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Functional Characterization of a Cathepsin L in Drosophila Melanogaster

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FUNCTIONAL CHARACTERIZATION OF A CATHEPSIN L IN DROSOPHILA MELANOGASTER

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The Faculty of the Department of Biology
Western Kentucky University
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Master of Science

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FUNCTIONAL CHARACTERIZATION OF A CATHEPSIN L IN DROSOPHLA MELANOGASTER

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The *Drosophila* dorsal Air Sac Primordium (ASP) is a tracheal tube that invasively grows toward and into the wing imaginal disc. The unfolding of *Drosophila* wing is a process following eclosion with a cuticular bilayer replacing epithelial cells originally packing the wing. We reasoned that protease functions might be needed for the invasion of ASP into the wing imaginal disc as well as the rearrangement of epithelia cells during wing unfolding. Our study is particularly focused on understanding the role of a Cathepsin L like cysteine protease (CP1) in the development of dorsal ASP and wing development of *Drosophila melanogaster*. To analyze the function of CP1, we overexpressed and knocked down CP1, respectively, using UAS-GAL4 system in combination with RNA interference technology.

We found that both the knockdown and overexpression of CP1 in ASP resulted in perturbed growth, migration and weakened invasion of ASPs. We further explored the mechanism by which CP1 regulates ASP development and found that CP1 is capable of degrading collagen IV, a component of extracellular matrix. For wing development, we observed that both the knockdown and overexpression of CP1 in wing imaginal discs interrupted with normal wing development.
In summary, our study demonstrated that CP1 facilitates the normal development of ASPs by degrading extracellular matrix and regulates wing development via a complex network of signaling pathways and protein interactions. Knowledge gained from this study has the potential to help us better understand the invasion of tumor cells through the extracellular matrix in humans.
1. INTRODUCTION

1.1 Development and extracellular matrix remodeling

The process of biological development from a single cell to a highly complicated living organism is an amazing feat of tightly regulated cellular behaviors including cell proliferation, specialization, interaction and movement. A single cell produces many cells and simultaneously creates cells with different characteristics at different locations in the organism. Cells also interact with each other and rearrange to form structured tissues and organs.

Outside of the cell, macromolecules like glycosaminoglycans, proteoaminoglycans and proteins including collagens, elastins, fibronectins and laminins interact with each other as well as cell-surface receptors to form interlocking networks. This extracellular matrix (ECM) serves to provide structural support for surrounding cells, acts as a cushion against mechanical stress, facilitates extracellular signal transmissions and determines the distribution of morphogens and growth factors (ROZARIO and DESIMONE).

Cellular behaviors especially cell migration are inevitably associated with breakage of cell-cell adhesion and cell-ECM adhesion, the maintenance of which is mediated by cell junction proteins (Figure 1A). Cadherins (Figure 1B), one of the most important cell adherens junction proteins, are named for their dependence on Ca\(^{2+}\) to function. They regulate cell-cell adhesion by connecting to cytosolic actin filaments via catenin and forming dimers in the extracellular compartment (GUAN et al.). Collagens, the most abundant components of the ECM, are connected to cell-surface integrins by binding to fibronectins (Figure
1C). Cellular behaviors are responsive to extracellular signals like morphogens and growth factors, the distribution of which is affected by molecules like heparan sulfate proteoglycans (HSPGs). Composed of a protein core and two unbranched heparin sulfate glycosaminoglycan (HSGAG) chains, they can either be tethered to the cell membrane or be a part of the ECM components.

The synthesis, modification and degradation of molecules mentioned above are in a balance to facilitate development. Changes of these molecules could either be the results of normal development or the causes of abnormal development (ROZARIO and DESIMONE 2010). Here, we will consider two possible roles of ECM remodeling—assisting cell movement and affecting morphogen distribution in the regulation of development.
Figure 1. Cell-cell adhesion and cell-matrix adhesion mediated by cell junctions

Five different types of cell junctions in epithelial cells are shown in (A). The basal surface of the cell is facing the extracellular matrix. (B) is the enlargement of adherens junction in (A). β-catenin connects actin filaments with E-cadherin dimers. (C) is the enlargement of focal adhesion in (A). One end of transmembrane integrin is attached to actin filaments in the cytoplasm, and the other end is connected to fibronectins, which is further interacting with collagens. (Adapted from Figure 1 in (JEFFERSON et al. 2004) and two figures from google images. Sources: [http://jonlieffmd.com/wp-content/uploads/2013/11/ECM-INTEGRIN-.jpg](http://jonlieffmd.com/wp-content/uploads/2013/11/ECM-INTEGRIN-.jpg); [http://pixgood.com/adherens-junction.html](http://pixgood.com/adherens-junction.html) )
1.2 Proteases

Proteases are enzymes that can degrade proteins by hydrolyzing peptide bonds. In the *MEROPS* database (an information resource for peptidases), they are classified by “clans” and “families”. For example, Cathepsin A belongs to family C1 within the CA clan (RAWLINGS *et al.* 2008). Based on optimal pH, they can be classified as acid proteases, neutral proteases and basic proteases. Based on catalytic residue, they can be classified as serine proteases, metalloproteinases, aspartic acid proteases, threonine proteases, cysteine proteases and glutamic proteases.

*Serine protease*

The active site of serine proteases is a catalytic triad, composed of three essential amino acids. These amino acids are located far from each other on the amino acid, but are closely assembled by folding. These amino acids are histidine, serine and aspartic acid with serine acting as nucleophile. In Figure 2A, aspartic acid forms hydrogen bonds with histidine. This linkage increases the potential of histidine to pull protons from serine. Lacking hydrogen, the oxygen of serine becomes more negative, ready to attack the terminal carbon of peptides. Also, some serine proteases only recognize specific amino acid residues. For instance, chymotrypsin, secreted by pancreatic cells, cleaves at the C-terminal side of tyrosine, phenylalanine and tryptophan. Besides playing multiple roles in digestion, blood clotting and immune response, serine proteases like plasmin, neutrophil elastase and Cathepsin G are also “active players” in ECM remodeling (CAWSTON and YOUNG 2010; SMITH and MARSHALL 2010).
Figure 2. The catalytic mechanisms employed by various proteases

(A) : Serine proteases
(B) : Metalloproteinase
(C) : Aspartic proteases
(D) : Cysteine proteases

Hydrogen bonds are shown in blue dotted lines.

The negative oxygen in (A), (B) and (C) and the negative thiol group in (D) are positioned to attack the terminal carbon of peptides. These are indicated by red arrows.

(Adapted from http://www.nature.com/scitable/content/protease-mechanisms-14462487)
**Metalloproteinase**

Three catalytic mechanisms have been proposed to explain the hydrolytic chemistry of zinc-dependent metalloproteinase: the interaction between a glutamate residue and the zinc ion, the interaction between a water molecule and the zinc ion and the interaction between a glutamate residue, a histidine residue and the zinc ion (shown in Figure 2B). Here, a molecule of water is recruited as an electrophilic agent to attack the carbonyl oxygen of the substrate. Matrix metalloproteinases (MMPs) are the main enzyme responsible for the degradation of extracellular matrix, thus, they play a central role in development, morphogenesis, tissue remodeling and metastasis, processes featured by ECM degradation. For example, MMP3 which degrades fibronectin, laminin and collagen IV, facilitates the collective epithelial migration of the mammary gland of female mice (WiseMAN et al. 2003). *Drosophila melanogaster* has two MMP homologs: MMP1 and MMP2. MMP1 is required for normal tracheal development by promoting tracheal tube elongation (GLASHEEN *et al.* 2010). MMP2, a membrane tethered protease targeting collagen IV and perlecan in the ECM, regulates Fly early embryo trachea branching morphogenesis (GUHA *et al.* 2009).

**Aspartic protease**

Similar to metalloproteinases, aspartic proteases also rely on a water molecule, which binds to aspartic residues, to catalyze substrate degradation (shown in Figure 2C). However, they are rarely reported to degrade extracellular
proteins. This is because the pH of extracellular space under physiological
conditions is between 7.2 and 7.5, whereas, aspartic proteases depend on acidic
pH to function. Aspartic proteases are involved in the regulation of blood
pressure (Renin), digestion (Pepsin) and HIV life cycle (HIV protease) (DAVIES
1990).

Threonine protease

A nucleophile threonine residue is harbored in the active site of a
threonine protease to execute degradation of the substrate. In 2012, SCHAUER et
al. reported that threonine protease participate in the regulation of the N- and C-
terminus degradation of ecdysteroid receptor isoforms (SCHAUER et al. 2012).

Cysteine protease

The strategy cysteine proteases use to degrade the substrate is similar to
that of serine proteases. The functional residue of cysteine protease is a cysteine
rather than a serine. Once histidine pulls hydrogen away from the cysteine thiol,
the cysteine thiol becomes anionic, ready to attack the substrate (described in
Figure 2D).

