Muscle contraction alters hemicentin dynamics at the B-LINK: a newly identified basement membrane adhesion system that connects tissues.

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Abstract

Basement membranes (BMs) are thin, dense sheets of extracellular matrix found covering most tissues in multicellular organisms. In some instances, BMs of adjacent tissues can become linked and attach tissues together. A better understanding of BM-BM adhesion can help elucidate the mechanisms of conditions like Alport syndrome, a human pathology characterized by a loss of kidney function due to a failed BM-BM linkage. In order to further characterize the linkage of neighboring tissues through their BMs, I investigated how the Basement Membrane Linkage complex (B-LINK), a complex that mediates BM-BM adhesion at the uterine-hypodermal juncture in *C. elegans*, responds to biomechanical force. To accomplish this, I determined the necessity of specific BM proteins to B-LINK structural integrity by performing gene knockdown with RNA interference (RNAi) and scoring for vulval rupture, a phenotype that results from a disrupted B-LINK. Type IV collagen was the only common BM component to be identified as an important factor in BM adhesion due to high vulval rupture percentages when it was knocked down at the L1 (80.5%) and L4 (20.0%) larval stages (Fisher's exact = 0.0001). Additionally, I used fluorescence recovery after photobleaching (FRAP) to measure the rate of the protein turnover of the B-LINK component hemicentin under different conditions. These FRAP experiments revealed that muscle contraction in animals significantly increases the hemicentin turnover rate when compared to immobilized worms over the same 15-minute time course (76.7% vs. 24.3%, p-value = 0.0041). These results provide a better understanding of which BM components are essential to B-LINK function and has revealed that muscle contraction influences B-LINK dynamics.

Introduction

Basement membranes (BMs) are thin, dense sheets of extracellular matrix found covering most tissues in animals that serve as scaffolds for cell adhesion and are therefore important for proper organ and tissue development (Yurchenco 2011). Adjacent tissues can adhere to one another through their juxtaposed BMs (Morrissey and Sherwood 2015). This BM-mediated tissue adhesion occurs at the glomerular BM in the kidney, the alveolar BM in the lung, and the pericyte BM in the brain (Abrahamson 1985; Vaccaro and Brody 1981). The Sherwood lab has recently discovered several components of a new cell directed adhesion system, the BM Linkage (B-LINK), that attaches tissues in *C. elegans* through their respective BMs (Morrissey et al. 2014). The B-LINK consists of the extracellular matrix (ECM) component hemicentin, the ECM receptor integrin, and the cytoskeleton linker VAB-10/plakin, each of which is strongly conserved in humans. Hemicentin is a unique matrix component only found between basement membranes and appears specific to B-LINKs, thus we chose specifically to study it. In addition to these B-LINK components, common BM components type IV collagen and fibulin provide the framework and stability for this tissue adhesion to become established.

One location of the B-LINK in *C. elegans* is the attachment of the uterine and hypodermal tissues, which are connected through their neighboring BMs. When the B-LINK is disrupted, BMs separate from one another, the junction fails, and the vulval and uterine tissues evert out of the vulval opening, resulting in an easily observable ruptured vulval phenotype, termed Rup. Since BM-BM adhesion junctions are found in most multicellular organisms, understanding how they are constructed and regulated is important to study. For example, Alport syndrome is a human pathology characterized by loss of kidney function due to separation and failure of the linked BMs that make up the endothelial-podocyte junction (Kashtan 2001). This BM-BM adhesion might be

a vertebrate B-LINK adhesion, and thus, examination of the B-LINK in *C. elegans* will likely be informative in understanding how BM-BM adhesions are built and maintained.

C. elegans permits an in depth study of the cell and molecular dynamics of BM adhesion *in vivo* since it is visually and genetically tractable. RNAi feeding, a post-transcriptional gene knockdown technique that works by inhibiting translation of mRNA, allows temporally controlled cell and tissue specific genetic perturbation. Additionally, the optical clarity of worms and the positioning of the B-LINK permit *in vivo* live imaging of BM adhesion in high resolution. The developmental stages of this model have also been extensively documented with four distinct larval stages, termed L1 through L4 respectively, before reaching a young adult stage capable of egg laying. This is helpful because it allows for temporal regulation and understanding of different developmental processes that specify different cells and construct tissues. Additionally, the composition of BMs in *C. elegans* is very similar to that of vertebrates with orthologs of all major BM components (Hutter et al. 2000). The *C. elegans* uterine-hypodermal junction is a strong model available to study the B-LINK due to its ease in visualizing through the transparent *C. elegans* body wall and the easily observable Rup phenotype, allowing key genes that regulate its formation to be identified through RNAi screening.

