Localization of Chitin Synthase in *Drosophila Melanogaster*

Masters Thesis

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1. Abstract

Proper cell-cell adhesion and communication are essential during development. Both are heavily maintained and regulated by the content present in the extracellular matrix (ECM), which composes the tough exoskeleton called the cuticle. An enzyme called Chitin synthase (CS) provides the exoskeleton with much of its strength and stability through the production of chitin. Chitin, a polymer of N-acetyl-ß-D-glucosamine, is an important element in the exoskeleton of invertebrates and functions much like cellulose in plants and keratin in vertebrates, that is, to provide hardness, strength, and protection against the external environment. The underlying component for both chitin and cuticle formation is CS, which is found across several different species. It is now known that nearly 5-6 different copies of CS in yeast and fungi have been condensed into only two copies in insects. During insect development, one of the two copies is involved in formation of the gut lining while the other is involved in epidermal tissue development, helping to produce the tracheal lining as well as the exoskeleton. The gene that encodes for the chitin synthase involved in the epidermal tissue in *D. melanogaster* is called *krotzkof verkehrt (kkv)*; we are specifically interested in its involvement in the formation of the exoskeleton. *kkv* is an important gene during development as it is involved in production of chitin by CS, which is then used to synthesize the cuticle found in the ECM. Mutant *kkv* results in detachment of the cuticle from the apical end of the cellular body, which then dilates and results in a lethal, curved, short embryo with a scrambled head that is unable to hatch. Not only does *kkv* need to function properly but CS must also be localized to the appropriate region in order to synthesize chitin. There are currently two hypotheses as to how that may occur: (1) CS rests on the apical membrane, and secrets chitin into the ECM, which is then guided to the proper location, and a cuticle is formed or (2) CS is carried around in vesicles termed chitosomes, which localize *kkv* to the right region while chitin synthesis is initiated inside. Upon proper localization, chitin is finally released. Recent discoveries in CRISPR/Cas9 have been used to facilitate understanding of this predicament.

Keywords: Arthropod, Exoskeleton, Cuticle, Chitin, Chitin Synthase, *kkv*, CRISPR/Cas9

2. Introduction to the Arthropod Exoskeleton

The word arthropod comes from the Greek words "arthro" and "podos" meaning "jointed legs" and rightfully describes a diverse group of invertebrate animals with an external skeleton, segmented body, and joined appendages. Much of the reason for their survival is due to the exoskeleton, which is composed of a tough element called the cuticle. The arthropod cuticle provides the organism with a number of properties ranging from stabilization of body and appendage shape, protection from predators, infection, and dehydration (10). In particular, the exoskeletons of insects are comprised of lightweight material that also provides the organism with fast locomotive skills both on land and in the air (76). The life cycle of insects is separated into two defined stages: larval and adult. Molting of the initial rigid, external skeleton does not hinder insect growth as the organism metamorphoses from a larva to an adult causing the cuticle to detach from the epithelial surface, shed, and be replaced with another (10). This allows insects to inhabit multiple ecological niches and specialize in different roles for each developmental stage: larval for feeding and adults for reproduction (76).

3. Cuticle Formation during Development

The insect cuticle as described by Neville (76) is a multi-laminate structure that is secreted by a single layer of epithelium in a variable time sequence allowing for the formation of several layers throughout the cuticle. Collectively, the insect cuticle forms the apical extracellular matrix (aECM) and is composed of lipids, waxes, glycosylated and unglycosylated proteins, and most importantly a polysaccharide called chitin (62). Despite the large amount of diversity among arthropods, the chitin containing cuticle is one element that has remained fairly conserved throughout. Chitin is not only found in the exoskeleton, but also in the internal head skeleton, foregut, hindgut, trachea, and mouthparts (27). Aspects of the cuticle vary within the organism's anatomical framework and even among

developmental stages. For example, the larval cuticle is usually soft and tender whereas the thoracic cuticle is stiff and dense (70).

The integument is a monolayer of epidermal cells that produce and secrete cuticular components. During embryogenesis, these cells undergo differentiation where a change in the shape of the cells produces an overall layer that displays strong cell-cell interactions and can withstand many sources of tension and pressure (70). Recent research on the ECM has made clear that this structure is not solely involved in maintaining organ shape but it also contributes to other aspects of cellular behavior and genetic programming.

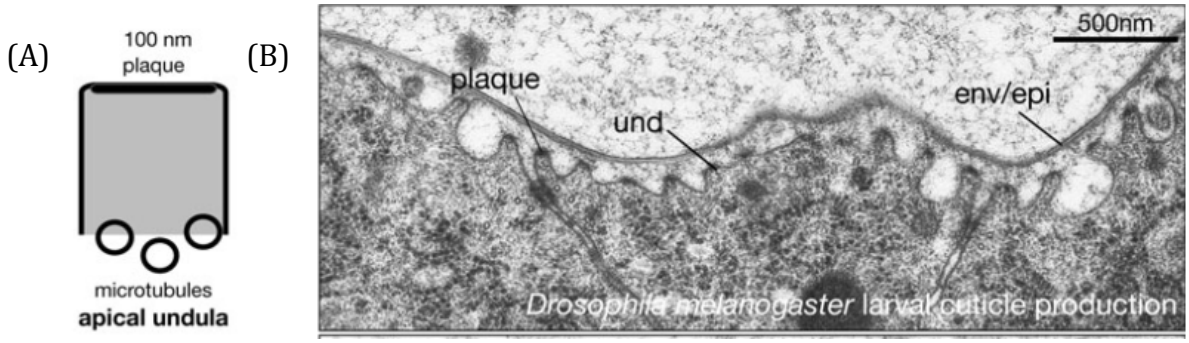

Figure 1 – Apical Undulae: (A) Model of apical undulae formed at the surface of epidermal cells. Microtubules help stabilize the longitudinal protrusions of the undulae, but the underlying interactions of the cytoskeleton with microtubule are unknown. (B) Zoomed-in model of the apical undulae showing *D. melanogaster* larval cuticle production. Plaques at the surface represent clusters of CS secreting chitin into the aECM perpendicular to the undulae (und) underneath the envelope (env) and epicuticle (epi) (10).

Overall, the cuticle is composed of three layers. Much has been written on the nomenclature of these different regions however in the following thesis, the cuticle will be divided up into the envelope, epicuticle, and procuticle. Before these layers are formed, the plasma membrane of the integument epithelial cells forms protrusions termed apical undulae (70) similar to microvilli that are stabilized by microtubules and run perpendicular to the horizontal laminae (Fig. 1A). Topology and correct localization of these undulae are thought to be essential for proper chitin microfibril orientation (Fig. 1B).

3.1 Cuticle Layers

3.1.1 Envelope

The outermost cuticle layer (Fig. 2), which faces the environment is comprised primarily of neutral lipids, wax esters, quinones, and long chain alcohols that give it a hydrophobic nature. This thereby provides the organism with a means of protection against dehydration and also acts as a pheromone in certain insects (10). The envelope can be further divided into the inner epicuticle and outer envelope or cuticulin, which is the major component of the envelope and composed of lipids and sclerotin (12). In *D. melanogaster*, the outer envelope is deposited in fragments at the tips of protrusions made by epithelial cells. These fragments fuse to form a single layer and are thickened by the addition of extra layers during cuticle differentiation (70, 12). A large discussion still remains as to how envelope components are transported across the plasma membrane of epithelial cells and through the several layers underlying the envelope. It has been suggested that pore canals that is, tubes that traverse throughout the entire cuticle from the apical epithelium to the tip of the cuticle, are responsible for transporting the material via an unknown mechanism (10)

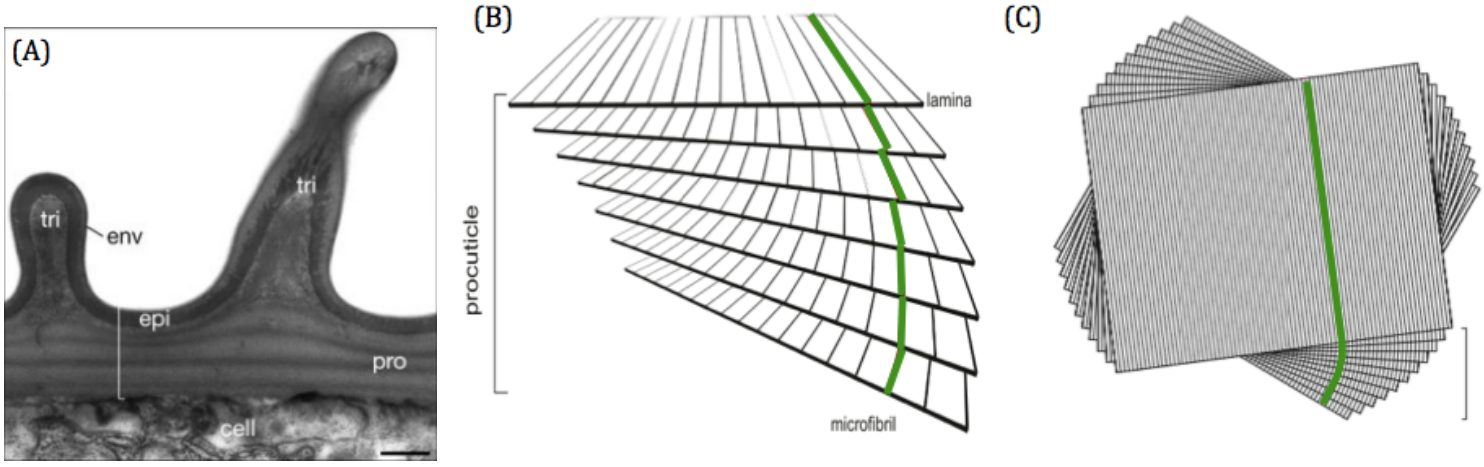

Figure 2 – Model of Cuticle Layers and Laminae Sheet Rotation: (A) The envelope is laid down in fragments that fuse together at the plasma membrane surface and proteins needed for the epicuticle are secreted through the valleys between crests. Both of these layers are thin relative to the procuticle. The final layer contains microfibrils of chitin fibers that form sheets of laminae. These sheets rotate with respect to one another as they are stacked up (17, 26). Epi, epicuticle; pro, procuticle; env, envelope; tri, Trichomes. (B) Zoom in on the procuticle where chitin polymers are lined up anti-parallel to form chitin microfibrils. These microfibrils are depicted here running parallel to form sheets of laminae. Tagging endogenous *kkv* with GFP (in green) would allow us to visualize this organization (12). (C) Model showing laminae arranged in a helical stack. Tagging *kkv* with GFP would allow us to visualize these microfibrils indicated in green (70).