1.3 Cysteine cathepsins/ proteases

Cysteine cathepsins were first described by Willstätter and Barmann in
1929 as acid proteases distinct from pepsin in the gastric mucosa of pig and dog.
Cathepsin means to “digest”. It belongs to the papain subfamily of clan CA
(RAWLINGS et al. 2010). In 1941, cathepsins were first classified as cathepsins I,
II, III. Later in 1952, they were reclassified as Cathepsins A, B, C with respect to
the substrate they act on. In humans, cysteine cathepsins are classified into Cathepsin B, C, F, H, L, K, O, S, V, W, and X (Rawlings et al. 2010), based on their structure, substrate specificities and catalytic mechanism. Cysteine cathepsins were first regarded as intracellular enzymes, because they localize within lysosomes, late endosomes and vesicular compartments. Lysosomes can protect the cysteine thiol from oxidation, and provide an acidic pH for cysteine proteases to function. Numerous studies, however, have shown Cathepsin B, K and L take part in the degradation of ECM proteins such as proteoglycan, Agreiccan, Elastin, Fibrillar, Fibril associated collagens with interrupted triple helices (Facit), Fibronectin, Laminin, Osteocalsin and Osteonectin. Particularly, Cathepsin L has also been shown to degrade basement membrane (type IV), a dense sheet lining the basal site of epithelial cells, in-vitro (Guinec et al. 1993). Potential explanations of why Cathepsin L can function in the ECM are proposed as follows: On one hand, the dependence of cathepsins on low pH is not that strict. For instance, Pro-Cathepsin L gains its active form at pH 3. However, with the presence of a negatively charged surface, the activation can occur even at pH 5.5 (Mason and Massey 1992). On the other hand, in some pathological processes, pericellular or extracellular environments could possibly be acidified and lead to activation of Cathepsin L. It was believed that oxidative ECM might inactivate cysteine cathepsins, however this is rejected by the fact that Cathepsin B, L, K degrade thyroglobulin at pH 7.4 and under oxidative stress (Jordans et al. 2009).
The importance of cysteine cathepsins

Cysteine cathepsins play a multitude of roles in human physiological processes. Cathepsin B and L are predicted to take part in digestion. Cathepsin K and Cathepsin S are confirmed to be involved in bone remodeling and MHC type II antigen presentation, respectively. Beers et al. mentioned the involvement of Cathepsin S in the late stages of invariant chain degradation in B cells, dendritic cells and macrophages (BEERS et al. 2005). Duong et al. described that Cathepsin K, produced by osteoclasts, mediates the degradation of type I collagen, the major constituent of bone organic matrix. He claimed that the inhibition of Cathepsin K may provide a possible way to cure osteoporosis by reducing bone resorption and maintaining bone formation (DUONG LE). Sun et al. demonstrated that Cathepsin L attenuates cardiac hypertrophy by autophagic lysosomal protein processing (SUN et al.). The dysregulation of cysteine cathepsins is also associated with human pathological processes especially cancer progression (shown in Table 1). A better understanding of cysteine cathepsins’ role in human pathogenesis would enrich our knowledge about how cancer progresses and bring us closer to a cancer cure. The knowledge gained could also be utilized to design potential therapeutics for diseases such as Alzheimer's disease, Cardiac disease.
<table>
<thead>
<tr>
<th>Name</th>
<th>Gene symbol</th>
<th>Clinical significance</th>
<th>References</th>
</tr>
</thead>
</table>
2. Esophageal adenocarcinoma and other tumors.  
3. Ovarian cancer.  
(HABIBOLLAHI et al. 2012)  
(LIU et al. 2006)  
(LIU et al. 2013) |
| Cathepsin C  | CTSC        | Function in the Activation of granule serine proteases in inflammatory cells. So it is involved in some inflammatory diseases.                                                                                             |                                     |
| Cathepsin H  | CTSH        | Malignant progression of prostate tumors                                                                                                                                                                                | (JEVNIKAR et al. 2013)             |
| Cathepsin K  | CTSK        | Breast cancer  
Osteoporosis                                                                                                                                                                                                       | (GOCHEVA and JOYCE 2007)  
(COSTA et al. 2011) |
| Cathepsin L  | CTSL        | Colorectal and breast cancer  
Heart diseases                                                                                                                                                                                                       | (SULLIVAN et al. 2009)  
(SUDHAN and SIEMANN 2013)  
(WANG et al. 2009) |
| Cathepsin O  | CTSO        | Not known                                                                                                                                                                                                              |                                     |
| Cathepsin S  | CTSS        | Heart diseases and cancer                                                                                                                                                                                               | (TSAI et al. 2013)                |
| Cathepsin W  | CTSW        | Not known                                                                                                                                                                                                              |                                     |
| Cathepsin Z  | CTSZ        | Cancer                                                                                                                                                                                                                 | (ZHANG et al. 2013)                |
Cathepsin L and Cancer

Cysteine cathepsins, particularly Cathepsin B and Cathepsin L, contribute to cancer metastasis and angiogenesis by degrading ECM. Secreted Cathepsin L promotes cancer metastasis by degrading basement membrane and ECM proteins like elastin. It has been shown to be the most efficient in degrading collagen XVIII, a component of vascular and epithelial basement membrane. Following the degradation of the terminal end of collagen XVIII, anti-angiogenic factor endostatin accumulates. In this case, Cathepsin L occludes angiogenesis. It can also facilitate angiogenesis by degrading endostatin fragments (FELBOR et al. 2000).

In addition to existing in lysosome and endosome, Intracellular Cathepsin L has also been localized to the nucleus and cytoplasm of the cell (BULYNKO et al. 2006; DUNCAN et al. 2008; GOULET et al. 2004; SEVER et al. 2007; VARANOU et al. 2006). Based on this, it was further predicted to regulate the expression of both oncogene and tumor suppressors due to the aberrant activity of intracellular proteases (GOULET et al. 2007).

1.4 Drosophila melanogaster: A useful genetic model organism to study

Cathepsin L

The fruit fly, Drosophila melanogaster, is an intensively studied genetic model organism with a short life cycle (Figure 3), easily identified morphological features and extensively studied genome. In 2001, it was first reported that approximately 75% of human disease-related genes have functional homologs in Drosophila melanogaster (REITER et al. 2001). Pandey et al. claimed that a
significant number of genes involved in fly development are also vital in mammalian development and that the function of adult fly’s structures mimicked mammalian heart, lung, kidney, gut and reproductive tract (PANDEY and NICHOLS 2011). These altogether place *Drosophila melanogaster* at the forefront of studies concerning development and diseases in addition to the study of genetics.

For investigating the function of cysteine cathepsins, *Drosophila melanogaster* turns out to be a superior model organism to others. This is because there are more cysteine proteases found in model organisms like rats and mouse, which makes it hard to control cathepsin gene expression. In 2004, PUENTE and LOPEZ-OTIN annotated 160 rat cysteine proteases and 153 mouse cysteine proteases (PUENTE and LOPEZ-OTIN 2004). Comparatively, only one Cathepsin L like cysteine proteinase is identified in *Drosophila melanogaster*. Taking advantage of the well-established UAS/ GAL4 system and in conjunction with RNA interference technology, we can easily overexpress and knock down the gene coding for Cathepsin L in *Drosophila melanogaster* to analyze how Cathepsin L functions in ECM remodeling during development.
Figure 3. The life cycle of *Drosophila melanogaster*

It takes about 1 day for the eggs to hatch into the 1\textsuperscript{st} instar larva. Then, at about 24 hours after hatching, it continues to molt twice (2\textsuperscript{nd} instar larva and 3\textsuperscript{rd} instar larva). Upon pupation, it will undergo metamorphosis and eclose as an adult fly.

(Adapted from https://www.flickr.com/photos/11304375@N07/2993342324/, Table 8.2 of http://www.d.umn.edu/~pschoff/documents/08Drosophila.pdf)
**UAS/ GAL4 system**

UAS/ GAL4 system was introduced by Andrea Brand and Norbert Perrimon in 1993 as a powerful technique to study gene expression and function in organisms like *Drosophila*. The system is composed of two parental lines: the driver and the responder. For the responder line, the initiation of the transcription of the targeted gene needs GAL4 protein to bind to the Upstream Activating Sequence (UAS). For the driver line, the expression of GAL4 is also controlled by a regulatory gene. Only by mating the two lines together can we get the targeted gene transcribed in a pattern that reflects the GAL4 expression pattern of the driver (DUFFY 2002).

