A Cathepsin-L is required for invasive behavior during Air Sac Primordium development in *Drosophila melanogaster*

Qian Dong 1, Breanna Brenneman 2, Christopher Fields, Ajay Srivastava 1,*

Department of Biology and Biotechnology Center, Western Kentucky University, 1906 College Heights Boulevard, TCCW 351, Bowling Green, KY 42101, USA

**Article Info**

Article history:
Received 13 July 2015
Revised 16 August 2015
Accepted 25 August 2015
Available online 2 September 2015

Edited by Lukas Huber

Keywords:
Invasive behavior
Air Sac Primordium
Cysteine Protease
Imaginal disc
Basement Membrane
Cathepsin-L
Lung development
*Drosophila*

**Abstract**

The *Drosophila* Air Sac Primordium (ASP) has emerged as an important structure where cellular, genetic and molecular events responsible for invasive behavior and branching morphogenesis can be studied. In this report we present data which demonstrate that a Cathepsin-L encoded by the gene CP1 in *Drosophila* is necessary for invasive behavior during ASP development. We find that CP1 is expressed in ASP and knockdown of CP1 results in suppression of migratory and invasive behavior observed during ASP development. We further show that CP1 possibly regulates invasive behavior by promoting degradation of Basement Membrane. Our data provide clues to the possible role of Cathepsin L in human lung development and tumor invasion, especially, given the similarities between human lung and *Drosophila* ASP development.

© 2015 Federation of European Biochemical Societies. Published by Elsevier B.V. All rights reserved.

**1. Introduction**

In adult *Drosophila*, the dorsal Air Sac is an important component of the adult respiratory system that supplies oxygen to flight muscles [1–3]. Located in the adult thorax, the Air Sac develops during the third larval instar from the Air Sac Primordium (ASP) [3]. During larval development, a tracheal branch associates with the wing imaginal disc and a group of tracheal cells begin to proliferate and form the ASP. The proliferation of tracheal cells and migration of the ASP is in response to FGF signaling [3] and the involvement of FGF signaling has been reported during the early stages of lung development [4,5]. As ASP develops, it invasively migrates from a superficial location over the wing disc to a more deep-seated location within the wing disc [6]. The regulation of ASP invasive behavior is not well understood.

Invasive cellular behavior is an important morphogenetic mechanism during normal animal development [7–10]. Tumor cells that become metastatic often hijack this normal cellular behavior and acquire the ability to break through a specialized form of Extracellular Matrix (ECM) [11,12] called Basement Membrane (BM) [13]. The degradation of BM is often dependent on Matrix MetalloProteases (MMPs) [14,15], which are found upregulated in human cancers [16,17], but other mechanisms of degradation have also been suggested [18–20]. Cathepsins or Cysteine Proteases are another class of enzymes that are associated with cancers and are also responsible for a diversity of roles during animal development [21–27]. What role, if any, do Cathepsins play during development of the ASP is not known. Furthermore, while Cathepsin-L is expressed during human fetal lung development [5] and during tumor invasion [21], what biological function it performs during these processes is not well studied. Here, we show that a Cathepsin L in *Drosophila* encoded by the CP1 gene [28] is necessary for ASP invasive behavior. Consequently, the ASP in the absence of CP1 fails to invade properly and assumes a superficial position over the wing disc. This could be due to defective degradation of the BM when CP1 is knocked down. We demonstrate that CP1 overexpression in the developing wing disc results in degradation of the BM.

**Author contributions:** Q.D. and A.S. contributed equally to this work. A.S. conceptualized, designed and performed the experiments, analyzed data and wrote the paper, Q.D. performed the experiments and helped write the paper. B.B. and C.F. performed experiments.

* Corresponding author at: Department of Biology and Biotechnology Center, Western Kentucky University, 1906 College Heights Boulevard, TCCW 351, Bowling Green, KY 42101, USA.

E-mail address: ajay.srivastava@wku.edu (A. Srivastava).