Biomechanical force has been found to be important in the development of many extracellular matrices and cell adhesions in tissues. For example, applied force stimulates the growth of focal contacts that adhere cells to extracellular matrix, strengthening cell-matrix adhesions and indicating their ability to serve as mechanosensors (Riveline et al. 2001). The actin microfilament-associated RhoGAP, RGA-2, and the Rho-binding kinase, LET-502/ROCK, have also been shown to balance actomyosin-dependent forces in order to regulate embryonic morphogenesis in *C. elegans* (Diogon et al 2007). Finally, the presence of directed actin

polymerization and reinforcement of cadherin-mediated adhesions in epithelial cell-cell junctions indicates that force is also deeply involved in cell-cell adhesion (Vasioukhin et al. 2000). Interestingly, the B-LINK component VAB-10/plakin has been shown to regulate the adhesion of epidermal-muscle attachments in response to force, suggesting that it might also be involved in force mediated reinforcement of the B-LINK (Bosher et al. 2003). It appears that the Rup phenotype only occurs after the point in development during which the uterine muscles begin to contract. Observation of VAB-10/plakin furthermore shows a slow increase in enrichment at the uterine-hypodermal junction prior to the start of muscle contraction followed by a sharp increase at the initiation of contraction. These pieces of preliminary data suggest that biomechanical force generated by muscle contraction may play an important role in the development and maturation of the B-LINK and the uterine-hypodermal junction.

Overall, the B-LINK in *C. elegans* is the first example of a BM-BM adhesion complex, a potentially widespread process. Further, linked tissues likely experience a great deal of force from fluid flow (e.g. at the blood brain barrier and the glomerular BM) and must commonly withstand and respond to force. My project is aimed at understanding how crucial specific matrix components are to resisting the mechanical stress of muscle contraction in *C. elegans* and to determine the dynamics of the key extracellular matrix B-LINK component hemicentin in response to muscle contraction.

Research Aims

1. Identifying which particular matrix components are necessary for structural integrity:

I will address the question of how essential hemicentin, VAB-10/plakin, and integrin as well as other common basement membrane components are to the structural integrity of the uterine-hypodermal junction. Employing RNAi feeding techniques to perform gene knockdown at

two different larval stages, I will determine if the Rup phenotype is caused by uterine muscle contraction as well as which components are necessary to buffer the force generated by these contractions throughout *C. elegans* development. If a B-LINK component is responsible for resisting biomechanical force and holding the junction together at a particular stage, I expect to see an increase in the Rup phenotype when that component is knocked down compared to controls. The data gathered from scoring the Rup phenotype at the two larval stages subjected to different RNAi will be statistically analyzed to highlight which basement membrane proteins are most essential to resisting the biomechanical force that exists throughout *C. elegans* development.

2. Dynamics of the B-LINK:

I will investigate the protein turnover rates of the B-LINK component hemicentin. It has been shown that mechanically active cell-cell adhesion complexes display different molecular dynamics when they are stressed versus when they are relaxed (Humphrey et al. 2014). Although the B-LINK adhesion complex experiences stress, it is unknown if it is a mechanically active complex. I will perform fluorescence recovery after photo-bleaching (FRAP), an imaging technique designed to assay molecular dynamics, on worms that are pharmacologically induced to either contract or relax their muscles. I will perform this technique in strains individually expressing GFP-tagged hemicentin, which will allow me to assess if hemicentin dynamics are altered in response to stress and to determine if the B-LINK is a mechanically active complex. I will also use polystyrene beads to physically immobilize animals due to the elevated level of friction as a control for the pharmacological agents and with the goal to determine an overall turnover rate for hemicentin at the B-LINK.

Methods

C. elegans Strain Maintenance

Culturing and handling of *C. elegans* was performed as previously described (Brenner 1974). Wild-type animals for the RNAi screen were strain Cas603, and the NK327 and NK368 strains were used for FRAP experiments due to their GFP-tagged hemicentin. Strains were stored at 20°C. Bacterial lawns of *Escherichia coli* OP50 strain, a food source, were grown on nematode growth media (NGM) agar plates when not listed otherwise (RNAi bacteria).

RNAi Feeding – Bacteria

To prepare the RNAi feeding plates, cultures of 1-3 mL of LB broth with a 1:1000 ampicillin concentration and a clonal culture of bacteria were transformed with the RNAi plasmid for the gene of interest. These tubes were then cultured for ~16 hours at 37°C in a shaking incubator. Afterwards, 2 μL of IPTG was added per mL of solution followed by an hour incubation at 37°C in order to induce expression of the RNAi construct. ~200 μL of thoroughly mixed solution was plated per NGM plates and let dry for 24 hours.

Once dry, 20-50 individuals of the Cas603 strain were plated on the RNAi plates at either the L1 or L4 larval stages. Individuals at these larval stages were isolated by bleaching the strains and leaving the fertilized eggs on a rocker in M9 buffer at room temperature for 12 hours, at which point all eggs have hatched into a synchronous population of L1 worms. The worms were then plated on RNAi plates at the L1 stage or on control OP50 plates and allowed to mature to the L4 stage before being washed and plated onto the RNAi bacteria plates.

The plates were then scored by calculating percentages of worms that exhibited the vulval Rup phenotype. This was done 24, 48, and 72 hours post-plating in order to ensure that the total effect of the RNAi was observed. Individuals expressing the Rup phenotype were removed from

the plate in order to ensure proper counting. The percentage of Rup phenotype was calculated accordingly for each possible combination of the strains, larval stages, and RNAi types.

Worm Immobilization

A number of approaches were taken in order to immobilize the animals and image them consistently. Worms were paralyzed using two drugs: levamisole, an agent that induces rigid paralysis, and tricaine, an agent that works to induce flaccid paralysis. Additionally, a combination of these two drugs was also used. Finally, polystyrene beads were used to increase friction between animals and an agar pad, resulting in sufficient immobilization for imaging (Kim et al. 2013).