3.1.2 Epicuticle

Not much is known about the epicuticle, which is composed of unidentified small proteins with low structure complexity (10). Unlike the envelope, the epicuticle is not formed in any sequential manner. Epicuticle material is deposited into the valleys between epithelial cell protrusions and slowly thickens during cuticle formation (12, 70)

3.1.3 Procuticle

Unlike the other two layers of the cuticle, the procuticle is the largest and harbors the polysaccharide chitin, whose microfibrils contain a specific organizational scheme (10). Chitin fibrils, arranged in an antiparallel manner, associate to form microfibrils, which are subsequently arranged parallel to one another (Fig 2B). These parallel microfibrils form a 2D sheet called laminae. These laminae form a helicoid pattern by which each new sheet is rotated by some degree from the previous sheet (Fig. 2C). This was originally discovered by Bouligand in 1965 in crustaceans and later confirmed in insects by Luke and Neville in 1969 (10). The orientation of laminae differs from organism to organism. The overall architecture of this layer is also stabilized through chitin-protein interactions. Resilin, one of the chitin-binding proteins found in this region, provides the cuticle with high elasticity (12).

3.2 Laying Down the Drosophila melanogaster Cuticle

While it was originally thought that each layer of the cuticle is temporally separated, recent work by Moussian (70) has shown otherwise. Through a series of images taken via light and fluorescence microscopy and transmission and scanning electron microscopy, Moussian demonstrated that the previously thought sequential layers were in fact not so temporally separated. The envelope precursor was seen in fragments at stage 15 of development at the tip of epithelial cell protrusions. These gaps fused and another layer was added during stage 17. Both the epicuticle and procuticle components are secreted during envelope development in stage 16. The

chitin filaments required for the procuticle is secreted mostly during the latter half of stage 17. In contrast, for adult cuticle there is a clear temporal separation in deposition, although the earlier deposited layers appear to be modified at later times. In comparison to exoskeleton, the chitin filaments found in the trachea are first seen at stage 15 and cover the entirety of the tracheal lumen. It isn't until stage 17 when chitin degradation occurs and the lumen of the trachea is cleared allowing for air to fill the space (70). Overall development thus involves establishment of the first three layers, thickening of the cuticle, and finally the formation of a helical structure by chitin laminae in the procuticle. .

4. Chitin background

Chitin, one of the components that constitute the procuticle layer, is the second most abundant polymer after cellulose. It is a linear polymer composed of ß-(1→4)-linked N-acetyl-D-glucosamine (GlcNAc monomers) where the reaction is catalyzed by an enzyme called chitin synthase (CS). Chitin is made up of alternating residues linked in ß-(1-4)-glycosidic bonds (7). Much research was implemented towards understanding the stereochemistry of the overall reaction. It was thought that in order to accommodate for the 180º turn between consecutive monomers, two GlcNAc residues were added during each catalytic cycle (1). Yeager's lab proved the presence of two active sites using dimeric inhibitors to prove greater overall inhibition over monomeric inhibitors (21). This provided a more in-depth look into the overall stereochemistry of the reaction where two GlcNAc monomers are used per catalytic cycle. GlcNAc monomers are essential sugars involved in various reactions, however one of their most important roles is contribution to the function and architecture of the ECM (18). The Leloir pathway (7) is used to convert a trehalose sugar into the most active form of GlcNAc, UDP-N-acetylglucosamine, where CS completes the final conversion step. This pathway is highly conserved in both arthropods and fungi (6).

Chitin exists in three different crystalline modifications called α, β, and γ chitin (7). The most prevalent form, α-chitin, is found in arthropod cuticles and contains chains in anti-parallel orientation. The anti-parallel orientation allows for

tight packing into chitin microfibrils, maximizing the number of hydrogen bonds and simultaneously minimizing room for any water. This is one of factors behind the strength and stability of arthropod cuticles. β chitin chains are arranged in a parallel orientation whereas γ-chitin chains contain two parallel strands with one that is anti-parallel. β and γ chains are more commonly found in cocoons. They lack the tightness and stability provided by α chains and therefore have an increased number of hydrogen bonds with water. This property gives them a more flexible and soft chitinous structure that is also found in the peritrophic membrane in the gut lining (8). This difference in chitin chains also results in different arrangement of chitin microfibrils later on. Whereas cuticle microfibrils are arranged in a helicoidal formation, peritrophic matrices and even those found in the trachea are structured as a random network of chitin fibrils and are very rarely found in an organized manner (7).

5. Chitin Synthase

Chitin synthase (CS) is the enzyme required for converting UDP-GlcNAc into chitin (8). CS is part of the glycotransferase family, which contains a group of enzymes that catalyze the transfer of sugar from donor to acceptor while forming a glycosidic bond. The overall reaction requires the presence of a divalent metal cation like Mg^{2+} or Mn^{2+} (7-8) Although much research has been done on fungal CS, especially yeast, the first CS sequence found in arthropods was identified by Tellam's lab in 2000 (1). He used degenerate primers with similarities to fungal chitin synthases to sequence the enzyme from *Lucilia cuprina* and further tested its presence with fungal CS inhibitors. From there, Tellam was able to repeat similar procedures with *C. elegans*, *D. melanogaster,* and arachnids. *In situ* localization of CS mRNA in 3rd instar larvae resulted in stained layers of epidermal cells underneath the procuticle.

From the sequence analysis, it was found that chitin synthases are relatively large proteins with 15-18 transmembrane segments. The enzyme can be split up into three domains: a, b, and c.

5.1 Domains A, B, and C

5.1.1 Domain A

Domain A, at the N-terminal, substantially varies in length when compared across different species. It also varies in terms of number of transmembrane segments which regulate whether this region is found intra- or extracellularly (7). Research has shown that a deletion of up to 389 base pairs in yeast CS1 and a 221 base pair deletion in yeast CS2 does not affect the enzymatic activity of either enzyme (75). From this, one may conclude that this region is rather insignificant. An alternative interpretation is that domain A has specific functions that have rapidly evolved leading to many segments.

5.1.2 Domain B

Domain B is known as the catalytic domain with hydrophobic properties and no transmembrane segments. Because UDP-GlcNAc is located in the cytosol, it is assumed that this domain faces the interior of the cell (7). Initial sequence analysis by Tellam's lab showed a conserved sequence among all four organisms; the sequence, QRRRW, is thought be a product-binding site. Point mutations in this region were implemented in yeast and resulted in decreased overall CS activity but had no effect on the K_m values for the substrate (8). A second conserved sequence was also found in the following domain: (S/T)WGT(R/K). Originally it was thought to be required for catalysis as any mutations resulted in a loss of activity, however because this region is located extracellularly in yeast, that idea was quickly abandoned. Although further experimentation is necessary, this second conserved sequence has been hypothesized to be part of the translocation process that moves chitin polymers into the extracellular matrix. (8). Other homologous sequences include Walker A and B motifs (Walker et al, 1982) and a GEDRxx(T/S) motif at the acceptor binding site (27).

5.1.3 Domain C

Domain C contains multiple transmembrane segments, which are conserved among *C. elegans, D. melanogaster,* and arachnids but not in yeast. Among

arthropods, the domain is fairly conserved with respect to location and spacing between transmembrane segments (27). One important feature of this domain involves five transmembrane segments that are located immediately after the catalytic domain with two further downstream near the C-terminal. These segments are thought to be involved in translocation of polymerized chitin chains.

5.2 CS Class A vs. Class B

While probing segments of digested DNA with a segment from the catalytic domain of *L. cuprina (Lc*CS-1), Tellam's lab (1) came across a new CS, which they termed *Lc*CS-2. Further analysis using the TBLASTN computer program and a similar probe revealed that this was also the case in *D. melanogaster* (DmCS-2) and *C. elegans* (*Ce*CS-2). Sequence analysis just on the catalytic domain region demonstrated a near 72% similarity between *Lc*CS-1 and *Dm*CS-1 genes and 98% similarity between *Dm*CS-1 and *Dm*CS-2. Cross species sequence analysis showed similar results.

Based on this information, researchers have grouped CS into two classes CS-A and CS-B. With the exception of a few arthropods, most insects have these two CS genes. In *Drosophila*, both of these genes are located on chromosome 3 and are thought to have evolved from a common ancestor via gene duplication (7). Class B is the more ancient form and expressed in the gut epithelial cells producing the peritrophic matrix. Much of the difference between class A and B can be seen at the c-terminal in Domain C (Fig 3). This domain contains a total of seven transmembrane segments. Class A genes are predicted to have a coiled coil region after the fifth transmembrane segment (27). This coiled coil is thought to face the extracellular matrix and might be involved in protein-protein interactions, vesicle fusion, or oligomerization (7, 49). Class B enzyme lacks this region.

Class A has two mutually exclusive exons that result in two mRNA splice variants (Fig. 3A). Both exons code for 59 amino acids and result in an additional site for N-linked glycosylation. This variation is located in Domain C, c-terminal to the five transmembrane segments. This change may result in different interactions with cytosolic or extracellular proteins, which can then regulate chitin synthesis,

localization, transport and/or organization (27). Gene studies done in *Lucilia, Tribolum, D. melanogaster* and *Manduca* have shown that Class A CS is expressed in the epidermis and trachea by different splice variants.

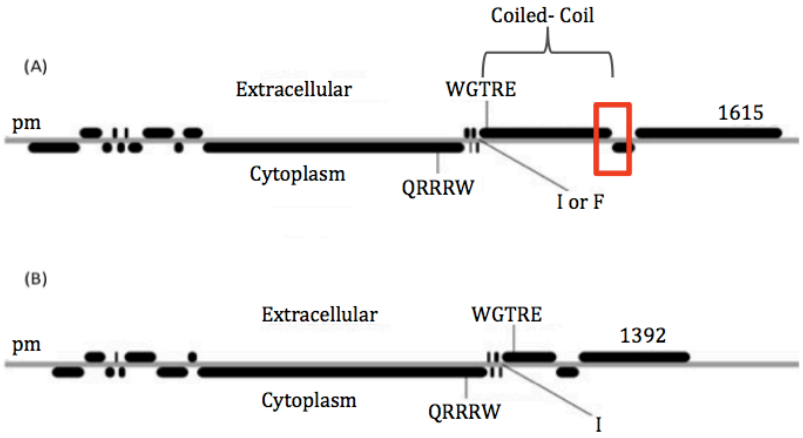

Figure 3 – Class A vs. Class B CS: (A) Class A CS (*kkv* indicated here) is expressed in the epidermis, trachea, and the fore- and hindgut. An additional region not present in class B enzymes is located after the catalytic domain indicated by the coiled-coil region. Class A also has two mRNA splice variants with differences near the c-terminal (indicated by red box) (B) Class B is expressed in the midgut, forming the peritrophic matrix. Both enzymes contain conserved regions of QRRRW and WGTRE in the catalytic domain (17).