1 Contributed equally to this work.

2 Present address: University of Virginia, 1335 Lee Street, Charlottesville, VA 22903, USA.

http://dx.doi.org/10.1016/j.febslet.2015.08.036

0014-5793/© 2015 Federation of European Biochemical Societies. Published by Elsevier B.V. All rights reserved.
Because Cathepsin-L is expressed during fetal lung development [5] and given the similarities between lung development and ASP development [4,29], it is our belief that data presented in this paper should aid in our understanding of lung development and branching morphogenesis – a process involved in the development of various organs like kidney, mammary gland, salivary gland etc [29]. Additionally, the demonstration in this paper that CP1 can degrade the BM and the fact that Cathepsin-L is expressed in invasive tumors suggests that one of the function of Cathepsin-L during lung development and tumor metastasis in humans might be to aid in the degradation of BM.

2. Results and discussion

2.1. Cell–Cell adhesion and CP1 expression during ASP development

The development of ASP begins during the early third larval instar as a group of tracheal cells respond to FGF signal emanating from the disc proper columnar epithelial cells [3]. As ASP develops, the epithelial cells proliferate, migrate over and invasively into the wing imaginal disc to form a tubular epithelial structure by the end of third larval instar (Fig. 1A) [1–3]. Because the ASP migrates and invasively grows towards the wing disc [6], we reasoned that the cells at the tip of the ASP must exhibit features of invasive cells. For example, invasive cells during normal development and tumor metastasis undergo an epithelial to mesenchymal transition where they lose their junctional contacts [30]. We visualized ASP in wing imaginal discs expressing GFP from a UAS-act5C GFP transgene driven under the control of a Btl-Gal4 driver (Fig. 1B green channel and merge) [31]. These GFP marked ASP also display actin rich tip cells of the late third larval instar ASP (Fig. 1B region bounded by the bracket and Fig. 1C arrow and arrowhead). The arrow points to more proximal cells of the ASP tip that have started to lose their Armadillo expression and the arrowhead points to loss of Armadillo in tip cells at the very distal end of the ASP suggesting that several rows of ASP tip cells from proximal to distal lose Armadillo suggesting that these cells possibly lose their junctional contacts as they migrate and invade the wing disc. At this point it is not clear which signals initiate the loss of junctional contacts in ASP tip cells.

It has been shown that MMPs are involved in the development of ASP [6,37] and are also found upregulated in invasive tumor cells [14,38,39]. We wondered if other proteases may be involved in ASP development also. We chose to understand the role of a single Cathepsin L in the Drosophila genome encoded by the gene CP1 [28]. This was motivated by the fact that Cathepsin L in humans is often found up regulated in cancer cells [25,40] and therefore offered the possibility that the Drosophila Cathepsin L, CP1, may be involved in supporting invasive behavior during ASP development. To understand CP1s role in ASP development and to assay for its expression in ASP, we utilized a previously characterized GFP Protein Trap in the CP1 gene (here after referred to as CP1-PT) [41–43]. This protein trap is expressed in tissues where CP1 expression has been reported by RNA in situ hybridization technique [44]. Because the overall expression from this protein trap is fairly low we stained wing discs from CP1-PT with an anti-GFP antibody and an Alexa 488 conjugated secondary antibody to enhance the GFP signal. Confocal scans of anti GFP stained CP1-PT wing discs revealed a strong expression of CP1 in the ASP (Fig. 1C). We also stained these wing discs with Anti-Armadillo antibody and found that indeed the tip cells lose their Armadillo (Fig. 1C, red channel) expression confirming our earlier observation from Btl-Gal4, UAS-act5C GFP wing discs. Because MMP2 in Drosophila is involved in the development of ASP [6,38] and because CP1 is also expressed in ASP, it is attractive to speculate that CP1 may be required for activation of MMP2. Future experiments could address this idea.

We next asked if CP1 was involved in the downregulation of Adherens junction markers in wing disc cells in general and in the ASP tip cells in particular. We first overexpressed CP1 from a UAS-CP1 transgene in a Ptc-Gal4 pattern along the anterior–posterior (A/P) compartment border in the wing disc and stained these discs for Adherens junction proteins Armadillo and E-Cadherin. Our data show that CP1 overexpression has no effect on Armadillo and E-Cadherin levels in the wing disc cells (data not shown). We also downregulated CP1 in the ASP tip cells using a UAS-CP1 RNAi transgene bearing stock (please see materials and methods) driven by Btl-Gal4, UAS-act5C GFP driver in the presence of UAS-DCR2 (to enhance the RNAi effect). We found that downregulation of CP1 in ASP had no effect on the Adherens junction proteins in the ASP tip cells either (data not shown) suggesting that the presence or absence of CP1 has no effect on the status of Adherens junctions. The molecular player(s) responsible for downregulation of Adherens junction proteins Armadillo and E-cadherin in ASP tip cells remain to be identified.

