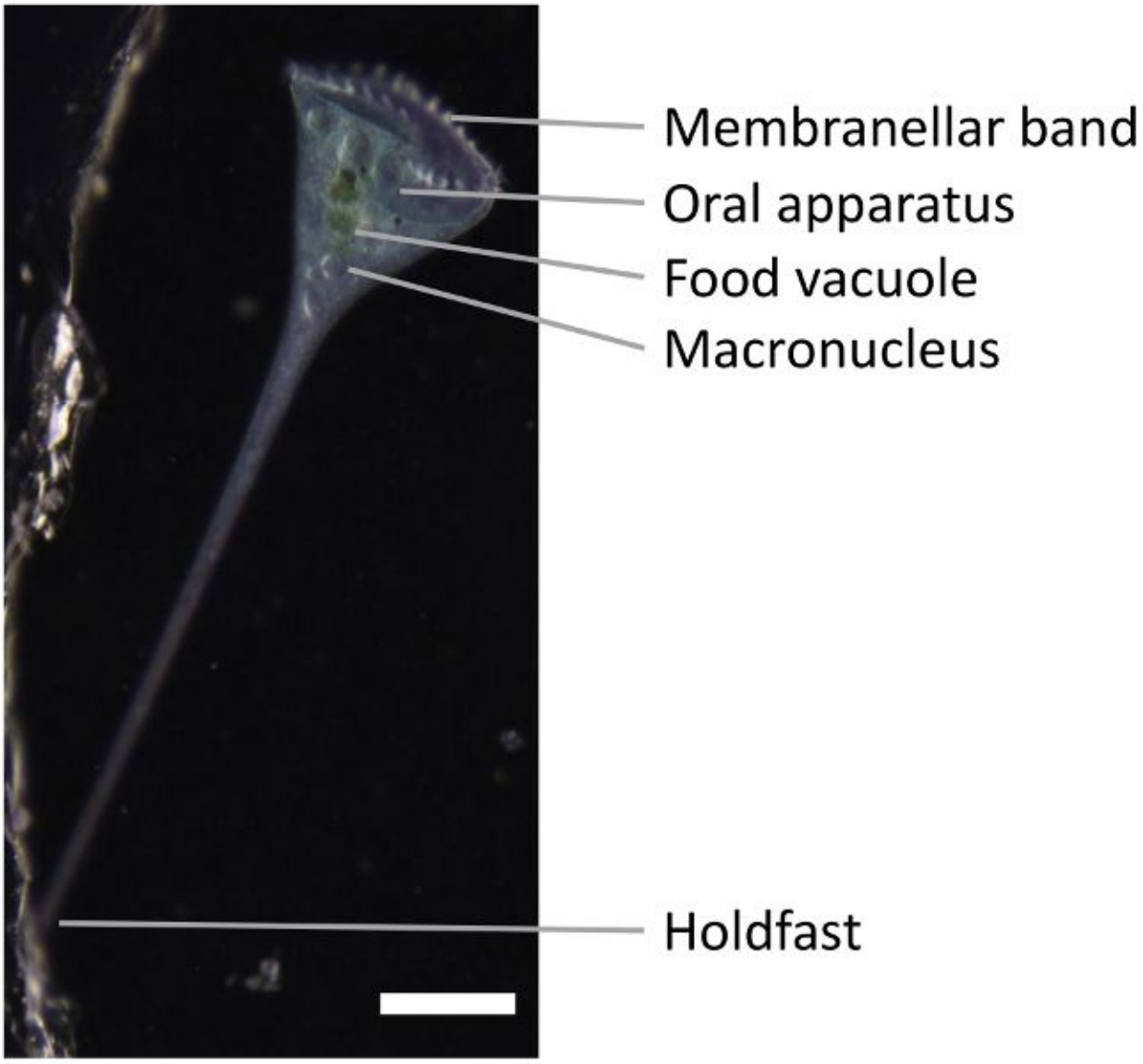

Figure 1.2. Snapshot of *Stentor*. The membranellar band and the oral apparatus are shown at the anterior end of the cell. The holdfast is at the posterior end of the cell. *Stentor* macronucleus is nodulated. A well-fed *Stentor* has green food vacuoles containing mostly *Chlamydomonas*. Scale bar is 0.5 mm.

If any part of *Stentor* is cut off, the missing piece of the cell can regenerate to yield a normal cell in a matter of hours. If a cell is cut in half, or even into much smaller pieces, each piece reorganizes into a normal-looking but smaller cell and restores proper proportionality between cell parts[8,9]. Even tiny fragments, 1/64th the size of the original cell, are able to regenerate into a small but normally proportioned cell, and then grow to the full size[8]. *Stentor* thus presents a unique

opportunity to study the mechanisms of organelle size scaling and cell growth regulation using surgical methods that are usually applied at the level of tissues or whole organisms.

One of the properties of *Stentor* that allows it to regenerate from a wide range of surgical operations is that it contains a single nodulated macronucleus (Figure 1.2) with about 50,000 copies of the entire genome[10]. As long as a cell fragment contains at least one macronuclear node, it has the ability to regenerate fully. Another property underlying *Stentor*'s regeneration ability is its prodigious wound-healing ability. Although many cell types are capable of healing their wounds[11], *Stentor* is able to recover from an extraordinary range of physical perturbations. An example of *Stentor* recovery from a drastic perturbation, along with the methods for visualizing cytoplasmic flow in *Stentor*[12] were previously reported. These methods allow the study of how wounding and subsequent regeneration affect the physical state of the cytoplasm.

Stentor's huge size, extraordinary regeneration ability, and the fact that it manifests many of the developmental phenomena seen in multicellular embryos (such as organizers, axiation, and patterning) attracted many developmental biologists during the turn of the last century, including Thomas Hunt Morgan[9]. During the 50's and 60's, microsurgical approaches demonstrated a startling array of regenerative and morphogenetic processes in this single-celled organism[13]. However, *Stentor* has been developed as a molecular biology model system only recently. During the past several years, the genome of *Stentor* was sequenced and assembled[10], and the method to perturb gene expression using RNAi by feeding was developed[14].

This dissertation covers three topics. First, we asked the question of how the timing of regeneration relates to cell division. Two ways to think about how a cell coordinates a huge process like

regeneration is the “domino effect” and the “master clock”. The “domino effect” is where one series of events trigger the next. The “master clock” is where there are checkpoints and a process is suspended until all the checkpoints are met. One system where the “master clock” is clear is in cell division. Several stop gaps are in place to prevent the continuation of cell division before those checks are completed. The morphological steps of regeneration and cell division are similar thus, we screened cell division inhibitors to find any that affect regeneration. We identify two aurora kinase inhibitors that were able to stop or prevent regeneration. This suggests that the “master clock” is how regeneration timing takes place.

Second, we discovered that Vaults were proteins that were very highly expressed in the membranellar bands of *Stentor* so we asked, what is the functionality of such a large structure, 670 angstroms, in tightly-woven sheets of long cilia. We knocked down Vaults via RNAi in *Stentor* and found that they do not affect the timing of regenerating the membranellar band and do not affect *Stentor* immune response. We noticed in the first rounds of RNAi, pigment leaching into their media and pigment granules in regeneration *Stentor*. We hypothesize that Vaults have a role in the distribution of pigment however, repeating these experiments were unsuccessful in showing the same phenotype.

Third, we would like to answer the question of where components of regeneration are drawn from during regeneration. One theory is that expression of those components happen immediately. Another theory is that there is a repository of components that is immediately exhausted and is replenished at a later time. Using previously published data, we identified membranellar band proteins that are differentially expressed during regeneration. We were surprised to find that most of them are expressed in the late stages of regeneration, which suggests that there is a regeneration component repository.

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Chapter 2 - Methodology for studying growth and regeneration in *Stentor*

Abstract

Cells need to be able to regenerate their parts to recover from external perturbations. The unicellular ciliate *Stentor coeruleus* is an excellent model organism to study wound healing and subsequent cell regeneration. The *Stentor* genome became available recently, along with modern molecular biology methods, such as RNAi. These tools make it possible to study single-cell regeneration at the molecular level. The first section of the protocol covers establishing *Stentor* cell cultures from single cells or cell fragments, along with general guidelines for maintaining *Stentor* cultures. Culturing *Stentor* in large quantities allows for the use of valuable tools like biochemistry, sequencing, and mass spectrometry. Subsequent sections of the protocol cover different approaches to inducing regeneration in *Stentor*. Manually cutting cells with a glass needle allows studying the regeneration of large cell parts, while treating cells with either sucrose or urea allows studying the regeneration of specific structures located at the anterior end of the cell. A method for imaging individual regenerating cells is provided, along with a rubric for staging and analyzing the dynamics of regeneration. The entire process of regeneration is divided in three stages. By visualizing the dynamics of the progression of a population of cells through the stages, the heterogeneity in regeneration timing is demonstrated.

Introduction

One of the reasons that *Stentor* was developed into a model organism for modern molecular biology only recently was the difficulty of growing large cultures due to its long cell cycle (3 to 5 days). However, modern genomic and proteomic methods require less material than they used to, and the volume of a single *Stentor* cell is sufficient for these methods, even without resorting to ultrasensitive methods that were developed for the analysis of single cells that are much smaller than *Stentor*. Section 1 of the protocol details the procedure for establishing a large culture from a single *Stentor* cell. The same approach can be used to establish a large culture from a cell fragment obtained by cutting a cell. Section 1 also provides the guidelines for maintaining healthy *Stentor* cultures over long periods of time. Section 2 of the protocol provides the methodology for inducing cell regeneration by cutting the cells manually with a glass needle. Section 3 of the protocol is dedicated to two methods of inducing the regeneration of specific cell structures (membranellar band and oral apparatus): treating the cells with either sucrose or urea leads to the shedding of these structures, followed by their regeneration. Section 4 of the protocol details a method for the imaging of individual regenerating cells over long periods of time. Section 4 ends with the description of the stages of regeneration and tips on the analysis of regeneration dynamics.

Protocol

1. Culturing *Stentor* and Establishing *Stentor* Cultures from Single Cells or Cell Fragments

1. Prepare *Chlamydomonas reinhardtii* culture to be used as food for *Stentor*.

i. Obtain *Chlamydomonas reinhardtii* cells from a commercial supplier (**Table of Materials**).

2. Establish a 500 mL liquid culture of *Chlamydomonas reinhardtii* in commercially available TAP media using sterile technique[13].

3. Keep the *Chlamydomonas* culture under a lamp at a concentration near saturation (at O.D. of about 1) by diluting it with TAP media twice a week. Note: The *Chlamydomonas* culture can be grown on a shaker.

4. Regularly check whether the *Chlamydomonas* culture is healthy by placing a drop of culture on a slide, covering it with a coverslip, and checking it under a microscope at 40X magnification. Note: Do not use the culture for *Stentor* feeding if it is contaminated with bacteria or if *Chlamydomonas* cells are aggregated into clusters. If either of these problems occurs, start a new *Chlamydomonas* culture.

2. Obtain *Stentor coeruleus* cells from a commercial supplier (**Table of Materials**).

1. If *Stentor* cells are needed from their natural habitat, collect them from a pond, lake, or river[11].
2. To collect *Stentor* from a pond, lake, or river, find an area with some vegetation where the water is relatively calm, shady, and clear. Note: There is a higher probability of finding *Stentor* at the locations where duckweed grows.
3. Collect at least 2 L of water in a container that is easy to pour from.
4. After detritus and particulate matter have settled to the bottom of the container, gently fill a few smaller containers to examine for *Stentor*, waiting for a few seconds after each collection for the detritus to settle in the large container.

5. Once the collection of samples is completed at a location, move to a new location that is at least 10 m away and repeat the collection of samples there. Note: Not every pond has a *Stentor* population, so multiple ponds may have to be sampled.
6. Return with the samples to the lab and transfer the water from the containers into individual Petri dishes.
7. In each Petri dish, search for *Stentor* under a stereomicroscope with oblique light at 5X magnification. Transfer individual *Stentor* cells using a 1 mL pipette into a well of a glass spot plate containing at least 100 µL of commercially available pasteurized spring water (PSW). Note: *Stentor* cells have a trumpet-like shape when they are attached to a substrate (Figure 1.1). Swimming *Stentor* cells are less extended than the cells attached to a substrate.
8. Wash *Stentor* in PSW at least 3x. Perform the washes by removing about 90% of the water from the well while keeping *Stentor* cells in the well, followed by adding 500 µL of PSW into the well. Note: Before handling *Stentor* using pipettes with pipette tips of 10 µL or smaller, cut about 0.5 mm off the end of the pipette tip with scissors to avoid wounding the cells due to shear forces that are generated as a large cell flows through too small of a tip opening.

3. Prepare *Chlamydomonas* before each feeding of *Stentor*.
    i. Transfer 1 mL of *Chlamydomonas* culture prepared as in Step 1.1 into a 1.5 mL microcentrifuge tube. Centrifuge at 2,095 x g for 3 min.
    ii. Remove the supernatant and resuspended the pellet in 1 mL of PSW. Centrifuge at 2,095 x g for 3 min. Remove the supernatant and resuspend the pellet in 500 µL of PSW. Note: Thus, washed and concentrated *Chlamydomonas* will be referred to as "prepared *Chlamydomonas*" in the following steps. TAP media is

detrimental to *Stentor*, thus washing *Chlamydomonas* before feeding *Stentor* is important.

4. If a clonal *Stentor* culture is needed, start the culture from an individual *Stentor* cell or a cell fragment.

   i. Since single *Stentor* cells do not grow well in PSW, prepare conditioned media by filter sterilizing 500 µL of media from an existing, healthy *Stentor* culture.

   ii. Transfer 500 µL of conditioned media to one of the wells of a glass spot plate.

   iii. Transfer one *Stentor* into the well of the glass spot plate containing conditioned media. Use as little medium as possible to make the transfer.

   iv. Feed each *Stentor* 5 µL of prepared *Chlamydomonas* every 48 h. When *Stentor* divide, count the number of cells in the well and add 5 µL of prepared *Chlamydomonas* per cell.

   v. Keep *Stentor* in a shaded place since the cells are sensitive to light (for example, in clear plastic boxes covered with paper towels).

2. Exchange *Stentor* medium in the well with fresh conditioned medium every 96 h.

   i. Prepare fresh conditioned media as in Step 1.5.1.

   ii. Make all *Stentor* cells detach from the bottom of the well by gently pipetting the liquid up and down in the well.

   iii. Carefully aspirate the liquid from the well using a 1 mL pipette, making sure all the cells remain in the well. Add 500 µL of fresh conditioned media to the well.

3. When the number of cells in the well exceeds 20, move the cells to a larger container, for example, a wide-mouth glass jar.

   i. Add 20 mL of PSW into an autoclaved wide-mouth glass jar. Note: Autoclaving of glassware for *Stentor* can be replaced with careful washing and rinsing.

ii. Carefully pipette the media up and down in the well to detach all the cells from the bottom of the well. Collect all *Stentor* cells using a 1 mL pipette tip and gently transfer them to the glass jar.

4. Do not tighten the lids on the jars with *Stentor* cultures, to allow sufficient access to air.

5. Add PSW to the jar every 48 h to keep *Stentor* density at about 20 cells/mL. Estimate the density of cells by eye. Note: Alternatively, use a 1 mL pipette to bubble air into the jar to make cells detach from the walls, pipette up 1 mL of the culture, and count the number of cells in the pipette tip.

6. Every 48 h, feed prepared *Chlamydomonas* to *Stentor* cultures in jars (see Step 1.4). Start with feeding the culture with 200 µL of prepared *Chlamydomonas*. As the volume of the culture increases, gradually increase the amount of prepared *Chlamydomonas* used for feeding up to 1 mL.

7. When the culture volume reaches about 90% of the jar's capacity, transfer the culture to a bigger container.

i. Pipette the culture inside the jar, up and down, with a 1 mL pipette to detach *Stentor* from the glass.

ii. Pour the entire contents of the jar into a 2-cup glass container.

iii. Rinse the jar with about 25 mL of PSW into the 2-cup glass container to collect the remaining *Stentor*.

5. Maintain healthy cultures in 2-cup glass containers.

i. Feed *Stentor* cultures 2 mL of prepared *Chlamydomonas* per 100 mL of culture every 4 - 5 days. Add PSW to the glass container every 4 - 5 days to keep *Stentor* density at about 20 cells/mL. Note: 450 mL is the maximum volume a 2-cup container holds.

ii. Once a week, inspect the cultures under a 5X dissecting microscope for rotifers, fungus, and other growth. To prolong the health of the culture, remove contaminating microorganisms along with abnormally shaped and colorless *Stentor* cells using a 1 mL pipette.

iii. When the glass container is about 90% full, split the culture.

iv. Add 25 mL of PSW to the glass container. Use a 25 mL pipette to pipette up and down to detach *Stentor* from the glass. Move about 50% of the culture into a new 2-cup glass container.

v. Add 25 mL of PSW to both cultures and continue maintaining the two cultures as described in this section of the protocol. Note: Since *Stentor* cells are sensitive to high temperature, maintain the temperature at 25 °C or lower in the room where the cultures are kept. Alternatively, the cultures can be kept in an incubator. Refer to **Table 1** for troubleshooting of *Stentor* culturing.

2. Inducing Regeneration by Cutting *Stentor* Cells

1. Use a needle puller to make several needles from capillary tubes using the program as follows: heat - 735, pull - 100, velocity - 110, time - 150, pressure - 400.

2. Prepare a 4% methylcellulose solution in 50 mL of PSW.

i. Add 1 g of methylcellulose (viscosity: 1500 cP) to 50 mL of PSW.

ii. Incubate at 4 °C for at least 8 h to facilitate the dissolution of methylcellulose.

iii. Keep 4% methylcellulose solution at room temperature.

3. Prepare a glass spot plate for storing the cell fragments after performing the cuts.

i. Filter sterilize media from a healthy culture, 500 mL per glass spot plate well needed.

ii. Transfer 500 mL of sterilized media in each of the wells needed.

4. Collect one healthy *Stentor* (having a defined trumpet shape, vibrant blue-green color, and no large vacuoles) in a 2 µL droplet and place it on a coverslip or slide. Add 2 µL of 4% methylcellulose (Figure 1). Let *Stentor* slow down before cutting it.

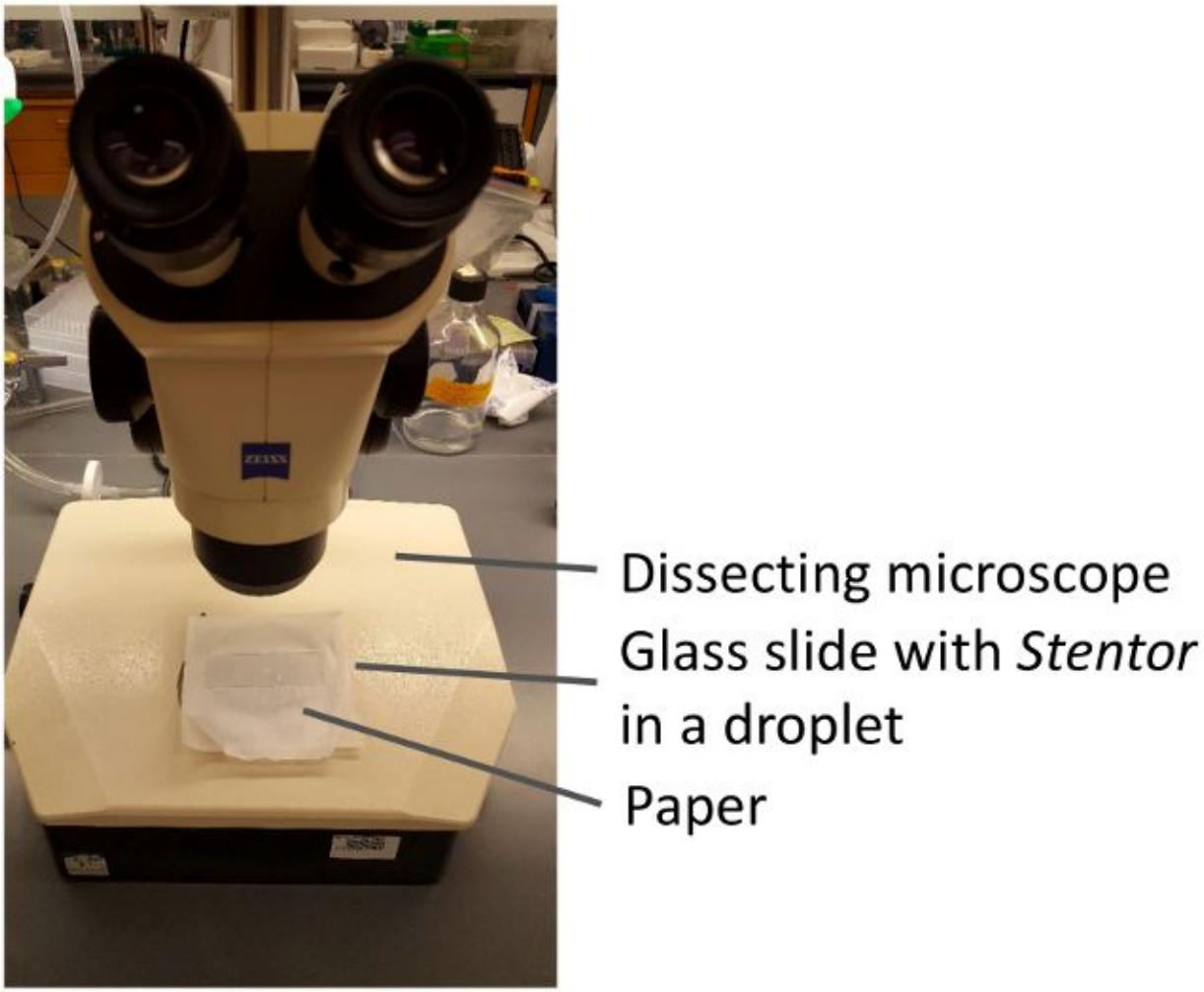

Figure 2.1. *Stentor* cutting set-up. Cell cutting is performed by manually manipulating a glass needle while looking at the cell using a commercially available dissecting stereo microscope. The purpose of the tissue paper is to provide the white background to see the cells better.

5. Hold the glass needle as parallel to the cutting surface as possible to prevent breaking the needle.

6. Using a stereo dissecting microscope, locate the tip of the glass needle and move it closer to the cell (Figure 2A). Observe the *Stentor* contract upon contact with the needle (**Figure 2B and C**).

Note: If the cell is in an orientation that makes separating the anterior end from the posterior end difficult, rotate it very gently with the side of the needle.

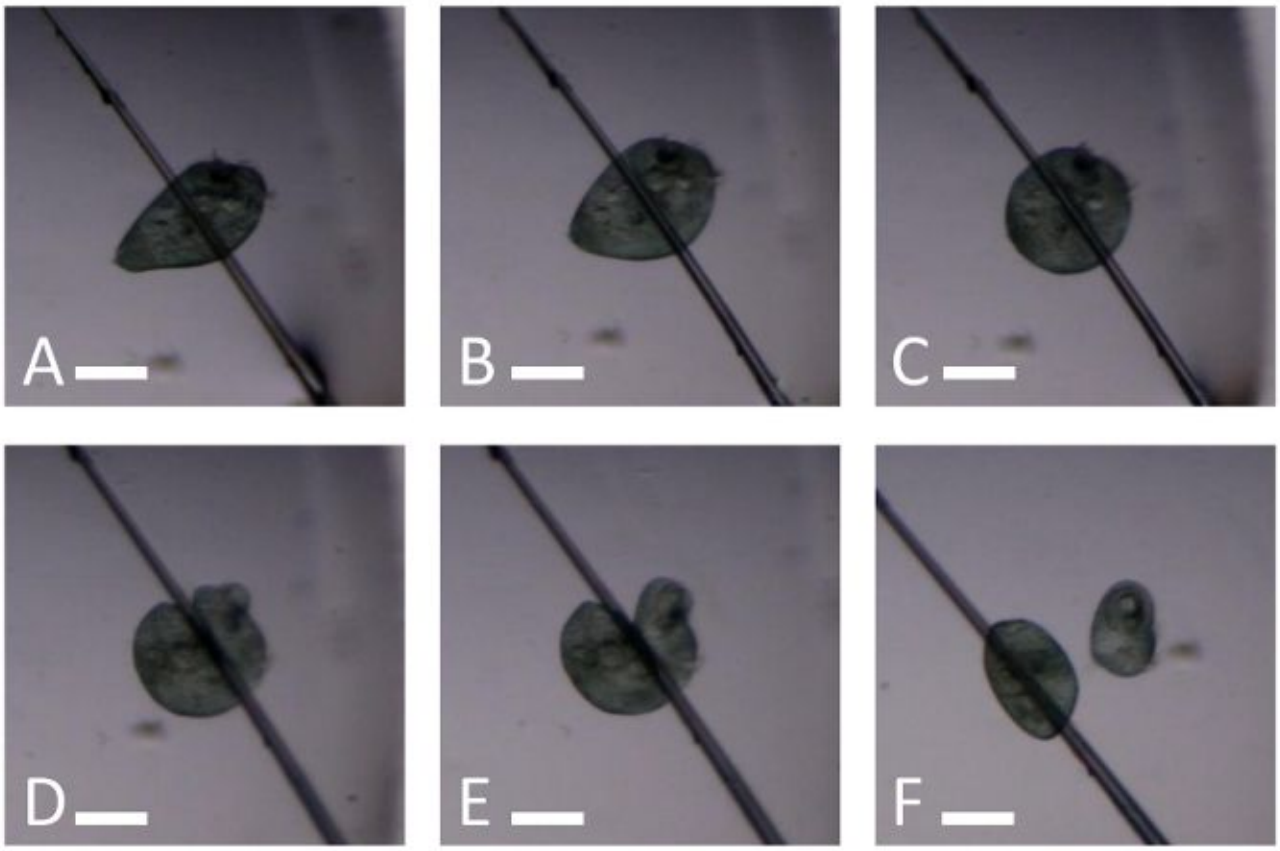

Figure 2.2. Snapshots illustrating how to cut a *Stentor* with a glass needle. (**A**) A *Stentor* immediately before the needle touches it. (**B**) A *Stentor* gently squeezed between a needle and glass slide. (**C**) A contracted *Stentor* reacting to the force of the needle. (**D**) A *Stentor* being cut by gently pressing on the cell with the side of the needle. (**E**) A *Stentor* now cut in two but not separated. (**F**) Two *Stentor* fragments. Scale bar is 0.25 mm.

1. Gently press on the contracted *Stentor* with the side of the glass needle to cut the cell in two (Figure 2D). Move the two fragments apart ensuring that there is no cytoplasmic connection between them, to avoid fragment fusion (**Figure 2F)**.