5.3 CS Organization at the Plasma Membrane

When it comes to trying to understand the overall organization of CS, most is left to speculation. Much of this is due to the fact that the arthropod CS has never been isolated in a pure and active form. Therefore its organization on the plasma membrane is based off comparisons conducted on CS and cellulose synthase. CS is thought to function as an oligomer at the plasma membrane. Cellulose synthase is organized as a hexagonal structure with a six-fold symmetry called a rosette (8). Each rosette is made up of six subunits, which can either be six monomeric or three dimeric synthetic units. This oligomerization aspect is suspected to be one of the many reasons why active CS has not yet been purified. The manner in which CS oligomerizes will also determine the formation of the active site (8). It's been speculated that oligomerization results in a pore in the plasma membrane that helps facilitate the transport of the hydrophilic chitin microfibril across the hydrophobic membrane. Partial purification of midgut CS from *Manduca* revealed a trimeric CS complex (8).

5.4 Krotzkopf Verkehrt

In *D. melanogaster,* the gene that encodes for the CS expressed in the trachea and epidermis is called krotzkopf verkehrt (3); it is also a vital component of proper exoskeletal cuticle formation (11). It was found through a mutant screen in 1984 by Nüsslein-Volhard and Wieschaus (2) who strived to characterize mutant alleles on the third chromosome involved in the larval cuticle. *kkv* mutants displayed a crumbled head skeleton, narrower denticle bands across the abdomen, and some embryos were inverted in the egg case. Further research found they contained a distinct "blimp" phenotype, whereby the cuticle of mutant embryos detaches from the body and dilates (3) (Fig. 4B). This phenotype is wider than the wildtype and indicates a loss of cuticle integrity (11). This became especially clear when these embryos were mechanically devitellinized. The denticle belt was stretched laterally and although the number of belts had not changed, the level of chitin within each belt had decreased. These embryos were also less pigmented (3, 11). Because *kkv* is also expressed in the trachea, that structure was no longer visible in the mutant embryos. The word *krotzkopf* means, "scrambled head" in German and rightfully describes the deformed and non-pigmented embryos. A scrambled head is not ideal for hatching and many times one will find that certain hyperactive embryos will have inverted in the egg case in an attempt to hatch (11).

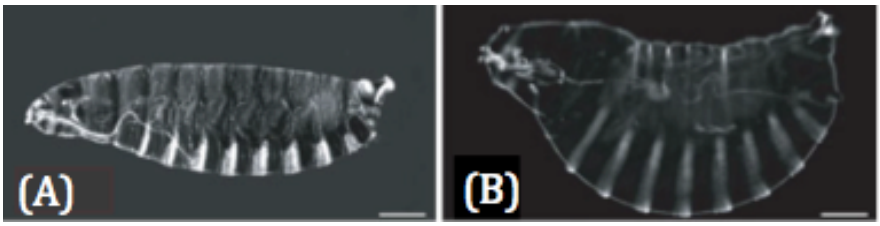

Figure 4 – Mutant *kkv*: Darkfield microscopy of cuticle preparations of (A) Wildtype *kkv* and (B) mutant *kkv*. Mutants show the characteristic blimp phenotype where the cuticle is detached from the apical surface losing their normal body morphology. *Kkv* mutants are seen with a scrambled head and larger body (11).

Further research on mutant embryo cuticles found it to be altered. The cuticle had variable thickness, particularly in the epicuticle and procuticle (Fig 5). The epicuticle was broadened, penetrating into the procuticle, which contained free procuticular chitin-binding proteins instead of a protein-chitin laminae. The overall

adhesion between the epithelial cells was also non-existent (11). The epidermis and cuticle depend on one another to form the exoskeleton and are required to stabilize body morphology.

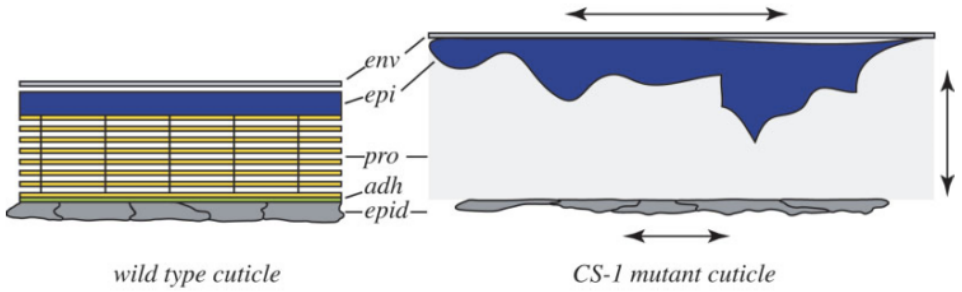

Figure 5 – Mutant Cuticle Model: Wildtype cuticle (left) shows laminae (yellow) stabilizing upper levels via association with the adhesion zone below. Mutant CS leads to a mutated cuticle where the layers are not separated into distinct regions. The epicuticle protrudes into the lower procuticle and appears larger than in the wt. The cuticle is not attached to the epidermal layers below, causing the blimp phenotype. Chitin laminae are required for proper cuticle formation. Env, envelope; epi, epicuticle; pro, procuticle; adh, adhesion zone; epid, epidermis (11).

5.4.1 Other Blimp Phenotype Genes

Through a collaborative effect, several labs during the 1980's were able to come across three genes that also produced the blimp phenotype of *kkv*. These include *knickkopf* (*knk*), *grainy head* (*grh*), and *retroactive* (*rtv*) (2). Another gene, *zeppelin* (*zep*) was added later (3). These five genes are vital for proper cuticle integrity. In terms of viability, *zep* mutants are the most hyperactive and therefore are able to hatch but die at roughly the same stage as *knk* and *rtv* mutants. *Kkv* and *grh* cause more severe damage to the head skeleton, denticle belt, and result in lower hyperactivity.

In situ hybridization of *knk* showed low levels of mRNA throughout all stages of development (3). *Knk* is thought to interact with the epidermis prior to cuticle formation along with *zep,* as both were found to be interacting with mutations in *Drosophila* E-cadherins encoded by *shotgun (shg*). They are needed for *shg* to form proper epithelial cell adhesion and subsequently the cuticle it secretes. Once it was sequenced, the *knk* gene was found to encode for an extracellular protein anchored to the plasma membrane via a GPI moiety with no enzyme domain (14). Much like *knk, rtv* is also an extracellular membrane-anchored protein and is thought to

coordinate binding with chitin via six aromatic residues. *Rtv* mutants show the standard blimp phenotype and mutant cuticle organization. It is thought to function in lamellar procuticle organization (14) via two possible methods: anchoring chitin chains to the plasma membrane, or binding other blimp phenotype proteins such as *knk* to organized chitin chains (63). Both *knk* and *rtv* are structural proteins assisting more with chitin filament assembly downstream of *kkv* and less with the overall chitin synthesis process.

Grh is a transcription factor that belongs to the GATA family of transcription factors. It is responsible for activation of several genes during development, one of them being *dopa-decarboxylase*, which is ultimately needed to produce the quinones required for proper crosslinking of cuticular proteins. While *grh* might increase expression of *kkv*, *grh* mutant cells have been shown to display normal *kkv* activity.

5.4.2 Genes Interacting with kkv

Recent work done by Moussian's lab has found two genes, *expansion* (*exp*) and *rebuff* (*reb*), which are required for *kkv* function. Without either gene, chitin deposition does not occur. Overexpression of both in chitin-devoid regions resulted in chitin deposition. *Exp* and *reb* are hypothesized to participate in chitin polymer translocation, microfibril formation, or in the direct or indirect posttranscriptional modification of *kkv* (13).

5.5 Forms of Regulation

CS regulation occurs at all stages of development. Tissue specific expression of *Lc*CS-1 mRNA was measured by RT-PCR and showed expression of CS in 1st, 2nd, and 3rd instar larvae, pupae, adults, and eggs (1). Any mutations would be detrimental to the overall growth of the organism. Several forms of regulation are in place to prevent such issues.

a. Hormonal control

   Insect molting and metamorphosis are controlled by ecdysterone, a steroid hormone that acts primarily on gene transcription. It exhibits a regulatory role over CS-A and CS-B transcript levels (4, 8). Experiments done with

Drosophila CS have shown that transcripts of either gene are not detected prior to and during late larval ecdysone pulses. Once the pulse ceases however, both genes are upregulated (4).

b. Transcriptional/post transcriptional control

kkv has five potential binding sites for the transcription factor *grh*, whose exact role in chitin synthesis is currently unknown. Post-transcriptional regulation includes phosphorylation, dephosphorylation, and N-glycosylation, which have been found to regulate the localization, activity, and stabilization of certain CS (8). CS activity can also be controlled via regulation of components in the Leloir pathway. In the pathway, the rate-limiting step is undergone by the glutamine-fructose-6-phosphate aminotransferase (GFAT). In *Drosophila* two GFAT genes, *Gfat1* and *Gfat2,* have been recognized (Adams et al 2000; Graack et al 2001). *Gfat1* is inhibited by UDP-GlcNAc via a feedback mechanism and stimulated by protein kinase A (PKA). This in turn controls levels of UDP-GlcNAc available to be converted into chitin by CS.

c. Chitinases

Insect chitinases belong to a family of glycohydrolases responsible for the catalysis of glycoside hydrolysis.

d. Zymogenic behavior

In some yeast and insects, it has been suggested that certain CS activity is regulated by trypsin and other proteases categorizing them as zymogens. Trypsin experiments have been done *in vivo* and appear to increase overall activity; to date however, no endogenous protease has been identified to cleave CS zymogens in insects (27).

e. Environmental factors

In certain mosquitos, it has been shown that CS regulation in the peritrophic matrix is dependent on a blood meal (8).