2.2. CP1 is necessary for ASP development and filopodia formation

The ASP can be divided into a proximal stalk and a distal tube like structure. The epithelial cells of the ASP are arranged around a central lumen with apical sides facing the lumen and basal sides facing away from the lumen [1,3,38]. The ASP also have filopodia at the tip region and the lateral region that respond to the FGF and DPP signals respectively [3,33]. Because CP1 is expressed throughout ASP we asked what, if any, role it might be playing in the ASP development. We chose to knock down CP1 levels in ASP by utilizing RNA interference (RNAi) technique. We used a UAS-CP1 RNAi transgene bearing line (see materials and methods for details) and crossed it to Btl-Gal4, UAS-act5C GFP fly line and enhanced the RNAi effect by simultaneously overexpressing DCR2 from a UAS-DCR2 transgene. Downregulation of CP1 resulted in a range of ASP developmental defects that we categorized on the basis of the shape of ASP into Normal (similar to wild type without any observable developmental defect), Short (stalk present but the tubular structure shortened) and Bud (lack of an identifiable stalk and most of the ASP present as a group of cells) categories, Fig. 2A and B. We analyzed 50 ASPs and quantified each of the categories from wild type ASPs and CP1 downregulated ASPs. While most of the ASPs are normal in wild type third instar larvae (96%, n = 50), the number of normal ASPs present in CP1 downregulated larvae is markedly low (14%, n = 50). The defective ASPs from CP1 downregulation represent 54% short (27/50) and 32% (16/50) bud like morphologies suggesting an important role for CP1 in the development of ASP (Fig. 2C and D).

Filopodia are actin based cellular projections often found in migrating and invasive cells. An increase in their numbers and size in cancer cells is associated with cancer metastasis [45]. Because the filopodia found around the ASP are involved in migration of the ASP and for capturing the BNL and DPP signals [3,33], we asked if CP1 had any effect on the ASP filopodia development and thereby its migratory and invasive ability. We counted the numbers of primary filopodia located around the ASP from wild type (Btl-Gal4, UAS-act5C GFP) and CP1 knockdown larvae and found that while ~13 ASP filopodia are found in wild type, the number is noticeably
reduced to ∼7 in CP1 knockdown ASPs (n = 50 ASP). Taken together these results implicate CP1 in playing important developmental roles in ASP morphology and filopodial development. It is possible that the loss of filopodia in CP1 knockdown ASPs may contribute to the observed ASP morphologies. Because the ASPs use filopodia to sense the source of BNL and DPP signals and also utilize filopodia for migration, a loss in the numbers of filopodia may result in inappropriate signal sensing resulting in the observed morphological defects and impeded migration. Alternatively, the filopodia may simply aid in the migration of ASP cells into the wing disc.
2.3. CP1 knockdown interferes with ASP invasive behavior

It is known that the ASP is located superficially over the wing disc during the early stages of larval development. However, by the late third larval instar stage the ASP invades and occupies a deep seated position in the wing disc [6]. Because filopodia are important for migration and invasion of normal and tumor cells [45], we reasoned that the loss of ASP filopodia numbers in the absence of CP1 may interfere with the ASP invasive behavior. To understand the role of CP1 in ASP invasive behavior we analyzed wild type (n = 6) and CP1 knockdown ASP (n = 8) by utilizing scanning electron microscopy of late third instar larval wing discs. Our data clearly demonstrate that while the wild type ASP are found embedded in the wing disc, in the absence of CP1 the ASP are superficial and located over the wing disc thereby implicating CP1 in the regulation of invasive behavior during ASP development.