**RNA interference theory**

RNA interference technology is used in combination with GAL4/UAS system to knock down the expression of targeted gene in *Drosophila melanogaster*. As shown in Figure 4, a DNA fragment from CP1 capable of generating RNA that folds onto itself is cloned downstream of the UAS site. Once GAL4 protein binds to the UAS site, double stranded RNA is generated. Dicer approaches the double stranded RNA and cleaves it into small interfering RNAs (siRNAs), which are then separated into single strands and integrated into the RISC complex. The siRNAs with RISC complex base-pair with targeted mRNA and cleave it, thus preventing translation (DUFFY 2002).
Figure 4. Map of the UAS-CP1 RNAi construct

A DNA fragment from CP1 capable of generating an RNA that folds onto itself (blue arrow) was inserted to the vector under the regulation of Upstream Activating Sequences (UAS). Once GAL4 protein binds to the UAS sites, the double stranded RNA is produced. The expression of double stranded RNA is regulated by an upstream hsp70 promoter. A SV40 terminator is placed downstream of the CP1 DNA fragment (blue arrow) to terminate the expression of the targeted gene. (DUFFY 2002)
1.5 CP1: a Cathepsin L like cysteine protease in *Drosophila melanogaster*

The protein CP1 consists of 341 amino acid residues. It is composed of a Cathepsin L like endopepdidase and an inhibitor I_29 domain at the N-terminus (shown in Figure 5). The presence of inhibitor I_29 prevents the substrate from approaching the active site and the subsequent cleavage of this domain can activate the zymogen. Besides, it is also involved in the proper folding, stabilization and transportation of newly synthesized CP1 (GROVES et al. 1996; OLONEN et al. 2003).

CP1 was first shown to be expressed in the gut cells, garland cells, salivary gland and macrophages of *Drosophila melanogaster* (MATSUMOTO et al. 1995). Two years later, Tryselius and Hultmark localized CP1 in small granules in hemolytic mbn-2 cells of *Drosophila melanogaster*, suggesting a possible participation of CP1 in the immune response (TRYSELIUS and HULTMARK 1997). Most recently in 2012, Kinser and Dolph demonstrated that CP1 is involved in the mediation of retinal degradation of several *Drosophila* mutants (KINSER and DOLPH 2012). On the basis of these expression data, CP1 was thought to be required for the normal development of *Drosophila*.

Here, we showed that CP1 is also expressed in *Drosophila* dorsal ASP and examined what role it plays in both ASP development and wing development.
Figure 5. The protein sequence of CP1

The blue region (residue 29~88) represents the inhibitor I_29. The red region (residue 125~339) represents peptidase C1A domains. The four orange diamonds denote active sites of CP1 and the six green squares denote the S2 subsites. The numbers above the bar and below the bidirectional arrow indicate the location of amino acids.

1.6 *Drosophila* Dorsal Air Sac Primordium Development

*Drosophila* air sacs are critical organs as they function like human lung to supply adult thoracic flight muscles with oxygen. The progenitor of air sacs, Air Sac Primordium (ASP), comes into being as an outgrowth (Figure 6) budding over the transverse connective (TC) from the early stage of 3rd instar. It grows into the wing imaginal discs at the very late stage of 3rd instar in response to FGF signaling. Developing ASP cells are, by nature, epithelial cells with apical sides facing the ASP lumen (CABERNARD and AFFOLTER 2005). At this stage, they form a single layer of long pointed sac-like epithelial sheet with a distal “tip”, characterized by tip cell filopodia (shown in Figure 6). Filopodia are actin-based lamellipodia extensions, the presence of which indicates directed cell migration. During late third instar larva, these actin-based lamelipodial extensions as well as ASP invade the basement membrane and are finally integrated into wing imaginal discs (Reviewed from (CABERNARD and AFFOLTER 2005; GUHA and KORNBERG 2005; GUHA et al. 2009; SATO and KORNBERG 2002).

To create an organized epithelia-like structure, neighboring cells need to coordinate themselves and adhere to each other in an appropriate way. This linking between epithelial cells is actually maintained by cell adherens junction complex (See Section 1.1), including E-cadherin. The extracellular domain of E-cadherin which mediates homophyllic adhesion and its intracellular domain indirectly connects to actin filament via β catenin. The *Drosophila* segment polarity gene *armadillo* is a homolog of the gene coding for vertebrate adherens junction protein β catenin (PEIFER et al. 1993). In *Drosophila*, E-cadherin and
Armadillo bind one cell to another. Any changes in the expression of these two proteins indicates changes in cell-cell interaction. In the case of ASP development, rearrangement of cell adherens junction proteins would be necessary for ASP migration.

Branchless, a *Drosophila* Fibroblast growth factor (FGF) homolog secreted from the columnar cells of wing imaginal discs (SATO and KORNBerg 2002), is the chemoattractant orchestrating the directional growth of ASPs. *Breathless*, a gene encoding for FGF receptors, is primarily expressed in *Drosophila* tracheal cells and ASP cells. In humans, FGF signaling is essential for angiogenesis, wound healing and the development of branched organs such as lungs and mammary glands (KNIGHTON et al. 1990; PETERS et al. 1994). Hence, the study of ASP development may also aid our understanding in how FGF signaling regulates normal development and tumorigenesis caused by abnormal cellular behaviors.

Heparan sulfate proteoglycan (HSPG), composed of a protein core and two unbranched heparin sulfate glycosaminoglycan (HSGAG) chains, is a modulator of FGF’s binding to FGFRs. The molecule can either be tethered to the cell membrane as the transmembrane syndecan and GPI anchored glypican, or be a part of extracellular components such as perlecan, agrin and collagen XVIII (BLACKHALL et al. 2001). As HSPG contains different structural motifs for FGFs and FGFRs, respectively, the balance between this binding dictates whether HSPG is a FGF signaling stimulator or a FGF signaling inhibitor. Dally-like, a *Drosophila* glypican, has been shown to be expressed in ASP cells. Yan
and Lin argued that Dally-like serves as co-receptors of Bnl signals (YAN and LIN 2007).
Figure 6. Schematics of air sac primordium development

Dorsal trachea (DT), transverse connective (TC) and air sac primordium (ASP) are shown green. Branchless (Bnl) is highlighted with orange. The region which will develop into adult wing hinge is shown in a black oval ring. The region which will develop into adult wing margin is shown in the centre line of the black oval ring.
1.7 *Drosophila* Wing Development

The *Drosophila* wing is derived from the wing imaginal disc, an epithelial sac consisting of up to 75,000 cells during late third instar (WUNDERLICH and DEPACE 2011). From a flat “map” to a complicated 3-D structure, wing imaginal discs comprise all the wing primordia including hinge, blade, margin and notum, the patterning of which is regulated by the activity of genes governing different compartments (GARCIA-BELDIO *et al.* 1973). Among these genes, *wingless* is one of the most extensively studied probably because it is required for the formation of wings and other adult body structures. More importantly, *wnt1*, the vertebrate homolog of *wg*, is a known proto-oncogene. Due to the fact that the mode of gene regulation is evolutionarily conserved among different species, it is advantageous to explore the role of WNT signaling pathway in human disease using *Drosophila* as the model organism.

In the wing imaginal discs of the third larva instar, *wg* is expressed in a specific pattern: two concentric rings, Dorsal/Ventral compartment boundary dividing the rings and particular region of the dorsal part of the wing imaginal disc (See Figure 7). Secreted from the D/V boundary, the *wg* ligand diffuses away and activates its target genes in a concentration dependent manner (SWARUP and VERHEYEN 2012). It has been proposed that the extracellular wingless gradient is regulated by the level of glypican, a heparan sulfated proteoglycan encoded by *Dally* gene and *Dally-like* (DLP) gene in *Drosophila melanogaster*. It would be interesting to further explore how glypican influences the distribution of wingless.
Cut, a gene encoding for a homeodomain transcriptional factor, is also expressed in the D/V boundary and functions to form the adult wing margin. In humans, cathepsin L has the ability to cleave Cut-like 1, the human homolog of Cut. The cleavage further impacts the transcriptional properties of Cut-like 1 (LYONS et al. 2014). We are interested in whether CP1 also affects the distribution and expression of Cut in Drosophila.

Under the guidance of a diversity of gene products, the wing imaginal disc evaginates with two layers of flattening cells apposed to each other as the dorsal and ventral surfaces of the wing during pupal metamorphosis. Upon eclosion, the wings previously folded within the pupal case expand to form a cuticular bilayer and the epithelial cells packing the wing are dissolved (HARTENSTEIN et al. 1997; KIGER et al. 2007). The degeneration of epithelial cells is inevitably associated with the breakdown of corresponding structural supporter (ECM) for cells. This suggests that CP1, which participates in the remodeling of ECM, could be required in the process of wing unfolding.
Figure 7. The expression pattern of *wg* in the wing imaginal disc of the third larval instar

*Wingless* is expressed in Dorsal/ Ventral compartment boundary, two concentric rings and part of the dorsal area (shown in yellow).
2. MATERIALS AND METHODS

2.1 Fly stocks and Culture

Fly maintenance and crosses were conducted at 25 °C (Unless stated otherwise) in vials and bottles according to standard procedures. A few pellets of Red Star® active dry yeast were added to bottles and vials before culturing to provide additional nutrition.