5.6 Localization

One of the many unknowns about *kkv* and CS in general involves attempting to understand the mechanism by which CS localizes itself. Attempts to purify CS from yeast have allowed for the discovery of chitosomes (5), or vesicles that contain CS on their plasma membrane. Although they have been recorded in yeast, evidence for their involvement in insects has not yet been found. Chitin synthesizing enzymes are thought to cluster at the tip of microvilli formed by epidermal cells. Even with that assumption, it is still not known whether CS is an integrated membrane protein or if it resides in vesicles that cluster near the apical membrane. One model based on the idea of chitosomes suggests that chitin is secreted into the lumen of specialized vesicles, which then fuse with the plasma membrane thus allowing for the secretion of chitin into the ECM (Fig 6A). This model however has a few shortcomings that should be considered. The vesicles are relatively small in size and may not provide adequate space on the membrane for a large enzyme nor may they contain the necessary space inside for chitin. Another aspect to consider is that CS is activate as it makes its way from the ER to the plasma membrane, thus leaving room for excess chitin production. If the catalytic domain of CS is inside the chitosome, a mechanism should exist for UDP-GlcNAc transport into the vesicle. The second model suggests intracellular vesicles that merely act as exocytotic conveyors transport CS from the

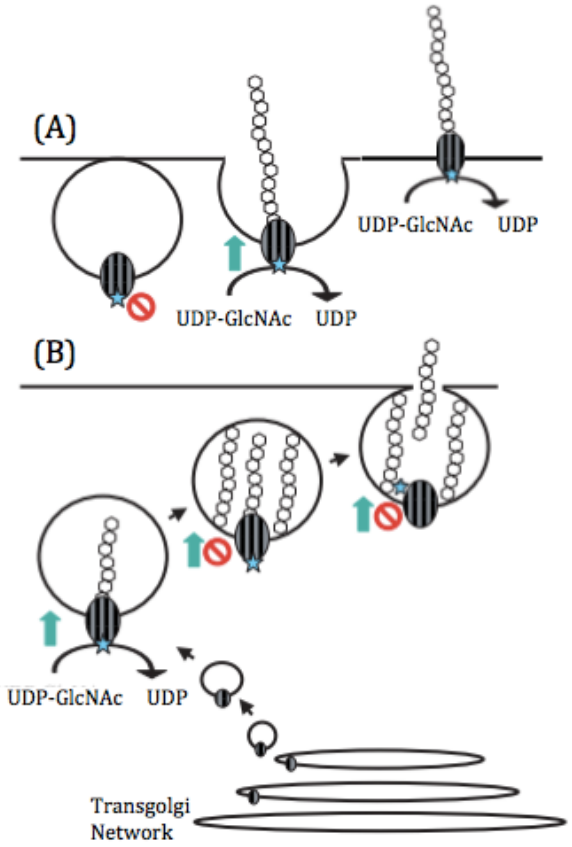

Figure 6 – CS Localization: (A) Vesicles loaded with CS are transported from the golgi to the plasma membrane at the apical surface, fuse and are then activated via unknown mechanism (proteolytic cleavage, oligomerization, etc.). (B) CS vesicles called chitosomes carry the activated enzyme through the cytosol, producing chitin fibrils into the lumen of the vesicles. Once the chitosome docks and fuses with the plasma membrane, the chitin fibrils are released into the aECM (7).

ER to the plasma membrane, where it could be activated by proteases or other proteins to form chitin (1, 7, 8) (Fig. 6B).

6.7 Fungal CS

The fungal genome contains somewhere between 2-20 genes per species, which have been categorized into five or seven classes (6). The most well studied species, yeast, contains three types of CS (22, 32, 71), whose activity is spatially and temporary dependent on the cell cycle (36). Domain A is quite variable among the different species with most lacking transmembrane segments. Classes V, VI, VII enzymes however contain a myosin motor domain (MMD) (Weiss et al 2006). With the exception of a number of transmembrane segments in domain C, domain B and C follow similar sequence schemes to those found in arthropods. Zymogenicity is variable but has been shown in crude extracts. When attempting to purify CS from *Mucor rouxii*, Ruiz-Herrera discovered the presence of chitosomes, vesicles that harbor CS on its membrane. These vesicles had a lower buoyant density than other exocytotic vesicles and were capable of producing chitin fibrils when substrate and activator were added to the extract (Bracker et al 1976). They also did not contain any yeast plasma membrane markers such as ß-1,3-Glucan synthetase (5).

6. Chitin applications and usages

Chitin polymers are the second most abundant polymers following cellulose. Investigation of these polymers would allow one to manipulate certain properties for other uses outside its natural involvement with the arthropod cuticle. Recent interest in chitosan, a deacetylated chitin derivative, has led to its usage in tissue engineering. Chitosan, a linear polysaccharide, is the second most abundant natural biopolymer commonly found in crustacean shell and fungal cell walls. It has variable solubility properties depending on the pH of the solution it is incorporated into. Tissue engineering advances in cartilage, bones, skin (55, 56) and even drug delivery systems (57) have made it clear that the material used has to be bioabsorbable, with certain porosity and degradable properties that do not hinder growth or normal physiology of the tissue within which it is placed. Chitosan, when

conjugated with other chemicals, helps facilitate those properties within these scaffolds. In one example, chitosan was combined with hyaluronan, a polysaccharide found in the ECM, to form a lightweight matrix for chondrocytes, cells found in cartilage. The combination of both chitosan and hyaluronan helped to increase not only the level of chondrocyte adhesion and proliferation but allowed for synthesis of collagen as well (56). The ability of chitosan to bind certain anionic molecules such as DNA and several growth factors also opens up the opportunity for further uses outside of tissue engineering, including developmental research (55).

Understanding the mechanism via which chitin is synthesized and deposited opens doors for ways to inhibit its production. These would prove extremely useful when developing insecticides for both agrarian and domestic purposes. Certain insecticides, which have been used substantially in research to understand chitin metabolism do not inhibit CS itself but other properties related to cuticle development. Nikkomycine Z, a potent competitive inhibitor of fungal chitin synthase, was tested on insects and found to be a growth regulator (1) without evidence of having direct effects on CS itself. Lufenuron, another fungal insecticide has also been used on insects and thought to affect chitin polymerization. While these and other insecticides may do the trick, affecting other reactions or properties related to the framework of the cuticle, they may have targets in vertebrates. Targeting CS itself would reduce such risks. This would also be extremely important when developing drugs for certain fungal infections that affect human lives (42).

7. CRISPR/Cas9 Overview

Targeted genome editing has become a powerful tool for biological research with great potential for therapeutic discoveries against genetic disorders. Precise editing in the past has been limited to certain organisms such as yeast and mice; even in those cases, complications arose with off-target effects, limited efficiencies, and high costs. An important first step to genome editing is creating DNA double-stranded breaks (DBS). These breaks can then be repaired via two mechanisms: (1) non-homologous end joining (NHEJ) and (2) homology directed repair (HDR). NHEJ is an easy method for inducing small insertions and deletions that cause changes in

the reading frame. HDR meanwhile takes advantage of a donor template to repair the damage. That template can be engineered in the lab to implement specific changes to the genome such as insertions or deletions of specific nucleotides, tags, and even resistance markers to name a few. In the past, these DSB have been induced via two methods: zinc finger nucleases (ZFN) and transcription activator-like effector nucleases (TALENs). Both are chimeric proteins made up of a nuclease, Fok1, which is guided by a programmable DNA-binding domain (61). Fok1 must dimerize to be active thereby requiring two ZFNs/TALENs to produce a DSB. While these techniques have been successful with modifications, they do present a significant number of drawbacks. Two ZFN or TALEN designs per modification require time and effort; sometimes, assembly of the separate parts alters the interaction between them (61). A new method called CRISPR/Cas9 has found a method by which to circumvent such issues.

The CRISPR system is the adaptive immune system used by bacteria whereby sequences from invading DNA are incorporated into CRISPR repeat sequences. When regions are transcribed, CRISPR RNAs (crRNAs) also known as protospacer sequences, are formed harboring both foreign bacterial DNA and parts of the CRISPR repeat. crRNAs bind to transactivating CRISPR RNAs (tracrRNA) and form a complex with the Cas9 nuclease. This complex can then cut foreign DNA if they are adjacent to a protospacer adjacent motif (PAM) (60). When it comes to implementing this in research, a cas9 nuclease and 20 nucleotide guide RNA (gRNA) target cut sites via RNA-DNA complementary base pairing. The targets must be 5' of a PAM sequence, which vary depending on the type of organism one is working with. This allows gRNAs to be of the general form, 5' 20 nucleotides –PAM 3', with a section of core RNA encoded by the gRNA vector.

This method has a wide variety of uses from bacteria to cell cultures to entire animals. Regions in the genome can be edited ranging from small to large insertions, deletions and replacements, to being modified with activation/inhibition domains, effector domains to produce conditional alleles, or fluorescent tags for a more precise understanding of localization and interactions during development. Delivery

of separate components has also been simplified down to merely injections instead of using viruses or electroporation like in the past (60).

7.1 Drawbacks

7.1.1 gRNA efficiency

gRNAs needed for this series of reactions are rather simple to make. They are made up of a 5' 20 nucleotide homology region upstream of a PAM sequence. Being that the specificity of the Cas9 is based on the PAM sequence, there is a great chance of off target cuts that must be repaired to prevent unknown mutations. Several websites have been set up to screen regions of interest for potential gRNA sequences and provide efficiency levels for each. The present issue concerns the discrepancy between sites and the fact that most do not contain distinctions between mismatches in their PAM sequences vs distal sequences in their algorithms (59). Data from each site should be compared to select the best gRNA. Also when choosing gRNAs, it has been found that those beginning with two guanine residues before the complementary sequence yield a better on-target to off-target ratio (60). The gRNA promoter should also be considered; recent work done in Port's lab demonstrated the importance of this requirement. CRISPR work done in *Drosophila* recommends using RNA polymerase III-dependent promoters from the *U6* snRNA genes, which include *U6:1, U6:2,* and *U6:3* (67). gRNA constructs were created using the three different promoters. Flies expressing each gRNA-y gene were then crossed to transgenic flies expressing *act-cas9*. The results showed that those gRNAs under *U6:1 and U6:3* promoters developed cuticles that were phenotypically more yellow than those under *U6:2* control.

7.1.2 Cas9 Nuclease Specificity

Modulating Cas9 activity will help reduce off target cuts and increase overall efficiency of a given experiment. It has been suggested that paired nucleases on adjacent strands in combination with two gRNAs provide greater specificity. This method also allows for equal levels of HDR vs NHEJ induction instead of one or the other (65).

The PAM sequence is a key requirement for the Cas9 target sights. Although a specific Cas9 recognizes specific PAM sequences, there are cases where regions of non-canonical PAM sites get cleaved (58). However if the Cas9 is modified, where its nuclease function is only partially active, that issue can be overcome. This would allow for nicks to occur in the genome instead of DSB and induce HDR instead of NHEJ (59).