2. Check whether both fragments have at least one macronuclear node each by examining the fragments under a dissecting microscope with oblique illumination. Note: Having at least one macronuclear node is essential for cell survival. The cell may shed its pellicle (transparent shell) along with the blue-green pigment during or after the cut. In most cases, this will not affect the long-term viability of the cell.

3. Move the fragments into wells of a glass spot plate prepared in Step 2.3.

4. Cut multiple cells because a fraction of the cut cells will not regenerate.

5. If performing multiple cuts in one session, replace the glass needle when it becomes heavily coated with *Stentor* residue or when its tip is broken. Alternatively, clean the needle by wiping it gently on a piece of silicon spacer. Note: Glass needles can be used for multiple cell cutting sessions.

6. Keep the glass spot plate with cell fragments in a humidity chamber.

7. Proceed to Section 4 of the protocol for details on imaging and interpretation of *Stentor* regeneration results.

3. Inducing Regeneration of Membranellar Band and Oral Apparatus by Sucrose or Urea Treatment

1. Membranellar band and oral apparatus removal using sucrose

    i. Prepare a 25% solution of sucrose in PSW.

2. Add 500 µL of 25% sucrose in PSW to a microcentrifuge tube with a snap cap.

3. Prepare 3 microcentrifuge tubes with snap caps with 1 mL of PSW in each tube for washing the cells after sucrose treatment.

4. Collect 30 - 60 *Stentor* cells in 1 mL of their culture media into a separate microcentrifuge tube using a 1 mL pipette (Figure 3, arrow A).

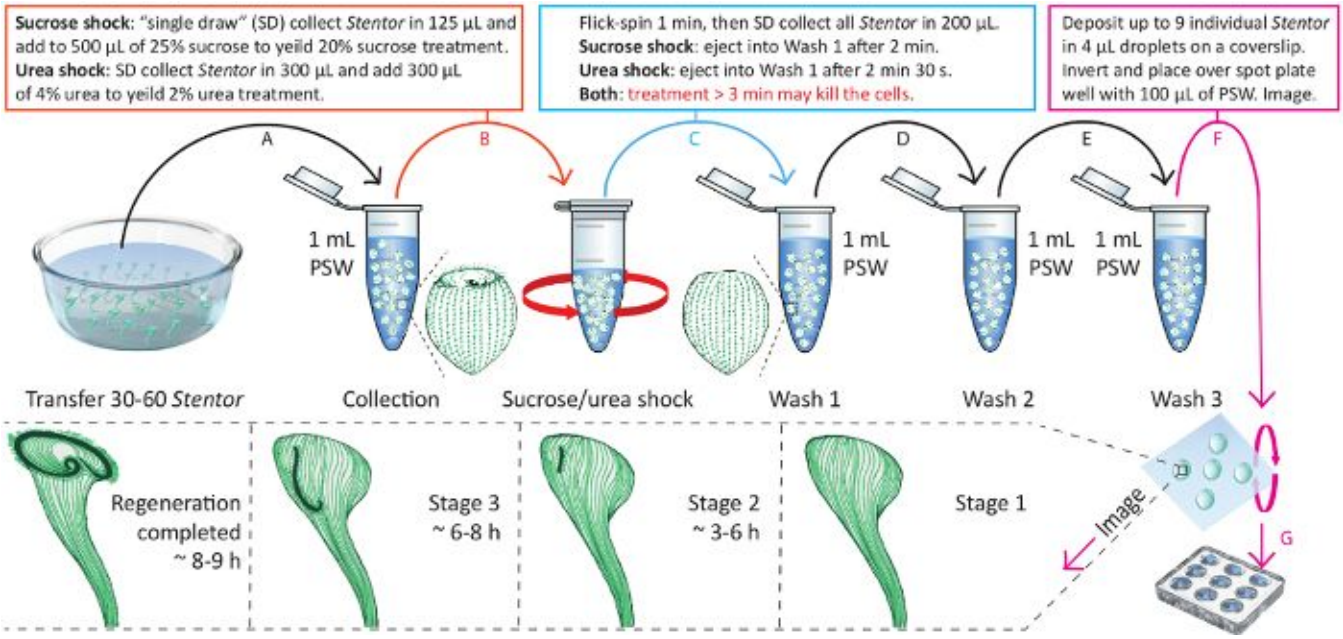

Figure 2.3. Schematic visualization of Section 3 and Section 4 of the protocol. This is an illustrated protocol for performing sucrose or urea treatment and observation of cell regeneration. The time indicated in the bottom panel is measured from the beginning of the Wash 1.

1. Collect all the cells from the tube in 125 µL final volume using a 200 µL pipette in a single draw. Transfer them to the tube with 500 µL of 25% sucrose (prepared in Step 3.1.2) to obtain 625 µL of 20% sucrose solution (Figure 3, arrow B). Start a stopwatch.

2. Incubate *Stentor* in this 20% sucrose solution and flick-spin the microcentrifuge tube in the rack for 1 min.

3. Collect all the cells in a single draw (adjust the pipette to 200 µL max capacity for easier collection).

4. Keep the cells in the pipette tip until the stopwatch shows 2 min of sucrose treatment.

5. Eject *Stentor* into one of the microcentrifuge tubes prepared in Step 3.1.3 (Figure 3, arrow C). Flick-spin the tube in the rack.

6. Wash the cells two more times, once in each of the two remaining microcentrifuge tubes containing PSW prepared in Step 3.1.3 (Figure 3, arrows D and E). Note: Single draw cell collection technique is not important in between the washes.

7. Proceed to Section 4 of the protocol for details on imaging and interpretation of *Stentor* regeneration dynamics (Figure 3, arrows F and G).

2. Membranellar band and oral apparatus removal using urea

1. Prepare a solution of 4% urea in PSW.

2. Add 300 µL of 4% urea in PSW to a microcentrifuge tube with a snap cap.

3. Prepare 3 microcentrifuge tubes with snap caps containing 1 mL of PSW in each tube for washing the cells after urea treatment.

4. Collect 30 - 60 *Stentor* cells in 1 mL of their culture media into a separate microcentrifuge tube using a 1 mL pipette (Figure 3, arrow A).

5. Collect all the cells from the tube in 300 µL final volume using a 1 mL pipette in a single draw. Transfer them to the tube with 300 µL of 4% urea (prepared in Step 3.2.2) to obtain 600 µL of 2% urea solution (Figure 3, arrow B). Start a stopwatch.

6. Incubate *Stentor* in this 2% urea solution and flick-spin the microcentrifuge tube in the rack for 1 min.

7. Collect all the cells in a single draw.

8. Keep the cells in the pipette tip until the stopwatch shows 2 min of urea treatment.

9. Eject *Stentor* into one of the microcentrifuge tubes prepared in Step 3.2.3 (Figure 3, arrow C). Flick-spin the tube in the rack.

10. Wash the cells two more times, once in each of the two remaining microcentrifuge tubes containing PSW prepared in Step 3.2.3 (Figure 3, arrows D and E). Note: Single draw cell collection technique is not important in between the washes.

11. Proceed to Section 4 of the protocol for details on imaging and interpretation of *Stentor* regeneration dynamics (Figure 3, arrows F and G).

4. Imaging and Analyzing Cell Regeneration

1. If using an upright microscope, use the hanging droplet method to image regeneration of individual cells.

    i. Put 100 µL of PSW in a well of a glass spot plate.

    ii. Isolate 1 *Stentor* cell in 4 µL of culture media (the media that they are in), using a 10 or 20 µL pipette. Deposit the droplet in the middle of a 22 x 22 mm2 coverslip (Figure 4). Note: If the cells that are 1) unhealthy (abnormally shaped or heavily vacuolated) or 2) still have membranellar bands or oral apparatuses (for chemical treatment experiments), do not use for imaging.

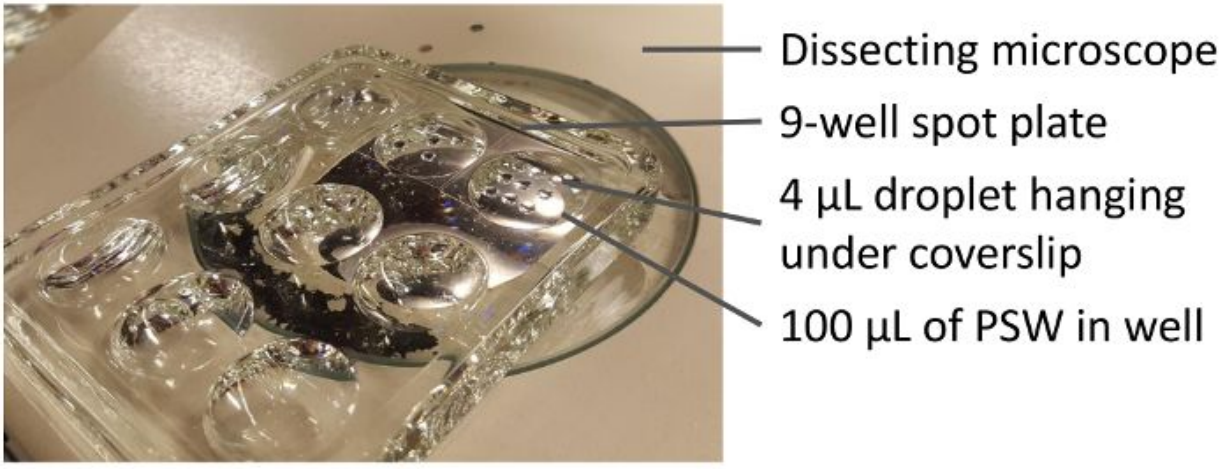

Figure 2.4. The setup for imaging and/or direct observation of regeneration with an upright microscope. In each 4 µL droplet hanging from the coverslip, there is one cell undergoing regeneration. Water in the wells of the glass spot plate limits evaporation of the droplets. This setup allows following the regeneration of multiple cells in parallel.

1. Arrange 4 more droplets of *Stentor* in the culture media around the previous droplet, while leaving enough space between the droplets.

2. Invert the coverslip with droplets and gently place it over the well of the glass spot plate to which PSW was added in Step 4.1.

3. Note: PSW in the well will minimize the evaporation of the droplets (Figure 4).

4. Prepare the remaining cells for imaging by following Steps 4.1.1 - 4.1.4.

5. Image the regenerating cells under a microscope with the desired time resolution. Alternatively, observe the regeneration using a dissecting stereo microscope. Note: Regeneration will begin immediately after cell cutting or treatment with sucrose or urea. However, visible new oral structures will form about 3 h after the beginning of regeneration.

2. For each time point, assign one of the three regeneration stages to each of the cells. To do this, compare each cell to the representative images illustrating the stages (Figure 3 and Figure 5).

1. Assign Stage 1 to the cells that do not have a membranellar band yet (Figure 5A).

2. Assign Stage 2 to the cells with a membranellar band. Note: A membranellar band appears 3 - 6 h after treatment (Figure 3, Figure 5B). At the very end of this stage, an additional curvature of the posterior end of the membranellar band will appear just before an oral primordium appears.

3. Assign Stage 3 to the cells with an oral primordium. Note: An oral primordium is an invagination appearing 6 - 8 h after treatment at the posterior end of the membranellar band (Figure 3, Figure 5C **and 5D**). Regeneration is completed when the cells have a membranellar band at their anterior end and the characteristic trumpet cell shape (Figure 3, Figure 5E). Most *Stentor* cells are fully regenerated within 8-9 h since the start of regeneration.

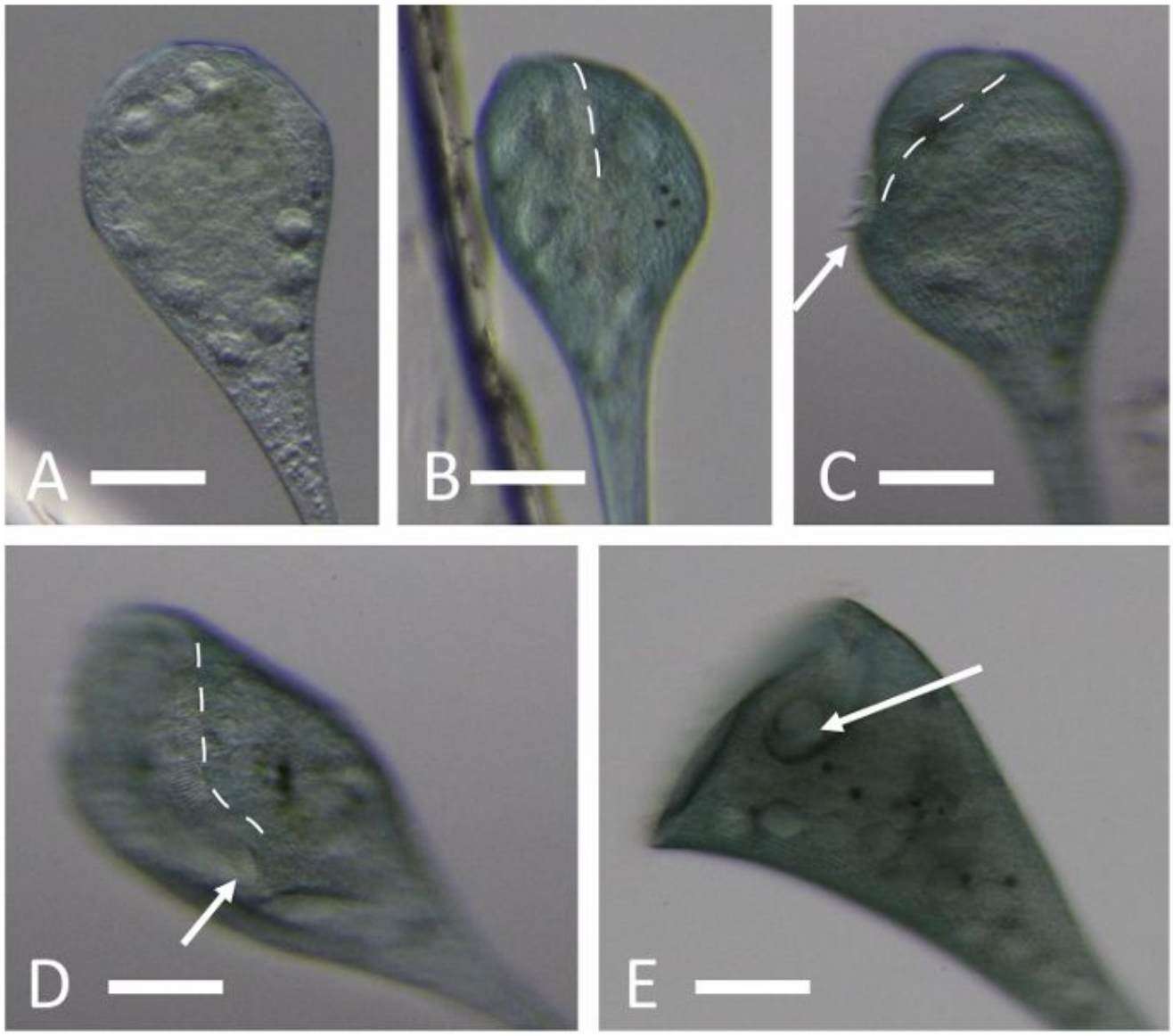

Figure 2.5. Snapshots of *Stentor* in each stage of the membranellar band and the oral apparatus regeneration. (**A**) Stage 1 is characterized by a teardrop-like cell shape and the absence of the membranellar band. (**B**) Stage 2 is characterized by the appearance of the membranellar band, a cilia-based structure that beats continuously. Membranellar bands are marked with white dashed lines in panels B - D. (**C and D**) Stage 3 is characterized by the appearance of oral primordium (marked with an arrow). Oral primordium looks like an invagination at the posterior end of the membranellar band. The oral primordium will then move up towards the anterior of the cell to become the oral apparatus. (**E**) Regeneration is completed when the cell has adopted the characteristic *Stentor* trumpet-like shape, and the oral apparatus (marked with an arrow) has widened at the anterior end of the cell. Scale bar is 0.25 mm.

Plot the percentage of cells in each of the regeneration stages for each time point in the form of a stacked box plot (Figure 6). Note: This type of plot allows the visualization of regeneration dynamics in a population of cells.

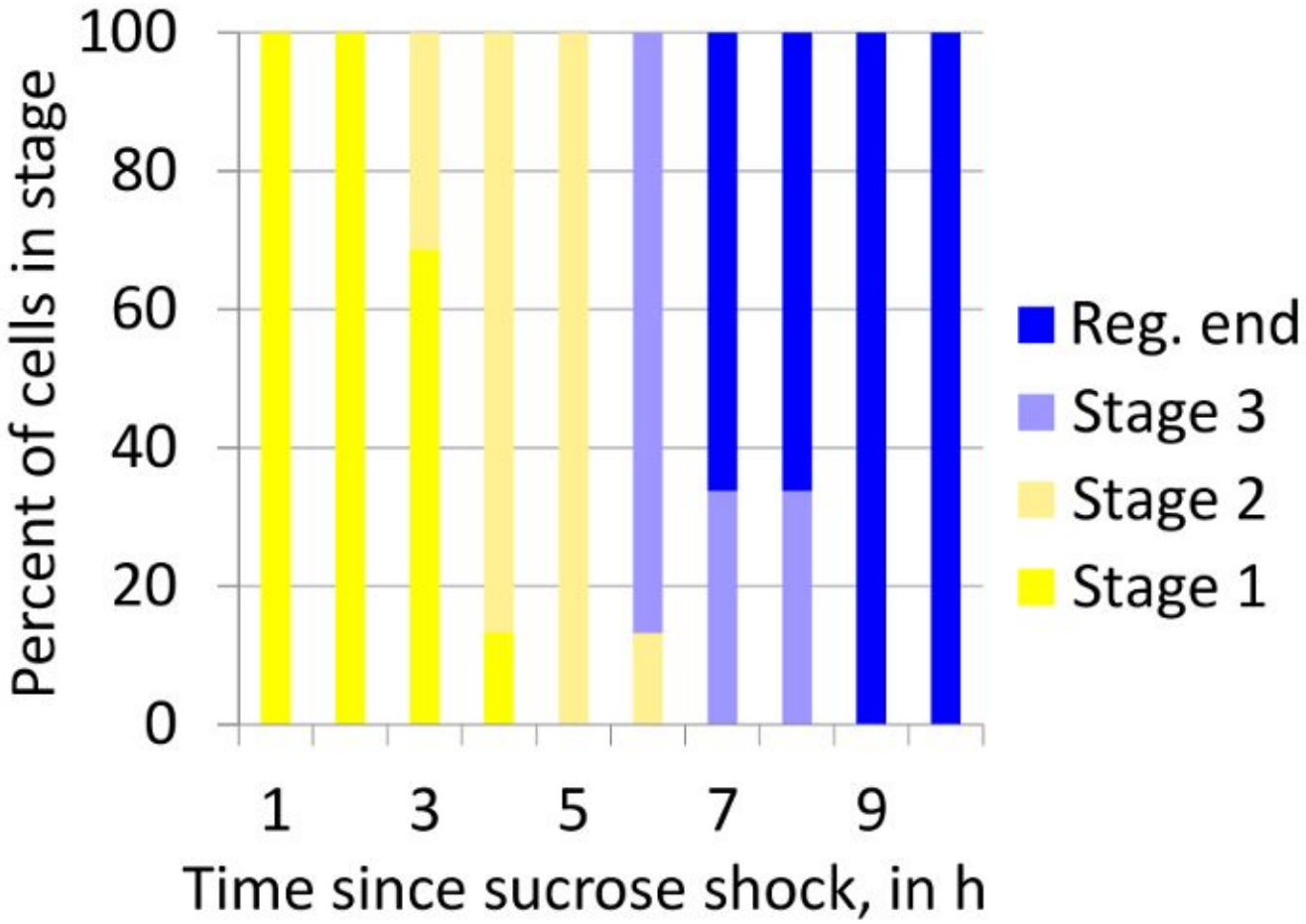

Figure 2.6. Stacked box plot showing how proportions of cells in all stages of regeneration after sucrose treatment changes over over time. The plot shows heterogeneity in the timing of regeneration stages within a population of regenerating cells. "Reg. end" indicates the completion of regeneration. The number of cells: 28.

Representative Results

Stentor cultures have been reliably established and maintained from individual cells or cell fragments using Section 1 of the protocol.

The time course of regeneration was measured in *Stentor* using the sucrose treatment method for initiating regeneration outlined in Step 3.1, combined with the imaging and analysis method discussed in Section 4 (Figure 6). This plot indicates that there is a one-hour-long spread in the

time taken by the population of cells to reach any particular stage. This type of analysis allows the study of temporal heterogeneity in the regeneration process in a population of regenerating cells.

The following is a summary of regeneration timing that has been observed thus far after dozens of sucrose treatments (Figure 3 and Figure 5). Stage 1 is when *Stentor* cells look like teardrops without any membranellar band (this stage starts immediately after sucrose washout). This stage lasts for 3 - 6 h. Stage 2 is when a membranellar band appears and grows (3 - 6 h after sucrose treatment). This stage lasts for 3 - 4 h. Stage 3 is when an oral primordium appears at the posterior end of the membranellar band (6 - 8 h after sucrose treatment), and both structures are moved toward the anterior end of the cell. This stage lasts for 1 - 2 h. When both the membranellar band and the oral apparatus reached the anterior end of the cell, this indicated the completion of regeneration. The cell has adopted characteristic *Stentor* trumpet-like shape. Cells were completely regenerated 8 - 9 h after sucrose treatment.

Discussion

Culturing *Stentor* presents a number of challenges. First, to perform experiments that require large numbers of cells, one needs to maintain a large number of *Stentor* cultures, as cultures become unhealthy when *Stentor* concentration exceeds 20 cells/mL. Second, the organisms that can contaminate *Stentor* cultures often divide faster than *Stentor* and overwhelm the culture (a common contaminant is rotifers). Thus, it is necessary to inspect the culture under a microscope periodically and remove the contaminants. Occasionally, a new culture needs to be started from a small number of cells rescued manually from a contaminated culture. This increases the time required to maintain healthy *Stentor* cultures. Third, expanding a single cell into a 400 mL culture

requires at least one month because *Stentor* cell cycle is 3 - 5 days. Fourth, unlike other model ciliates, *Stentor* rarely go into cyst form and they cannot be frozen.

An important aspect of culturing *Stentor* is the selection of an appropriate food organism. Various methods for culturing *Stentor* were previously described. One of them suggests to use skim milk to culture bacteria which then feed *Stentor*. Such a technique is effective in culturing *Stentor*; however, application of genomic techniques requires pure samples to avoid confusion resulting from genomic reads from undefined food organisms. Using the current protocol, *Stentor* can be grown in mass and, because their food, *Chlamydomonas*, has been sequenced, the presence of genomic contamination from the food organism can be detected and controlled for. For unknown reasons, Tartar had to replenish his stocks by returning to where he found *Stentor* before. With the current protocol, we have been able to keep *Stentor* for years.

Regeneration experiments with *Stentor* are generally straightforward, but there are a few critical details to keep in mind. In regard to Section 2, cutting *Stentor* cells in half can be mastered in minutes. Mastering more advanced microsurgery procedures of *Stentor* may require a week of practice. While performing a sucrose or urea treatment (Section 3), if at the beginning of imaging most cells still have their membranellar bands, increase incubation time by 10 - 30 s for when sucrose treatment is performed next. Do not increase the time of sucrose treatment beyond 3 min incubation because it will result in cell death.

Imaging of *Stentor* regeneration requires methods to image large cells over long periods of time. The imaging method detailed in Section 4 can only be used with an upright microscope. If an inverted microscope is available instead, then cells can be placed on a slide or coverslip in small chambers. One method is to create a chamber out of petroleum jelly and cover with another coverslip to prevent evaporation. When imaging, if *Stentor* are not in the correct orientation to

observe the characteristic features of each stage of regeneration, tap the plate firmly to make the cell contract. Watch the cell extend to identify the stage of regeneration (full extension takes about 45 s). If the cell is still in the wrong orientation, repeat the tapping. The images shown in Figure 1 and Figure 6 were taken by a stereo zoom microscope; however, all experiments detailed can be performed using a 5X dissecting stereo microscope.

Another challenge besides culturing and imaging *Stentor* is the tracking of regeneration, specifically the amount of time necessary to identify stages. If the regenerating cell is perfectly oriented with the region of the oral primordium clearly visible, identification of the stage takes a few seconds. Sometimes, however, the *Stentor* cell is in an orientation preventing clear assignment of regeneration stage, thus taking more time to identify. The significant amount of time required to stage individual regenerating cells might delay the quantification of all regenerating cells, thus decreasing the temporal precision of staging and requiring the observer to wait and re-image the cell after it has moved to a new orientation. Consequently, this experiment is both time and labor intensive. For these reasons, it would be highly desirable to develop automated methods for detecting *Stentor* cells and assigning stages of regeneration in video microscopy data. These would also allow for more reproducible experiments, increases in the sample size, and the removal of human bias.

The emergence of highly sensitive genomic and proteomic methods has begun to put studies of single cells into reach. For such single-cell analyses, the giant size of a *Stentor* cell makes it a desirable test subject for proof-of-concept experiments. For such experiments to be possible, culturing *Stentor* is fundamental, and so the methods described here should play a role in further development of more advanced single-cell techniques.

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Chapter 3 - Aurora kinase inhibitors delay regeneration in *Stentor coeruleus* at an intermediate step

Abstract

The giant unicellular ciliate *Stentor coeruleus* can be cut into pieces and each piece will regenerate into a healthy, full-sized individual. The molecular mechanism for how *Stentor* regenerates is a complete mystery, however, the process of regeneration shows striking similarities to the process of cell division. On a morphological level, the process of creating a second mouth in division or a new oral apparatus in regeneration have the same steps and occur in the same order. On the transcriptional level, genes encoding elements of the cell division and cell cycle regulatory machinery, including Aurora kinases, are differentially expressed during regeneration. This suggests that there may be some common regulatory mechanisms involved in both regeneration and cell division. If the cell cycle machinery really does play a role in regeneration, then inhibition of proteins that regulate the timing of cell division may also affect the timing of regeneration in *Stentor*. Here we show that two well-characterized Aurora kinase A+B inhibitors that affect the timing of regeneration. ZM447439 slows down regeneration by at least one hour. PF03814735 completely suppresses regeneration until the drug is removed. Here we provide the first direct experimental evidence that *Stentor* may harness the cell division machinery to regulate the sequential process of regeneration.