Fly stocks were obtained from Bloomington Drosophila Stock Center. The UAS-CP1 RNAi line used in this study was obtained from the Vienna Drosophila RNAi Center. The UAS-CP1 3XHA was ordered from Fly ORF. Genotypes of stocks used in this study are given in Table 2 and Table 3. Genotypes used in figures are given in Table 4.
**Table 2. Stocks used for ASP development**

<table>
<thead>
<tr>
<th>Lines</th>
<th>Description and references</th>
</tr>
</thead>
<tbody>
<tr>
<td>CP1 protein trap</td>
<td>The distribution of CP1 was observed using CP1 protein trap (BUSZCZAK et al. 2007)</td>
</tr>
<tr>
<td>Btl-Gal4, UAS-actin5C</td>
<td>Btl-Gal4 (SHIGA et al. 1996) is a promoter that expresses gal4 in trachea and ASP. It is used to drive the expression of UASp-Act5C GFP (KELSO et al. 2002). The flybase ID: FBst0008807</td>
</tr>
<tr>
<td>GFP/CyO-lacZ</td>
<td></td>
</tr>
<tr>
<td>UAS-CP1 RNAi/CyO; UAS-DCR2/TM6Tb</td>
<td>UAS-CP1 RNAi on the second chromosome can effectively knock down CP1. This was found by Breanna Brenneman. UAS-DCR2 (FBti0100276) is described in the indicated Flybase reference. The described line is created in this study.</td>
</tr>
<tr>
<td>UAS-CP1 3XHA</td>
<td>UAS-CP1 3XHA (FBst0500649) is used to overexpress CP1(SCHERTEL et al. 2013)</td>
</tr>
<tr>
<td>Ptc-Gal4, UAS-Src-RFP/CyO</td>
<td>Ptc-Gal4 (FBti0002124) line is described in the indicated Flybase reference. This line was created by Dr. Ajay Srivastava.</td>
</tr>
</tbody>
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### Table 3. Stocks used for wing development

<table>
<thead>
<tr>
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<th>Description and references</th>
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<td>Sd-Gal4</td>
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<td>Cg-Gal4</td>
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<tr>
<td>Vg-Gal4 (BF2)</td>
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<tr>
<td>GMR-Gal4/CyO</td>
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<tr>
<td>Bx-Gal4</td>
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</tr>
<tr>
<td>Tub-Gal4/TM6Tb</td>
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<td>Vg-Gal4, UAS-SrcRFP</td>
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<tr>
<td>Pnr-Gal4</td>
<td>Flybase ID: FBti0004011</td>
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<td>Btl-Gal4, UAS-actin-5cGFP/CyO-lacZ</td>
<td>Flybase ID: FBst0008807</td>
</tr>
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<td>LSP2-Gal4, UAS-GFPnls/TM6Tb</td>
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<td>Twist-Gal4</td>
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<td>Vg(Q)-Gal4</td>
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<td>En-Gal4</td>
<td>Flybase ID: FBst1001713</td>
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<td>w^{1118}, UAS-DCR2; nubbin-Gal4</td>
<td>UAS-DCR2 (FBti0100276) and Nubbin-Gal4 (FBti0150342) are described in the indicated Flybase reference.</td>
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<tr>
<td>UAS-CP1 RNAi</td>
<td>UAS-CP1 RNAi on the second chromosome can effectively knock down CP1. This is found by Breanna Brenneman.</td>
</tr>
<tr>
<td>UAS-CP1 3XHA</td>
<td>UAS-CP1 3XHA (FBst0500649) is used to overexpress CP1 (SCHERTEL et al. 2013)</td>
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<tr>
<td>Ptc-Gal4, UAS-Src-RFP/CyO</td>
<td>Ptc-Gal4 (FBti0002124) line is described in the indicated Flybase reference. This line is created by Dr. Ajay Srivastava.</td>
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Table 4. Genotypes of strains shown in figures

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<th>Figure No.</th>
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<td>Figure 8</td>
<td>(A-F): Btl-Gal4, UAS-actin-5cGFP/CyO-lacZ</td>
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<td>Figure 9</td>
<td>(A-C) and (A’-C’): CP1 protein trap</td>
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<td>Figure 10</td>
<td>(A): Btl-Gal4, UAS-actin-5cGFP/CyO-lacZ</td>
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<td>(B): Btl-Gal4, UAS-actin-5cGFP/UAS-CP1 RNAi; UAS-DCR2/+</td>
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<td>Figure 11</td>
<td>(A): Btl-Gal4, UAS-actin-5cGFP/CyO-lacZ</td>
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<td>(B): Btl-Gal4, UAS-actin-5cGFP/+; UAS-CP1 3XHA/+</td>
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<td>Figure 12</td>
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<td>Btl-Gal4, UAS-actin-5cGFP/UAS-CP1 RNAi; UAS-DCR2/+</td>
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<td></td>
<td>Btl-Gal4, UAS-actin-5cGFP/+; UAS-CP1 3XHA/+</td>
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<td>Figure 13</td>
<td>(A and C): Btl-Gal4, UAS-actin-5cGFP/CyO-lacZ</td>
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<td></td>
<td>(B and D): Btl-Gal4, UAS-actin-5cGFP/UAS-CP1 RNAi; UAS-DCR2/+</td>
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<td>Figure 14</td>
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<td>(F-I, I’): Ptc-Gal4, UAS-Src-RFP/+; UAS-CP1 3XHA/+</td>
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<td>Figure 15</td>
<td>(A-C): Btl-Gal4, UAS-actin-5cGFP/CyO-lacZ</td>
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<td>Figure 16</td>
<td>(A-C): Btl-Gal4, UAS-actin-5cGFP/CyO-lacZ</td>
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<td>(D-F): Btl-Gal4, UAS-actin-5cGFP/UAS-CP1 RNAi; UAS-DCR2/+</td>
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<td>Figure 17</td>
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<td>Figure 18</td>
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<td>(B): Sd-GAL4/+; UAS-CP1-3XHA/+</td>
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<td>(D): UAS-CP1-3XHA</td>
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<tr>
<td></td>
<td>(E): GMR-gal4/+; UAS-CP1X3HA/+</td>
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(F): Vg(Q)-GAL4/+;UAS-CP1-3XHA/+ 
(G): Vg-GAL4/+;UAS-CP1-3XHA/+ 

**Figure 19** 
(A): UAS-CP1 3XHA 
(B): Ptc-gal4,UAS-GFP/+;UAS-CP1 3XHA/+ 
(C): En-Gal4/+;UAS-CP1 3XHA/+ 
(D): Sd-gal4/+;UAS-CP1 3XHA/+ 
(E): Nubbin-gal4/+;UAS- CP1 3XHA/+ 
(F): Salm-gal4/+;UAS-CP1 3XHA/+ 

**Figure 20** 
(A-C): Ptc-Gal4, UAS-Src-RFP/CyO 
(D-F): Ptc-Gal4, UAS-Src-RFP/+; UAS-CP1 3XHA/+ 

**Figure 21** 
(A-C): Ptc-Gal4, UAS-Src-RFP/CyO 
(D-F): Ptc-Gal4, UAS-Src-RFP/+; UAS-CP1 3XHA/+ 

**Figure 22** 
(A): Ptc-gal4, UAS-Src RFP/ CyO 
(B): Nubbin-gal4/+;UAS- CP1 3XHA/+
2.2 Immunohistochemistry

Third instar larvae were dissected in cold 1XPBS (phosphate buffered saline), fixed for 10 minutes in new fixative (SRIVASTAVA et al. 2007), washed two times in PBTA for 20 minutes each, and blocked for 30 minutes at room temperature in PBTA with 5% normal donkey serum, and incubated with primary antibodies in blocking solution overnight at 4°C. In the morning, the samples were washed three times in PBTA for 15 minutes each, blocked for 30 minutes in blocking solution described above, and incubated with secondary antibodies in blocking solution for 90 minutes at room temperature. Following 4X 15 minute washes in PBTA, the samples were mounted in a drop of Vectashield-DAPI (Vector Laboratories, Burlingame, CA). All the incubating procedures were conducted on a gyro shaker. The primary antibodies including mouse anti-Armadillo, rat anti-E-cadherin, mouse anti-Wingless, mouse anti-Cut and mouse anti-Dally-like were purchased from the Developmental Studies Hybridoma Bank at the University of Iowa. Rabbit anti-GFP was purchased from Abcam Inc., Cambridge, MA. Secondary antibodies applied were Alexa –Fluor 488, Alexa-Fluor 546 and Alexa-Fluor 568. Concentrations of antibodies used in this study are shown in Table 5. A Zeiss LSM 510 Confocal Microscope was used for image acquisition and data analysis. Confocal scans were acquired by Dr. Srivastava.