Catalytically dead Cas9 have also been fused with Fok1. Two gRNAs and chimeric Cas9 nucleases would be required for this process. Because Fok1 needs to dimerize, it wouldn't be constitutively active; this combines the honing in aspect of CRISPR/Cas9 with a more target specific nuclease. One would have to consider the effect of the size of fok1 on the overall transduction efficiency of the plasmid DNA (69).

8. Methods

8.1 Fly care

All flies used were grown on standard fly food at 25ºC. OregonR (OreR) wildtype flies, and y*v*; *CyO/Gla* and *w hs-flp; TM3/TM6* balancers were from the Adler lab stock collection. The various chromosomes were originally obtained from the Bloomington Drosophila Stock Center. *act-Cas9*, and *vas-Cas9* stocks were obtained from the Bloomington Drosophila Stock Center. *Nos*-cas9 was obtained from BestGene and *kkv* ^{PB} from the Exelixis Collection at Harvard Medical School.

8.2 Yeast Overexpression via Gateway Cloning

We overexpressed transcript D of *kkv* (Flybase ID: FBtr0301398) in yeast cells using the gateway system (Fig. 7). This procedure takes advantage of two site-specific recombination events through the use of *att*P sites. Using Accuprime pFx DNA Polymerase (Catalog number: 123444-024), we flanked transcript D with forward and reverse primers.

Forward: ***att*B1**; translation start codon**;** 18-20 gene specific nucleotides
GGGG **ACAAGTTTGTACAAAAAAGCAGGCT** TC ATG TTCAGTTTAGCGAAGACAACGAACCCGAAA
Reverse: ***att*B2**; translation stop codon; 18-20 gene specific nucleotides (reverse complement)
GGGG **ACCACTTTGTACAAGAAAGCTGGGT** C TTA CTGTTTGATGCTTCTATTTATTGTTTTAAA

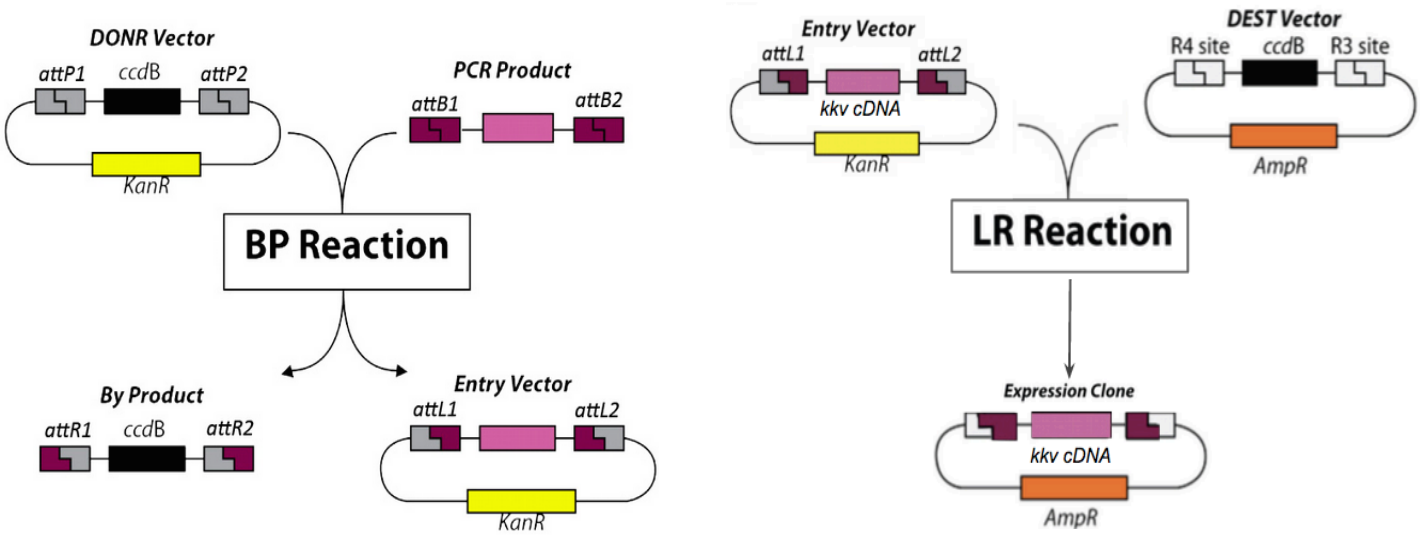

Figure 7: Overview of Gateway System (19)

Gateway® BP Clonase® II Enzyme mix and Gateway® LR Clonase® II Enzyme mix were used (procedures included). Entry vector pDONR 221 (invitrogen) was used during the BP reaction and the destination vector pAG426GPD-ccdB, a gift from Susan Lindquist (Addgene plasmid # 14156), was used during the LR reaction. The destination vector was than transformed into yeast cells, KKY1035 and KKY1037 (provided by Keith Kosminski at the University of Virginia) and transformant yeast colonies were picked from URA$^{(-)}$ plates. The pYES2 Yeast Expression Vector manual (Cat. no. V825–20) was used to express our protein in yeast cells.

8.3 kkv Antibodies and Western Blots

Two *kkv* antibodies T01812 and G3453 (both anti-rabbit) were made using SDIX and LLC services. We isolated newly formed OreR pupa (wt) from their vials and placed them in 25ºC incubators for 48hrs. These pupae were removed from the pupal case and separated into three e-tubes with two containing five flies and one with two flies. 250µl of SDS sample buffer was added to each tube and the content was ground up. 25µl of 2-mercaptoethanol was added to each mixture and the tubes were heated accordingly for 10 min:

Tube A of 5: 90ºC

Tube B of 5: 60ºC

Tube C of 2: 90ºC

The samples were spun down at 14000 rpm for 5min and the supernatant transferred to fresh e-tubes and used for western blot. All antibodies were diluted 1:1000. The secondary antibody was goat anti-rabbit (purchased through Li-Core https://www.licor.com/bio/products/reagents/secondary_antibodies/).

8.4 Injections

Rainbow Transgenic Flies Inc. provided all injection services (http://www.rainbowgene.com/default.html).

8.5 Vectors

8.5.1 pUAST-attB GAL4 construct

kkv transcript D was cloned from a cDNA clone in lab using the following primers*. The pUASTattb kkvR and pUASTattb GFPF primers were designed to create an overhang at the end of *kkv* and beginning of GFP, which could subsequently be annealed together. pUASTattb kkvF and pUASTattb GFPR primers were designed to allow for insertion of the fragment *kkv*-GFP between EagI and XhoI restriction sites in the vector, pUAST-*att*B (Bischof et al, 2007) (Fig 8).

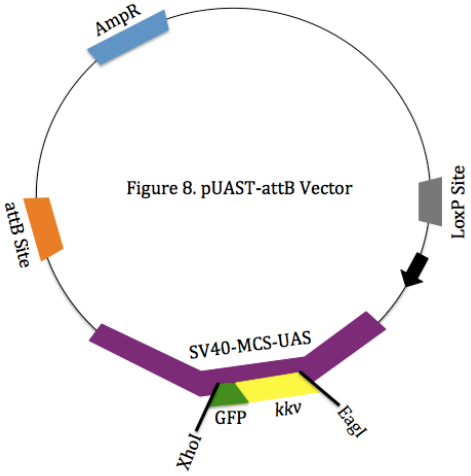

Figure 8. pUAST-attB Vector

pUASTattb kkvF: 5' ATCG **CGGCCG** CTGTTTGATGCTTCTATTTA
pUASTattb kkvR: 5' reverse complement CCTTGCTCACCATTTCAGTTTAGCGA
pUASTattb GFPF: 5' TCGCTAAACTGAAATGGTGAGCAAGG
pUASTattb GFPR: 5' reverse complement GTCA **CTCGAG** TTACTTGTACAGCTCGTCCA

*Green designating GFP sequences; bold represent **restriction sites**; underlined are regions of overlap

Ligation was done using the Gibson Assembly Cloning Kit (New England BioLabs catalog#: E5510S). Although the protocol does not specify, 5µl of each flanked gene was mixed with 10µl of Gibson Assembly Master Mix and left overnight on ice at 4ºC before proceeding with the protocol provided. This construct underwent a standard phiC31-attP40 injection on the second chromosome.

8.5.2 pHD-DsRed HDR construct

For the homology directed repair construct, pHD-DsRed (ADDGENE plasmid #51434) was used. For this construct, three separate fragments, *kkv*a, GFP, and *kkv*b were ligated together using Gibson Assembly. *kkv*a and GFP regions of overlap were then annealed together and inserted between the restriction sites SacII and NdeI. *kkv*b was inserted between PstI and XhoI (Fig. 9). The following primers* were used. All annealing and ligation was done via Gibson assembly. *kkv*a and *kkv*b were cloned from the genomic clone BCR34M23 (created by the Berkeley *Drosophila* Genome Project) and GFP was cloned from pUAST*attB*-GFP.

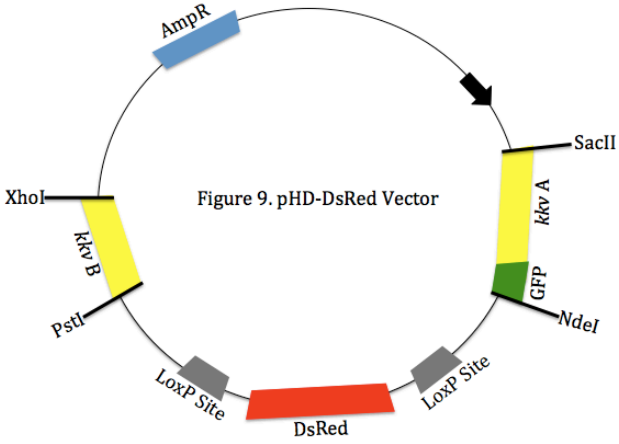

Figure 9. pHD-DsRed Vector

*kkv*a 5' **CCGCGG** TAGCATGCTGTGGAGATCGT
5' reverse complement CCTTGCTCACCATGCTAAGCATAATG
GFP 5' CATTATGCTTAGCATGGTGAGCAAGG
5' reverse complement **CATATG** TTA CTTGTACAGCTCGTCCATGC
*kkv*b 5' **CTGCAG** ATGTACTATACTATCATTTG
5' reverse complement **CTCGAG** GCACAGTTCGCTGTGGGGTC
* Green designating GFP sequences; bold represent **restriction sites**; underlined are regions of overlap