Introduction

The ability to heal wounds and regenerate damaged structures is essential for an organism's survival. Multicellular organisms mostly rely on cell division to patch wounds and regenerate lost structures with newly proliferated cells, but when a single cell is damaged, it must be able to

recognize and repair that damage without being able to rely on other cells. Nowhere is this challenge more dramatic than in the giant unicellular ciliate *Stentor coeruleus,* for when cut into pieces, each piece will fully regenerate into a healthy, full-sized individual[1]. *Stentor* cells are a millimeter long with a wine glass shape, and have a complex and intricate ultrastructure. *Stentor* are binucleate ciliates with two morphologically distinct nuclei. The micronuclei is used for germline reproduction and the macronucleus is transcriptionally active throughout the cell cycle. *Stentor* has an oral pouch, a cilia-lined pore to intake food at its wide anterior and a holdfast, the structure by which the cell attaches to a surface, at its posterior. Connecting these two are a series of microtubule rows called cortical rows that resemble pinstripes. The oral pouch and the holdfast can each fully regenerate if removed, and a bisected cell can regenerate two normal-looking cells[1]. The molecular mechanism for how *Stentor* regenerates missing parts is a complete mystery. This study focuses on regeneration of the oral apparatus, which consists of a circular band of cilia-based structures known as the membranellar band, connected to an oral pouch located at a defined position. During feeding, the membranellar band creates a fluid flow to bring food to the anterior end of the cell, where it is engulfed through the oral pouch.

Regeneration in *Stentor coeruleus* can be induced by sucrose shock. This leads to shedding of the oral apparatus, which is comprised of the oral pouch and membranellar band (Figure 1A). After sucrose shocking, *Stentor* look tear-drop shaped and stay stationary for approximately three hours. After three or four hours of regeneration, *Stentor* begin to form a membranellar band in the middle of the cell body, initially oriented parallel to the body axis. The membranellar band grows simultaneously towards the top and bottom of the cell. At the top of the cell, the membranellar band will continue growing across the top. After six or seven hours of regeneration, the posterior end of the membranellar band will begin to curl to form the oral pouch and a physical indentation

of the cell surface can be seen. Within the last two hours of regeneration, the oral pouch will be moved to the top of the cell along with the membranellar band. *Stentor* usually completes regeneration within 8 hours.

The process of regeneration shows striking similarities to the process of cell division. When a *Stentor* cell divides asymmetrically along its vertical axis, the anterior daughter cell retains the oral apparatus from the mother cell and the posterior daughter inherits a *de novo* oral apparatus that forms just prior to cytokinesis. This *de novo* creation of an oral apparatus during regeneration proceeds through a series of morphological steps virtually identical to those seen during the creation of a new oral pouch during division[1], namely, the formation of a membranellar band parallel to the body axis[2], curling of the band, and formation of the oral pouch. During division, the macronucleus undergoes a dramatic shape change from a moniliform string of small spherical nodes into a short tube, when then re-elongates just prior to mitosis. This same nuclear shape change also takes place during regeneration, further suggesting a similarity of the two processes[3].

Similarity between regeneration and division has also been reported at the transcriptional level, based on studies of the RNA transcriptome during regeneration. Genes encoding elements of the cell division and cell cycle regulatory machinery, including Aurora kinases, are differentially expressed during the later stages of regeneration compared to the earlier stages of regeneration[4]. Such similarities suggest that there may be some common regulatory mechanisms involved in both regeneration and cell division. Since aurora kinase signaling indicates that a spindle is properly assembled[5], a similar mechanism could be at work in *Stentor* to signal the correct assembly of one or more structures during regeneration. But it is also possible that the similarity has nothing to do with regeneration and instead plays some other role. For example, the micronuclei undergo

mitosis during both cell division and regeneration[6], so perhaps the transcriptional changes in cell cycle-related genes have only to do with the micronuclear mitosis and not regeneration itself. However, if the cell cycle machinery really does play a role in regeneration, then inhibition of proteins that regulate the timing of cell division may also affect the timing of regeneration in *Stentor*. The use of drugs has historical precedence. Actinomycin D[7], puromycin[7], concanavalin A[8] and DNA synthesis inhibitors[9] have been shown to slow or stop regeneration in *Stentor*. Here we show that two well-characterized Aurora kinase A+B inhibitors slow or stop regeneration in *Stentor*, providing the first direct experimental evidence that *Stentor* may harness the cell division machinery to regulate the sequential process of regeneration.

Objective

The general objective is to learn whether regeneration and division may harness conserved molecular mechanisms. The specific objective is to test whether inhibition of the Aurora kinases, well known regulators of cell division, alters the process of regeneration in *Stentor*.

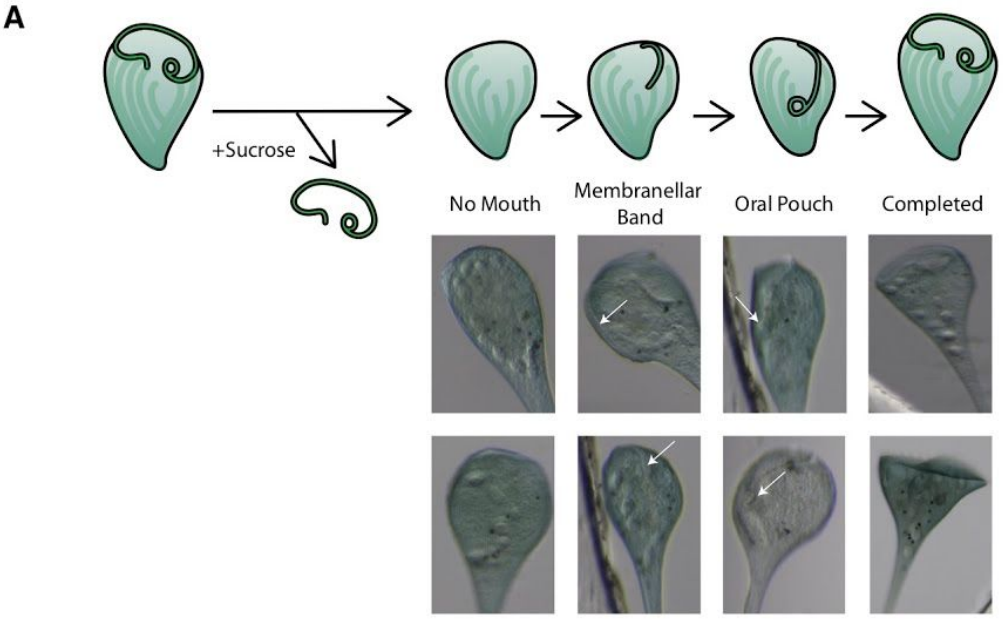
Results and Discussion

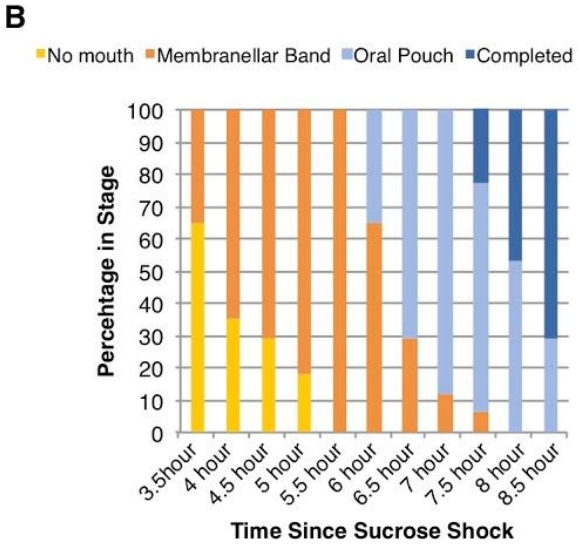
Compared to the timing of events in untreated cells (Figure 1B), addition of Aurora kinase A+B inhibitor ZM447439[10] caused regeneration to be delayed by at least one hour. 10% of treated cells did not form a membranellar band until 4 hours into regeneration (Figure 1C). Treated cells spent more time forming a membranellar band and the first oral pouch did not appear until seven or eight hours after starting regeneration, compared to untreated cells where oral pouches appear in the 6.5 hour time point. The first fully regenerated *Stentor* did not form until eight and a half hours later (Figure C). We have observed the same pattern of delay three times in separate experiments (data not shown).

Although ZM447439 is known to be a highly specific inhibitor of Aurora kinases in mammalian cells, any chemical inhibitor can show off-target effects, especially when applied in a different cell type. To confirm our result that Aurora inhibition delays regeneration, we tested a second highly specific and reversible Aurora kinase A+B inhibitor, PF03814735[11]. We found that with this inhibitor, regeneration was suspended at the membranellar band stage (Figure 1D). 38% of *Stentor* still had no oral pouch by six hours, and none of the Stentor had regenerated by ten and a half hours. Regeneration was paused at the membranellar band stage for the duration of the experiment.

PF03814735 is reversible in other systems[11], therefore we questioned whether the block on regeneration could be reversed after the inhibitor is removed. After a two hour incubation and three subsequent washes, *Stentor* were able to regenerate in a timely fashion, forming membranellar bands after five hours, oral pouches after seven hours and fully regenerating in ten hours (Figure 1E).

Our results indicate that Aurora kinase function may normally be required to drive a specific step of regeneration that takes place after the membranellar band has formed but before it moves to the anterior of the cell and forms an oral pouch. Such a temporal requirement is reminiscent of the requirement of Aurora kinases for specific stages of mitotic progression. As with the cell cycle, the ability to reversibly arrest regeneration and then analyze timing of events after the arrest is alleviated may, in the future, provide a way to determine whether regeneration is timed by a series of domino-like events, each triggering the next, or a master clock like that used in the cell cycle.


A
+Sucrose
No Mouth
Membranellar Band
Oral Pouch
Completed


B
No mouth
Membranellar Band
Oral Pouch
Completed
Percehtage in Stage
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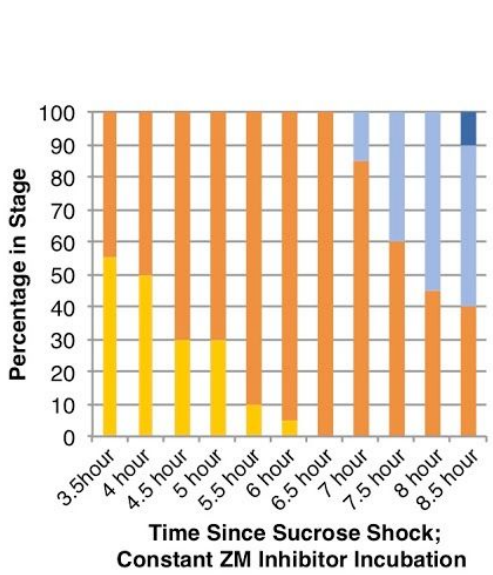
C
Percentage in Stage
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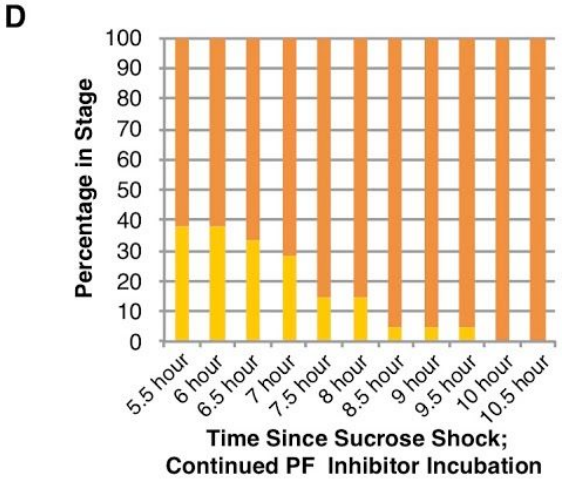
Constant ZM Inhibitor Incubation


D
Percentage in Stage
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Continued PF Inhibitor Incubation

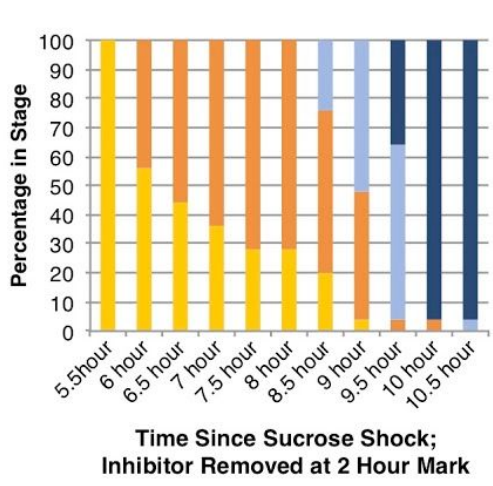
E
Percentage in Stage
Time Since Sucrose Shock;
Inhibitor Removed at 2 Hour Mark

Figure 3.1: Aurora kinase A + B inhibitors slow or stop regeneration.
(A) *Stentor* exhibits three, distinct, chronological, morphologies during regeneration. After sucrose shocking, they first adopt a tear-drop shape, then form a membranellar band (dotted line) parallel to their body axis. Next, they form an oral pouch (arrow) at the posterior end of the membranellar band. Finally, they move the oral pouch to the top of the cell.

(B) Under normal conditions, *Stentor* need approximately eight hours to regenerate. After about three hours a membranellar band starts to appear, and after another three hours the oral pouch becomes visible, after which two more hours are spent moving the membranellar band and the pouch to the correct position to complete regeneration.

(C) Aurora kinase A+B inhibitor, ZM447439, has little effect on the first phase of regeneration, formation of the membranelle band, but dramatically slows down the second phase of regeneration, formation of the oral pouch.

(D) Aurora kinase A+B inhibitor, PF03814735, permits formation of the membranellar band but completely blocks regeneration at the stage of oral pouch formation.

(E) Aurora kinase A+B inhibitor, PF03814735, can be removed and regeneration occurs subsequently within eight hours.

Conclusions

Our results indicate that regeneration in *Stentor* takes place in two separately regulated steps, with Aurora kinase possibly regulating the second step. These results support the idea that regeneration in *Stentor* is regulated by components of the cell division machinery, suggesting that the similarity between the two processes more than just a superficial coincidence. These small molecule inhibitors provide new tools to perturb the process and study its effects.

Limitations

Bioinformatic analysis of the aurora kinase family in *Stentor* indicates that there are 44 different aurora kinases[12], and that these cannot be clearly mapped onto the Aurora classes A, B, and C in mammals. Consequently, it is not currently clear which of the Aurora kinases in *Stentor* is actually being affected by the inhibitors during regeneration.

Conjectures

Both mitosis and regeneration proceed through a series of distinct steps that must take place in the correct order, and each step must not start until the preceding steps are completed. We conjecture that the cell cycle machinery, which has evolved to regulate the sequential steps of division, may provide the necessary timing and ordering of events that allows proper regeneration. For example, regeneration might require a series of checkpoints, one of which is mediated by Aurora signaling. Early observations of washing out the competitive inhibitor suggested that subsequent events took place more synchronously. However, measurements of regeneration timing will be needed to confirm this impression.

Methods

Sucrose shock

Cells were gathered by pipette individually and washed with pasteurized spring water (PSW; Carolina Biological Supply). An equal volume of 25% (w/v) sucrose was added to cells in PSW to give a final concentration of 12.5% sucrose. Cells were incubated for approximately 3 minutes or until the membranellar band was shed. Sucrose was then diluted 50x by addition of PSW. After 20-30 minutes, cells that have rounded up (indicating imminent death) or that still had membranellar bands present were discarded.

Identification of stages

Cells were examined at 30 minute or one hour intervals, using a Zeiss Stemi 2000 at 5x to identify cells that retained a non-spherical shape. Three hours after sucrose shock, the presence of a membranellar band was assessed by looking for a faint band of randomly beating cilia in the middle of the cell. Since these may be facing away from the camera lens, it was important to look

at the other side of *Stentor*. If the cells had been starved, the membranellar band was more likely to be visible through the cell. To locate the oral pouch, the most posterior part of the membranellar band was examined for the presence of an indentation that represents the oral pouch. It was observed that immediately before the oral pouch first appeared, the membranellar band began curling. Cells were considered to have completed regeneration if the oral pouch was present and the membranellar band had migrated to the anterior end of the cell.

Inhibitor treatment

The inhibitors ZM447439 and PF03814735, purchased from Selleck Chemicals, were dissolved in DMSO at concentrations of 5.0mM and 2.1mM respectively. These stock solutions were then diluted to final concentrations of 0.1nM and 42pM in wells containing *Stentor* cells in PSW.

References

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Chapter 4 - Proteomic Dissection of *Stentor*

Stentor coeruleus is a useful model organism to study single-cell regeneration. They have a distinctive cell shape and large size. They can stretch to 1mm in length. At their anterior end, they have a membranellar band consisting of tightly woven layers of long cilia that beat in synchrony. They have an oral apparatus where they phagocytose unfortunate microorganisms caught in their flow. On the posterior end, they have a holdfast to attach themselves to underwater surfaces (Figure one). And, when cut in half, they can regenerate. The process of regeneration takes eight hours and their transcriptome during regeneration has been characterized[1]. The next step to understanding their regeneration is to have an account of proteins so that we can see the building blocks of *Stentor*. Because of the size of Stentor, we are able to dissect them into parts and look for enrichment and depletion of proteins in each part. We analyzed over 4000 proteins, providing an inventory of proteins that *Stentor* will use for regeneration, as well as the proteins required to make a new membranellar band.

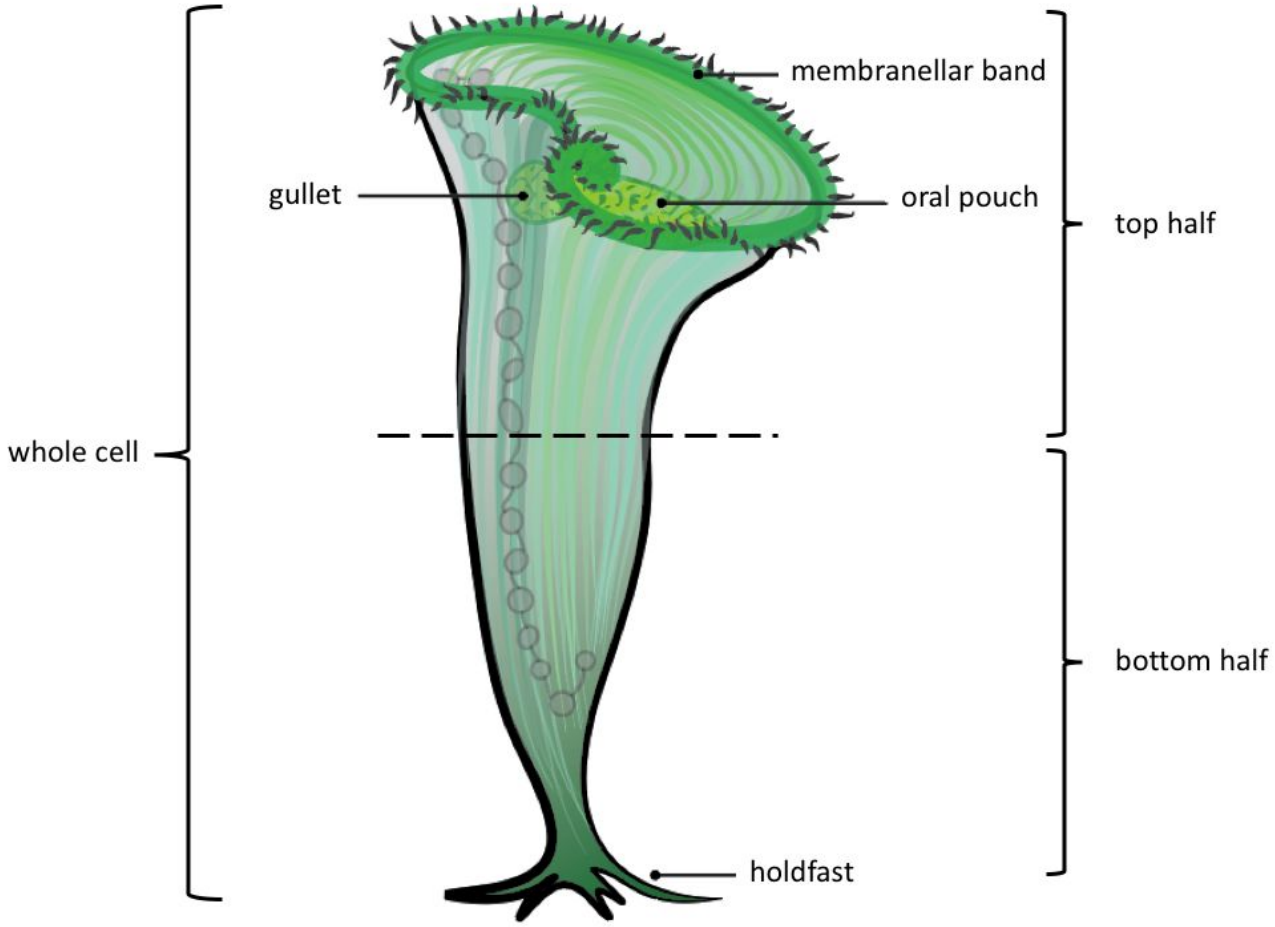


Figure 4.1: Diagram of *Stentor*. *Stentor* have a distinct anterior and posterior structures. The membranellar band and the oral pouch is at the anterior of the cell. The holdfast is at the posterior. If the cell is cut in half, the anterior portion is referred to as the top half and the posterior portion is referred to as the bottom half.

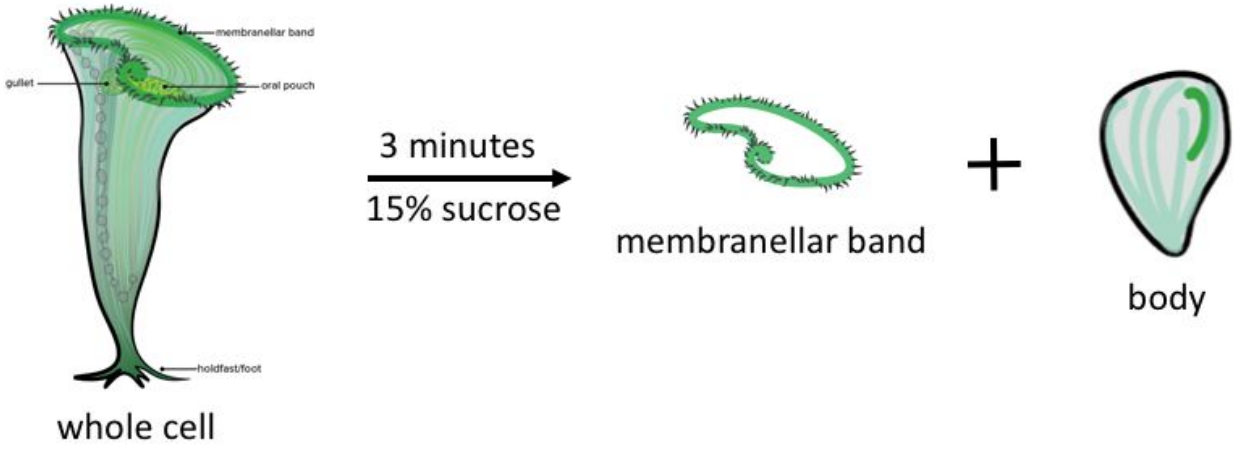


Figure 4.2: Obtaining membranellar bands. Incubating *Stentor* in a sucrose solution, causes them to shed their membranellar bands. The subsequent pieces are membranellar bands that can be collected and the bodies can be observed to study regeneration.

Results

One of the structures that *Stentor* have to regenerate is the membranellar band. To induce shedding of the membranellar band, we incubate the *Stentor* in 15% sucrose (Figure 2). To approximate the number of proteins in the membranellar band, we collected 100 membranellar bands and ran them on an SDS-PAGE gel (Figure 3). Here we see that there are two large bands: one at 50 kd and one at 250 kd. We suspected that the 50 kd band was mostly tubulin, which is shown in the adjacent western blot. These two gel slices were then sent to Applied Biomics for mass spectrometry (Table 6: 50kD and 7: 250kD). Applied Biomics confirmed that tubulin was a large subset of proteins in the 50kD band and that dynein heavy chain was the most abundant prortein in the 250 kD band. The presence of Aurora kinases (g35501 and g25039) were curious because of chapter three of this manuscript, however these were of very low abundance in this band and there is no evidence that these proteins are enriched in the MB fraction.

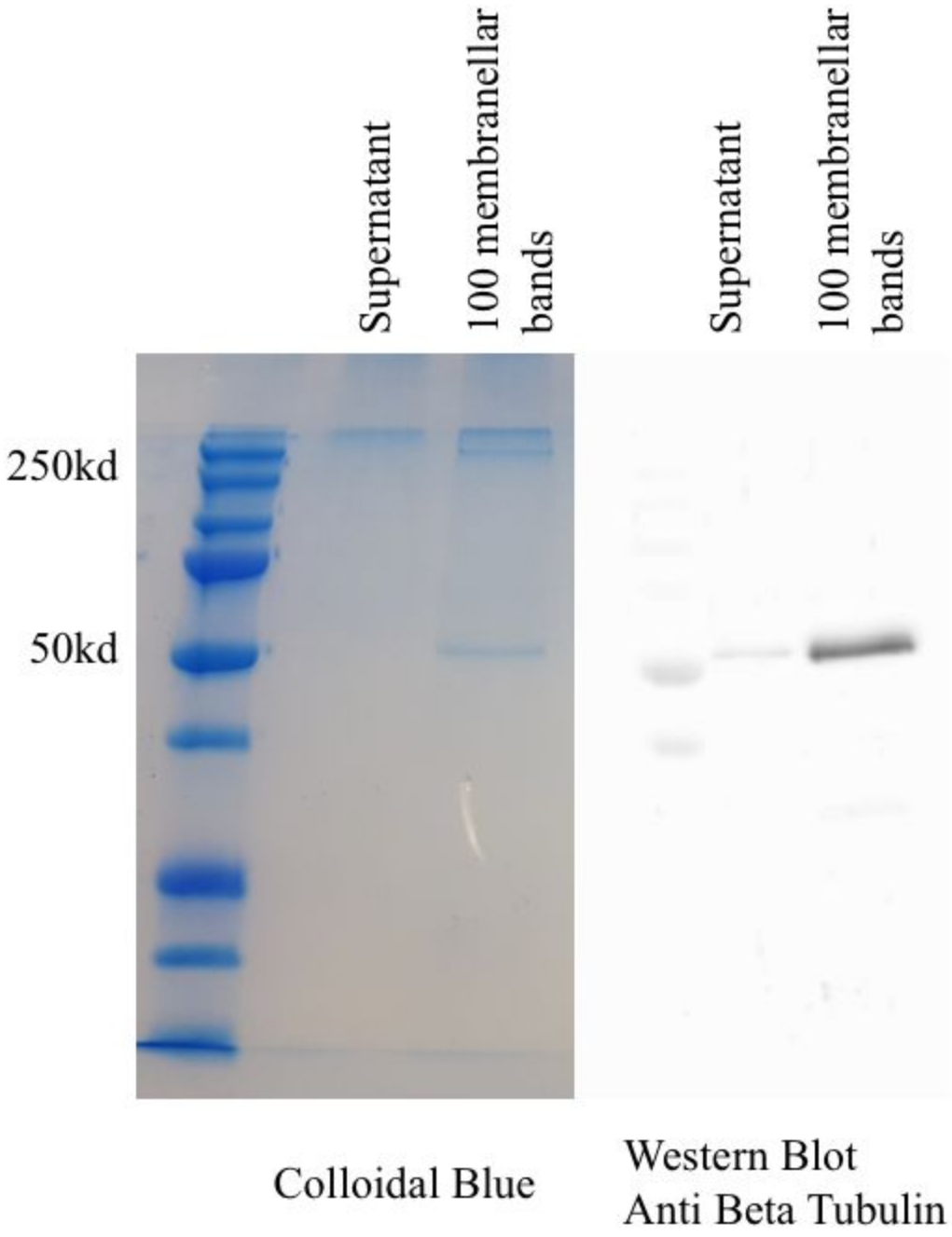


Figure 4.3: SDS-page gel of membranellar bands and western blot against beta tubulin.