Fluorescence Recovery After Photo-bleaching (FRAP)

Photo-bleaching of the uterine seam cell (UTSE) in the NK327 and NK368 stains was performed on an inverted Zeiss LSM 510 confocal with a 40x Plan-Apochromat objective. The photo-bleaching itself was performed using a 60mW 405nm Omicron Luxx diode laser, controlled by the iLas 488 Argon/2, to knockdown GFP-tagged hemicentin. Regions of interest were selected and then bleached at 10% power.

Worms were imaged immediately before and after photo-bleaching and at intervals of two or five minutes afterwards in order to monitor fluorescence recovery. The time period between acquisitions was determined based on length of the overall time course. Images were acquired with a gain of 200 and an exposure time of 1000ms.

Image processing and analysis

The acquired images were processed to enhance brightness/contrast and to register the stacks for maximal alignment consistency using FIJI/ImageJ v2.0.0. In order to measure the level of recovery, the bleached region of interest was compared to a control region of fluorescence of

the same size. Percent recovery was then calculated from the normalized ratios of these bleached and control regions.

Statistical Analysis

The statistical analysis for the results of the experiments was performed either with a two-tailed paired Student's T-test or a two-tailed Fisher's exact test. Confidence intervals were calculated at the 95% level, and captions in the corresponding figures specify which test was used.

Results

BM Component Rup

To better understand the role of force in BM adhesion at the uterine-hypodermal junction, I performed gene knockdown of the B-LINK components integrin, type IV collagen, hemicentin, and VAB-10A/plakin as well as proteins essential to BM formation and structure, including laminin, fibulin and teneurine-1 in the *C. elegans* strain Cas603, and the Rup phenotype percentage was recorded. This was done using RNAi bacterial feeding at different times in the development of *C. elegans*, either the L1 or L4 larval stage, in order to specify the role of these molecules temporally in the *C. elegans* system. I hypothesized that those worms where key BM components for B-LINK integrity were knocked down would exhibit a higher Rup phenotype than the other RNAi plates as well as the OP50 control, assuming consistent RNAi penetrance. Also, I expected that the percentage of Rup phenotype would be higher when plated at the L1 stage than when plated at the L4 stage because the uterine tissue would form poorly since it has yet to be fully developed. At the L4 stage, the uterine tissue is formed, but the B-LINK does not yet exist so the results are more likely to indicate the effect only on the B-LINK.

The percentages of Rup phenotype in the various iterations of this RNAi screen are presented in Figures 1 and 2. In both the L1 and L4 plating's, the Rup percentage was significantly higher for the type IV collagen alpha1 subunit *Emb-9* than the control (p-value = 0.0001 for L1 and L4). It is also higher than that of every other BM component at the L1 plating with a rupture percentage of 80.5% (Figure 1). In the L4 plating, only the *VAB-10A* RNAi Rup percentage of 30.2% was higher than the *Emb-9* percentage of 20.0% (Figure 2).

The Rup percentage for the *Lam-1* RNAi was considerably less than that of the *Emb-9* RNAi at both the L1 (2.5%) and L4 (1.6%) larval stages despite the fact that they are both major BM components (Figures 1 and 2). This indicates the potential importance of type IV collagen to proper B-LINK structure and formation. The B-LINK components also had Rup percentages less than that observed with *Emb-9* RNAi. *Ina-1* RNAi resulted in Rup percentages of 15.6 and 15.1, *VAB-10A* RNAi resulted in Rup percentages of 1.1 and 30.2, and hemicentin RNAi resulted in Rup percentages of 2.9 and 6.1 at the L1 and L4 larval stages respectively. These indicate the importance of type IV collagen, EMB-9, to development of the B-LINK.

Hemicentin Dynamics

As hemicentin is a specific matrix component of the B-LINK, I was next interested in understanding the dynamics of this unique B-LINK component in response to muscle contraction. I performed fluorescence recovery after photo-bleaching (FRAP) on GFP-tagged hemicentin on animals in the L4 larval stage as well as young adults. In order to image worms in a FRAP time course, the animals need to be properly immobilized so that photos are taken reliably of the same region. To induce immobilization in the worms, I used a mix of levamisole, a drug that induces rigid paralysis via continued muscle contraction, and tricaine, a drug that induces a flaccid paralysis due to decreased neural functioning. While imaging worms immobilized with levamisole

and tricaine, in some animals a hemicentin recovery of 85.6% was observed in two minutes while the majority of animals did not recover at all (Figures 3 and 4).

To determine if this effect was caused by levamisole induced muscle contraction, I repeated the experiment, immobilizing worms with varying doses of levamisole. At high doses these treatments caused lethality and at low doses did not immobilize the worms sufficiently. I next used polystyrene beads as a way to immobilize worms with no anesthetic. Polystyrene beads are nanoparticles that immobilize worms due to an increased friction between the animals and the agar pad. We thought that polystyrene beads would allow us to recapitulate the fluorescence recovery phenotype, but they did not do so initially. Only when we rescued the worms and re-imaged them later were we able to replicate the dynamic recovery. For this recovery to be observed, animals were either bleached and imaged in polystyrene beads for the entire time course or bleached and then rescued to a plate where they moved freely, permitting muscle contraction. Strikingly, I found that hemicentin recovered rapidly when the worms were allowed to move (less than 15 minutes for more than three-fourths recovery, Figure 5), suggesting that muscle contraction results in rapid turnover of hemicentin at the B-LINK. The recovery percentage was 76.7% for the unrestricted condition and 24.3% for the immobilized condition after 15 minutes (n=6, p-value = 0.0041, Figure 6).