Recipes of solutions used: (New fixative: 0.1 M PIPES (pH 7.2) + 4% Paraformaldehyde. PBTA: PBS+0.1% TritonX100+1% Bovine serum albumin (BSA) + 0.01% Azide.)
Table 5. Antibodies and their concentrations used in this study

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<tr>
<th>Primary Antibodies</th>
<th>Secondary Antibodies</th>
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<tr>
<td>Mouse anti-Armadillo (N2 7A1)</td>
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<td>Rat anti-E-cadherin (DCAD2)</td>
<td>1: 25</td>
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<tr>
<td>Mouse anti-Dally-like (13G8)</td>
<td>1: 50</td>
<td></td>
</tr>
<tr>
<td>Mouse anti-Wingless (4D4)</td>
<td>1: 50</td>
<td></td>
</tr>
<tr>
<td>Mouse anti-Cut (2B10)</td>
<td>1: 50</td>
<td></td>
</tr>
<tr>
<td>Rabbit anti-GFP (ab290)</td>
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<td>Alexa –Fluor 488</td>
<td>1: 800</td>
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<td>Anti-rabbit 488</td>
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<tr>
<td>Alexa-Fluor 546</td>
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<tr>
<td>Alexa-Fluor 568</td>
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</table>
2.3 Collagenase Assay

Third instar larvae were dissected in cold 1XPBS, incubated in staining solution (100μg/mL DQ™ Gelatin in 1X PBS) for 90 minutes, fixed in new fixative for 30 minutes, and washed two times in PBTA for 20 minutes each at room temperature. DQ™ Gelatin was obtained from life technologies. Samples were mounted in a drop of Vectashield-DAPI (Vector Laboratories, Burlingame, CA) and imaged using Carl Zeiss Axioplan 2 Imaging Fluorescent Microscope.

2.4 Scanning Electron Microscopy

Late stage third larval instars were prepared for scanning electron microscopy (SEM) by a first fixation in 1% glutaraldehyde (10 min) and a second fixation in 2% osmium tetroxide (OsO₄) (1 h). Samples were dehydrated in different percentage of ethanol up to 100%, and incubating in fresh 100% EtOH. Critical point drying, mounting on sticky tapes, sputter coating and SEM at 20 kV were performed according to standard procedures using JSM-6510LV with a LaB6 gun.

2.5 Wingless Extracellular Staining

Third instar larvae were dissected in cold 1XPBS, incubated with mouse anti-Wingless primary antibody (dissolved in 1XPBS, 1: 17) in a 4 °C cold room for 60 minutes, rinsed in 1XPBS three times, fixed for 20 minutes in new fixative, washed two times in PBTA for 20 minutes each, and blocked for 30 minutes at room temperature in PBTA with 5% normal donkey serum. Secondary antibody staining was conducted according to the original protocol.
3. RESULTS

3.1 Cell adherens junction proteins are down regulated at the tip of late stage ASP

The line Btl-Gal4, UAS-actin-5cGFP/CyO-lacZ was chosen to help us investigate the process of ASP growth toward the wing imaginal disc.

The Breathless gene encodes a Drosophila homolog of the fibroblast growth factor (FGF) receptor. As it is expressed in both trachea and ASP cells, it was used as the driver of Gal4 protein, the presence of which further initiates the expression of a fusion protein of actin and green fluorescent protein (GFP) by binding to the upstream activating sites (UAS). The actin-GFP outlining ASP cells enabled us to morphologically identify the late stage ASP shown in Figure 8A and 8D.

Based on the fact that the development of ASP is a combination of both cell growth and cell migration, we hypothesized that the remodeling of cell-cell junction protein complex is required for ASP development. To examine whether this rearrangement occurs and at what time point it occurs, we tested the expression of cell adherens junction proteins – armadillo and E-cadherin, in various stages of ASP development by applying primary antibodies first and adding corresponding secondary antibodies later. We found that both Armadillo and E-cadherin are expressed at the apical side of ASP cells during all the stages (Figure 8C and 8F). More significantly, we observed that, at the tip of late stage ASPs, the expression of both armadillo and E-cadherin are weakened (Figure 8B and 8E, arrows).
Figure 8. The distribution of cell adherens junction protein in late third instar larva ASP

(A) and (D): The outline of ASP cells are labeled with GFP. (B) Armadillo and (E) E-cadherin, cell adherens junction proteins, are expressed at the apical surface of neighboring cell interfaces. Loss of both (B red) Armadillo and (E red) E-cadherin are observed at the tip cells (region with arrow). (C) and (F): (C) is the overlay of (A) and (B). And (F) is the overlay of (D) and (E). The arrowheads region at the tip of ASP lack the expression of (B) Armadillo and (E) E-cadherin. ASP is framed in (A-F). These images are generated by confocal scanning.
3.2 CP1 is expressed in *Drosophila* dorsal ASP

CP1, the only homolog of human Cathepsin L in *Drosophila*, has previously been shown to be expressed in the gut cells, garland cells, wing imaginal discs, and macrophages of all larva stages as well as mbn-2 cell lines (TRYSELIUS and HULTMARK 1997). Utilizing a CP1 protein trap line, we were able to better understand the distribution of CP1 in *Drosophila*. Confocal scan of the ASP area from CP1 protein trap demonstrated that CP1 is localized predominantly in the cytoplasm of ASP cells (Figure 9A and 9A'). Antibody staining against Armadillo for the late stage ASP confirmed our previous observation that Armadillo is down regulated at the tip of late stage ASP (Figure 9B and 9B').
Figure 9. The expression of CP1 in the ASP

(A, A’) CP1 (green) expression assayed with a CP1 protein trap line, is localized to the ASP. In (B) and (B’), Armadillo is stained with an anti-Armadillo antibody. (C) is the overlay of (A) and (B). (A’-C’) are enlargement of (A-C). Region with arrow in (B’) and region with arrowhead in (C’) show the loss of Armadillo expression. ASP is framed in (A-C). These images are generated by confocal scanning.
3.3 Categorization of *Drosophila* ASPs (late stage) when CP1 is knocked down

Having established that CP1 is expressed in ASP cells, we analyzed the function of CP1 in ASP development by knocking down and overexpressing CP1, respectively. We first tested the silencing effects of three *UAS-CP1 RNAi* lines we ordered from the Vienna *Drosophila* RNAi Center. These RNAi lines differ from each other in that the *UAS-CP1 RNAi* was placed on different chromosomes. It turned out that only when *UAS-CP1 RNAi* is on the second chromosome, the silencing effect is obvious (This was found by a summer REU student, Breanna Brenneman). To create a CP1 RNAi line with enhanced silencing effect, we modified the line by crossing in a *UAS-DCR2* transgene on the third chromosome, because DCR2 can cleave the double stranded RNAs. The genotype of this line is: *UAS-CP1 RNAi/CyO; UAS-DCR2/TM6Tb*. We further confirmed that the addition of *UAS-DCR2* to the third chromosome induced the silencing effect we expected. Previously, we crossed the *UAS-CP1 RNAi* (2nd chromosome) line with a nubbin-gal4 driver line, in which *UAS-DCR2* is on the first chromosome. We observed defective folded wing phenotypes in adults. Now, as we crossed *UAS-CP1 RNAi/ CyO; UAS-DCR2/TM6Tb* with a nubbin-gal4 driver, we observed similar wing unfolding defects. With both *UAS-CP1 RNAi* and *UAS-DCR2* in the same line, we are able to induce RNAi silencing effect using different gal4 drivers.

To knock down CP1 in the ASP, we overexpressed the *UAS-CP1 RNAi* in combination with *UAS-DCR2* using Btl-gal4 driver. For wild type late stage ASPs,
they should be shaped like long-pointed with a typical tip and stalk as shown in Figure 10A. When CP1 is knocked down, 27 out of 50 late stage ASPs are short in comparison with normal shaped late stage ASPs and 16 out of 50 late stage ASPs display like buds without stalks (shown in Figure 10B). The numbers of different shaped late stage ASPs out of 50 late stage ASPs are displayed on the top of the graph shown in Figure 10C (for the wild type) and Figure 10D (for CP1 knockdown). This demonstrated that the knockdown of CP1 in the ASP impedes the growth of ASPs.
Figure 10. The categorization of late stage ASPs when CP1 is knocked down

(A) Wild type ASP expressing GFP is shown in white, (B) ASPs with CP1 knocked down are labeled with btl-gal4 driving expression of GFP.

50 ASPs were categorized in 3 groups (normal, short and bud) according to their shapes in (A) wild type and (B) a line with CP1 knocked down, respectively. (C) The number of categorized ASPs from wildtype larvae. (D) The number of ASPs in each group mentioned in (B).
3.4 Categorization of *Drosophila* ASPs (late stage) when CP1 is overexpressed

CP1 was overexpressed in ASP by using a UAS-CP1 3XHA line under the control of a *Btl-Gal4* driver (trachea and ASP). We observed five categories of different shaped late stage ASPs (Figure 11B). The numbers of different shaped late stage ASPs out of 50 late stage ASPs are displayed in the graphs in Figure 11C (for the wild types) and in Figure 11D (for CP1 overexpression). Our results suggest that CP1 overexpression also blocks the normal growth of ASPs.
Figure 11. The categorization of late stage ASPs when CP1 is overexpressed

(A) Wild type ASP expressing GFP is shown in white, (B) ASP with CP1 overexpressed are labeled with btl-gal4 driving expression of GFP.