Table 4.1: 50kd band from Figure 3 sent for mass spectrometry.

Gene Model Identifier	Description
SteCoe_14841	Alpha tub like 1
SteCoe_22851	Beta tub like 1
SteCoe_24853	Tubulin
SteCoe_38337	Tubulin_C
SteCoe_3761	Alpha tub like 8
SteCoe_38102	Tubulin
SteCoe_5158	Hypothetical
SteCoe_36121	Obscurin-like
SteCoe_37527	MARK subfamily kinase
SteCoe_11034	Hypothetical
SteCoe_14800	Hypothetical
SteCoe_27590	Kinectin
SteCoe_11594	Hypothetical
SteCoe_19482	Tubulin-tyrosine ligase
SteCoe_34786	Cyclic nucleotide-binding
SteCoe_31542	EF-hand_8

Table 4.2: 250kd band from Figure 3 sent for mass spectrometry.

Gene Model Identifier	Description
SteCoe_36121	Obscurin-like
SteCoe_10941	Myosin 2 HCL
SteCoe_5158	Hypothetical
SteCoe_4972	RNA dependent RNA polymerase
SteCoe_37527	MARK subfamily
SteCoe_35501	Aurora kinase
SteCoe_25039	Aurora kinase
SteCoe_721	Vacuolar Protein Sorting 35
SteCoe_11197	Dynein Heavy Chain N1
SteCoe_36091	Centromere J-like
SteCoe_17904	Hypothetical
SteCoe_28480	NDR kinase
SteCoe_16556	Hypothetical
SteCoe_7806	Kinesin motor
SteCoe_7626	Mg transporter
SteCoe_13060	Phospholipid transporter
SteCoe_3850	Zinc-Finger-B box
SteCoe_28636	NIF domain

Next, we initiated a collaboration with Pacific Northwest National Labs (PNNL). We sent then 5 samples: Whole *Stentors*, *Stentors* without membranellar bands (bodies), membranellar bands, top halves and bottom halves (Figure 1 and 2). Due to the comprehensive annotation of the *Stentor* genome[2], PNNL was able to identify 2751 proteins between the 5 samples. We identified those proteins via the *Stentor* genome database. Then, we trimmed the data set to include only proteins

that have been identified more than once so that we can look for enrichment and calculated relative abundance. Last, comparing those relative abundances between every sample, we identified enriched proteins in each sample (Figure 4a). The top 5 most enriched proteins are listed in Figure 4b. Then, each sample was compared to each other by scatter plot. Proteins that are along the y=x line are about the same relative abundance, and the further away the protein is from y=x, the more enriched in a sample it is (Figure 4c-4l).

We were encouraged to find dynein heavy chain in the membranellar band which contains abundant, long motile cilia, and histones in the bodies, which contain the macronucleus. We were curious about CENP-T in the bodies because they coordinate complexes to the kinetochore during mitosis. This suggests that *Stentors* are ready to coordinate microtubules at the mark of a phosphorylation[3]. We were surprised to observe four proteases in the bottom half of *Stentor*, suggesting that catabolism may occur towards the posterior end.

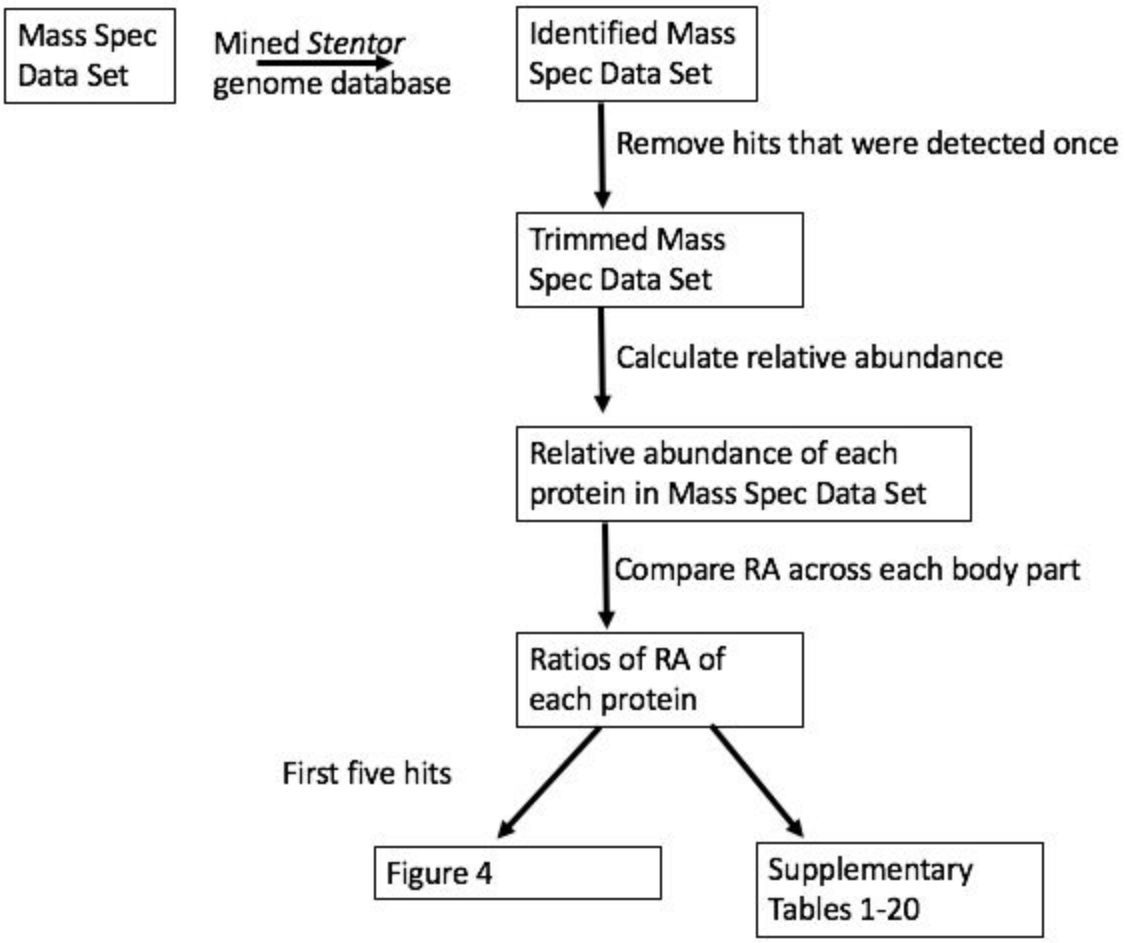
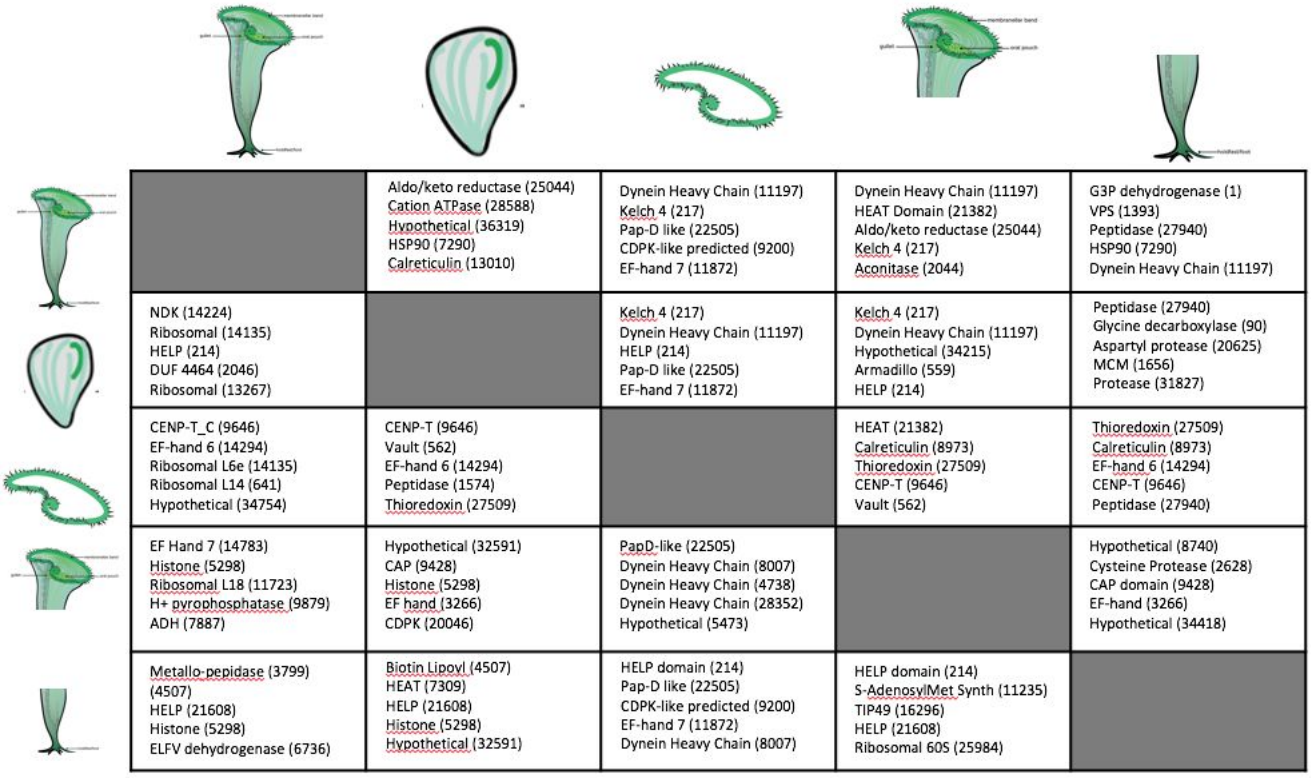


Figure 4.4a: Mass spectrometry analysis flow chart.



	Aldo/keto reductase (25044) Cation ATPase (28588) Hypothetical (36319) HSP90 (7290) Calreticulin (13010)	Dynein Heavy Chain (11197) Kelch 4 (217) Pap-D like (22505) CDPK-like predicted (9200) EF-hand 7 (11872)	Dynein Heavy Chain (11197) HEAT Domain (21382) Aldo/keto reductase (25044) Kelch 4 (217) Aconitase (2044)	G3P dehydrogenase (1) VPS (1393) Peptidase (27940) HSP90 (7290) Dynein Heavy Chain (11197)
NDK (14224) Ribosomal (14135) HELP (214) DUF 4464 (2046) Ribosomal (13267)		Kelch 4 (217) Dynein Heavy Chain (11197) HELP (214) Pap-D like (22505) EF-hand 7 (11872)	Kelch 4 (217) Dynein Heavy Chain (11197) Hypothetical (34215) Armadillo (559) HELP (214)	Peptidase (27940) Glycine decarboxylase (90) Aspartyl protease (20625) MCM (1656) Protease (31827)
CENP-T_C (9646) EF-hand 6 (14294) Ribosomal L6e (14135) Ribosomal L14 (641) Hypothetical (34754)	CENP-T (9646) Vault (562) EF-hand 6 (14294) Peptidase (1574) Thioredoxin (27509)		HEAT (21382) Calreticulin (8973) Thioredoxin (27509) CENP-T (9646) Vault (562)	Thioredoxin (27509) Calreticulin (8973) EF-hand 6 (14294) CENP-T (9646) Peptidase (27940)
EF Hand 7 (14783) Histone (5298) Ribosomal L18 (11723) H+ pyrophosphatase (9879) ADH (7887)	Hypothetical (32591) CAP (9428) Histone (5298) EF hand (3266) CDPK (20046)	PapD-like (22505) Dynein Heavy Chain (8007) Dynein Heavy Chain (4738) Dynein Heavy Chain (28352) Hypothetical (5473)		Hypothetical (8740) Cysteine Protease (2628) CAP domain (9428) EF-hand (3266) Hypothetical (34418)
Metallo-pepidase (3799) (4507) HELP (21608) Histone (5298) ELFV dehydrogenase (6736)	Biotin Lipoyl (4507) HEAT (7309) HELP (21608) Histone (5298) Hypothetical (32591)	HELP domain (214) Pap-D like (22505) CDPK-like predicted (9200) EF-hand 7 (11872) Dynein Heavy Chain (8007)	HELP domain (214) S-AdenosylMet Synth (11235) TIP49 (16296) HELP (21608) Ribosomal 60S (25984)	

Figure 4.4b: Top 5 enriched proteins for each sample compared to all other samples. Table is read horizontal/vertical. Example: Aldo/keto reductase is the most enriched protein in the body compared to whole cells.

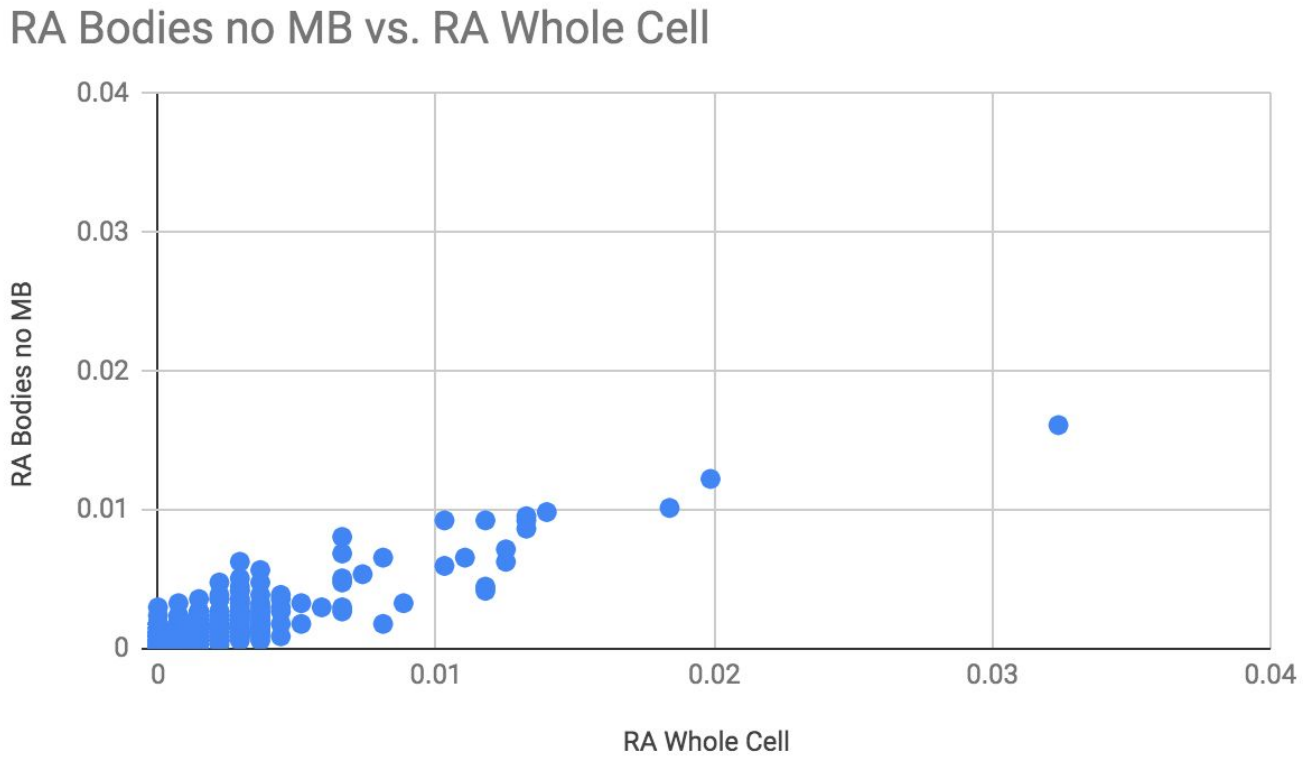


Figure 4.4c: Scatter plot of proteins found in bodies and whole cells.

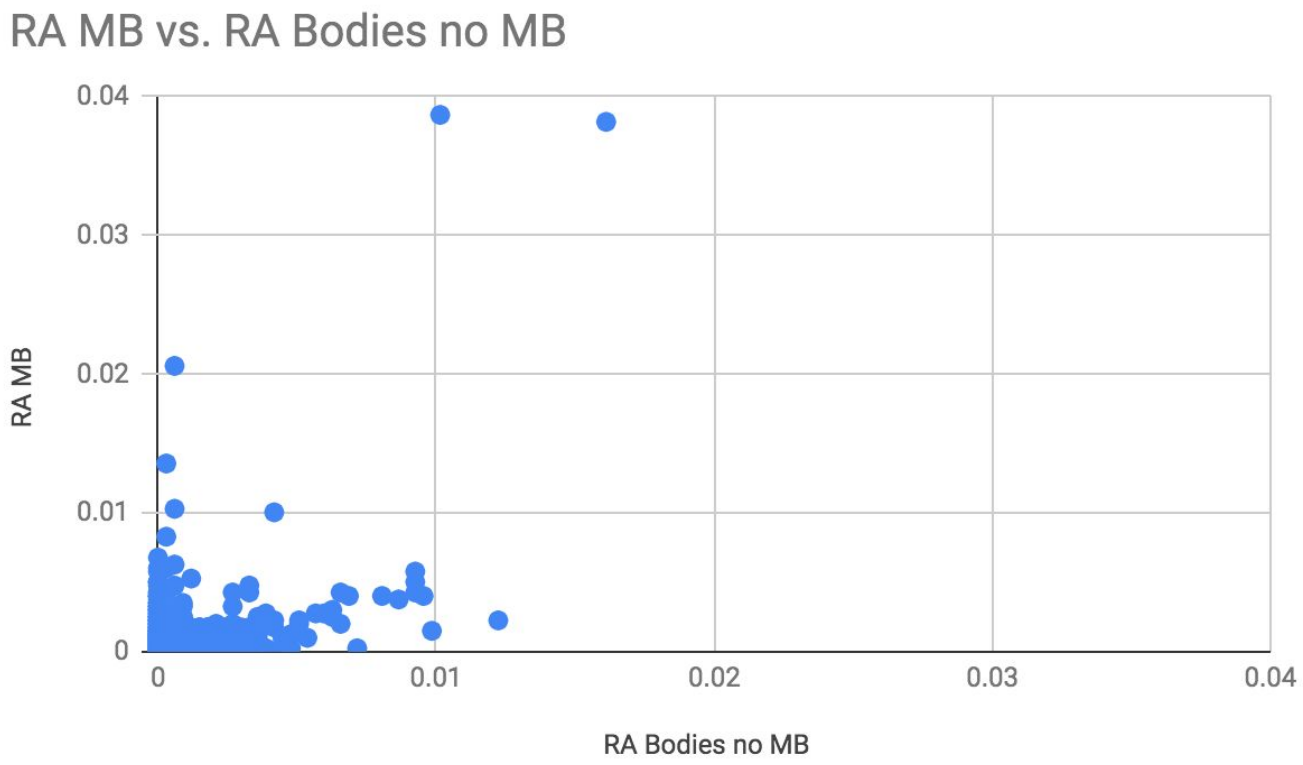


Figure 4.4d: Scatter plot of proteins found in membranellar bands and bodies.

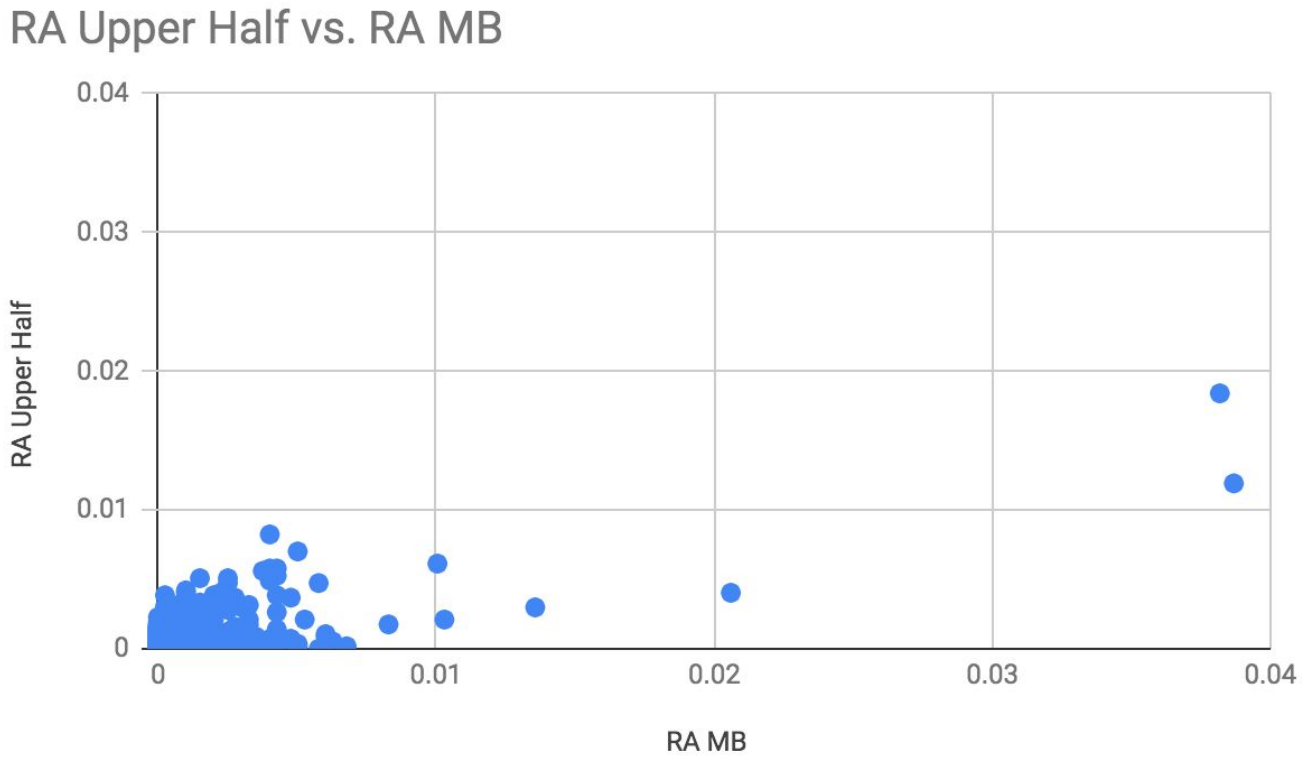


Figure 4.4e: Scatter plot of proteins found in membranellar bands and top halves.

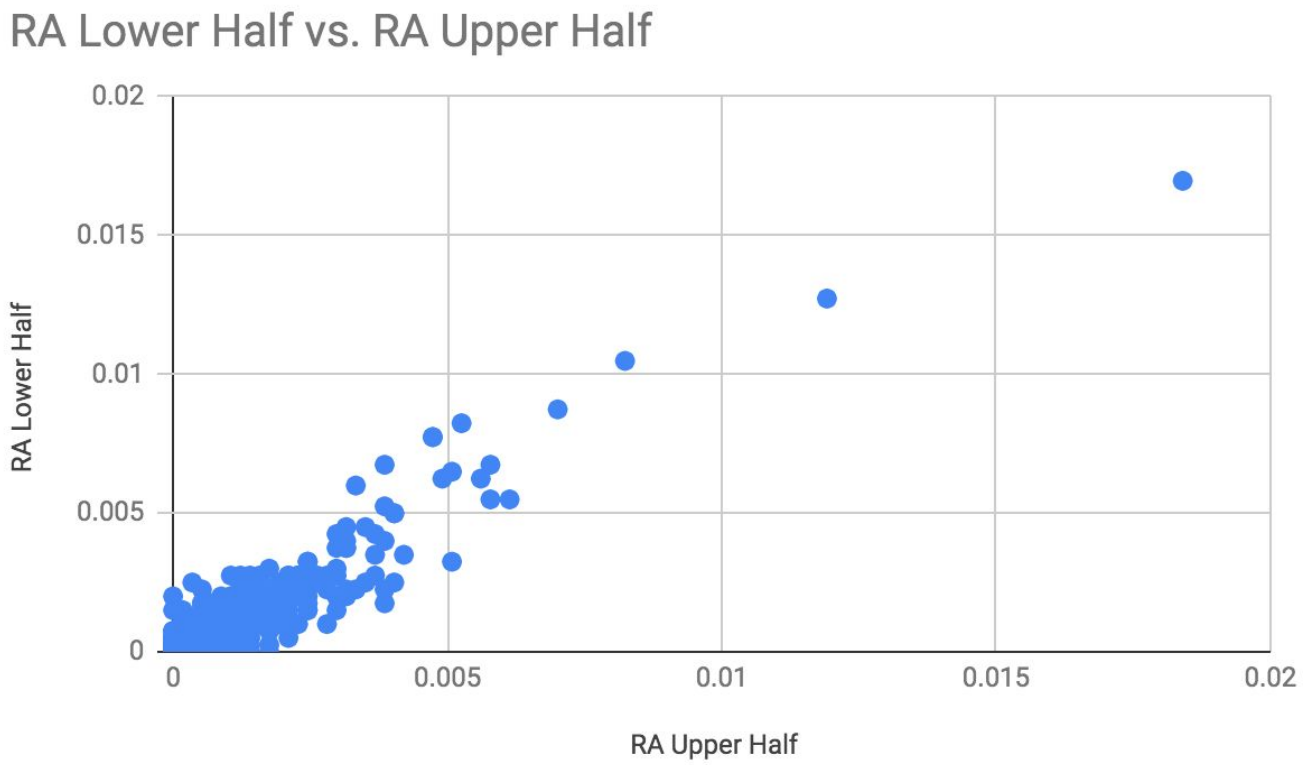


Figure 4.4f: Scatter plot of proteins found in bottom halves versus top halves.