Discussion

The B-LINK in *C. elegans* serves as a useful model to study BM-BM adhesion complexes, but the way in which it responds to biomechanical force has not been well characterized (Morrissey et al. 2014). To uncover more about the contribution of BM and B-LINK components to the overall integrity of the adhesion complex, I performed an RNAi screen at both the L1 and L4 larval stages. This permits temporal control for the BM components under consideration. My hypothesis for

these RNAi experiments was the Rup percentages for the genes of proteins that are most essential to the integrity of the B-LINK would be significantly higher than those of other proteins. All known B-LINK components ruptured at the expected frequency, indicating proper RNAi feeding. As depicted in figure 1, the observed Rup percentages for the gene *Emb-9* are high, 80.5% and 20% at the L1 and L4 larval stages respectively, highlighting the importance of EMB-9, or the protein type IV collagen, to the integrity of the B-LINK (Figures 1 and 2). This suggests that type IV collagen most likely plays an important role in BM adhesion.

The gene *lam-1*, which encodes a subunit of laminin, another common BM component like type IV collagen, had Rup percentages of 2.5% and 1.6% at the L1 and L4 stages respectively (Figures 1 and 2). This is in stark contrast to the high Rup percentages seen in RNAi for type IV collagen. Since we expect that a BM component essential to B-LINK integrity would exhibit a significant Rup phenotype, these results suggest laminin is not essential to B-LINK dynamics. This observation offers strong evidence that the high Rup percentages of the *emb-9* RNAi are not due to a general BM phenotype and that type IV collagen is important to BM adhesion complexes.

BM components have long thought to be long lived, and recent data collected by the Sherwood lab suggest that type IV collagen is highly stable with a 50% turnover rate in approximately 18 hours. Since hemicentin is a B-LINK component and a part of the extracellular matrix, we decided to investigate its turnover rates in more detail and hypothesized that hemicentin should also have a longer turnover rate (Hynes 2012; unpublished data). However, as pictured in Figure 3, hemicentin showed an incredibly fast turnover of 85.6% in only 2 minutes under a mixture of levamisole and tricaine anesthetics. I experience some difficulty reproducing results seen in the aforementioned rapid turnover (Figure 3). In order to perform FRAP, the worms need to be immobilized so that photos can be taken consistently of the same region. Dissecting the

mechanism of action for both levamisole and tricaine proved difficult. I performed a dose response with levamisole and found that high doses killed worms while low doses didn't immobilize the worms properly or reproduce the observed phenotype.

To better characterize this observed hemicentin turnover, polystyrene beads were used to immobilize the worms in the absence of anesthetic to perform FRAP. These beads increase the friction between the animal in the agar pad, which has previously been shown to inhibit muscle function in the pharynx (Kim et al. 2013). Worms immobilized on beads were compared to worms that were photobleached, returned to an NGM plate for a defined amount of time, and then imaged for recovery of HIM-4::GFP, hemicentin tagged with GFP. The difference between those animals that were immobilized in polystyrene beads and those that were recovered to a NGM plate is the ability for muscle contraction to occur.