50 ASPs are categorized in 5 groups (normal, tip branched, no stalk, tip budding and stick like) according to their shapes in (A) wild type and (B), a line with CP1 overexpressed. (C) The number of categorized ASPs from wild type larvae. (D) The number of ASPs in each group mentioned in (B).
3.5 Filopodia counting of late stage ASPs

Filopodia are actin-based cellular projections that ASP cells use to sense the environment and guide the movement toward FGF signals from the wing imaginal disc. In humans, increases in the number or size of filopodia indicate a higher rate of cancer metastasis (Nilufar et al. 2013). This suggests that changes in ASP filopodia may reflect the migration ability and invasiveness of the ASP. To examine whether CP1 is capable of affecting ASP migration, we manually counted the number of late stage ASP filopodia for the wild type, the CP1 overexpression line and the CP1 knockdown line, respectively. We found that the average number of filopodia decreased when CP1 expression was knocked down. However, when CP1 was overexpressed, we did not see remarkable changes in the average of filopodia. These are shown in the graphs in Figure 12. To detect how CP1 overexpression impacts ASP migration, we compared the normal shaped ASP obtained from the CP1 overexpression line (described in Figure 11B) and the wild type ASP (described in Figure 11A). We observed that the ASP filopodia from CP1 overexpression line is shorter than that from the wild type. Altogether, both knockdown and overexpression of CP1 in ASP hamper the migration of ASP toward wing imaginal discs.
Figure 12. Number of filopodia in late stage ASPs for wildtype, CP1 knocked down line and CP1 overexpression line, respectively.

50 ASPs were used for each line. For each sample, filopodia counting was done for three times every 24 hours to avoid statistical errors. The average of those three time counting was used to generate the average filopodia number of 50
3.6 CP1 knockdown weakens the invasiveness of ASP

To detect the invasiveness of ASP to the wing imaginal disc, we observed and compared the position of ASP within wing imaginal discs in both the wild type (shown in Figure 13A and 13C) and CP1 knockdown flies (shown in Figure 13B and 13D). From the 6 wild type samples we obtained, all ASPs were deeply embedded, juxtaposed and fused with wing imaginal discs. In contrast, out of the 8 CP1 knockdown samples we obtained, 3 ASPs remain at more superficial location within the wing imaginal discs. 2 ASPs are embedded at medium depth. And three showed a wild type phenotype, deep inside the wing imaginal discs. These indicate the knock down of CP1 negatively regulates the invasion of ASPs.
Figure 13. SEM images of late stage ASPs from wild types and CP1 knockdown line

(A and C): Late stage ASP from wild type larvae. (B and D): Late stage ASP from CP1 knockdown larvae. Late stage ASPs are deeply embedded in the wing imaginal disc in (A) and (C) with extremely unclear boundaries between ASP and wing disc. In contrast, late stage ASPs are located superficially on the wing imaginal disc when CP1 is knocked down in (B) and (D). The abnormal shaped ASPs in (B) and (D) correlate with data from independent experiment in Figure 10. B. ASPs are framed and pointed with white arrows.
3.7 CP1 has the ability to degrade collagen

The integrity of wing imaginal discs is maintained by basement membrane (BM), a specialized extracellular structure on the basal side of polarized epithelial cells (Srivastava et al. 2007). Hence, the degradation of basement membrane is necessary in the process of ASP invading and growing into the wing imaginal disc. We have demonstrated that CP1 plays a role in ASP invasion. We reasoned that the slowdown of ASP invasive growth described in section 3.6 is due to the attenuated degradation of basement membrane of wing imaginal discs caused by reduced function of CP1 in ASP. Additionally, numerous studies have reported that Cathepsin L degrades collagen, a component of BM, *in-vitro*. We tested the interaction between CP1 and collagen IV by employing a collagenase assay. DQ gelatin is a denatured form of collagen IV. Heavily labeled with fluorescein, the fluorescence of the reagent is quenched without collagenase digestion. Proteolysis of DQ gelatin generates highly fluorescent peptides. The increase in the fluorescence is proportional to collagenase activity (Della Porta et al. 1999). CP1 is expressed all over the wing imaginal discs. The collagenase assay applied to the wild type wing imaginal disc exhibited a uniform distribution of green fluorescence at wing pouch region (Figure 14C and 14D). In order to demonstrate CP1’s contribution to the degradation of DQ gelatin, we overexpressed *UAS-CP1 3XHA* in a *ptc* pattern (Figure 14E red curve) using a *Ptc-gal4* driver. The collagenase assay showed that there were scattered dots with enhanced fluorescence at the wing pouch region where CP1 is
overexpressed (Figure 14H, 14 I and 14I’). This confirmed that CP1 degrades collagen.
Figure 14. The interaction of CP1 and collagen

(A-D) wild type wing imaginal discs.

(E) Schematics. DQ gelatin is applied all over the wing imaginal disc. CP1 (black curve) is overexpressed in ptc pattern (red curve).

(F-I, I’) wing imaginal discs with CP1 overexpressed in ptc pattern.

(A, F) ASP outline is shown by DAPI. (B, G) ptc pattern is shown in red by ptc-gal4 driving RFP expression. (C) The distribution of DQ gelatin in wild type wing disc. (H) The distribution of DQ gelatin when CP1 is overexpressed in ptc pattern. (D) The overlay of (A), (B) and (C). (I) the overlay of (F), (G) and (H). (I’) the enlargement of (I). Regions where DQ gelatin degradation occurs are respectively framed out in (H), (I) and (I’).
3.8 Dally-like proteoglycan (DLP) is expressed in ASP

Previously, Yan and Lin et al. showed that Dally-like is expressed in ASP tracheoblasts and performs as a co-receptor for FGF signals from the wing imaginal discs (YAN and LIN 2007). We confirmed this observation by immunostaining Dally-like in ASPs of early stage, mid stage and late stage, respectively. The outline of ASP is marked using *Drosophila* line: Btl-gal4, UAS-actin5cGFP/CyO-lacZ. We further demonstrated that the expression of Dally-like is weakened in late stage ASP as shown in Figure 15C. We defined late stage ASP as highly developed and deeply embedded within the wing imaginal discs. Hence, we predicted that the weakened expression of Dally-like proteoglycan could be the result of reduced need for FGF signals during late stage of ASP development.
Figure 15. Dally-like proteoglycan is expressed through all the stages of ASP development

(A): Early stage ASP; (B): Mid stage ASP; (C): Late stage ASP

The expression of DLP is shown in red in (A), (B) and (C). In (C), the expression of DLP is weak in comparison with that in (A) and (B).
3.9 CP1 regulates the level of Dally-like proteoglycan in late stage ASP

Having demonstrated that CP1 regulates ASP development by degrading collagen IV, we explored whether CP1 affects the expression of Dally-like. Dally-like immuno-staining for wild type late stage ASP is shown in red in Figure 16B and 16C. We observed that both the knockdown and the overexpression of CP1 in ASP resulted in an up regulation of Dally-like in the late stage ASP as shown in Figure 16E (CP1 knockdown) and Figure 16H (CP1 overexpression).
Figure 16. Both the knockdown and the overexpression of CP1 lead to the up regulation of Dally-like proteoglycan in late stage ASP

(A-C) The expression of DLP of late stage ASPs

(D-F) The expression of DLP of late stage ASPs when CP1 is knocked down.

(G-I) The expression of DLP of late stage ASPs when CP1 is overexpressed.

In (A), (D) and (G), the outline of late stage ASPs are shown in green. In (B), (E) and (H), the expression of DLP are shown in red. (C) is the merge of (A) and (B). (F) is the merge of (D) and (E). (I) is the merge of (G) and (H). These images are generated by confocal scanning.
3.10 CP1 knockdown causes defects of wing unfolding

Previously, Breanna Brenneman found that the CP1 RNA interference effect is strong only when *UAS-CP1 RNAi* is on the second chromosome. She also observed a defective wing phenotype at 25 °C when she crossed a *CP1 RNAi* line with a *UAS-DCR2* line. This confirmed that the RNAi silencing effect is strong when DCR2 is overexpressed. However, DCR2 can only be overexpressed with the presence of *nubbin-gal4* in this case since the line we used here is: *w*¹¹¹⁸, *UAS-DCR2; nubbin-gal4*. If we place *UAS-CP1 RNAi* and *UAS-DCR2* in the same line, we are able to cross it with different gal4 driver lines. So, I created a *Drosophila* line with *UAS-CP1 RNAi* on the second chromosome and *UAS-DCR2* on the third chromosome (*UAS-CP1 RNAi/CyO; UAS-DCR2/TM6Tb*). When I crossed it with *nubbin-gal4* line, we observed the same wing-unfolding defect at 25 °C as shown in Figure 17A. This data suggested that this line has the expected RNAi silencing effects and supported the application of it in all the CP1 knockdown experiments throughout this study.