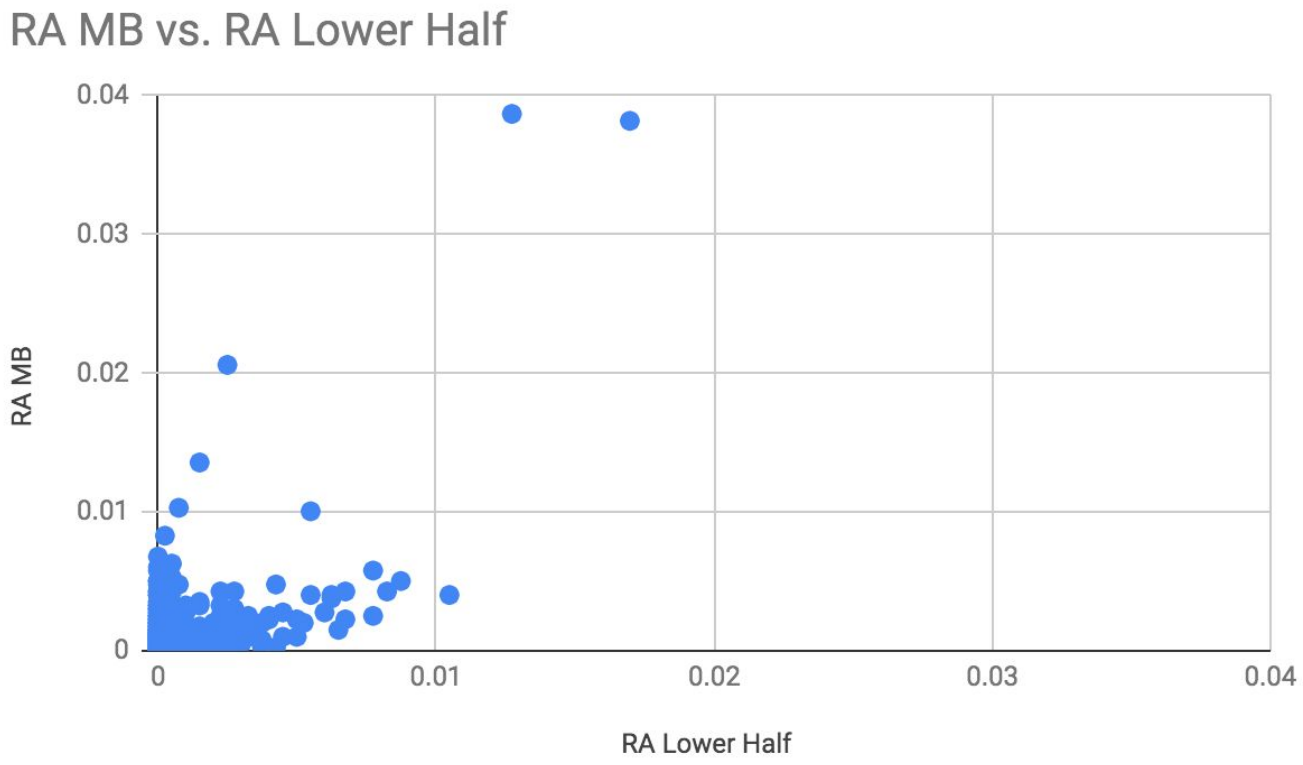


Figure 4.4g: Scatter plot of proteins found in membranellar bands and bottom halves.

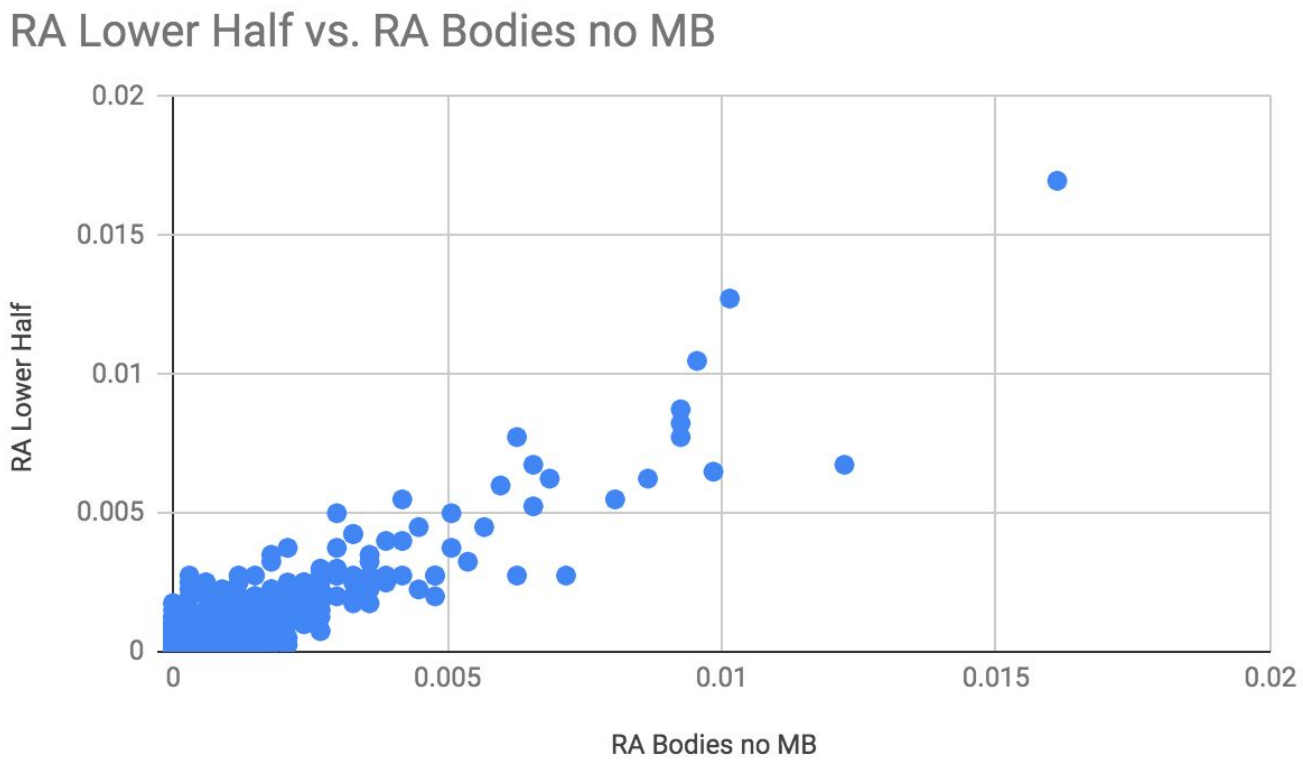


Figure 4.4h: Scatter plot of proteins found in bottom halves versus bodies.

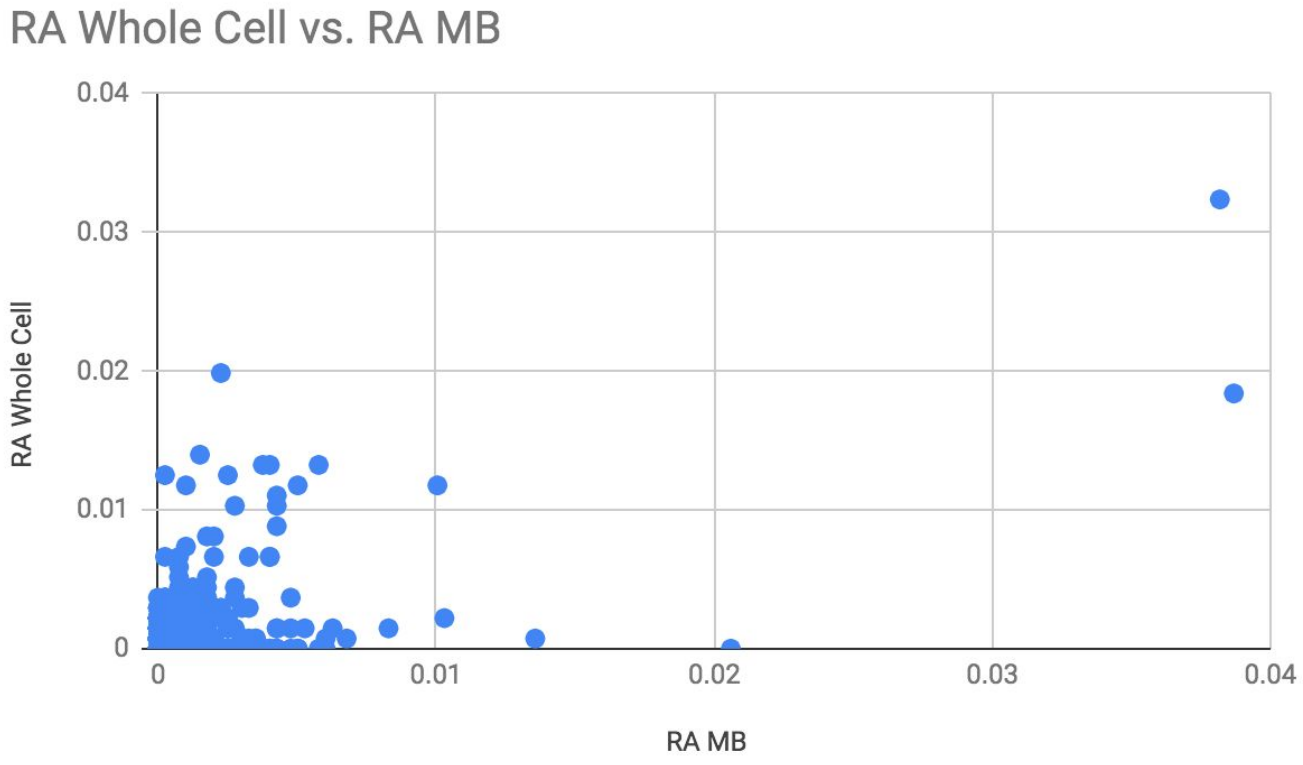


Figure 4.4i: Scatter plot of proteins found in whole cells and membranellar bands.

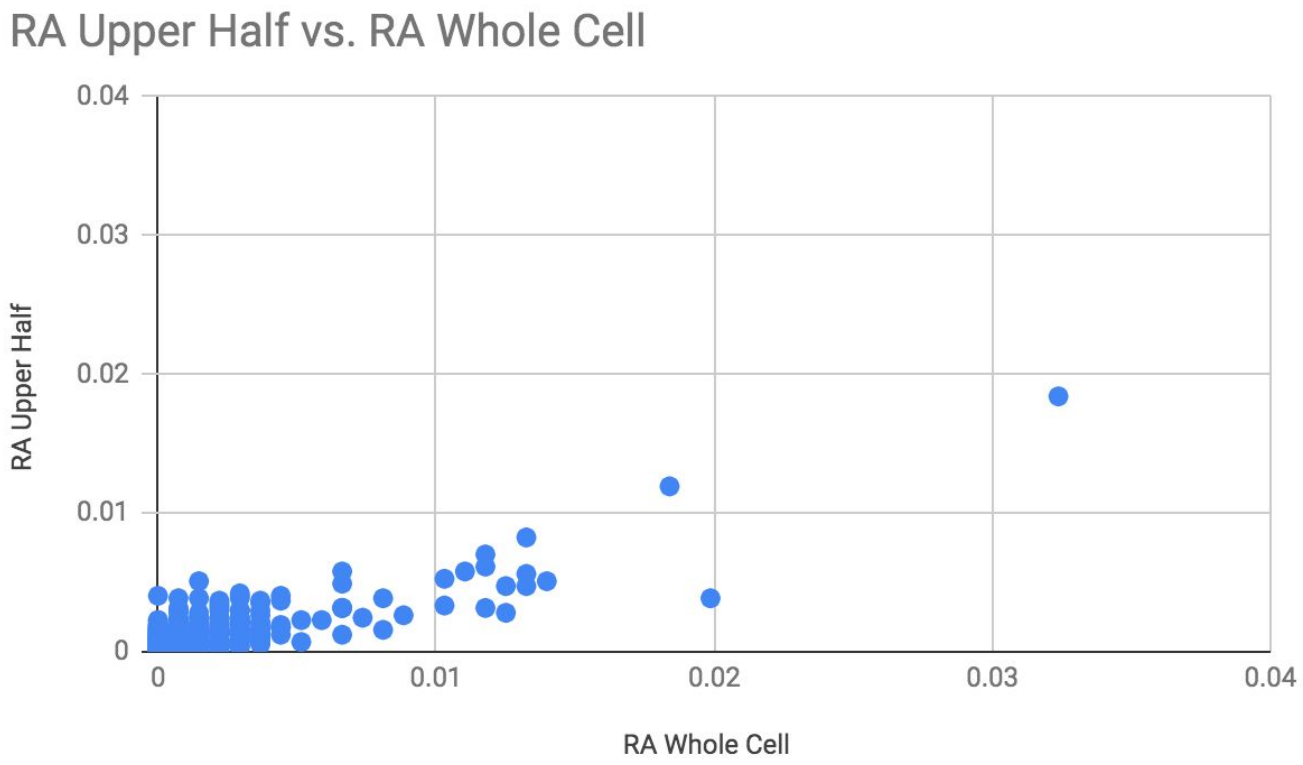


Figure 4.4j: Scatter plot of proteins found in top halves versus whole cell.

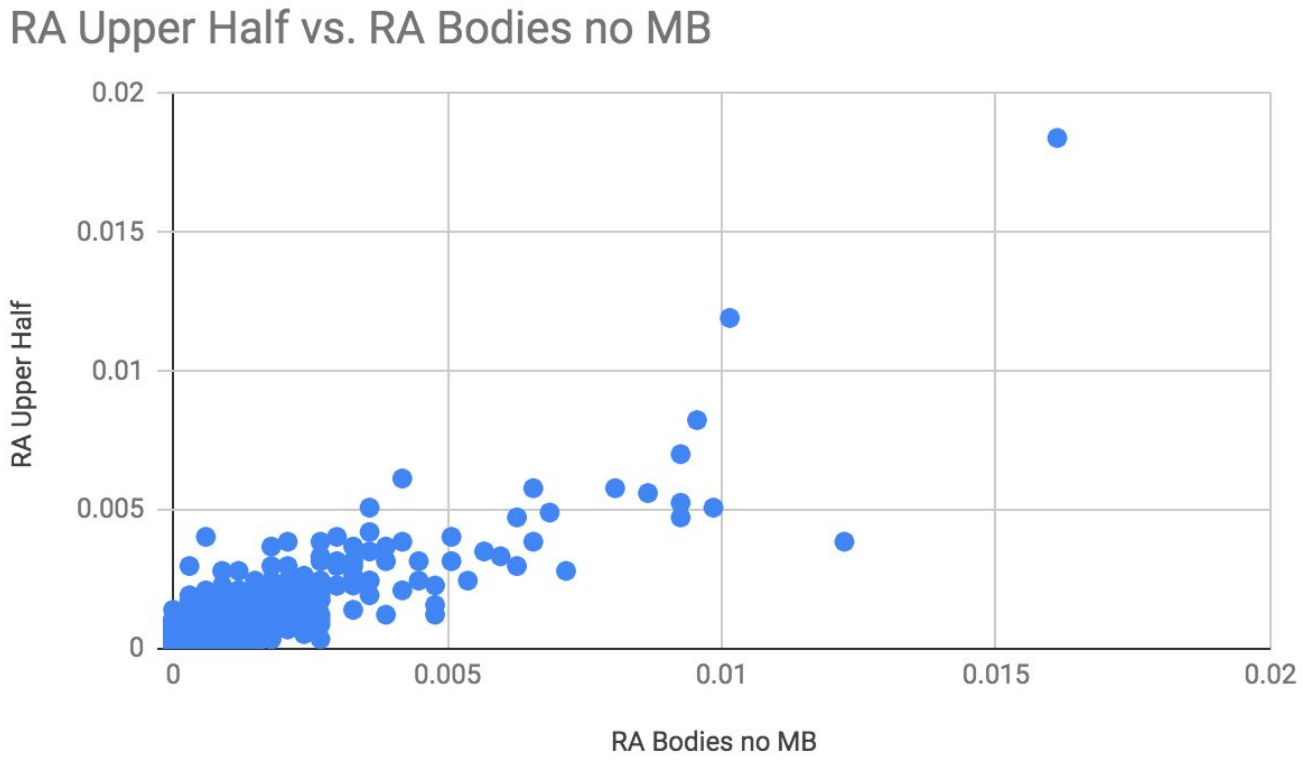


Figure 4.4k: Scatter plot of proteins found in top halves and bodies.

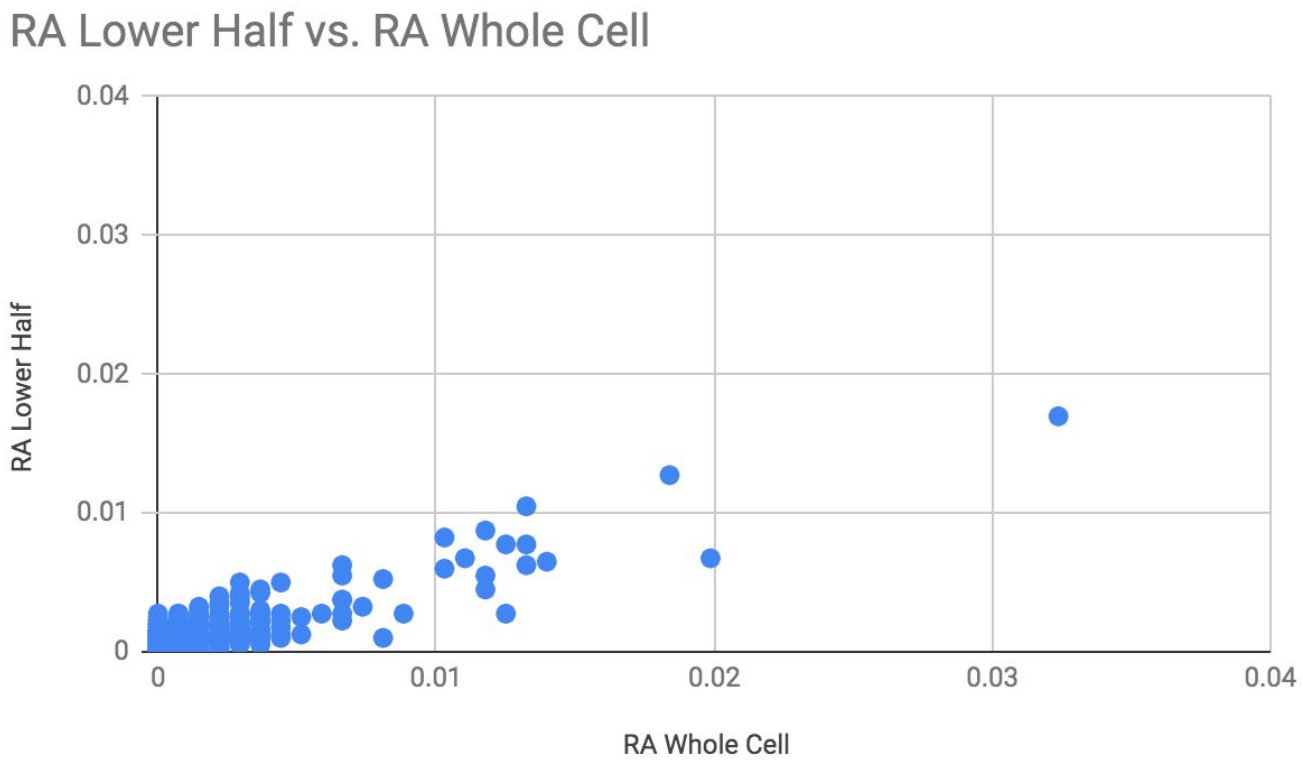


Figure 4.4l: Scatter plot of proteins found in bottom halves and whole cells.

After identifying the proteins in the membranellar band, we compared the list of proteins to genes that were differentially expressed during *Stentor* regeneration. This yielded a subset of 51 proteins (Figure 5). These genes were previously organized into 5 clusters, thus we cataloged the identified proteins into the corresponding clusters (Table 8-12). We were surprised to see that the majority of highly abundant proteins in the membranellar band were mostly expressed towards the end of regeneration, which suggests that there is a protein repository that *Stentor* uses to immediately regenerate and then subsequently restores said repository.

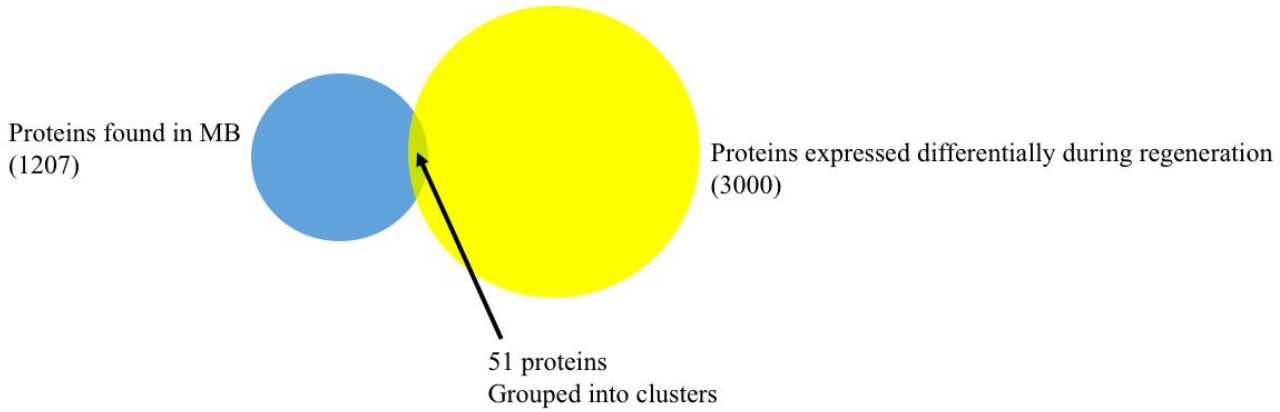


Figure 4.5: 51 proteins were found both in the membranellar band and the top 3000 differentially expressed during regeneration.

Table 4.3: The 51 proteins found in figure 5 were categorized into their corresponding clusters. This is cluster one.

Gene Model Identifier	# hits from MS	Description
SteCoe_7647	7	Glutamate/Leucine/Phenylalanine/Valine dehydrogenase
SteCoe_12639	5	Phosphoglycerate kinase
SteCoe_3054	5	CDPK
SteCoe_853	3	Phosphoenolpyruvate carboxykinase
SteCoe_3075	3	ATP synthase alpha/beta family; nucleotide-binding domain
SteCoe_21359	3	E1-E2 ATPase
SteCoe_5774	3	Nuclear movement
SteCoe_9879	2	Inorganic H+ pyrophosphatase
SteCoe_1989	2	Histidine phosphatase
SteCoe_25044	2	Aldo/keto reductase
SteCoe_419	2	PNTB domain protein
SteCoe_11507	2	Aldo/keto reductase

Table 4.4: The 51 proteins found in figure 5 were categorized into their corresponding clusters. This is cluster two.

Gene Model Identifier	# hits from MS	Description
SteCoe_26963	11	Repeat of unknown function

Table 4.5: The 51 proteins found in figure 5 were categorized into their corresponding clusters. This is cluster three.

Gene Model Identifier	# hits from MS	Description
SteCoe_23544	12	Radial_spoke domain protein
SteCoe_32978	8	Region in Clathrin and VPS
SteCoe_6780	8	Putative intraflagellar transport

Table 4.6: The 51 proteins found in figure 5 were categorized into their corresponding clusters. This is cluster four. Hits lower than 10 are shown in the supplementary.

Proteins found in MB and differentially expressed in regeneration		
Gene Model Identifier	# hits from MS	Description
SteCoe_11872	24	EF-hand_7 domain protein
SteCoe_18966	16	Dynein heavy chain; region D6 of dynein motor
SteCoe_23868	14	EF-hand_7 domain protein
SteCoe_559	13	Armadillo/beta-catenin-like repeat
SteCoe_33155	12	Adenylate kinase
SteCoe_28006	11	WD domain; G-beta repeat
SteCoe_14094	10	WD domain; G-beta repeat

Table 4.7: The 51 proteins found in figure 5 were categorized into their corresponding clusters. This is cluster five. Hits lower than 10 are shown in the supplementary.

Proteins found in MB and differentially expressed in regeneration		
Gene Model Identifier	# hits from MS	Description
SteCoe_1580	41	HELP
SteCoe_21613	40	EF-hand_7
SteCoe_214	33	HELP
SteCoe_9200	24	CDPK-like predicted pseudokinase with ADK
SteCoe_1748	21	EF-hand_7
SteCoe_5473	17	EF-hand pair
SteCoe_29163	16	hypothetical
SteCoe_12657	15	WD40
SteCoe_23966	14	EF-hand_7
SteCoe_19756	14	hypothetical
SteCoe_21611	13	DUF1126
SteCoe_2776	13	ParcG
SteCoe_16409	13	HELP
SteCoe_702	13	GAS2
SteCoe_12857	13	EF-hand_7
SteCoe_36556	12	EF-hand_8
SteCoe_499	12	WD40
SteCoe_1167	10	Radial_spoke
SteCoe_1022	10	WD40
SteCoe_13619	10	hypothetical

This proteomic analysis used a solubilization method that dissolved all proteins in the cell and did not leave a visible pellet. In Chapter 5, we will discuss analysis of a soluble fraction released by gentle cell lysis methods.

	Solute transporter (26316) Bacterial globin (23757) Proteasome (10971) Peptidase C1 (27940) EF hand 7 (18895)	Solute transporter (26316) EF hand 6 (23757) Bacterial globin (23757) DEAD (12603) TCTP (3808)
Hypothetical (14740) Hypothetical (32782) Vault (11087) DUF2475 (10313) Superoxide dismutase (3192)		Ribosome 60S (30079) Hypothetical (29764) Elongation factor (29966) Cysteine protease (1171) Hypothetical (35443)
V-ATPase G (31243) GAS2 (12001) Clatherin (17091) SNARE (15528) RNA recognition (19984)	V-ATPase G (31243) MORN (18028) Hypothetical (32910) Hypothetical (16036) Peptidase C1 (27940)	

Figure 4.6: Top 5 enriched soluble proteins for each sample compared to all other samples. Table is read horizontal/vertical. Example: Solute transporter is the most enriched protein in the body compared to whole cells.