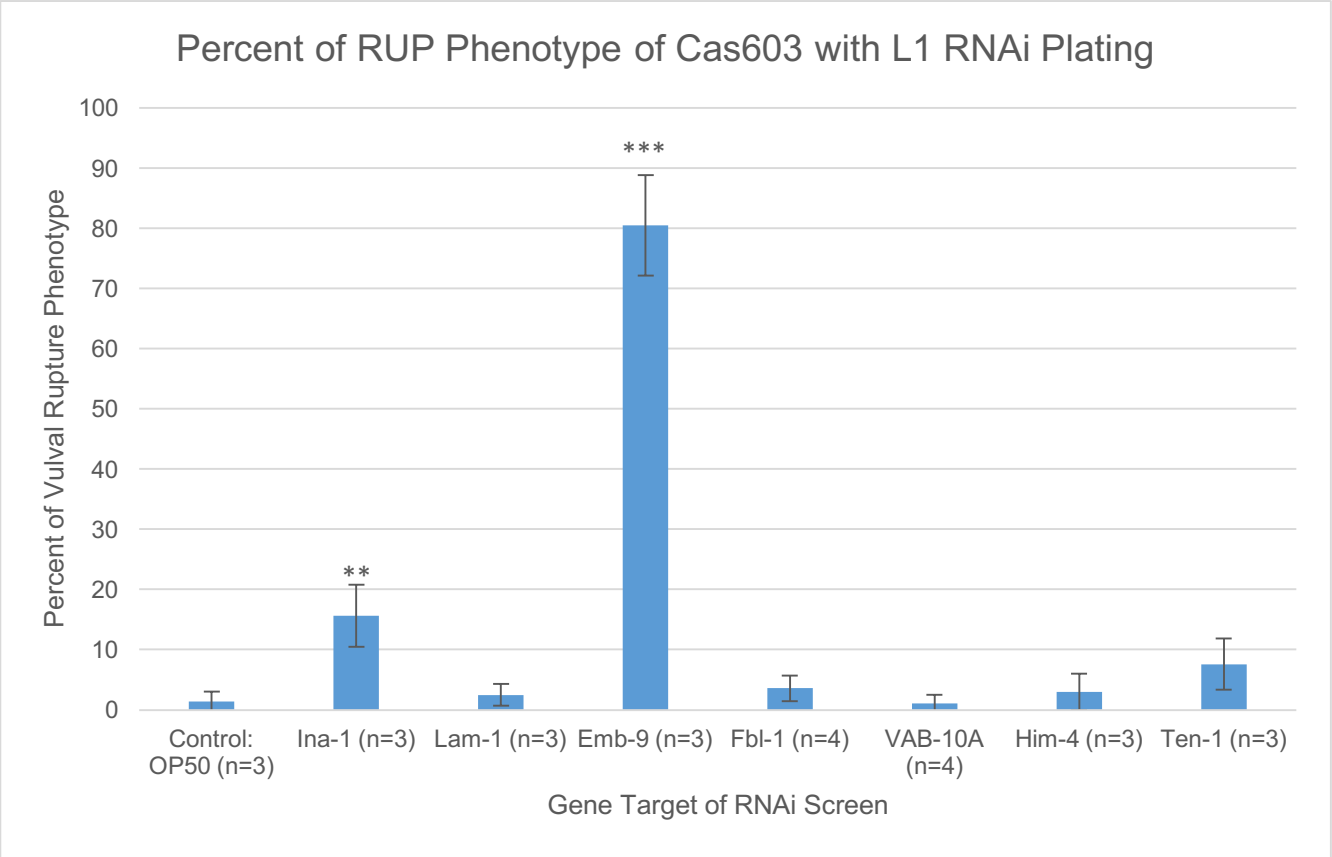
The data presented in figure 6 was reliably collected as a result of this more consistent immobilization agent. Since hemicentin is believed to be highly stable (Vogel and Hedgecock 2001; Yurchenco et al. 2004), the rapid recovery of 76.7% of the original hemicentin fluorescence after only 15 minutes in unrestricted animals is extremely surprising (Figure 6). This is more than a three-fold difference from the 24.3% recovery of the worms that were immobilized over the entire time course (Figure 6). The contraction of muscles may lead to increased hemicentin recovery because it requires additional proteins to withstand the increased biomechanical stress at this juncture. This observation needs to be further characterized however in order to better understand the exact dynamics behind its recovery.

Additional future directions for this project include crossing a strain with a GFP-tagged hemicentin into a background that experiences vulval contractions induced by blue light. Since blue light is used to image GFP, this would result in the induced contraction of polystyrene bead-

immobilized worms when imaged and confirm contraction of the vulval muscles is specifically responsible for rapid hemicentin turnover. This should therefore result in a recovery similar to that presented in figure 6 when this strain is imaged using confocal microscopy. This would allow for a more complete characterization of the hemicentin turnover at the B-LINK, and it could analyze the level of muscle contraction necessary to express a rapid hemicentin turnover. Additionally, the turnover rates of integrin and fibulin at the B-LINK should also be investigated to determine whether this is an observation specific to hemicentin alone or whether it could apply to other BM or B-LINK components.