8.5.3 pCFD4 Double gRNAs

The *pCFD4* vector (addgene 49411) (Port et al, 2014) combines together two promoters, *U6:1* and *U6:3,* adjacent to the gRNA core sequence allowing for the expression of two gRNAs at one time (Fig. 10). Forward and reverse primers* were provided in the supplementary figure 3D. gRNA primers were purchased from http://www.operon.com/ and the vector *pCFD4* was used as the template during PCR. The primers contain a 3' homology to the vector backbone to allow for PCR amplification, which can then be inserted

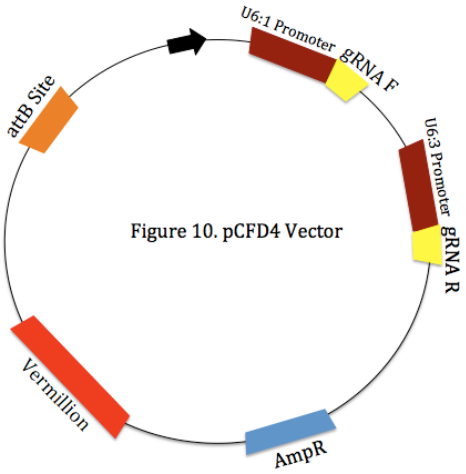

Figure 10. pCFD4 Vector

into a BbsI-digested *pCFD4* backbone. Ligation was done via Gibson Assembly. The two gRNAs F and R were chosen from regions of *kkv*a and *kkv*b respectively using https://chopchop.rc.fas.harvard.edu/. This *pCFD4* construct will be co-injected with the HDR construct into nos-Cas9 flies on the second chromosome (attP40). *pCFD4* will also be injected separately into *P{nos-phiC31\int.NLS}X, P{CaryP}attP40*.

pCFD4 gRNA F
5' TATATAGGAAAGATATCCGGGTGAACTTCG CCCATTCTCACCCGCCGCCTGAC GTTTTAGAGCTAGAAATAGCAAG
pCFD4 gRNA R (reverse complement)
5' ATTTTAACTTGCTATTTCTAGCTCTAAAAC CCTGCGACAACGAGCATATGTTC CGACGTTAAATTGAAAATAGGTC
* Underlined regions represent the two gRNAs

8.5.4 *Single Stranded Oligonucleotide (ssODN) Template for Homology Repair*

Protocol for designing the oligo came from the FlyCrispr website (http://flycrispr.molbio.wisc.edu/) provided by labs at the University of Wisconsin. The Oligo consists of four parts. Our ssODN* targeted a transposable mutation called *kkv*^{PB} marked with w^{+}. We induced mutations in the homology arm to prevent cuts by our gRNA. The oligo was made through http://www.operon.com/ (Fig. 16).

5'— (~60-nt 5' homology) **CCNNNN(attP sequence)NNNNGG** (~60-nt 3' homology) — 3''

(1) **PAM sequence**
(2) **NNN** corresponds to the 3-nt adjacent to the **PAM** site that remain following Cas9 cleavage. In this example, the 5' PAM site is on the antisense strand and the 3' PAM site is on the sense strand.
(3) **attP sequence (50-nt):**
GTAGTGCCCCAACTGGGGTAACCTTTGAGTTCTCTCAGTTGGGGGCGTAG
(4) Homology arms on either side

CACCAAACAGCTAATGATGCTCTTTGTTCTCGGTACTCCCGTTTACCTTGTTAGCCCCAT
CCTAATGTAGTGCCCCAACTGGGGTAACCTTTGAGTTCTCTCAGTTGGGGGCGTAGCCAA
GGATAAG**GAAGTT**CCGAACCCACCTTCGGTAGTGGAGACCCTACCCCAAGTTTAGACCA
ATACCCGCATATTTTCGGCT
* Underlined region represents the homology arm with four base pair mutations to prevent cutting by the gRNA

8.5.5 *pCFD3 Single gRNA constructs*

The *pCFD3* vector (Addgene 49410) (67) contains the *U6:3* promoter adjacent to the gRNA core (Fig. 11). Two separate vectors were used to form gRNA

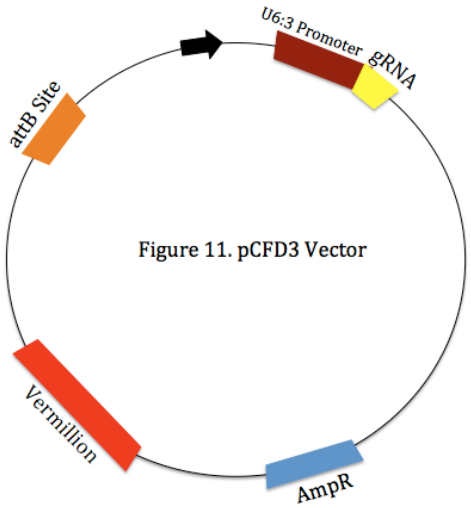

Figure 11. pCFD3 Vector

constructs to target regions around the *kkv* ^{PB} transposable mutation with one gRNA inside a *kkv* intron and the other outside of *kkv* near its 5' start. https://chopchop.rc.fas.harvard.edu/ was used to find the most efficient gRNA, made through http://www.operon.com/, and annealed together using the following protocol. 20µl of each primer was mixed together, placed on a 90ºC heat block for 2min, and then cooled down to room temperature. Annealed primers were inserted into a BbsI-digested *pCFD3* backbone via Gibson Assembly. The primers* are listed below. These gRNAs will be co-injected with the ssODN repair template into *nos-Cas9;kkv* ^{PB} and *yw vas-Cas9;kkv*^{PB} flies.

gRNA-Out	5' forward: **GAAGAC** ATG GTTCGGATGTAGCTTATCCTTGG
	5' reverse complement: **GAAGAC** TTA CCAAGGATAAGCTACATCCGAAC
gRNA-In	5' forward: **GAAGAC** ATG GATAAACAGCCTGAACCATTAGG
	5' reverse complement : **GAAGAC** TTA CCTAATGGTTCAGGCTGTTTATC

* Bold regions represent **BbsI restriction sites** followed by either a start or stop codon and then the underlined gRNA sequence

8.5.6 *pattB Repair construct*

This construct consists of annealing the region between gRNA-Out and gRNA-In with a GFP tag and inserting it into the EagI and XhoI digested backbone of pattB. The region between the two gRNAs was cloned from the genomic clone BCR34M23 and GFP was cloned from pUASTattB-GFP (Fig 12A). The following primers* were used and ligated via Gibson Assembly. These flies will undergo a phiC31-attP40 injection on the 2nd chromosome.

GFP repair	5' Forward: ATCG **CGGCCG** ATGGTGAGCAAGGGCGAGGA
	5' Reverse complement: CATCCGAACCCAC CTTGTACAGCTCG
kkv repair	5' Forward: CGAGCTGTACAAG GTGGGTTCGGATG
	5' Reverse complement: GTCA **CTCGAG** CCATCCTAATGGTTCAGGCT

* Bold regions represent **restriction sties**; green represents GFP; underlined regions are areas of overlap.

A second *kkv* pattB construct without GFP was also constructed using the following primers inserting *kkv* between EagI and XhoI (in bold) (Fig. 12B).

5' Forward ATCG **CGGCCG** GTGGGTTCGGATGTAGCTTA
5' Reverse GTCA **CTCGAG** CCATCCTAATGGTTCAGGCT

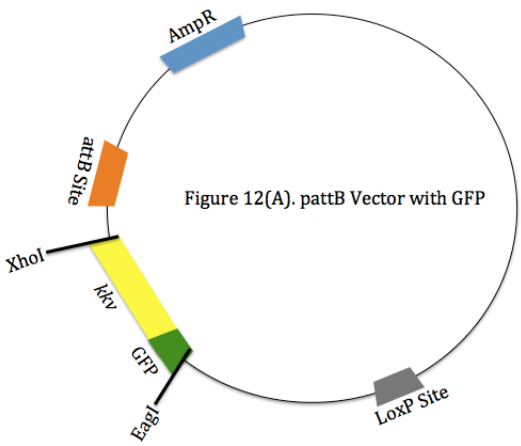

Figure 12(A). pattB Vector with GFP

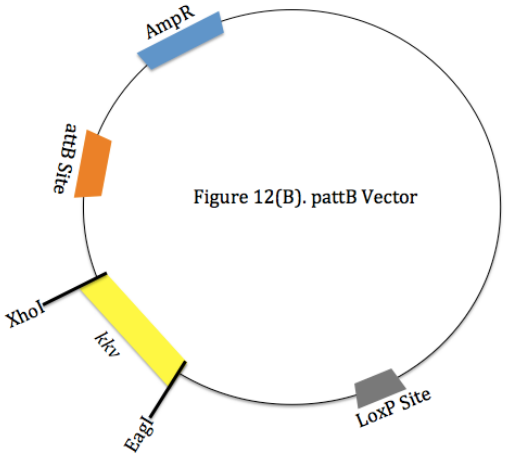

Figure 12(B). pattB Vector

9. Predicted Results and Discussion

9.1 Yeast overexpression

We cloned *kkv* transcript D into a destination vector via Gateway Cloning. This procedure avoids the use of restriction enzymes and thereby bypasses problems related to excess cutting or inversions of the template DNA. The procedure consists of two recombination reactions, the first being called BP, which inserts the fragment into an entry vector. These vectors are quite useful as they allow for the movement of a sequence of interest into a destination or expression vector via a second recombination reaction called LR. A wide variety of destination vectors exist with different fluorescent tags, resistance markers, etc.

Once we had transformed our yeast vector (verified via DNA sequencing), we induced expression using both liquid induction medium and plates. We had hoped to observe some morphological change within the yeast cells, however that did not occur. From what we have discussed regarding CS activity, this could be caused by an array of things. It is possible that the enzyme needed to be phosphorylated or required the presence of other transcription factors and proteins. CS also doesn't function properly without the presence of a divalent cation Mg^{+2} or Mn^{+2}.