Interestingly, when I crossed the *UAS-CP1 RNAi* line with *w*¹¹¹⁸, *UAS-DCR2; nubbin-gal4* line at room temperature, not all the progeny exhibited the wing-unfolding defect. Statistical analysis showed that 14.84% progeny flies had a defective wing unfolding phenotype as shown in Figure 17B.
Figure 17. Defective wing phenotypes when CP1 expression is knocked down

(A): CP1 knockdown in developing wing results in a failure of the wing to unfold at 25 °C. (B) Adult fly with incompletely unfolded wing observed at room temperature. (C) The ratio of folded wing phenotype and incompletely unfolded wing phenotype under room temperature.

(A) and (B): w^{1118}, UAS-DCR2; nubbin-gal4/UAS-CP1 RNAi. (A) is Breanna Brenneman’s previous observation.
3.11 CP1 overexpression in different patterns leads to various phenotypes

To systematically analyze CP1’s function in wing development, we overexpressed CP1 by crossing UAS-CP1 3XHA line with different GAL4 drivers listed in Table 6. Corresponding phenotypes are shown in Figure 18. In Figure 18A, 18B and 18G, wing bristles are lost at random spots along the wing margin. In Figure 18F, more wing vein like material is observed in comparison with the wild type (Figure 18C). We reasoned that the overexpression of CP1 interrupted signaling pathways necessary for the normal patterning of Drosophila wing margin. Hence, we sought to examine the interaction of CP1 and proteins essential in wing margin development.
Table 6. Phenotype description when CP1 is overexpressed using different Gal4 drivers

<table>
<thead>
<tr>
<th>Gal4 drivers</th>
<th>Progeny phenotypes</th>
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<tbody>
<tr>
<td><strong>Sd-gal4</strong></td>
<td>Wing notching (female)</td>
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<tr>
<td><strong>Cg-gal4</strong></td>
<td>Wild type</td>
</tr>
<tr>
<td><strong>Vg-gal4(BF2)</strong></td>
<td>Wild type</td>
</tr>
<tr>
<td><strong>GMR-gal4/CyO-Acf-GFP</strong></td>
<td>Rough eye</td>
</tr>
<tr>
<td><strong>Bx-gal4</strong></td>
<td>Wild type</td>
</tr>
<tr>
<td><strong>Tub-gal4/TM6Tb</strong></td>
<td>Lethal</td>
</tr>
<tr>
<td><strong>Vg-gal4,UAS SrcRFP</strong></td>
<td>Some wing notching</td>
</tr>
<tr>
<td><strong>Pnr-gal4</strong></td>
<td>Wild type</td>
</tr>
<tr>
<td><strong>Btl-gal4,UAS-actin-5cGFP/CyO-lacZ</strong></td>
<td>Wild type</td>
</tr>
<tr>
<td><strong>LSP2-gal4,UAS-GFPnls/TM6Tb</strong></td>
<td>Wild type</td>
</tr>
<tr>
<td><strong>Twist-GAL4</strong></td>
<td>Wild type</td>
</tr>
<tr>
<td><strong>Vg(Q)-gal4</strong></td>
<td>More wing veins</td>
</tr>
<tr>
<td><strong>En-gal4</strong></td>
<td>Wing notching (posterior)</td>
</tr>
</tbody>
</table>
Figure 18. Phenotypes when CP1 is overexpressed using different Gal4 drivers

(A): Wing notching (white arrows) at the posterior (En-GAL4; UAS-CP1 3XHA).
(B): Wing notching (Sd-GAL4; UAS-CP1 3XHA). (C): Wild type wing (UAS-CP1 3XHA). (D): Wild type eye (UAS-CP1 3XHA). (E): Rough eye (GMR-gal4; UAS CP1X3HA). (F): More wing vein like material (Vg(Q)-GAL4;UAS-CP1 3XHA). (G):
Wing notching (Vg-GAL4; UAS-CP1 3XHA). Phenotypes of wing notching are indicated with white arrows.

3.12 The expression of wingless when CP1 is overexpressed using different Gal4 Drivers

*Drosophila* adult wing margin can be traced back to a three cell wide stripe (Figure 7 D/V boundary) expressing wingless in late third larva instar wing imaginal disc. Couso *et al.* found that wing margin hairs are lost in mutants with inactivated wingless ([COUSO *et al.* 1994]). The phenotype we observed when CP1 is overexpressed is similar to what they described. Thus, we estimated the expression of wingless by immunostaining (Figure 19) when CP1 is overexpressed in wing imaginal discs using various Gal4 drivers. Images taken using fluorescence microscopy indicated some downregulations of wingless when CP1 is overexpressed in *sd* and *salm* pattern, however, not in *patch*, *engrailed* and *nubbin* pattern. The interaction of CP1 and wingless distribution is still not clear due to the factors that he downregulation of wingless is not uniform when we chose other gal4 drivers and that Wingless exists in both cytoplasm of the cell and the extracellular matrix. Traditional staining exhibits the overall staining of wingless rather than a dynamic change in certain compartments. Additionally, the distribution of wingless in the extracellular matrix is facilitated by Dally and Dally-like glypican. We predicted that diffusion of wingless signals might occur in response to the up regulation of CP1. To confirm this prediction, we overexpressed CP1 in *ptc* pattern in late third larval instar wing imaginal discs and stained these discs for Dally-like glypican (Figure 20) and extracellular
wingless distribution (Figure 21), respectively. Immunostaining for Dally-like glypican showed that the expression of DLP is elevated in response to CP1 overexpression, which matches our finding in the ASP where the overexpression of CP1 in ASP was observed to upregulate DLP. However, we did not observe any changes in extracellular wingless distribution when CP1 is overexpressed in tissues where ptc is expressed. We conclude that the expression of wingless is not regulated by CP1. Therefore, the overexpression of CP1 might affect the development of wing margin via other pathways.
Figure 19. The expression of wingless in the late stage wing imaginal discs when CP1 is overexpressed using different Gal4 drivers

(A) Wild type: wingless is expressed in D/V boundary and concentric rings at wing pouch area. (B) The expression of wingless when CP1 is overexpressed in patch pattern. (C) The expression of wingless when CP1 is overexpressed in engrailed pattern. (D) The expression of wingless when CP1 is overexpressed in sd pattern. The absence of wingless expression is shown with white arrowheads. (E) The expression of wingless when CP1 is overexpressed in nubbin pattern. (F) The absence of wingless when CP1 is overexpressed in salm pattern. The breakage of wingless expression is shown with white arrowheads.
Figure 20. The expression of Dally-like glypican when CP1 is overexpressed

(A-C) Wild type: Ptc-gal4, UAS-Src RFP/CyO. (D-E) CP1 overexpression line: Ptc-gal4, UAS-Src RFP/ +; UAS-CP1 3XHA/+.

The patch pattern is shown in red in (A) and (D). The expression of Dally-like glypican is shown in green in (B) and (E). Regions with white arrows in (E) showed more DLP expression than regions with white arrows in (B). (C) is the merge of (A) and (B). (F) is the merge of (D) and (E). These images were generated by confocal scanning.
Figure 21. The expression of extracellular wingless when CP1 is overexpressed

(A-C) Wild type: Ptc-gal4, UAS-Src RFP/CyO. (D-E) CP1 overexpression line: Ptc-gal4, UAS-Src RFP/ +; UAS-CP1 3XHA/+ . The patch pattern is shown in red in (A) and (D). The expression of extracellular wingless is shown in green in (B) and (E). (C) is the merge of (A) and (B). (F) is the merge of (D) and (E). These images are generated by confocal scanning.
3.13 The expression of Cut when CP1 is overexpressed using different Gal4 drivers

Cut, a homeodomain transcriptional factor in Drosophila and a target of Wingless, is also expressed at the presumptive wing margin area, overlapping with part of wingless expression pattern in late third larval instar wing imaginal discs. Because Cut has been thought to mediate wingless function, we also detected the expression of Cut in wing imaginal discs when CP1 is overexpressed in different patterns. Nubbin, sd and salm are genes expressed at specific regions of the wing imaginal discs during late third larva instar. For instance, when CP1 is overexpressed at the region where nubbin is expressed, we would be able to see the effects on the expression of Cut at this particular region in comparison to the same region without CP1 overexpression. Fluorescence microscopy showed that the Cut expression is down regulated in response to CP1 overexpression in tissues where nubbin, sd and salm are expressed. Confocal microscope imaging of the expression of Cut confirmed our observation with a higher resolution. Confocal images are shown in Figure 22 (A wild type and B: CP1 overexpression line).
Figure 22. The expression of Cut when CP1 is overexpressed