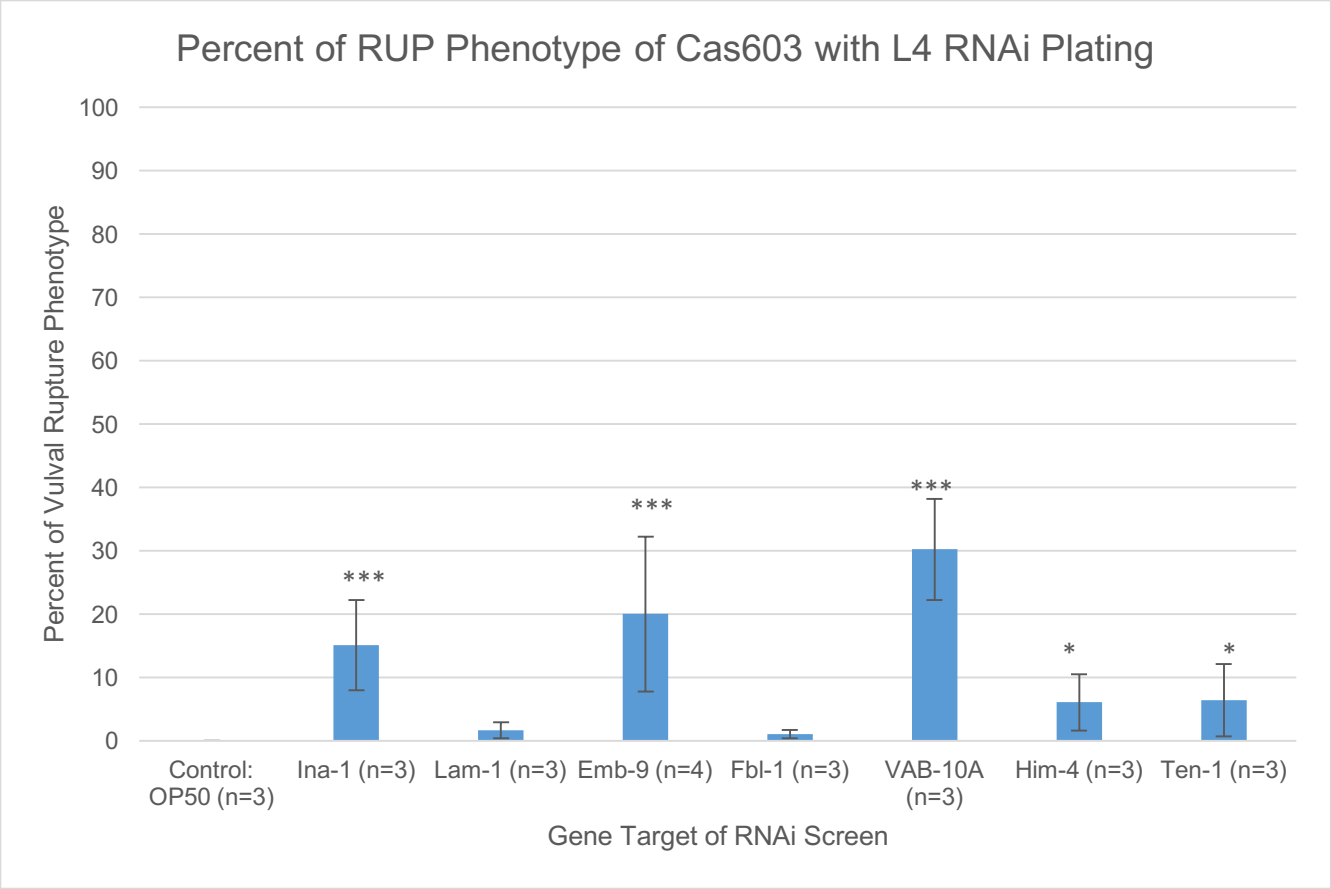
Appendix

Figure 1:



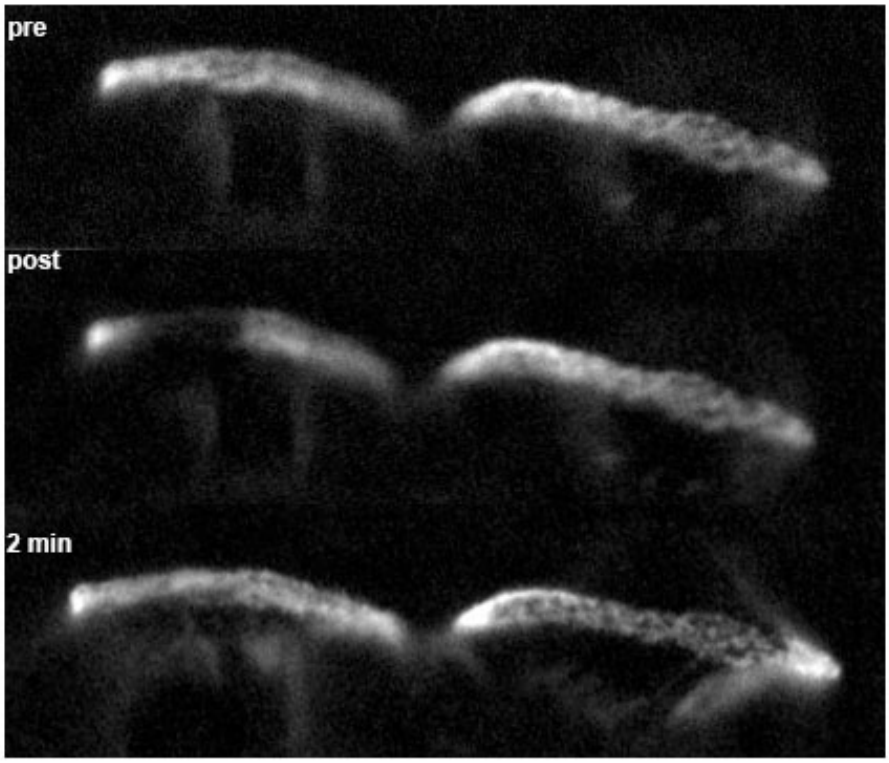
The results of the RNAi screen at the L1 larval stage for BM and B-LINK components. These percentages of Rup phenotype highlight the necessity of integrin and type IV collagen for the proper uterine-hypodermal junction development. Even though plakin is known to be an important B-LINK component, the low *VAB-10A* plakin rupture percentage reported here was likely due to an overall paralysis of the animals, disabling a vulval rupture. A two-tailed Fisher's exact test was used to analyze the rupture percentages from these experiments.