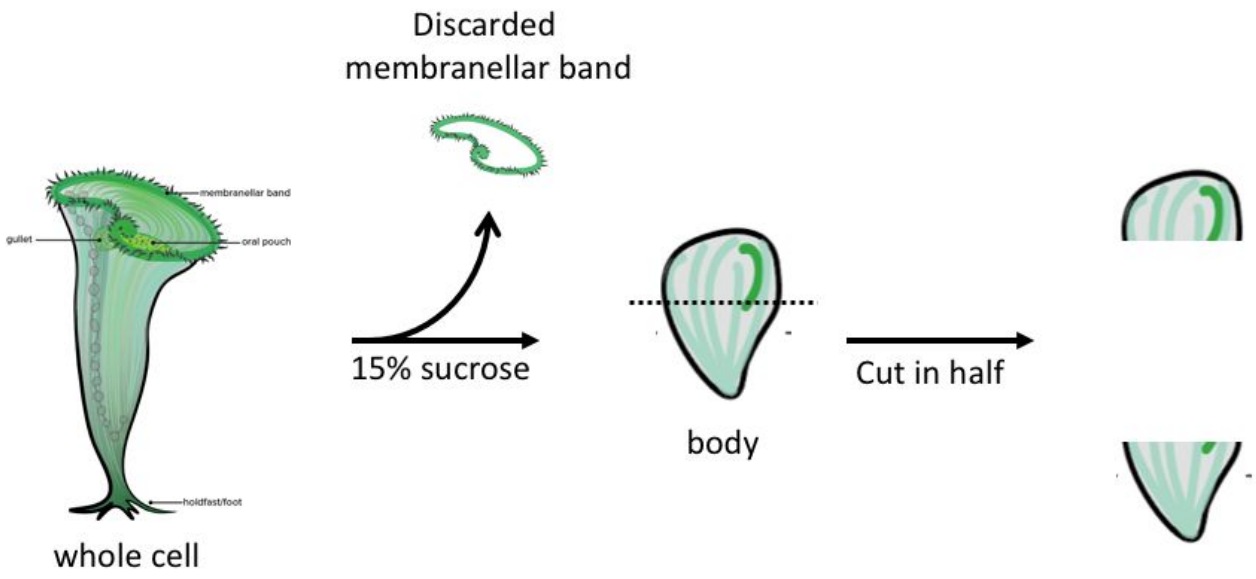


Figure 4.7: Experimental process of collecting top and bottom halves that have their membranellar band removed.

Tubulin and dynein heavy chain dominated the mass spectrometry. 5% of all hits were tubulin. Therefore, in order to identify the proteins that are in the upper half of *Stentor*, we dissected Stentor in half after sucrose shock (Figure 7) and prepared the samples for deeper reads (Figure 8). The number of proteins were equally distributed between all twelve fractions of High pH reversed phase peptide fractionation (Figure 9). Then to get a higher number of overall identifications, the samples were processed through 2D liquid chromatography (Figure 10). Hits were then compared using a student's t-test and clustered into upper and lower (Figure 11). Finally, the proteins were further categorized into functional groups (Figure 12).

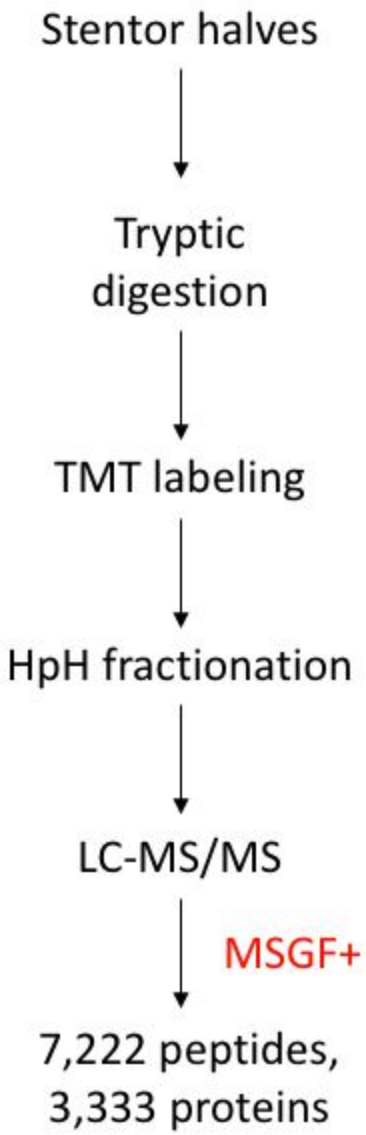


Figure 4.8: Mass spectrometry flow chart for processing samples from Figure 7.

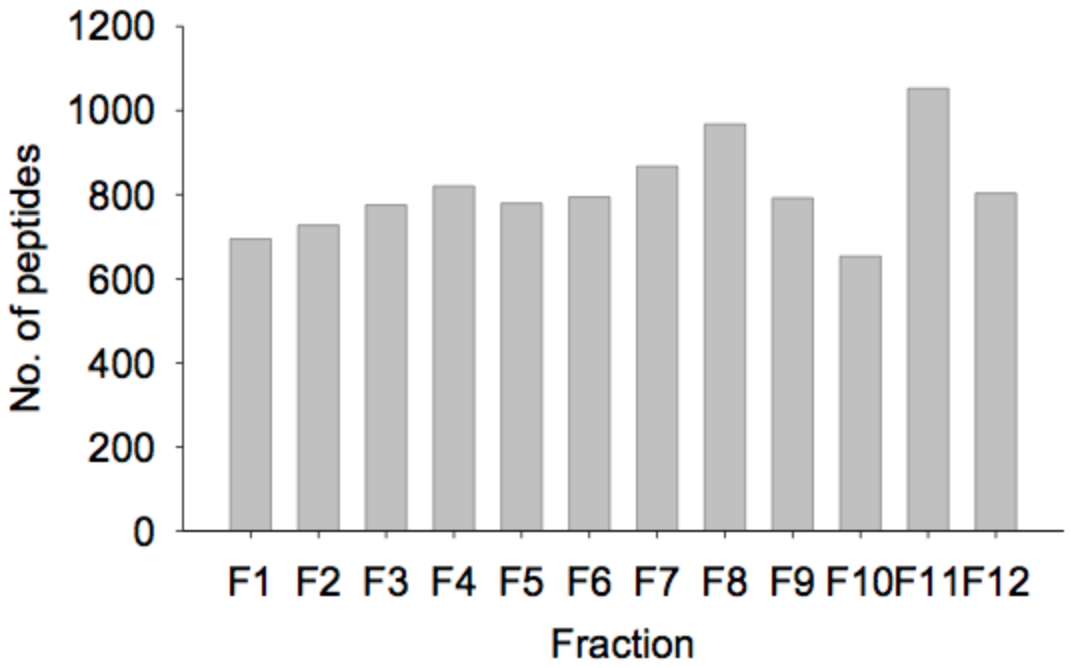


Figure 4.9: Estimated number of peptides from HpH fractionation.

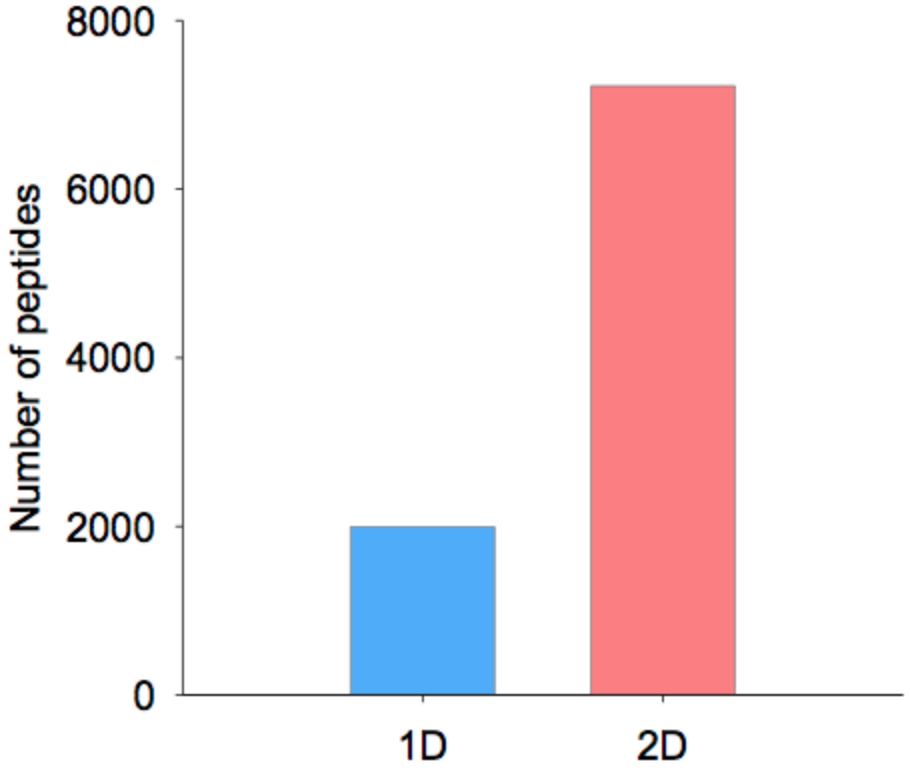


Figure 4.10: Estimated of number of peptides identified after 1D-LC and 2D-LC.

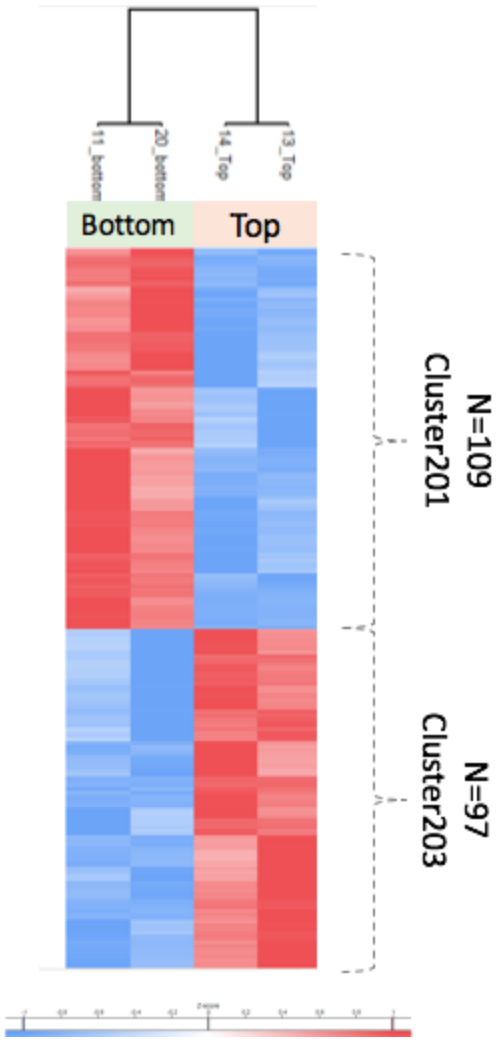


Figure 4.11: Proteins resulting from mass spectrometry were analyzed and clustered using Student's t-test.

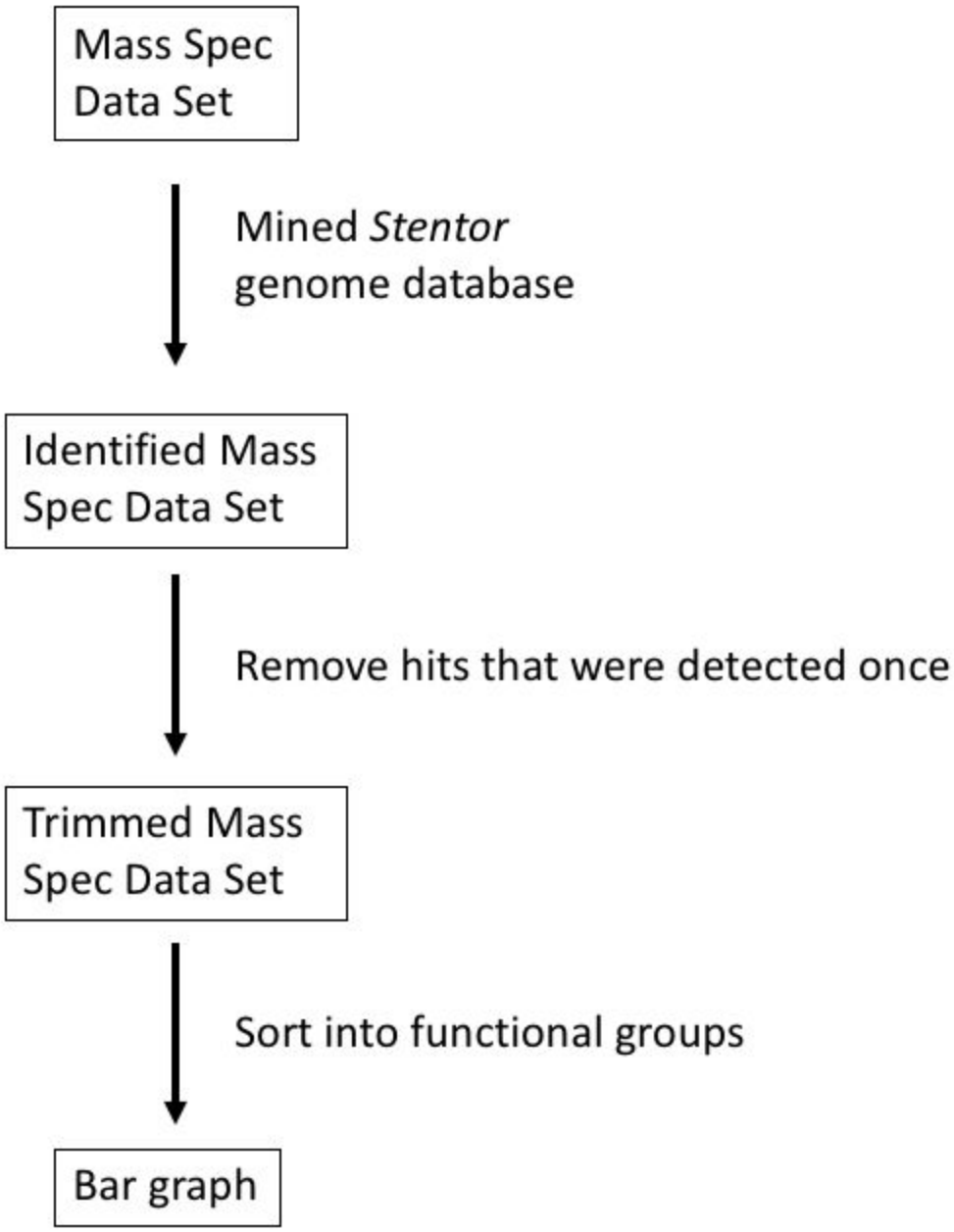


Figure 4.12: Analysis flow chart of proteins found in Figure 11.

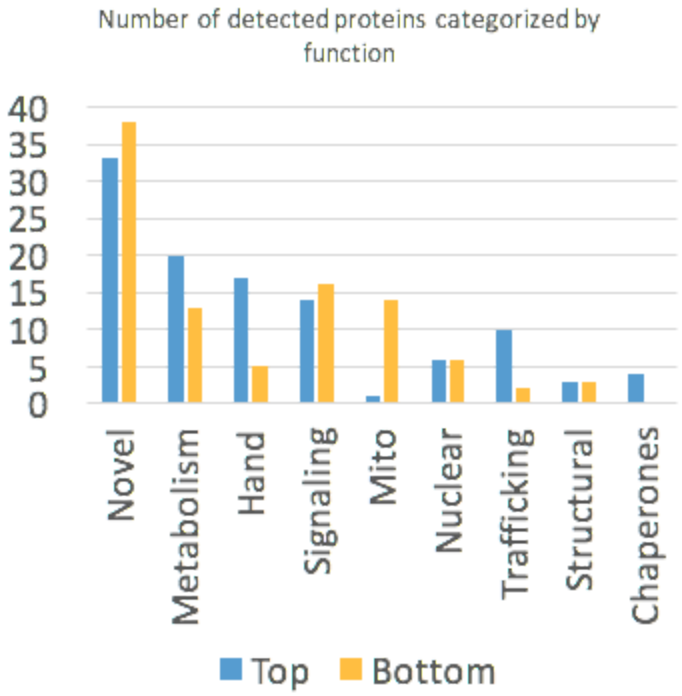


Figure 4.13: Enriched proteins, that were found in Figure 11, classified into functional groups.

Discussion

Using proteomics to classify regeneration-related genes

The analysis presented here is, to our knowledge, the first reported documentation of the Stentor proteome. The ability to determine proteomic content of different cellular regions highlights the unique features of Stentor as a model system, in that its large size enabled me to reproducibly cut similar fragments from cells. Knowing the protein content of different cellular regions now provides a key piece of information for future dissection of the regeneration process, for the following reason. We have already begun analyzing regeneration in terms of transcriptional programs. However, there are at least two possible reasons why a given gene might be up-regulated during regeneration. First,the gene might play a role in some process that facilitates regeneration, such as biosynthesis, trafficking, or signaling. Second, the gene might encode one of the actual precursor proteins that incorporates into the regenerated structure. These two types of upregulated genes play very different roles in regeneration, and so it is critical to classify upregulated genes

into these two groups. By determining the proteome of the membranellar band, we can now identify at least some of the upregulated genes as encoding protein components of the structure being regenerated.

Re-use versus new synthesis of regenerated structures

One surprising result of this analysis is that many of the genes encoding the MB proteome show a peak of expression hours after the MB has formed. One hypothesis to explain this result is that regeneration may primarily involve re-use of existing proteins or structures, which are then replaced later on by new protein synthesis. This might make sense if regeneration has to happen so quickly that there isn't enough time to synthesize all the proteins that are needed. Since the MB is composed largely of an array of basla bodies with associated cilia, one possibility is that the MB is formed using pre-existing basal bodies. An example of such re-use already is known in the division of a different type of ciliates, the oxytrichids. These ciliates move using cirri, which are clusters of ciliary axonemes within a single ciliary membrane. When the cell divides, existing cirri dissociate, with their component basal bodies rearranging themselves into a row. Then, each of these pre-existing basal bodies templates the formation of one or more new basal bodies, and then the old and new basal bodies re-group themselves into new cirri (Jerka-Dziadosz 1980). Thus, creation of new cirri involves a combination of re-utilization of existing basal bodies and formation of new ones. The big difference is that in Stentor, the MB does not form near the old one, and clearly does not incorporate any basal bodies from the old MB. This is even more clearly true during regeneration, in which the MB has been entirely removed. It remains possible, however, that MB formation may use existing basal bodies already present in the cortical ciliary rows.

Regional differences in cytoskeletal proteins

It is already known that the membranellar band contains long, densely packed, motile cilia. For this reason, we expected that the MB proteome would be enriched for tubulin and axonemal proteins even though the entire surface of the cell is covered with cilia. This was indeed the case as we found that the MB was enriched for tubulin, axonemal dynein heavy chains, and radial spoke proteins. It has also been known from

prior immunofuorescence studies (Maloney) that the posterior of the cell contains extensive contractile fibers composed of centrin-related EF hand proteins. Consistent with this expectation, we found many EF hand proteins enriched in the poster of the cell body. One unexpected protein family enriched in the posterior was GAS2, a protein family involved in linking microtubules to actin filaments in other organisms. The role of actin in ciliates is generally not well understood, and typical approaches for detecting F-actin do not work in ciliates, raising the question of whether actin does in fact assemble long filaments in these organisms. It is thus not clear what GAS2 would be doing in Stentor, but in any case it represents a clear regional difference within the cortex of the cell.

References

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Chapter 5 - On Vaults in *Stentor*

Abstract

It has been proposed that the membranellar band might contain a soluble factor that acts as a signal to block regeneration under resting conditions. Based on this idea, I extended the proteomic analysis to specifically examine the soluble protein fraction from the membranellar band. This analysis showed that the soluble fraction was highly enriched in proteins with homology to the Major Vault Protein (MVP). MVP is known in other systems to self-assemble into large protein-RNA complexes known as vaults. Despite their conservation through many species, Vault's function as ribonucleic protein remains unknown. Many theories including signaling, immunity, and drug resistance have been questioned. Here we explored their role in *Stentor*. Knockdown of vaults had no visible effect on regeneration in *Stentor*. In some experiments, knockdown of vault proteins increased sensitivity to holospora, a ciliate parasite, and led to spontaneous inking of the water with Stentorin pigment, possibly arguing against roles in cellular defense pathways. However these results were inconsistent between experiments, possibly due to variable efficacy of the RNAi response with these constructs.

Introduction

A major question concerning regeneration in Stentor (or any other organism for that matter) is what signal or cue triggers the regeneration process? How is the loss of a structure detected in order to know when to start making a new one? Hyvert et al (1972) reported that isolated membranellar bands, when surgically implanted into intact cells, will prevent the cell from regenerating when its own membranellar band is removed. This result suggested a "beacon" model, in which a pre-existing membranellar bands emits a signal that would block regeneration.

In this model, removal of the membranellar band triggers regeneration because the blocking signal is lost. The simplest biochemical version of such a model would be that the membranellar band contains one or more signaling proteins that generate a soluble signal that travels to the rest of the cell and blocks regeneration.

Results

To identify a candidate a signal, I performed a proteomic analysis specifically of soluble proteins contained in the membranellar band. In Chapter 4, we focussed on proteins identified after dissolving the entire sample using harsh conditions capable of releasing even highly insoluble proteins. Here, instead, samples were prepared under conditions of gentle lysis that leave insoluble proteins in the pellet. These insoluble pellets were removed and only the soluble fraction was analyzed. In collaboration with Pacific Northwest National Labs, we sent three parts of *Stentor* for mass spectrometry (as described in Chapter 4), in this case, whole cells, shed membranellar bands, and cell bodies collected after shedding their membranellar bands. The reason to analyze both membranellar bands and MB-less cell bodies was that for a regeneration blocking signal to be useful, it would have to be relatively unique to the MB and not highly abundant in the rest of the cell, otherwise it would not be informative about the presence or absence of the MB. By looking for proteins that are enriched in the MB and also depleted from cell bodies lacking the MB, I could double check the specificity of the protein for the MB itself.

Hits from mass spectrometry were analyzed for enrichment and depletion. One interesting class of protein was Major Vault Protein (MVP). MVP orthologs were found to be among the top 10 proteins enriched in the soluble fraction from isolated membranellar bands compared to whole

cells, and were also among the top 10 proteins that were depleted from the soluble fraction of cell bodies after membranellar band removal in (Table 1 and 2).

Vaults are ribonucleoprotein complexes that are found in many eukaryotic model organisms like humans, mice and c.elegans however they are lost in D. melanogaster[14]. Their conservation suggests that they play an important role. However, their function has not been discovered. Immunity, drug resistance, signaling and nucleo-cytoplasmic transport have been hypothesized. Vaults were discovered as contaminants of vesicle preparations[15]. There are three parts that make up the Vault complex. MVP (Major Vault Protein) is a highly conserved protein[16] whose repeats form a large 670 angstrom barrel[17]. Inside the vault are two accessory proteins, vault poly(ADP)ribo polymerase[18] (vPARP) and telomere associated protein[19] (TEP), whose functions remain unclear.

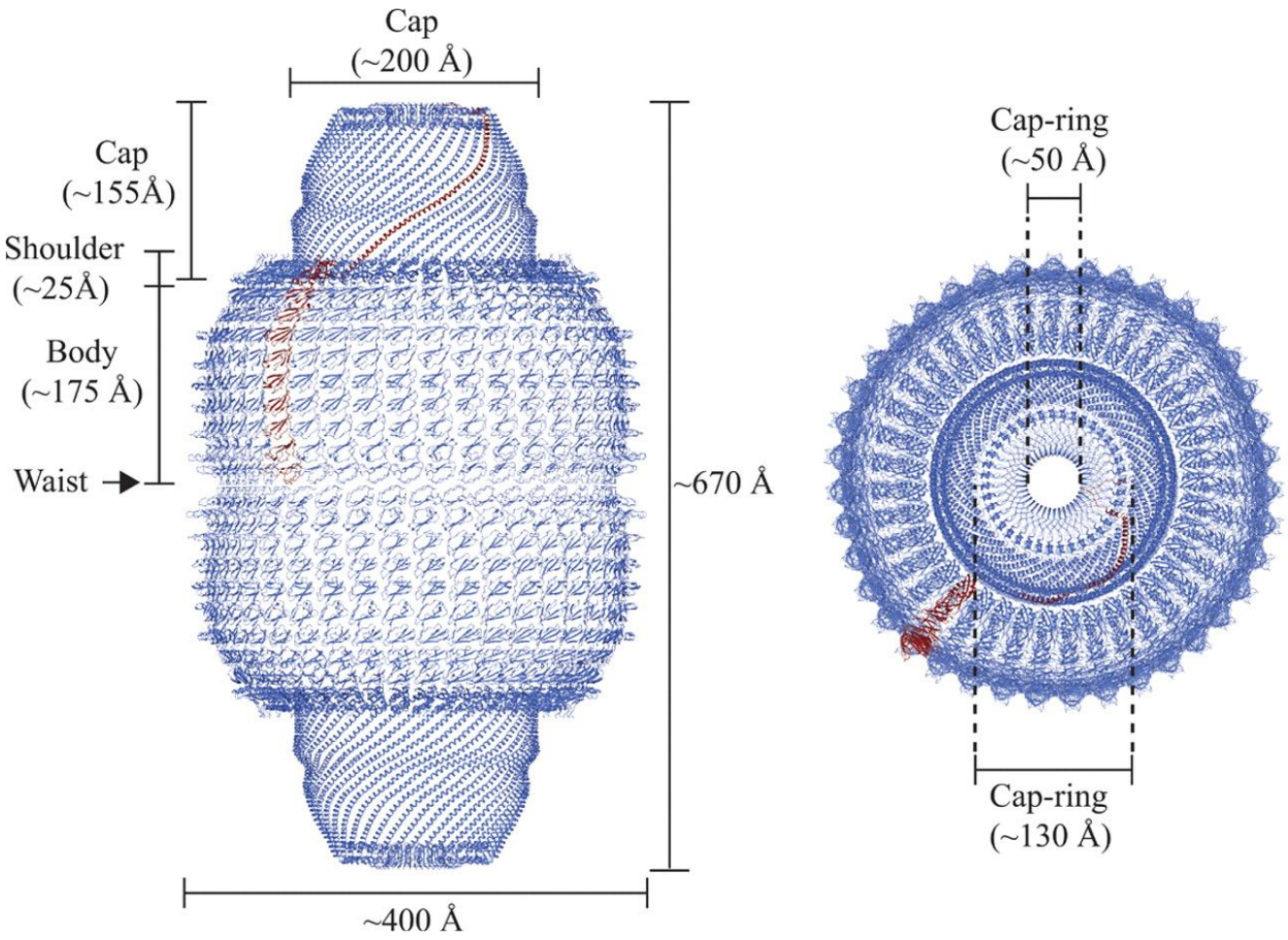

Figure 5.1: The whole vault structure is made from 78 oligo-mers of Major Vault Protein (MVP). One oligo-mer is in red. The overall size is 670 angstroms. TANAKA 2009

The highly specific enrichment of MVP proteins in the MB suggests that vaults might be, or carry, a signal that regulates regeneration. Seven predicted MVP orthologs were found in the Stentor genome,, and all seven were cloned to be tested via RNAi using the protocol described in Appendix 1.

Table 5.1 and 5.2: Top 10 enriched and depleted proteins in soluble fraction showed Vaults as top candidates for RNAi.