---

**Fig. 2.** CP1 is necessary for ASP development (A and B) Fluorescence images of ASP tagged with GFP expression from a UAS-actin5c-GFP transgene under the control of a Btl-Gal4 driver. The wild type ASP is shown in (A), the presence of filopodia are marked with an arrow. Various phenotypes generated as a result of CP1 knockdown by driving a UAS-CP1 RNAi line are shown in (B) and categorized as Normal (similar to wild type), short (the ASP fails to develop), bud (ASP stalk is missing and ASP appears like a bud). (C and D) Quantification of the ASP phenotypes (n = 50) in wild type, C, and CP1 knockdown, (D) Error bars represent standard deviation. (C) Most of the ASPs appear normal, (D) majority of ASPs display a short and a bud type phenotype and fail to develop like normal ASPs. (E) Quantification of the per ASP filopodia numbers in wild type and CP1 knockdown ASPs. In wild type -13 filopodia are found per ASP whereas CP1 knockdown results in ~7 filopodia.
Given the similarities between normal invasive behavior and tumor invasive behavior, our results are suggestive of an important role for the CP1 Cathepsin-L in invasive behavior during pathogenic conditions like cancer.

2.4. CP1 overexpression results in degradation of the Basement Membrane

The Drosophila wing disc is surrounded by a specialized form of ECM called the BM [14]. Because ASP invasion is suppressed in the absence of CP1, we reasoned that for ASP invasion into the wing disc CP1 might be required for degradation of the BM. To test whether CP1 can degrade the BM, we overexpressed CP1 along the Anterior/Posterior compartment boundary in the third instar wing disc using the Ptc-Gal4 driver [14]. We also treated these discs with a Fluorescein conjugated DQ Gelatin (Please see Materials and Methods) according to manufacturer’s instructions and a published report [46]. The fluorescence from DQ Gelatin is intensified when this denatured form of Collagen IV is degraded in the presence of collagenases and gelatinases. Our results demonstrate that only background fluorescence is observed in wing discs treated with DQ Gelatin (Fig. 4A–E). However, discs treated with DQ Gelatin where the CP1 transgene is overexpressed in the Ptc pattern result in considerable increase in fluorescence suggesting the presence of collagenase activity (Fig. 4F–I). Because Collagenases degrade Collagens, and because the DQ Gelatin assay results in degradation of labeled gelatin, we conclude that CP1 degrades BM through collagenase activity.

2.5. Concluding remarks

As mentioned earlier, human lung development and Drosophila tracheal development share similarities. They both require FGF signaling for their development and both are a result of branching morphogenesis [4,29]. In Drosophila, ASP is a good model to understand the molecular events responsible for tracheal development and knowledge gained from this could be extrapolated to better understand lung development in humans. Furthermore, Cathepsin-L has been shown to be expressed during lung development and during tumor metastasis but what roles it plays during these processes is not well studied. In this study we demonstrate that a Cathepsin-L encoded by the gene CP1 is expressed in Drosophila ASP, required for invasive/ migratory behavior during ASP development and is capable of promoting degradation of the BM. In the light of the facts, that Cathepsin-L is expressed during human fetal lung development, and because it is also expressed in invasive tumor cells in humans, the present study provides novel insights into the role of Cathepsin-L in normal development and invasive behavior during ASP development.

3. Materials and methods

3.1. Drosophila stocks and culture

Fly crosses were conducted at 25 °C (Unless stated otherwise) in vials and bottles according to standard procedures supplemented with a few pellets of Red Star® active dry yeast. For overexpression of transgenes we utilized the UAS/Gal4 system in flies [47].
Fly stock w; Btl-Gal4, UAS-actin 5C-GFP/ CyO-LacZ (FBst0008807) was obtained from Bloomington Drosophila Stock Center. The line Ptc-Gal4, UAS-Src-RFP/CyO has been previously described [14]. The UAS-CP1 RNAi line used in this study was obtained from the Vienna Drosophila RNAi Center [48] and was tested for specificity based on a previously reported mutant wing phenotype where the mutant wings failed to unfold [49]. Downregulation of CP1 in the wing disc using the UAS-CP1 RNAi line driven by a nubbin-Gal4 driver in the presence of UAS-DCR2 results in a similar wing unfolding defect and is presented in Supplementary Fig. 2. The UAS-CP1 3XHA (FBst0500649) overexpression line was obtained from Fly ORF and has been described previously [50]. CP1 Protein Trap has been previously described (FBst0051555) [41–43].