Figure 2:



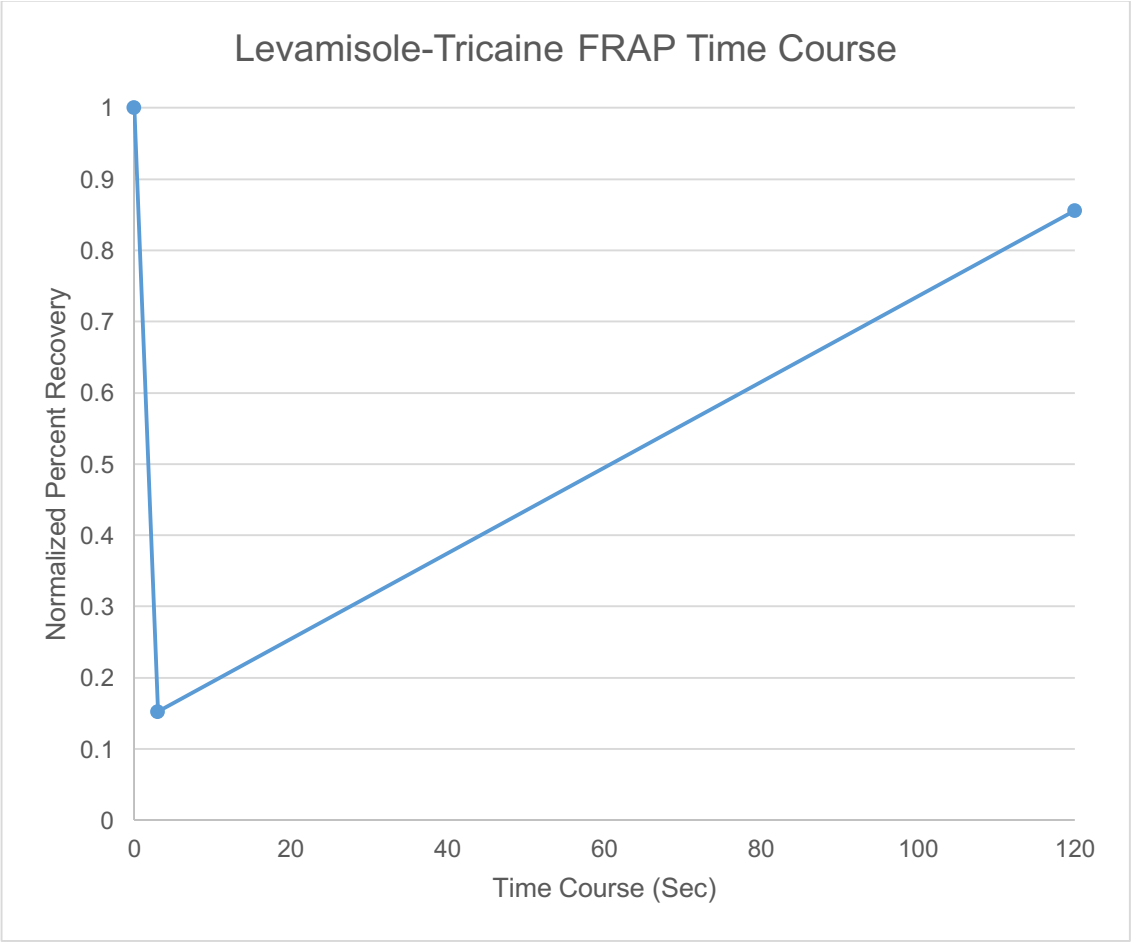
The results of the RNAi screen at the L4 larval stage for BM and B-LINK components. Integrin, type IV collagen, and *VAB-10A* plakin are shown here to result in significant vulval rupture when knocked down by RNAi at the L4 larval stage. Additionally, knockdown of hemicentin and teneurin appears to result in an elevated Rup phenotype, showing the importance of these proteins to proper uterine-hypodermal junction development. A two-tailed Fisher's exact test was used to analyze the rupture percentages from these experiments.

Figure 3:



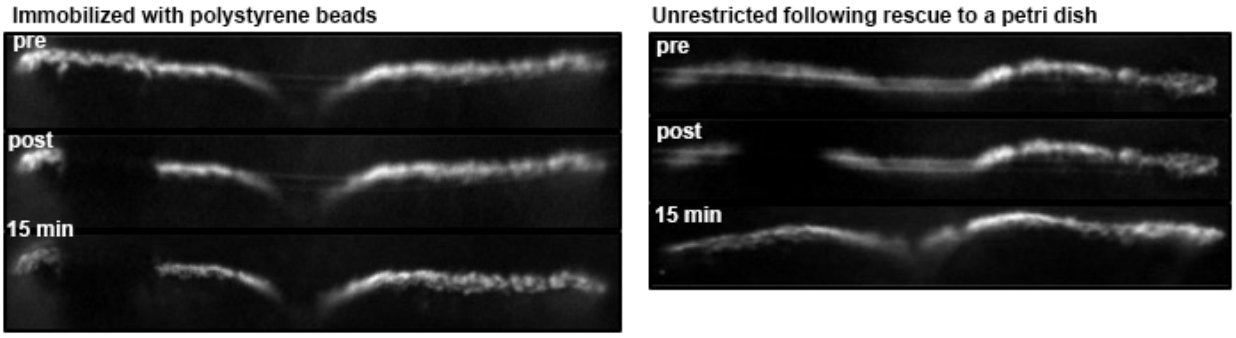
Photos of rapid hemicentin fluorescence recovery under levamisole-tricaine anesthetics. This time course of a knockdown and recovery of GFP-tagged hemicentin at the B-LINK was done under a mix of levamisole and tricaine anesthetics. In only two minutes, the recovery is clearly very robust, and, as depicted in figure 4, recovers to about 85.6%. This observation was surprising due to how highly conserved BMs are considered to be. However, it was extremely difficult to replicate despite attempting multiple dilutions of both anesthetics alone.