Top 10 enriched	
SteCoe_11087	Vault
SteCoe_32782	hypothetical
SteCoe_19427	Fibrillarin
SteCoe_36319	hypothetical
SteCoe_7621	Vault
SteCoe_16600	Cpn60_TCP1
SteCoe_13619	hypothetical
SteCoe_28588	Cation_ATPase_N
SteCoe_2997	hypothetical
SteCoe_16973	Proteasome

Top 10 depleted	
SteCoe_14740	hypothetical
SteCoe_11087	Vault
SteCoe_32782	hypothetical
SteCoe_3192	Sod_Cu
SteCoe_10313	DUF2475
SteCoe_13619	hypothetical
SteCoe_19984	RRM_1
SteCoe_15528	SNARE
SteCoe_2881	MBF1
SteCoe_7621	Vault

The function of Vaults in *Stentor* has never been previously examined. One hypothesis was that the vaults might encode a signal to prevent regeneration when the MB is present. An alternative hypothesis, based on the proposed role in host defense and immunity in other systems, was that vaults might be involved in protecting the cell against pathogens. RNAi of vaults was used to test these hypotheses. Knockdown of vaults does not affect regeneration or its timing (Figure two). The first round of RNAi suggested that vaults play a role in retaining pigment because the media would turn slightly blue (Figure 3, 4, and 5). However, this phentype was not seen in subsequent replica experiments. In this first round, pigment granules were seen during the middle of regeneration (Figure 6). We hypothesized that vaults are involved in the distribution of pigment however, these results were not seen in follow up experiments..

The other hypothesized role for the vaults was in host defense. In this case, the enrichment of vaults in the membranellar band might be explained by the fact that Stentor are predatory cells that

feed on bacteria and protists, such that the membranellar band and associated oral apparatus is the part of the cell where foreign organisms are first introduced into the interior of the cell. It might thus be logical to have host defense factors enriched in this structure. In order to explore the possibility that Stentor vaults were functioning in host defense, I tested the role of MVP in defense against, *holospora*, a parasite that infects ciliates. To carry out these experiments, I travelled to SUNY New Paltz where Professor Lydia Bright studies holospora infection in *Paramecium*. Early data showed that one vault knockdown was sensitive to the parasite however, repeated experiments did not show the same sensitivity (Figure 7-24).

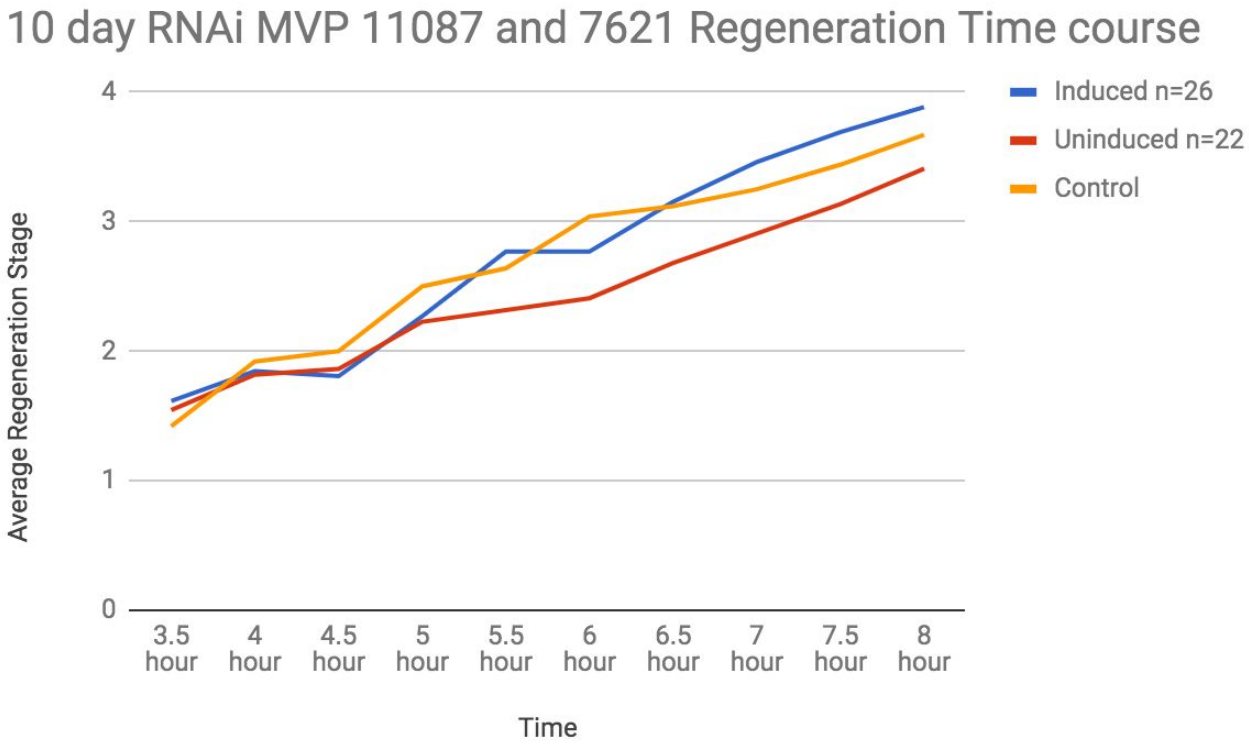

Figure 5.2: Using the cloning protocol written above, regeneration time course of g11087 (Vault) and g7621 (Vault) was performed however, nothing significant was observed. Regeneration timing was unaffected.

While knocking down genes using RNAi, we grow *Stentor* in a large concentration in a few milliliters of spring water and results in blue-pigmented media. Knockdown by RNAi in *Stentor* requires a few days to see robust expression. While growing RNAi against g7621, on day seven, a slightly blue pigment in the

water was visible by eye. Taking pictures of this media was difficult since the media had only a hint of blue. This minor coloration was confirmed by three other individuals. On day eleven, we were able to see a spike in 630nm absorption on the nanodrop (Figure three). However, repeats of this experiment resulted in LF4 control population also showing pigmented media on day 11 of RNAi (Figure four and five). Thus blue pigmented media may be only be a signifier of population.

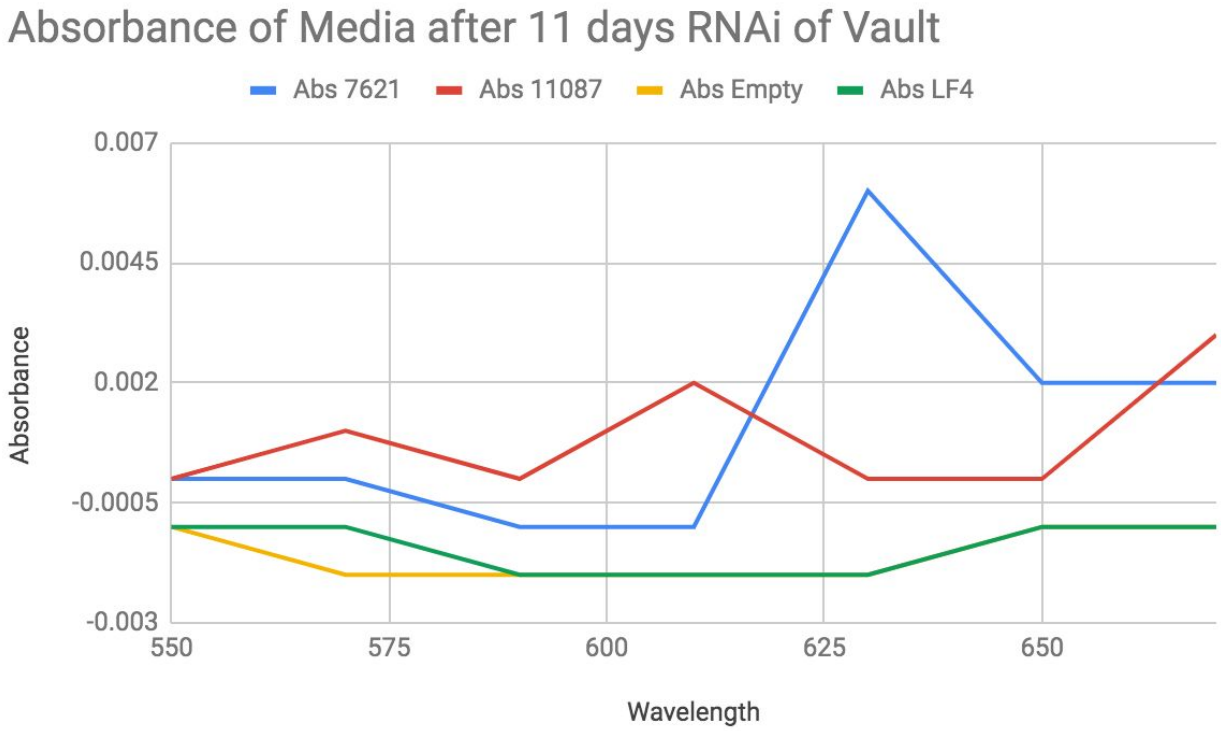

Figure 5.3: RNAi of g7621 for 11 days resulted in the deposition of blue pigment into the media. RNAi of controls and g11087 did not result in pigmented media. This was measured by nanodrop.

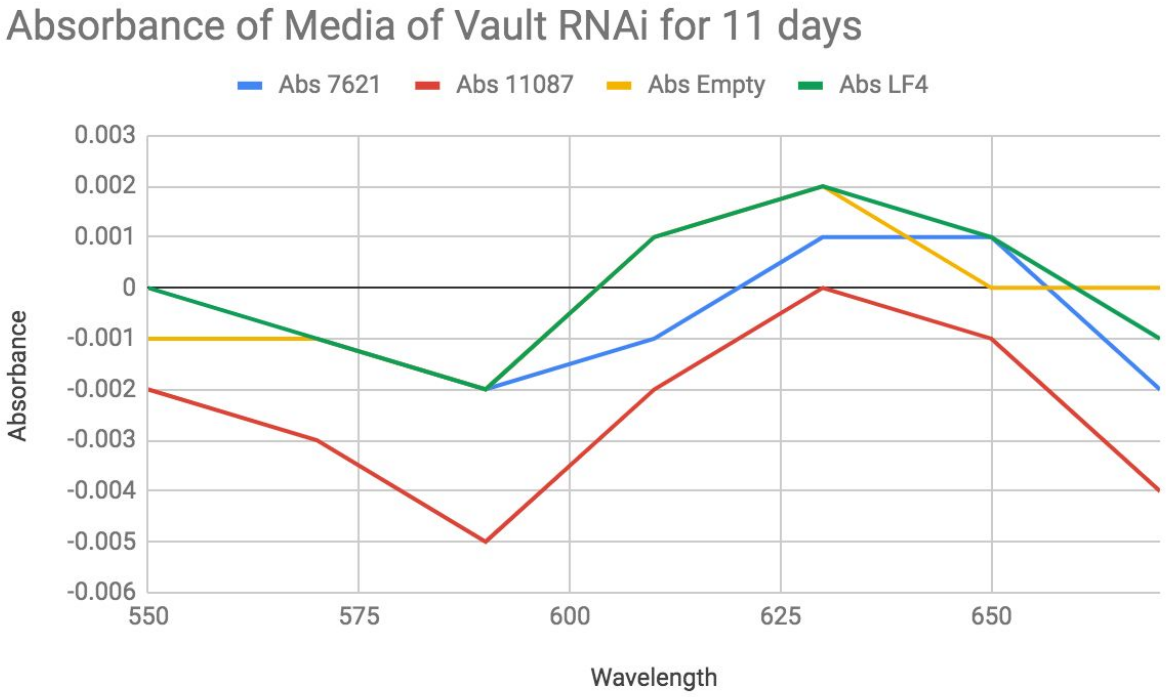

Figure 5.4: Repeated RNAi of Vaults for 11 days did not result in the same deposition of blue pigment into the media. This was measured by nanodrop.

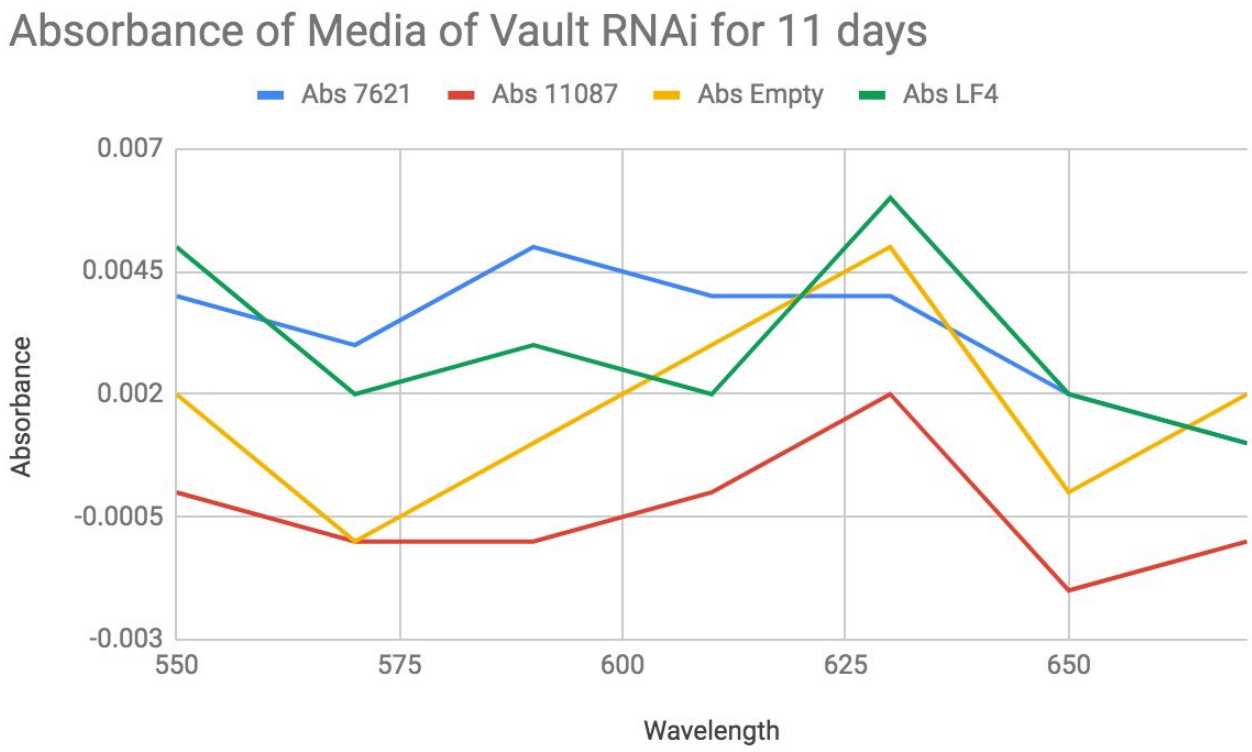

Figure 5.5: Repeated RNAi of Vaults for 11 days did not result in the same deposition of blue pigment into the media. This was measured by nanodrop.

After 14 days of RNAi against vaults, we tested the RNAi Stentor's ability to regenerate (Figure two). Even though they regenerated within 8 hours, there were large, dark-blue, granules inside of regenerating *Stentor* (Figure 6). These granules will be expelled sometime shortly after completing regeneration. This was not seen in repeat experiments. One lab member claims that these granules are seen often in regeneration.

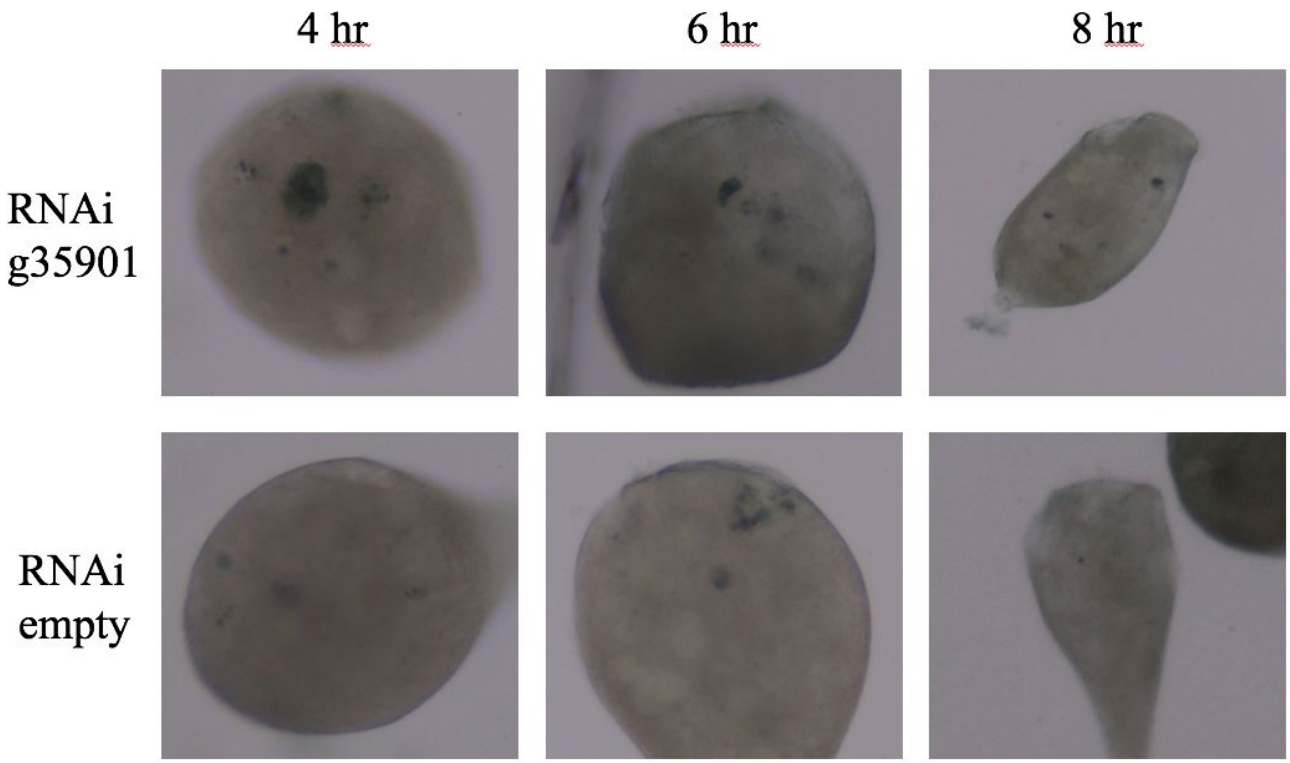

Figure 5.6: The first trial run of RNAi of g35901 (Vault) for 14 days resulted in pigment granules inside of regenerating *Stentor*.

Vaults have been implicated in immune response[14]. To test this in *Stentor*, we found a paramecium parasite *holospora*, who invade host nuclei and lyse the host cells[20]. Therefore, provided a clear, binary readout of alive or dead. In collaboration with the Bright lab at SUNY New Paltz, we tested knockdowns of four vaults with one negative control (Figure 7). Three vaults: 28400, 6764, and 32983, Mob1/positive control, and empty vector/negative control did not survive the flight to New York. Two different infection methods were tried. In the Bright lab, *holospora*-infected *Paramecium* were lysed to continue the lineage. This method was modified to use *Stentor's* favored media. Because *Stentor* are avid filter-feeders, we also attempted to

feed *holospora*-infected Paramecium as a whole. Vault knockdown g36083 initially showed increased sensitivity to *holospora*, having a higher death rate compared to the control (Figure 7, 9). The first round of experiments resulted in some bleaching of cells or loss of pigment (Figure 8, 10). Subsequent experiments were performed by the Bright lab and the initial results were not seen again (Figures 11-24).

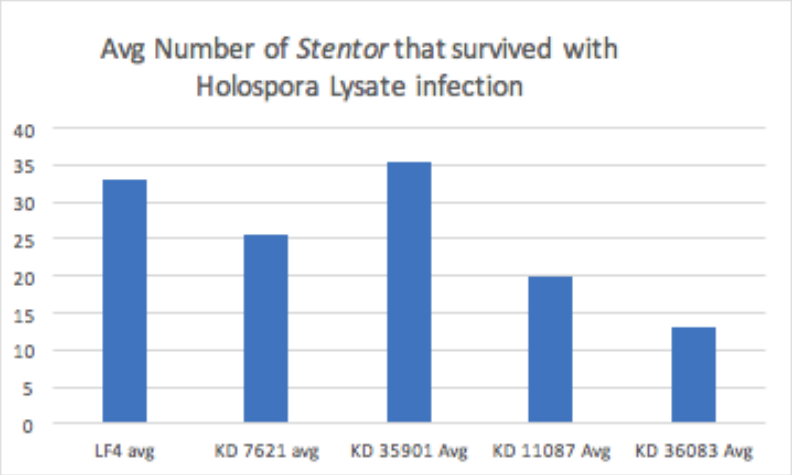

Figure 5.7: Three replicates of 40 cells were incubated with *holospora* lysate, 14 day RNAi of g36083 (Vaults), showed the most significant death rate, with one sample completely dying.

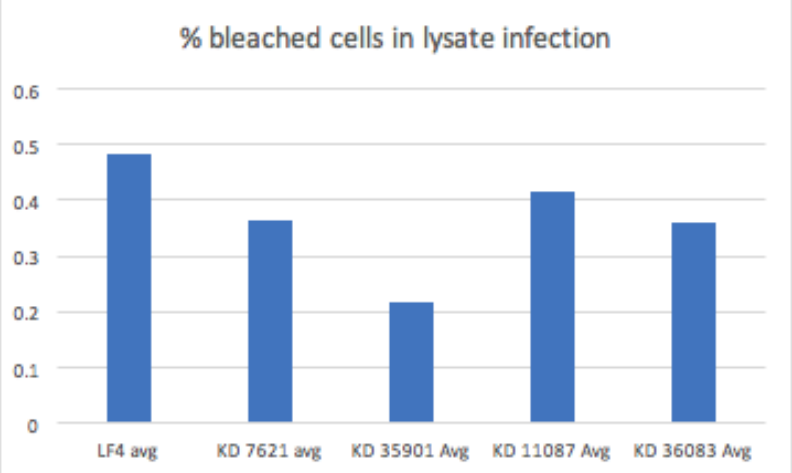

Figure 5.8: Observations of lysate infection included a bleaching effect where *Stentor* lost pigment. These were accounted for in the above graph.

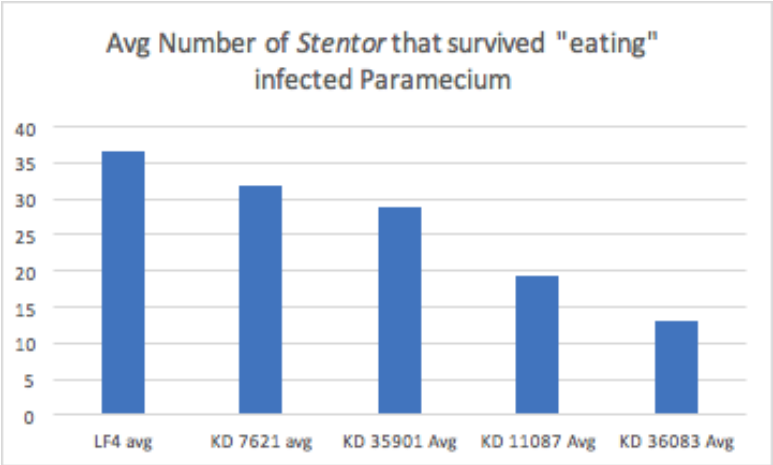

Figure 5.9: 14 day RNAi *Stentor* were starved for 12 hours and subsequently fed *holospora* infected Paramecium. Again, g36083 showed sensitivity to the infection compared to LF4 control.

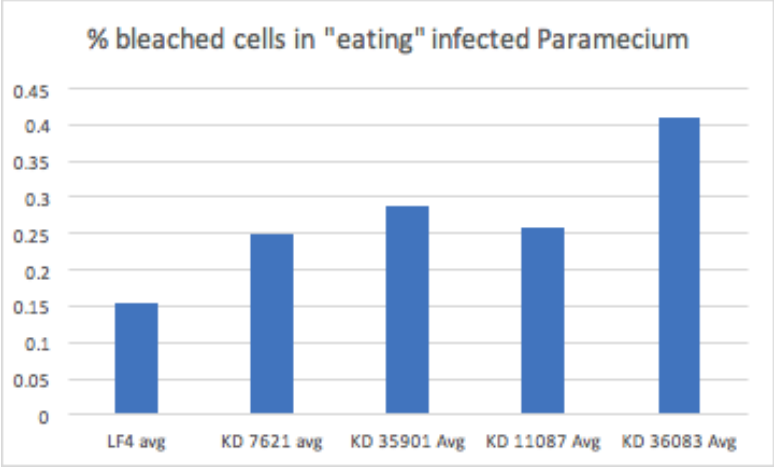

Figure 5.10: Bleaching was more prevalent in control *Stentor*. The remaining *Stentor* that lived through the holospora infection maintained their blue pigment.

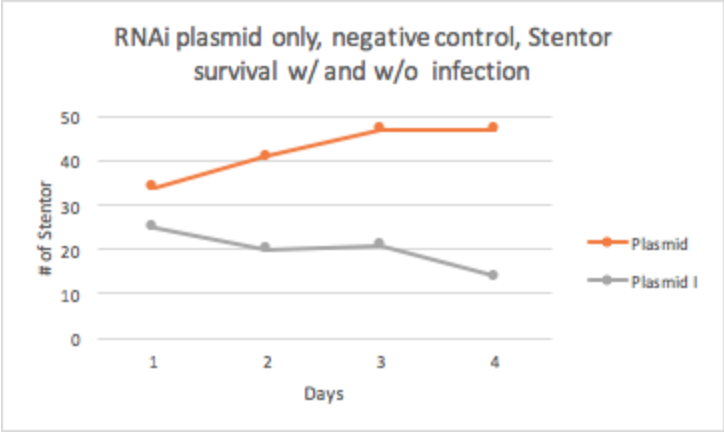

Figure 5.11: RNAi of *Stentor* with plasmid only (no gene control) survival rate with infection decreased over time.

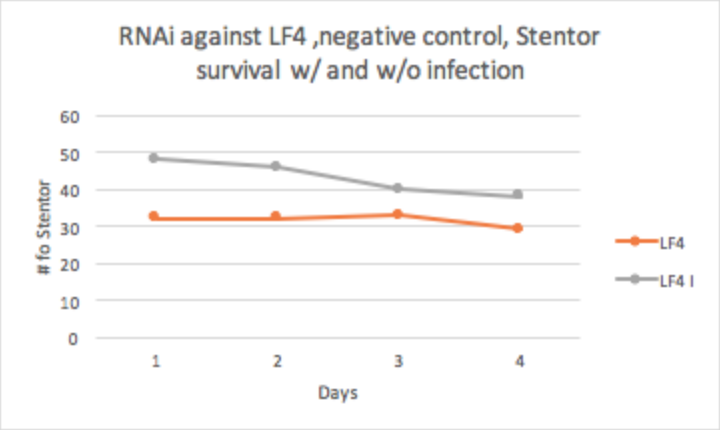

Figure 5.12: 14 days of RNAi of *Stentor* against LF4 (control) had no significant difference between survival rates with and without infection.