(A) Wild type: ptc-gal4, UAS-Src RFP/ CyO. (B) The expression of Cut when CP1 is overexpressed in *nubbin* pattern. The expression of Cut are shown in red. White arrowheads indicate gaps in Cut expression. These images were generated by confocal scanning.
4. DISCUSSION AND FUTURE DIRECTIONS

*Drosophila melanogaster*, for centuries, has been used as an excellent model for genetic research. Our study correlated the development of *Drosophila* dorsal air sac primordium with cancer progression. Cancer is distinguished from benign tumors for its ability to metastasize to other organs in the body. The movement of cancer cells from primary tumor to secondary tumor needs the participation of blood or lymphatic circulation. To enter blood or lymphatic vessels, cancer cells penetrate the basement membranes (BM) lining vessels and do the same when they target a site to form a new tumor. It is widely accepted that tumor growth is particularly dependent on angiogenesis to supply oxygen for cell respiration. The invasive growth of air sac primordium towards wing imaginal discs during late third larva instar mimics the process of cancer metastasis in several aspects. On one hand, as the progenitor of a lung-like structure of the adult flies, ASP migrates in response to Branchless, which is the homolog of mammalian angiogenesis inducer FGF signals. On the other hand, invasion of ASP into wing imaginal discs is inevitably accompanied with breakage of basement membranes (BM) lining wing disc epithelial cells. Epithelial-to-mesenchymal transition (EMT) is a process critical to tumor metastasis. It is characterized by the loss of the expression of E-cadherin at cell-cell junction and the translocation of β catenin from cytoplasm to nucleus (JEANES et al. 2008). Our data exhibited that both E-cadherin and Armadillo (the homolog of mammalian β catenin) are down regulated at late stage ASP tip cells. Further
study on the expression of Armadillo and E-cadherin in ASP development may give us hints on how EMT occurs in human cancer.

CP1 is the only Cathepsin L like cysteine protease found in *Drosophila melanogaster*. Cathepsin L has been reported to regulate tumor formation and metastasis by a multitude of mechanisms including degrading extracellular matrix, assisting in angiogenesis and affecting the expression of oncogenes (when it is in the nucleus). Our study demonstrated that CP1 is expressed in *Drosophila* dorsal air sac primordium. The analysis of CP1’s function in ASP development exhibited that abnormal growth and inhibited migration of air sac primordium occurred in both cases when the expression of CP1 in ASP is knocked down and overexpressed. It is also noteworthy that the invasiveness of ASP toward wing imaginal discs is weakened when CP1 is knocked down in ASP. A collagenase assay further confirmed that CP1 regulates ASP development by degrading collagen IV, which is a component of extracellular matrix. The degradation of ECM makes the migration of ASP toward wing imaginal discs possible. The interaction of CP1 and collagen IV could also account for the observation that wing fails to unfold when CP1 is knocked down in nubbin pattern. This is due to the fact that the unfolding of adult wings is initiated with the degeneration of epithelial cells, in which the breakdown of extracellular matrix is required (HARTENSTEIN et al. 1997; KIGER et al. 2007).

The detection of the interaction between CP1 and molecules including Dally-like glypican, wingless and cut suggests other mechanism of how CP1 affects both ASP development and wing development.
4.1 Future Directions

CP1, Glypican and FGF signaling:

Dally-like and Dally proteins are GPI-anchored glypican indispensable for Breathless signaling. They perform like co-receptors for Branchless signals in the process of larval air sac primordium development (Yan and Lin 2007). In our study, we observed that both the overexpression and the knockdown of CP1 lead to an up regulation of Dally-like in late stage ASPs. Previously, as we counted the number of filopodia for late stage ASPs to detect how CP1 affects the migration of ASPs, we found that the migration of ASPs is impeded as a result of both the overexpression and the knockdown of CP1. It is possible that the up regulation of Dally-like is a compensatory effect. The incomplete migration of ASPs triggers the overexpression of co-receptors for Branchless signals. In this way, the responsiveness of ASPs toward Branchless could be enhanced, such that the invasive growth of ASP into wing imaginal discs could be accomplished.

We considered the potential interaction of CP1 and Dally-like. An in vitro study of Cathepsin L revealed that Cathepsin L promotes the heparanase activity, aiding the release of HSPG from the cell surface and extracellular protein cores (Aboud-Jarrois et al. 2008). When CP1 is knocked down, it could be possible that the normal release of Dally-like from the cell surface is blocked, leading to an accumulation of Dally-like at the cell surface. However, as we ectopically overexpressed and knocked down CP1 in patch pattern in wing imaginal discs, respectively, the expression of Dally like along patch pattern is elevated only when CP1 is overexpressed. It could be possible that the
mechanism how CP1 regulates the expression of Dally-like in wing imaginal discs is different from that in ASPs. It could also be possible that CP1, *in-vivo*, interacts with Dally-like in a distinct way from what is published in 2008 (Aboud-Jarrois *et al.* 2008). Besides, we have to take into account the difference between human and *Drosophila*. Finally, in addition to Dally-like, Dally is also a glypican serving as low affinity receptors for Branchless in *Drosophila*. We should consider that there could be collaboration between Dally-like and Dally in directing ASP migration. In this case, the expression of Dally should be detected as well to figure out what role glypican plays in ASP development.

Based on the discussion above, our future study should deal with problems listed below:

1. The interaction of CP1 and glypican at molecular levels.

2. How Dally and Dally-like respond to the change of CP1 level in ASPs.

3. The interaction and collaboration of Dally and Dally-like in ASP development.

**CP1, Cut, and ASP development:**

When we overexpressed CP1 using different drivers, we found that the expression of Cut is down regulated, which leads to defective wing margin phenotype. We also found that Cut is expressed in the ASP (Figure 23). Based on these observations, Cut could also be down regulated, when CP1 is overexpressed in the ASP. We have already known that Breathless encodes for the receptor of FGF signals and that the expression of Breathless is regulated by Cut, a homeodomain transcriptional factor in *Drosophila* (Pitsouli and Perrimon
Hence, we predicted that the overexpression of CP1 could possibly affect the expression of *Breathless* via Cut, resulting in a lower responsiveness toward FGF signals. This explains one of our observations that late stage ASP filopodia is shorter when CP1 is overexpressed.

To confirm this prediction, we should first detect the expression of Cut and Breathless in the ASP by antibody staining when CP1 is overexpressed. It is also necessary to measure the lengths of late stage ASP filopodia when CP1 is overexpressed and compare them with those of normal late stage ASP filopodia.
Figure 23. Cut is expressed in Drosophila dorsal air sac primordium

The expression of Cut is shown in red, ASP is framed out.

Genotypes: $w^{118}, UAS-DCR2; nubbin-Gal4$

This image is generated by confocal scanning.
5. REFERENCES


HOOK, V. Y., M. KINDY and G. HOOK, 2008 Inhibitors of cathepsin B improve memory and reduce beta-amyloid in transgenic Alzheimer disease mice
expressing the wild-type, but not the Swedish mutant, beta-secretase site of the amyloid precursor protein. J Biol Chem 283: 7745-7753.


### 6. ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tbody>
<tr>
<td>Arm</td>
<td>Armadillo</td>
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<tr>
<td>ASP</td>
<td>Air sac primordium</td>
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<tr>
<td>BM</td>
<td>Basement membrane</td>
</tr>
<tr>
<td>Bnl</td>
<td>Branchless</td>
</tr>
<tr>
<td>Btl</td>
<td>Breathless</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
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<tr>
<td>CP1</td>
<td>Cysteine protease 1</td>
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<tr>
<td>DCR2</td>
<td>Dicer2</td>
</tr>
<tr>
<td>DLP</td>
<td>Dally-like proteoglycan</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
</tr>
<tr>
<td>EMT</td>
<td>Epithelial-mesenchymal transition</td>
</tr>
<tr>
<td>Facit</td>
<td>Fibril associated collagens with interrupted triple helices</td>
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<tr>
<td>FGF</td>
<td>Fibroblast growth factor</td>
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<tr>
<td>FGFR</td>
<td>Fibroblast growth factor receptor</td>
</tr>
<tr>
<td>GFP</td>
<td>Green fluorescent protein</td>
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<tr>
<td>GPI-anchored glypican</td>
<td>Glycophosphatidylinositol-anchored glypican</td>
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<td>Heparan sulfate proteoglycans</td>
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<tr>
<td>HSGAG</td>
<td>Heparan sulfate glycosaminoglycan</td>
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<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
</tr>
<tr>
<td>MMPs</td>
<td>Matrix metalloproteinases</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate-buffered saline</td>
</tr>
<tr>
<td>PBTA</td>
<td>PBS+0.1% TritonX100+1% BSA+ 0.01% Azide</td>
</tr>
<tr>
<td>RISC</td>
<td>RNA-induced silencing complex</td>
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<td>SEM</td>
<td>Scanning electron microscope</td>
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<td>siRNA</td>
<td>Small interfering ENA</td>
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<td>TC</td>
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