Fig. 4. CP1 overexpression can degrade the Basement Membrane (A–D and F–I) Confocal scans of third instar wing imaginal discs. (E) Schematic of third instar wing imaginal disc with the position of Ptc-Gal4 expression marked in red and CP1 overexpression overlapping Ptc-Gal4 domain marked with a black line. The staining of collagen is marked with DQ-Gelatin (green). (A–D) Discs overexpressing RFP from a UAS-RFP transgene under the control of a Ptc-Gal4 driver. (A–C) are individual channels, blue (DAPI), red (RFP) and green (DQ-Gelatin). The overlap is shown in (D). (F–I) Third instar wing imaginal disc confocal scans from larvae overexpressing RFP and CP1 from a UAS-RFP and UAS-CP1 transgene respectively under the control of a Ptc-Gal4 driver. Individual channels are presented in (F–H) as blue (DAPI), red (RFP) and green (DQ-Gelatin). The overlap is shown in (I and I’) (at a higher magnification). BM is not degraded in (D) but upon overexpression of CP1 is degraded (bounded box in (I and I’)).
3.2. Genotype used in various figures

Fig. 1.
(B) w; Btl-Gal4, UAS-actin-5cGFP/CyO-LacZ
(C) w; Cyp1aC01377 / Sm6a

Fig. 2.
(A and C) w; Btl-Gal4, UAS-actin-5cGFP/CyO-lacZ
(B and D) w; Btl-Gal4, UAS-actin-5cGFP/UAS-CP1 RNAi; UAS-DCR2/+;

Fig. 3.
(A and B) w; Btl-Gal4, UAS-actin-5cGFP/CyO-lacZ
(C and D) w; Btl-Gal4, UAS-actin-5cGFP/UAS-CP1 RNAi; UAS-DCR2/+;

Fig. 4.
(A–D) w; Ptc-Gal4, UAS-Src-RFP/CyO
(F–I) w; Ptc-Gal4, UAS-Src-RFP/+; UAS-CP1 3XHA/+;

3.3. Immunohistochemistry

Third instar larvae were dissected in cold 1XPBS and processed for immunohistochemistry as described previously [14]. The primary antibodies including mouse anti-Armadillo (N27A1, used at a dilution of 1/100), rat anti-E-cadherin (DCAD2, used at a dilution of 1/25) were purchased from the Developmental Studies Hybridoma Bank at the University of Iowa. Rabbit anti-GFP (ab290, used at a dilution of 1/800) was purchased from Abcam Inc., Cambridge, MA. Appropriate Secondary antibodies applied were conjugated to either Alexa – Fluor 488, Alexa-Fluor 546 or Alexa-Fluor 568. A Zeiss LSM 510 Confocal Microscope was used for image acquisition and data analysis.

3.4. Scanning electron microscopy

Late stage third larval instars were prepared for scanning electron microscopy (S.E.M.) 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 (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 S.E.M. at 20 kV were performed according to standard procedures using JSM-6510LV with a LaB6 gun.

3.5. Collagenase assay

Third instar larvae were dissected in cold 1XPBS, incubated in staining solution (100 µg/mL DQ™ Gelatin in 1 x PBS) for 90 min, fixed in 4% paraformaldehyde fixative for 30 min, and washed two times in PBTA (1XPBS + 0.1% TritonX100 + 1% Bovine serum albumin + 0.01% Sodium Azide) for 20 min each at room temperature. DQ™ Gelatin was obtained from life technologies. Samples were mounted in a drop of Vectashield-DAPI (Vector Laboratories, Burlington, CA) and imaged using Carl Zeiss Axioplan 2 Imaging Fluorescent Microscope.

Conflict of interest

The authors declare that they do not have any conflict of interest.

Acknowledgements

The BDSC and VDRC are acknowledged for various reagents used in this study. Part of this work was supported by a Graduate School Grant to Q.D. B.B. was supported by an NSF – REU internship. Research in my laboratory at WKU is supported by the WKU Department of Biology startup funds, WKU Research Foundation RCAP-I grant # 11-8032 and by a KBRIN-AREA grant funded through a parent grant from the National Institute of General Medical Sciences of the National Institutes of Health under award number 5P20GM103436-13.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.febslet.2015.08.036.

References