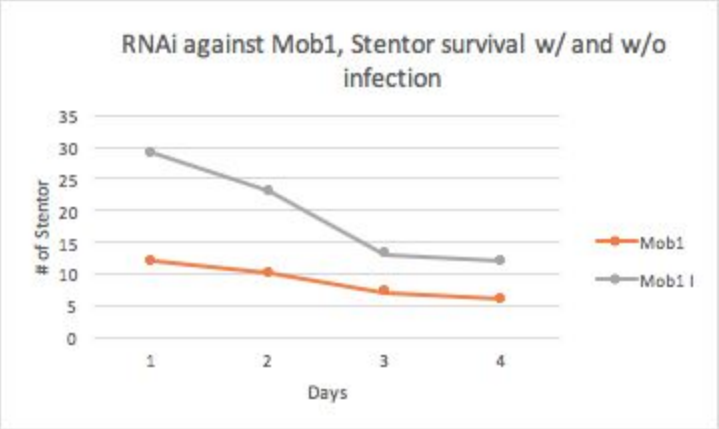

Figure 5.13: RNAi against Mob1, showed decreased survival rate with and without infection.

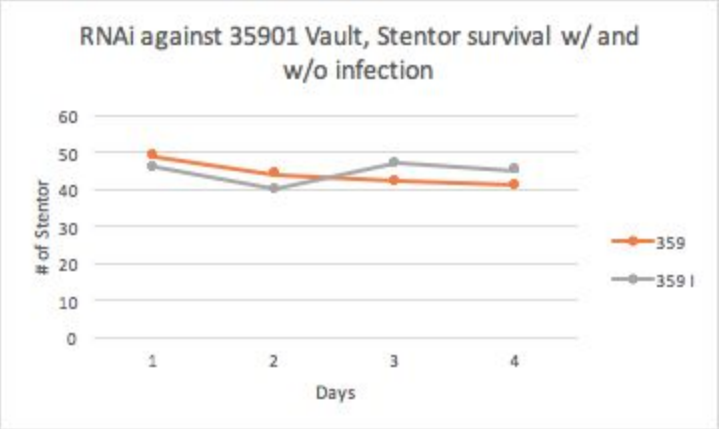

Figure 5.14: RNAi against g35901 (Vault) showed no significant difference between survival rates with and without infection.

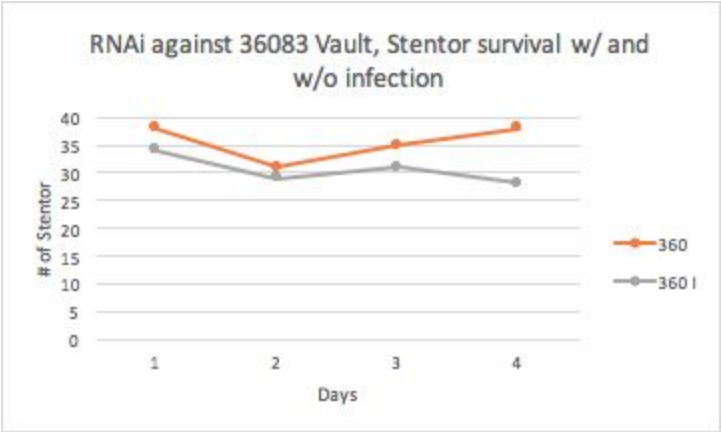

Figure 5.15: RNAi against g36083 (Vault) showed no significant difference between survival rates with and without infection.

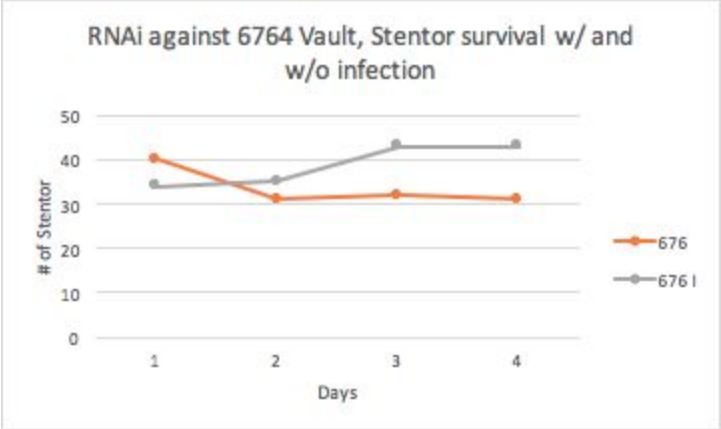

Figure 5.16: RNAi against g6764 (Vault) showed no significant difference between survival rates with and without infection.

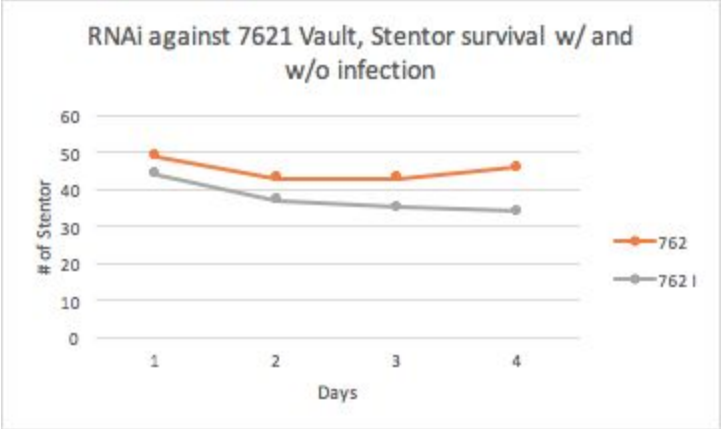

Figure 5.17: RNAi against g7621 (Vault) showed no significant difference between survival rates with and without infection.

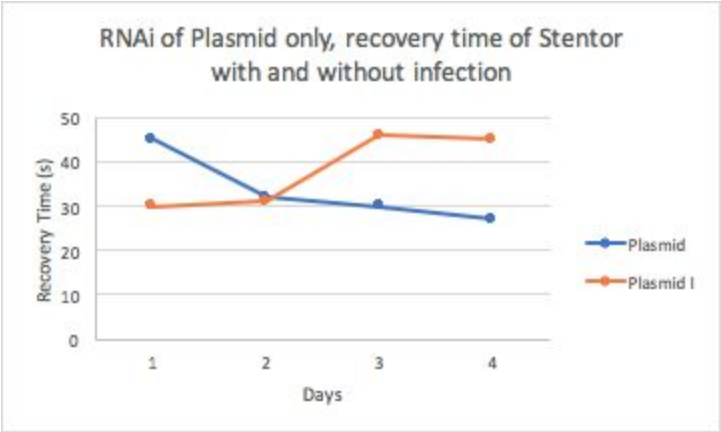

Figure 5.18: RNAi against pPR-T4p (empty vector) showed better recovery time with infection compared to without infection.

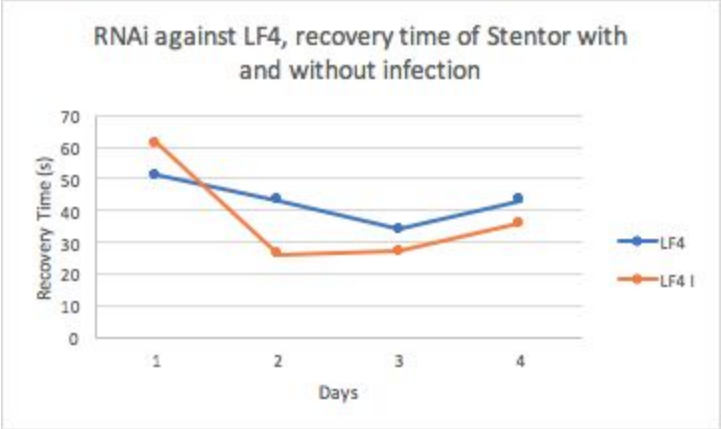

Figure 5.19: RNAi against LF4 (control) showed no significant difference between recovery time with and without infection.

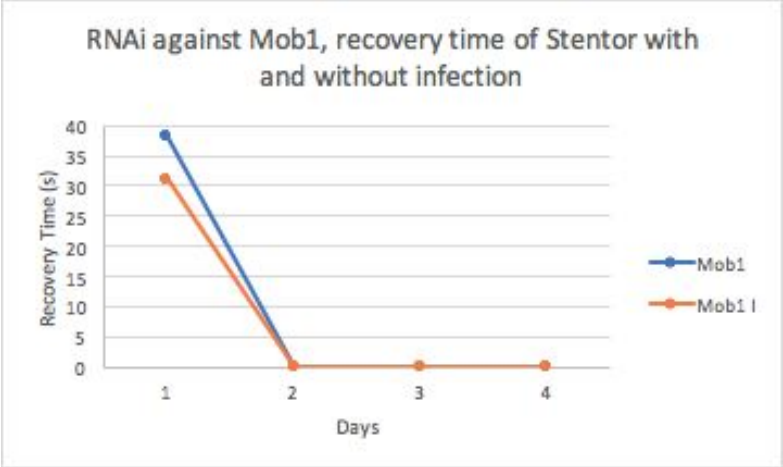

Figure 5.20: RNAi against Mob1 (positive control) showed no significant difference between recovery time with and without infection. They all died.

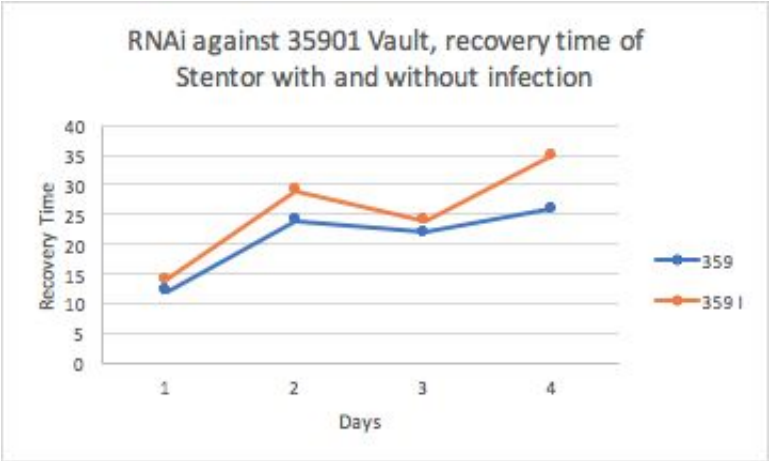

Figure 5.21: 14 days of RNAi against g35901 (Vault) showed no significant difference between recovery times with and without infection.

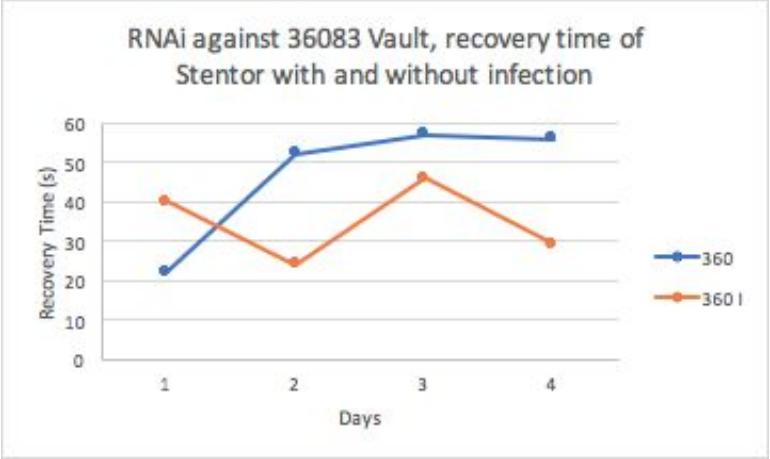

Figure 5.22: 14 days of RNAi against g36083 (Vault) showed an initial decrease in recovery compared to controls however recovered their shape after 72 hours. Subsequently, lost their shape again in 96 hours.

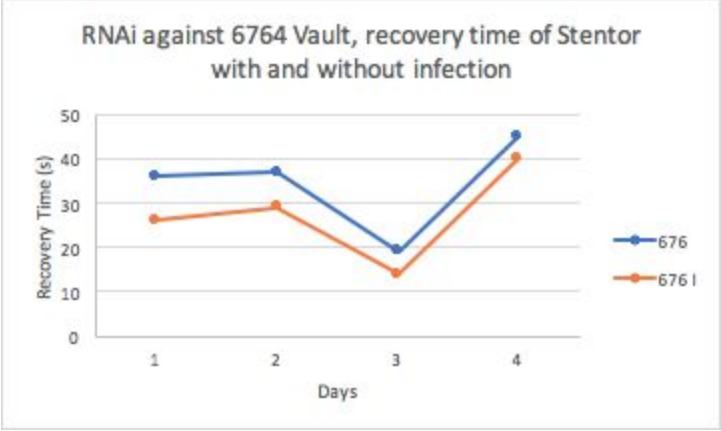

Figure 5.23: RNAi against g6467 (Vault) showed no significant difference between recovery times with and without infection.

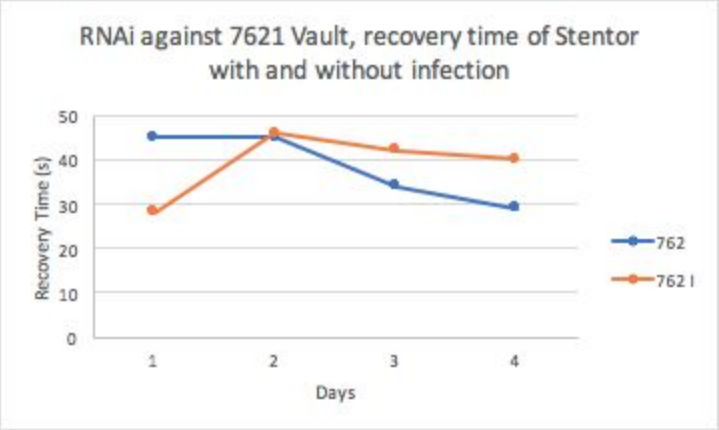

Figure 5.24: RNAi against g7621 (Vault) showed no significant difference between recovery times with and without infection.

Discussion

The function of vaults in *Stentor* remains unknown. RNAi phenotypes were highly variable between experiments, suggesting that variation in cell growth conditions may affect the experimental outcome. Without understanding the nature of this variation, we cannot yet rule out the idea that vault proteins may play a role in either host defense or pigment release. However, in no case did RNAi of any vault protein give any indication of an effect on regeneration. I therefore suspect that vaults are not likely to be a key signal controlling regulation. Further experiments will be needed to analyze the possible functions of other proteins identified in the soluble fraction.

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Chapter 6 - Dissertation Discussion

Several aspects of my work provide new perspectives on ideas reported in pre-existing literature, and raise new questions. With respect to Chapter 3, it has been long noted that the steps of membranellar band regeneration in Stentor are highly similar to those observed during membranaller band formation during normal cell division (Paulin 1971; 1975; Targar 1961). The fact that aurora kinase inhibitors, which target a conserved regulator of the cell division cycle, also affect the timing of specific steps in regeneration (Chapter 3), suggests that the similarity between division and regeneration extends beyond visual similarity to the molecular level. The question now is how far does this conservation extend. It might be the case that cell cycle proteins play a role in regulating timing of the steps of regeneration, but that the steps use mechanisms unrelated to division. On the other hand, given that some of the later stages of regeneration involve the regulated growth and shrinkage of microtubules, as the membranellar band primordium is shifted to an anterior position, it is interesting to speculate whether some elements of the kinetochore machinery responsible for regulating microtubule dynamics or attachment may play some role in the microtubule length changes during oral primordium migration. An alternative, and simpler, hypothesis is that the cell cycle machinery is involved simply because of the fact that micronuclei undergo mitosis during regeneration (Raikhel 1981). In this case, inhibition of aurora kinase may be arresting micronuclear mitosis, and then somehow this arrest leads to a delay in regeneration. This result would be extremely interesting since it would indicate that some mechanism exists to entrain steps of regeneration to steps in the micronuclear division cycle.

With respect to chapter 4, previous literature has shown that membranellar band regeneration requires protein synthesis and transcription (James 1967). The straightforward interpretation for this result had been that cells have to make the protein building blocks from which to construct a new membranellar band. However, my observation that the most enriched proteins of the membranellar band correspond to genes that are expressed after the membranellar band is formed, suggest that this simple idea may not be correct. Instead it appears that the membranellar band may form from pre-existing building blocks, and then re-synthesize what was consumed later on. But in that case, how can we explain the result of James and others showing that translation and transcription are required throughout regeneration? One hypothesis is that the requirement for protein synthesis is not for the purpose of building precursors, but for synthesizing regulatory molecules that signify specific stages of regeneration. In this regard, it may be particularly interesting, in the future, to investigate genes that are upregulated during regeneration but which do not encode enriched components of the membranellar band based on my proteomic analysis. Some of these may encode regulatory molecules that control the timing of the different stages of regeneration that I delineated in Chapter 2.

The possibility that protein synthesis during the early stages of regeneration is important for producing regulatory molecules that signify stages of regeneration, which stems from my observations in Chapter 4, has implications for my results in Chapter 3. Perhaps some of these key proteins synthesized during early stages of regeneration are needed to activate aurora kinase activity in later stages of regeneration.

In my opinion, the big question moving forward is what is the initial trigger that drives the whole regeneration process. The surgical implantation result of Hyvert et al. suggested that a soluble

factor in the membranellar band might be the key signal, and I was therefore very excited to see that the soluble proteome of the membranellar band was dominated by a single class of proteins, the major vault protein family. However, as discussed in Chapter 5, while I did observe some intriguing but poorly reproducible phenotypes when knocking down MVP by RNAI, in no case did I observe an effect on the initiation of regeneration. I speculate that the key signal might not be a protein that is itself enriched in the membranellar band, but rather some signal that is produced in the membranellar band and then diffuses out. For example, a kinase that is located inside the membranellar band could produce a signal in the form of phosphorylated substrate proteins. This type of signaling is used in the spindle checkpoint, and so it is reasonable to speculate that such a signal may be acting here. Therefore, I believe that one of the highest priorities for future experiments is to analyze the function of any kinases or other signal-generating molecules that I have found in my proteomic analysis of the membranellar band. My dream, therefore, is that my work reported in this dissertation may serve as the starting point for answering the biggest unsolved mystery of Stentor regeneration, namely, the nature of the triggering signal for the regeneration process.

Appendix 1. Protocol for generating RNAi constructs for Stentor

1. Obtain gene sequence from StentorDB for RNAi target gene.
2. Go to http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi

a. Enter in gene sequence from #1.

b. Max Tm difference should be 1-5

c. GC content should be min 40 opt 50 max 60.

d. Tm 58 opt 60 max 65 works well

e. Product size 1000-2000 2000-3000 750-1000 500-750

f. Under advanced settings:

i. Max poly X: 4

ii. CG clamp: 1

g. Click pick primers and grab the first ones

3. Order primers

a. https://www.idtdna.com/pages

b. Click "Custom DNA oligos"

c. Click "Order now" next to "DNA oligos" -> Order in Tubes

d. For ease name primers as g###_F and g###_R

e. Don't forget to add primer extensions in front of the sequence:

i. Forward: CATTACCATCCCG

ii. Reverse: CCAATTCTACCCG

f. Click "Order now" next to single stranded DNA

i. This address has been working:

UCSF

600 16th St Rm N376

Genentech Hall

SAN FRANCISCO, California 94143

USA

g. Use PO number: B000301178

h. Wait for primers to arrive (48 hours). A guy wearing headphones will deliver them to our lab.

i. Make 100uM (100x) solution when primers arrive by adding 10 times the nmol amount in uL.

i. Example: Primers are at 32.1nmol

ii. Add 321ul of ddH2O to make 100uM

j. Then make 10uM (10x) solution of primers

i. 90ul of ddH2O + 10ul of above

k. *If teaching this protocol, this is a great warmup question for calculating concentrations… and it makes students feel good about themselves if they do it without paper. On the other hand, if they sit down and write it out, they might feel really dumb… proceed with caution.

4. PCR

a. Each tube has to contain these things:

i. 10ul of 5x HF buffer

ii. 1ul of 10mM dNTP

iii. 1ul of 10uM Fwd primer

iv. 1ul of 10uM Rvs primer

v. 0.1ul of Template - I usually end up adding about 0.5ul

vi. 0.25ul of Phusion

vii. 36.65ul of ddH2O

b. This is usually my master mix formula for 8 constructs:

i. 90ul of 5x HF buffer

ii. 9ul of 10mM dNTP

iii. 1ul of Template

iv. 2ul of Phusion

v. 324ul of ddH2O

vi. Mix all of the above in one 1.8mL eppie tube

vii. Divide them into 8 PCR tubes at 48ul each. Then add each of the primers separately.

c. Run PCR -

i. Right machine has ATI1 program in the Main folder.

5. While PCR is going, digest plasmid and pour gel

a. For the plasmid, combine these things:

i. 17ul of plasmid

ii. 2ul of CutSmart Buffer

iii. 1ul of Sma1

iv. Each construct needs 20ng of plasmid AND we are running it through the gel so make double at least

1. 34ul of plasmid

2. 4ul of cutsmart buffer

3. 2ul of sma1

b. Pour Gel

i. Our gel boxes are designed to cast gels while having running buffer inside.

ii. Weigh out 0.8g agarose and add 80mL of TBE to a flask

iii. Stuff paper towel in the top so that it doesn't spill

iv. Microwave for 45 seconds or until boiling

v. Add 4ul of ethidium bromide (20x)

vi. Make sure gel box is sealed (turn 90 degrees)

vii. Pour gel

viii. Add comb

ix. Wait until it's jelly time

6. Run gel, gel purify and PCR purify

a. Remove comb and submerge gel in TBE

i. Note: Make sure that the positive end is towards the bottom!

b. Load 10ul of 1000kb ladder

c. Load 10ul of PCR product plus 2ul of loading buffer (use a piece of parafilm to mix on and make your life easier, it's hard enough as it is)

d. Load ALL of plasmid at the end

i. 40ul plasmid + 8ul 6x loading buffer

e. Run gel - ~70W - 90W

f. While gel is running, there is enough time to PCR purify, we have a kit for that…

g. After gel has ran, double check that there are bands and then cut out plasmid for gel extraction, we have a kit for that too

i. Chou lab has a nice UV transilluminator next to their nanodrop

h. Measure concentration for all PCR products and plasmid

i. Note: when you are more familiar with this protocol, PCR cleanup and gel purification is similar so they can be performed at the same time.

7. T4 polymerase reaction

a. For all the PCR reactions, in separate PCR tubes:

i. 1ul of T4 buffer

ii. 0.5ul of CTP (10uM) 100uM?

iii. 1ul of 0.1M DTT

iv. 1ul of 10x BSA

v. 0.5ul T4 polymerase

vi. 6ul PCR product

b. For plasmid, if you have more than 4 constructs, I would suggest make double, also in PCR tubes:

i. 2ul of T4 buffer

ii. 1ul of GTP (10uM) 100uM?

iii. 2ul of 0.1M DTT

iv. 2ul of 10x BSA

v. 1ul T4 polymerase

vi. 12ul gel purified, digested plasmid

c. Incubate both separately for 40 min RT and heat inactivate for 20 min at 75 C

i. Use the right PCR machine with the program RT-75 in the Main folder

d. Mix 2ul of PCR product with 2ul of plasmid

e. Incubate for 10 min RT

f. Add 1.5ul 25mM EDTA

g. Incubate 5 min RT

h. Add 14.5ul of ddH2O and use **5ul** to transform

8. Transform

a. Thaw DH5alphas on ice **(grab a new 500ul aliquot)**

b. Add the **5uL** from above

c. Incubate on ice for 20 min

d. Heat shock at 42C for 40 sec

e. Add 1ml LB and shake em at 37C for 1 hour

f. Warm up KAN plates in the meantime

g. After 1 hour, spin down the cells, decant the supernatant and resuspend the pellet in the remaining LB

h. Spread on KAN plates

i. Grow O/N (8 hours if you want to pick colonies under the microscope)

j. END DAY ONE

9. Pick colonies

a. If you have colonies on your plate, pick one using a pipette tip and add it to 5mL of LB + 5ul of KAN

b. Grow for 6+ hours (if you picked tiny colonies, grow O/N)

c. **END DAY TWO**

d. Miniprep the whole 5ml - yes, we have a kit for that

10. Send for sequencing

a. Go to https://www.elimbio.com/dna_sequencing.htm

b. Next to sequencing it should say, "**Schedule a Local Sample Pickup, then Fill Out**

c. **Order Form for the Corresponding Order**" Click on "Go" to the right of it.

i.I prefer Non-mixed, but you do you

1. Tube 1: 3ul Fwd sequencing primer + 7ul ddH2O
2. Tube 2: 3ul Rvs sequencing primer +7ul ddH2O
3. Tube 3 and on: 10ul of plasmid (500 ng)

ii. Pre-mixed

1. Add together - total 15ul

a. 8ul plasmid

b. 7ul 10uM primer

d. Prepare samples and fill in the sheet

e. Use this PO number: B000377850

f. Print out the sheet with your sample information

g. Then tape samples on to the print-out

h. Leave it in the green envelope to the left of the cold room (and to the left of the pipette tips).

11. Double check that the sequence matches with your gene of interest

a. Go to https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE_TYPE=BlastSearch

b. Paste sequencing results into **Enter accession number(s), gi(s), or FASTA sequence(s)**

c. Make sure that the first hit is the stentor gene that you cloned.

12. Triple check that you have the right construct

a. Using ape or a similar program, make a plasmid map

b. Pick two enzymes, preferably one that cuts in the middle of your PCR insert AND would result in two different sizes. (*Teaching moment: What would happen if they resulted in the same size?)

i. Alternatively, the vector pPR-T4P has a two BamH1 cut sites that sandwich the insert so then you can digest with just BamH1 and look for a band that is your construct size.

c. Go to Step 6 and 7 for the gel

13. Transform into HT115 cells (KAN TET plates)

a. Thaw HT115 on ice

b. Add the 5ul from the miniprep

c. Incubate on ice for 20 min

d. Heat shock at 42C for 40 sec

e. Add 1ml LB and shake em at 37C for 1 hour

f. Warm up KAN plates

i. Spread 60ul of 10mg/ml TET

ii. Allow plates to dry for approximately one hour in the 37.

g. Spin down cells for 1 minute at 13,000 RPM

h. Remove ~900ul of supernatant

i. Resuspend pellet in remaining supernatant and spread on KAN/TET plates

j. Grow overnight at 37 degrees in the shaker

14. Pick colonies and grow ~5ml

a. Save glycerol stocks (~15% glycerol)

i. Use 750ul of culture

ii. Add 250ul of 60% glycerol

iii. Store in -80

15. Follow the Marshall lab protocol for RNAi by feeding.

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