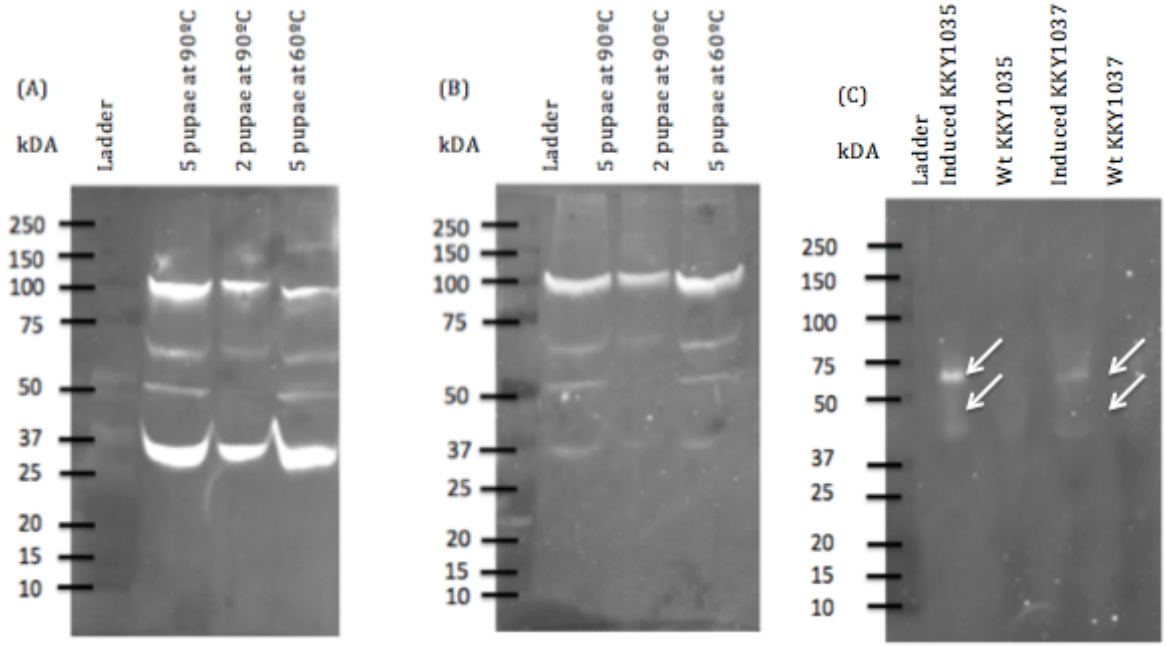

Figure 13 – Western Blot Results: (A) Antibody G3454 tested on wt OreR pupae incubated at 25ºC for 48hrs (B) Antibody T01812 tested on wt OreR pupae incubated at 25ºC for 48hrs (C) Antibody T01812 test on transformed yeast cells and wt yeast cells

To check for the expression of *kkv*, we performed a western using two *kkv* antibodies, G3453 and T01812. Before attempting to do so, we confirmed their activity on first instar pupa. Post 2-mercaptoethanol addition, the mixture was heated up to either 90ºC or 60ºC due to the unknown but potential polymerization property of CS, which may affect the speed with which it travels on a gel. The molecular weight of transcript D is around 182kDA but because the enzyme was cleaved, we did not see one concise band but rather several bands (Fig 13A, 13B). Cleavage might have occurred in areas where the protein passes through the plasma membrane, with the heaviest band around 100kDA being the catalytic domain. Temperature did not have any effect on polymerization of the protein. These antibodies were then tested on our transformed yeast cells, and we were able to show the presence of *kkv*. The antibodies used were specific for *kkv* and therefore should not have stained any endogenous yeast CS; indeed, no bands were seen for wt KKY1035 or KKY1037 (Fig 13C).

9.2 Overexpression of kkv in Drosophila

Continuing with the idea of overexpression, we wanted to attempt to do one of two things within *Drosophila*: overexpress *kkv* or express *kkv* in certain patterns. To do so, we cloned *kkv* from a cDNA clone and GFP from a pUAST-attB-GFP vector and flanked each with overlapping regions. These two separate pieces were ligated together using Gibson Assembly into the pUAST-*att*B vector. This vector is particularly useful in our case because it has an upstream activation sequence that is driven by a yeast transcription activator called GAL4 (Fig. 14A). This will allow us to express the gene and fluorescent tag in controlled manners. Because CS is so vital for survival, it is very difficult to turn this gene on and off. We could for example use vestigial-GAL4 for expression all over the wing or patched-GAL4 to express *kkv*-GFP in a stripe along the wing (Fig. 14B). We could then try to induce knockouts in that same region and see if the phenotype can be rescued. We do not expect to see much change in cell morphology, however there could be an excess production of chitin polymers. In the thoracic region, it has been previously shown that excess chitin is deposited early on during development that disappears at later stages. We expect to see less of these polymers disappearing (Adler et al, 2015). Future antibody staining of this region for other proteins would allow us to decipher certain protein-protein interactions such as those between CS and potential chitin-organizing proteins. Presently, this construct has been sent out for injection into a pC31 fly line.

An attempt to create this construct using Gateway Cloning did not work properly. Donor221 was used as the entry vector and PTWG was used as the destination vector from the Drosophila Genomic Resource Center (https://dgrc.bio.indiana.edu/product/View?product=1076). The PTWG vector is useful in the sense that it already had a GFP tag and the overall construct was made using just two recombination reactions. The construct was sent in for a p-element injection twice, and the G_0 generation was crossed to a *whshep; TM3/TM6* balancer. This however did not produce any transformants. While the first group of injected flies was kept at 25ºC, the second was moved to the 21ºC incubator. This was done in case *kkv* expression *was* temporally sensitive; the slower growth rate of the 21ºC incubator would allow us to observe this. While no transformants were recorded,

there was a greater level of fertility in injected females when crossed with a male balancer than with female balancers crossed with injected males. This lack of transformation could be due to enhancer trapping of the insert leading to expression lethality.

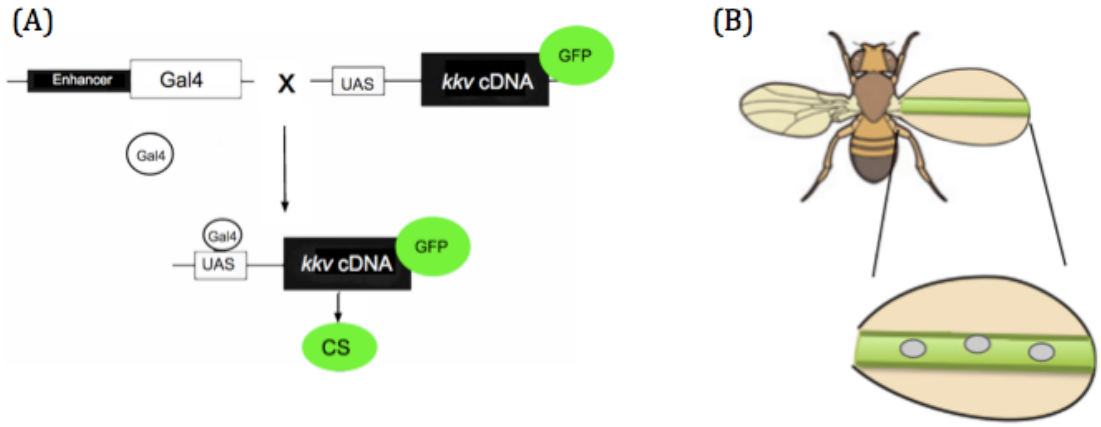

Figure 14 – Gal4/UAS: (A) Gal4 is a regulatory gene found in yeast. When produced, it acts as a transcription activator that binds an upstream activation sequence (UAS), which drives the production of genes that may follow. Our overexpression vector will help to tag endogenous *kkv.* (B) Different types of transcription activators can be used to express a gene. Patched GAL4 will express *kkv*-GFP in a stripe along the wing. Knockouts (blue circles) can be induced to see if the wt phenotype can be rescued.

9.3 Tagging Endogenous *kkv*

9.3.1 HDR and 2gRNAs

We are attempting to tag *kkv* by taking advantage of a cell's DNA repair mechanism that is, homology directed repair. By injecting our HDR and *pCFD4* gRNA constructs together into *nos-Cas9 attp40* flies, our gRNA will produce two cuts near the c-terminal (Fig. 15). Once these cuts have been produced, the HDR construct we provide will repair regions adjacent to both cuts and tag *kkv* with a GFP tag. The pHD-DsRed vector has a DsRed marker that will be expressed in the eyes and will allow us to identify transformants. We should also be able to detect the GFP under a fluorescent dissection scope, as CS is such a vital enzyme in the exoskeleton. Currently, these constructs have been sent out for co-injection into a *nos-Cas9 attP40* line.

Tagging *kkv* would allow us to understand several aspects of CS activity particularly localization. There are thought to be two manners in which CS can

localize at the apical membrane. One model, based on the idea of chitosomes, suggests that chitin is secreted into the lumen of specialized vesicles, which then fuse with the plasma membrane secreting the chitin content out into the ECM. The second model suggests intracellular vesicles that merely act as exocytotic conveyors to transport CS from the ER to the plasma membrane, where it could potentially be activated by proteases or other proteins to form chitin. Based on the literature discussed, the latter model holds great potential. With chitosome-like vesicles, one would need to consider how a large enzyme like CS could fit into a small vesicle, how the size of the vesicle might limit the level of chitin production, manners for transporting UDP-GlcNAc into the lumen of the vesicle where the catalytic domain resides, and finally what the advantages are of having CS constitutively active in the cytosol. Normally large amounts of chitosomes are seen in EM studies done in Fungi and presently, no evidence of such vesicles has been found in EM studies done in *D. melanogaster*.