Figure 4:



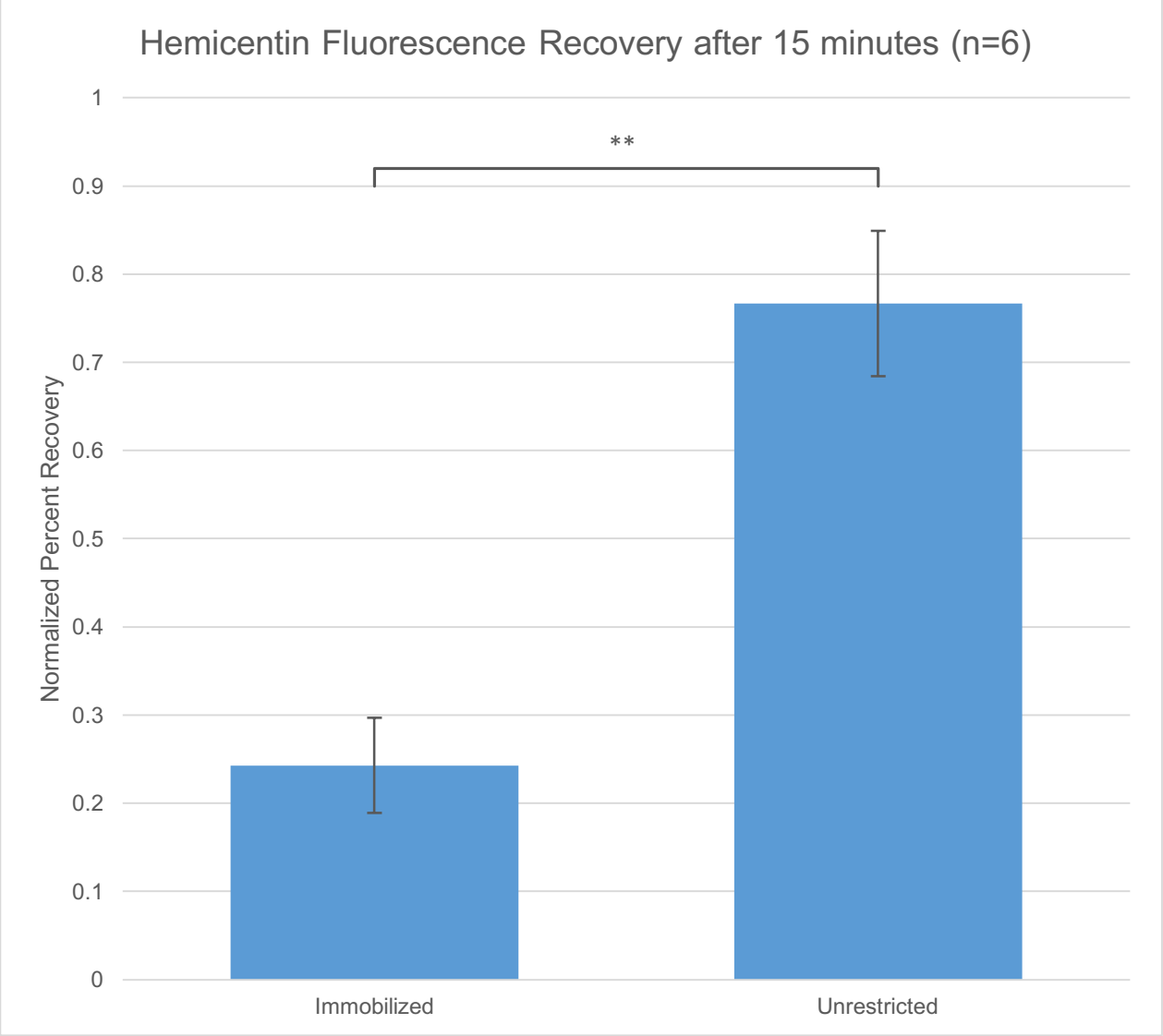
Normalized percent recovery of hemicentin fluorescence recovery under levamisole-tricaine anesthetics. This graph highlights how dramatically the hemicentin recovered in animal presented in figure 3. This observation led us to investigate the role of immobilization and muscle contraction has on the turnover of hemicentin at the B-LINK.

Figure 5:



Representative photos of hemicentin fluorescence recovery with polystyrene beads after 15 minutes. This figure shows the difference in the recovery of fluorescence in animals that were allowed to have unrestricted movement (right) as opposed to those that remained immobilized due to the increased friction between the agar pad because of the presence of polystyrene beads. This observation was reproducible and striking in its severity and extent.

Figure 6:



Normalized percent recovery of hemicentin fluorescence recovery with polystyrene beads after 15 minutes. This graph presents the data processed from six simultaneous FRAP experiments, showing a significantly higher level of fluorescence recovery after 15 minutes in immobilized animals versus those with unrestricted muscle contraction and movement (p-value = 0.0041). This observation works to better characterize the initial observation that muscle contraction might aid in the recovery of hemicentin at the B-LINK. Although the percent recovery in the immobilized worms is well above zero, this might be due to an incomplete and varied immobilization from experiment to experiment depending on how the slide was prepared and where the worm ended up on a given slide. A two-tailed paired Student's T-test was used to analyze the percent recovery from these FRAP experiments.

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