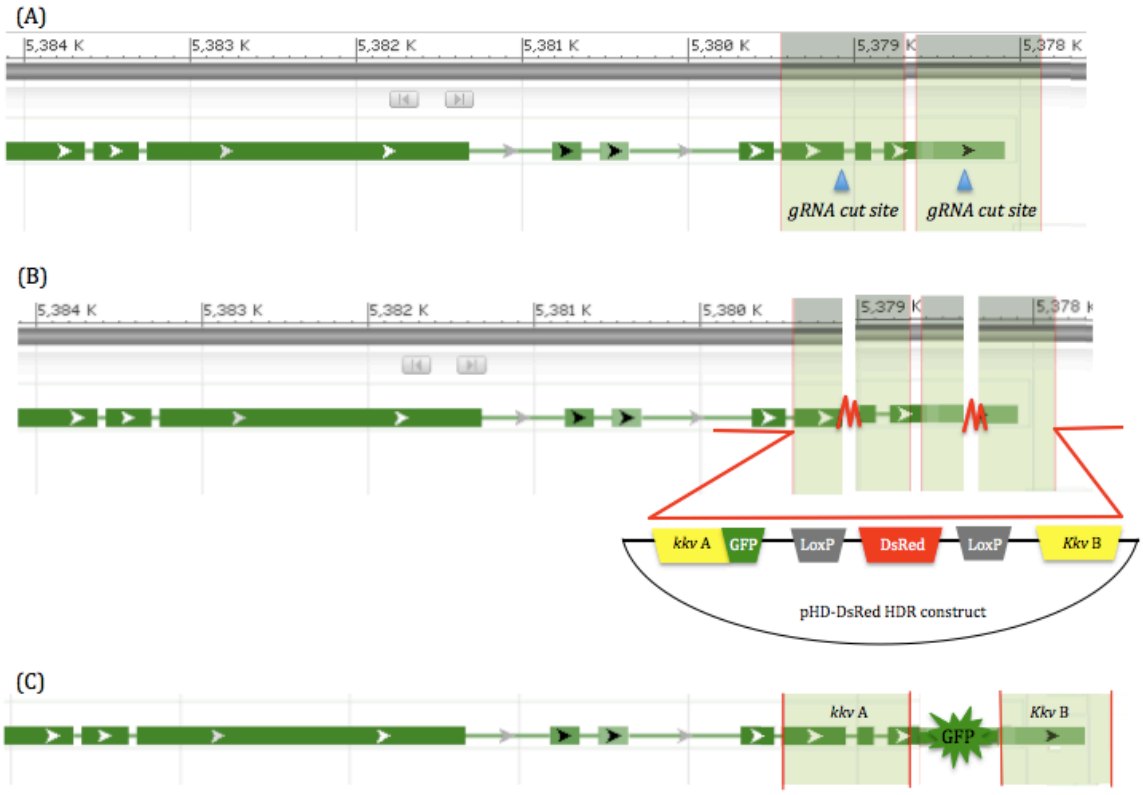

Figure 15 – HDR and 2gRNA: 2 gRNAs will be used to target regions in Domain A of *kkv* to induce double strand breaks. A HDR template with homology arms to *kkv* and a GFP tag will be provided to repair these regions while tagging our enzyme.

This construct could also allow us to understand the rotational configuration of the cuticle (Fig. 2B, 2C). When the chitin polymer is secreted into the ECM, it arranges in a pattern to form sheets called laminae. These sheets stack up on each other with a small rotation in each layer allowing for the formation of a helicoidal structure. An endogenous GFP tag could potentially allow us to visualize this stacking effect. One attempt to co-inject a *pCFD4* with two gRNAs along with the HDR construct has been made, however, we did not obtain any transformants. Both constructs were checked again via sequencing and PCR to confirm that they were appropriately made. There is also the issue with efficiency of a given gRNA. Although there are a great variety of websites available to provide potential gRNA sequences, there is very little overlap between them. While a particular gRNA may have a high efficiency score on one site, the same gRNA may prove insufficient when cross-checked with a second source. This proved to be true with our first set of gRNAs. We checked the efficiency of these gRNAs in lab by injecting them separately into a *P{nos-phiC31\int.NLS}X, P{CaryP}attP40* fly line (on the 2nd chromosome). The G_0 transformant siblings with vermillion eyes, a marker present on the vector, were crossed to each other in order to increase progeny count. F_1 virgin females were then crossed with *yv; CyO/Gla* balancer males and virgin female progeny with *yv; gRNA/CyO* was collected. These flies had wt red eyes with curly wings. This stock was then crossed to one of three Cas9 nucleases, *act-Cas9, nos-Cas9*, and *vas-Cas9*. If the gRNA worked as expected, larvae should be seen but no flies; we did however see progeny. Female progeny were observed under the scope and equal ratios of curly to straight winged flies were seen. Those with straight wings showed wt phenotypes however, due to the low efficiency values of these gRNAs, 1-2 flies with bent wing hinges were seen per vial. CS does produce chitin in that particular region so it is possible that the gRNAs were cutting the CS expressed there. It is also possible that the wing was damaged in the fly vial. These results suggest that the gRNAs were not efficient. Further imaging of these wings must be done to understand other possible phenotype changes. Moving forward, the regions we targeted with our HDR were reevaluated for other gRNA targets using both http://crispr.mit.edu/ and https://chopchop.rc.fas.harvard.edu/; two new gRNAs

with the highest efficiency scores from both sites were used to make a second *pCFD4* construct described in the method section above.

Besides gRNA efficiency levels, there is a chance that our gRNA construct is targeting our HDR construct instead of the genomic *kkv* since both the gRNA and HDR construct are injected together. This issue might be resolved if the amount of gRNA and Cas9 nuclease injected is reduced or if several base pairs in the homology arms are mutated. Although slightly rare, there is the potential for polymorphisms to exist in the region being targeted. One could resolve this issue through PCR and sequencing of several injected flies.

9.3.2 ssODN Repair Template and gRNA constructs

Another way to endogenously tag *kkv* is through the use of an attP site. attP sites are regions for site specific recombination reactions. One can insert this site through the use of an ssODN repair template. It works exactly like the HDR and gRNA constructs but instead of inserting a tag, we are inserting an attP site for future purposes and the gRNAs are in separate *pCFD3* vectors instead of one. The *pCFD3* constructs are slightly easier to make and contain the optimal *U6:3* promoter. The ssODN construct and two gRNAs will be co-injected into a *nos-Cas9;kkv*^{PB} and *yw vas-cas9;kkv*^{PB} fly line developed in our own lab. For screening purposes, we choose to target the attP to a region that also contains a transposable element insertion named *kkv* ^{PB} marked with white^{+} near the 5' end (Fig. 16A). Flies with this mutation are viable. The oligonucleotide provided as a repair template contains an attP site and four point mutations to the homology arm. We expect insertion of the oligonucleotide will result in deletion of the transposon, hence all transformants with the attP site insert should show a loss of eye color (Fig. 16B). In the future, we could use the attP site to insert any desired DNA that contains an attB site. A separate repair construct using the pattB vector will be made encompassing the region between the two gRNAs used in the *kkv* ^{PB} injection along with a GFP tag. This construct will rescue the wt phenotype for *kkv* and tag the gene at the same time (Fig. 16C).

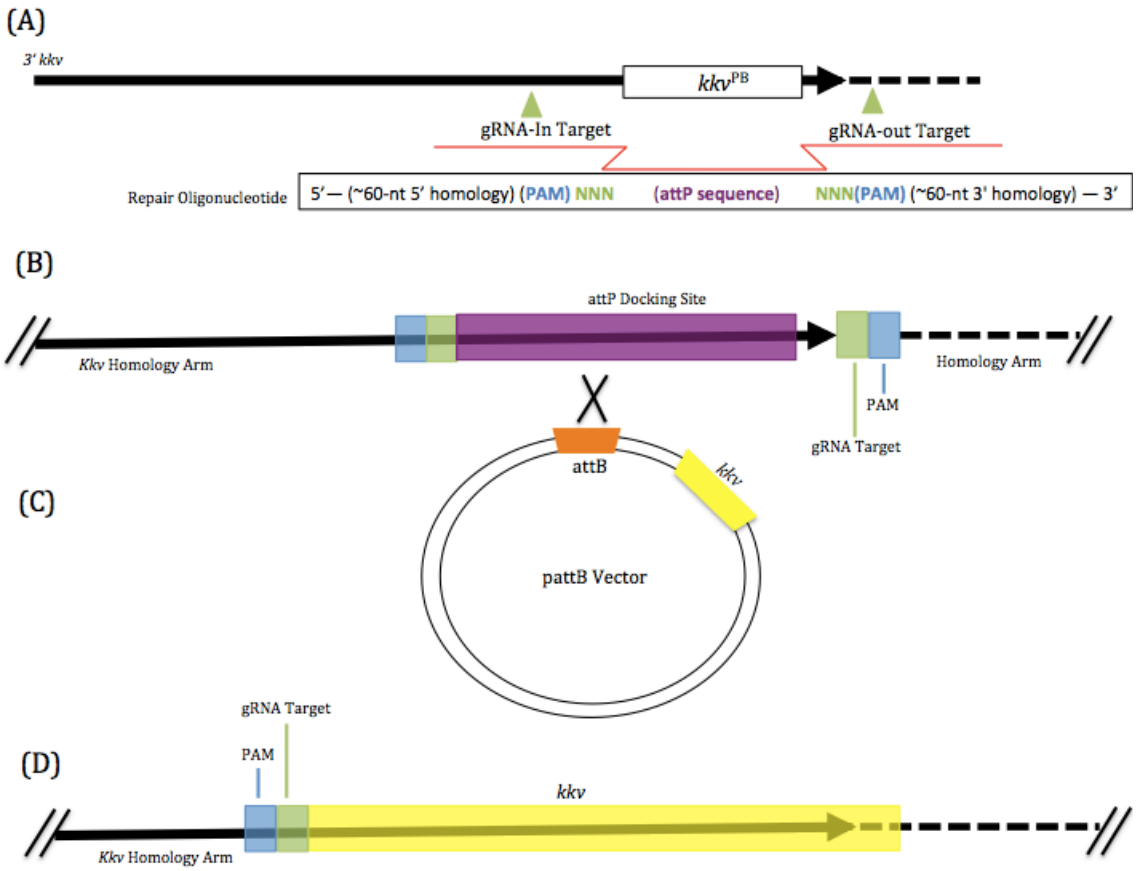

Figure 16 – *kkv*^{PB} ssODN gRNA and Repair Template – (A) *kkv*^{PB} is a non-lethal transposable mutation near the 5' end of *kkv*. It is targeted to provide a method of screening for insertion of an attP site. Two gRNAs incorporated into separate *pCFD3* vectors will target regions surrounding this mutation creating two double stranded breaks. The repair template is an oligo that contains homology arms for the break, and the attP docking site. (B) The oligo will repair the cut sites while incorporating an attP-docking site in between. This attP site will allow for site specific recombination reactions whereby the attP site can be replaced with a specific marker, mutations, etc. (C) One such recombination reaction includes replacing the attP site with *kkv* incorporated into an pattB vector. (D) This would result in replacement of attB with the wt *kkv*.

10. Conclusion

Arthropods are a very abundant and diverse species. A common element among them however is a tough exoskeleton that helps to protect the organism from external harm, dehydration, etc. The underlying element of the exoskeleton is the cuticle, made up of a polymer called chitin produced by chitin synthase. Understanding the mechanism via which CS functions and localizes will open doors to (1) analyzing the fundamental aspect of the cuticle itself and (2) means of controlling its activity. The latter will be especially important because CS is also found in the cell walls of fungi, a common parasite to livestock, agriculture and

humans. The means to understanding this information will come through the use of CRISPR/Cas9 among other molecular and biochemical techniques. While our projects are only just beginning, it leaves us much room to understand the fundamentals of both CS and CRISPR/Cas9 while improving on the techniques for the latter.

11. Citation

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