The Characterization of The Function(s) of Crag in Basement Membrane Polarity

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ABSTRACT

THE CHARACTERIZATION OF THE FUNCTION(S) OF Crag IN BASEMENT MEMBRANE POLARITY

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Department of Biological Sciences
Northern Illinois University, 2021
Olivier Devergne, Director

The abundant epithelial cells play various roles in the development and maintenance of an organism, and their apical-basolateral polarity is a unique characteristic that is imperative to their function and integrity. This polarity is maintained via various environmental cues and tightly regulated intracellular trafficking, where the presence of the basement membrane (BM) provides a basal cue. The BM is a specialized extracellular matrix (ECM) secreted by the epithelial cells, which accumulates on the basal side and serves as their mechanical anchorage. Although the BM is of paramount importance in the apical-basolateral polarity and the loss of BM’s integrity is a hallmark of cancer, little is known regarding the basal deposition restriction of BM components. Crag is a regulator of the biological pathway dedicated to the basal restriction of BM components, however, its interactors and the exact mechanism responsible for its polarized intracellular localization and its activity remain yet to be elucidated. In this thesis, a structure-function analysis of Crag was performed using the follicular epithelium (FE) of the Drosophila melanogaster ovary as the model system to determine the specific Crag’s domain(s) responsible for localization and activity. In this multidomain protein, we determined that the CBS domain plays an important role in the localization of Crag to the apical and lateral domains, and the DENN domains play a role in the activity of Crag in restricting BM components to the
basal side. These results allowed us to begin to understand which domains of Crag are important for its localization and its activity of regulating the polarized deposition of BM protein. Next, we will continue this structure-function analysis of Crag to precisely determine the relative importance of each of the domains present in its localization and activity. Overall, this study will help us to further understand how Crag works in the biological pathway that regulates the polarized deposition of BM proteins.
THE CHARACTERIZATION OF THE FUNCTION(S) OF CRAG IN BASEMENT MEMBRANE POLARITY

BY

HEMIN PANKAJKUMAR SHAH
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A THESIS SUBMITTED TO THE GRADUATE SCHOOL IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE MASTER OF SCIENCE

DEPARTMENT OF BIOLOGICAL SCIENCES

Thesis Director:
Olivier Devergne
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Introduction

I. The Epithelium

In complex multicellular organisms, organs and tissues need to be composed of specialized cells to perform specific functions. Epithelial cells are one such type of specialized cells which make up epithelial tissue. Found in over 150 different types in mammals, epithelial cells are of paramount importance in the generation and function of body systems including digestive, respiratory, reproductive, circulatory, nervous, and endocrine (Rodriguez-Boulan & Macara, 2014).

Epithelia are found as semipermeable sheets of uniformly polarized contiguous cells, organized either in a monolayer or multilayer such as in developing embryos or in skin, respectively (Guillot & Lecuit, 2013). These cells display a special type of polarity called the apical-basolateral (AB) polarity. Conversely, cells lacking AB polarity which are usually found loosely as single cells, or in aggregates, are known as mesenchyme (Fristrom, 1988). The external epithelium, or epidermal epithelium (skin), forms a barrier between the organism and its external milieu while the internal epithelia line the cavities and organs both mechanically and chemically separating two different environments (Figure 1) (Baroni et al., 2012).
Figure 1: Epithelial tissues.
The abundance of epithelial tissues suggests its various functions throughout the body organ systems. These functions include (A) gas exchange in the alveoli of the lungs via diffusion, (B) blood filtration as glomeruli filtration in the kidney to eliminate waste in form of urine, (C) absorption of nutrients and secretion of certain hormones in intestines, (D) sweeping dust particles, pollutants, and various corrosive agents and pathogens to protect underlying tissue of the respiratory tract, (E) and protection of underlying tissue against abrasion in the esophagus. Adopted from Epithelial Tissue – Zoology.
Polarity in Epithelium

Crucial for structure, function, and morphogenesis, cells usually display polarity. Epithelial cells display the apico-basolateral polarity which results in morphological and functional asymmetry in these cells. They are polarized into discrete apical and basolateral (a joint term referring to apical and basal domains due to similar constituents) plasma membrane domains, responsible for aggregation and sorting of various lipids and proteins (Cao et al., 2012; Rodriguez-Boulan et al., 2005). While looking at a traditional epithelium or an epithelial cell, the apical domain faces the lumen, or body cavity, while the basal domain is connected to the basement membrane (BM), or basal lamina, which is a specialized sheet of Extracellular Matrix (ECM) (Figure 2). The lateral domain is responsible for the cell-cell interactions between adjoining cells via tight junctions, adherens junctions, and spot desmosomes, which hold the cells together (Giepmans & van IJzendoorn, 2009). AB polarity is not only crucial for the vectoral functions such absorption and diffusion of proteins in the epithelial cells, but also for the morphogenesis and maintenance of epithelia during development (St Johnston & Sanson, 2011). Disruption of the AB polarity leads to epithelial-mesenchymal transition (EMT), resulting in various pathologies including malignancy and metastasis of tumor, with metastasis resulting from loss of polarity being a hallmark of cancer (Hanahan & Weinberg, 2000, 2011; Macara & McCaffrey, 2013; Moreno-Bueno et al., 2008; Royer & Lu, 2011).
Figure 2: Epithelial cells and domains.

(A) Pseudo-colored transmitted electron micrograph (TEM) of a human fallopian tube where the epithelial cells can be seen anchored to the BM (green), with basal side facing the BM. Adapted from Science Source. (B) Schematic representing a traditional epithelial cell and its domains. The apical domain (red) faces the lumen, basal domain (blue) adheres to the BM (green), and the lateral domains are present connecting neighboring cells (purple). The junctional complexes can be seen on the lateral domains.

The focus of this thesis is the study of one critical component of the epithelial cell polarity: the deposition of BM proteins to the basal surface. My thesis focuses on the role of Crag in the polarized deposition of BM proteins. Specifically, Crag (Calmodulin-binding protein related to a Rab3 GDP/GTP exchange protein), a multidomain protein responsible for this polarized deposition, is being investigated with the aim to determine the domain(s) necessary for its localization and activity (Denef et al., 2008). Using the follicular epithelium (FE) of Drosophila melanogaster as a model system, one of the few tissues known to synthesize all the BM components in Drosophila, the effects of various forms of Crag (Crag lacking single or multiple domains) on its localization and the polarized deposition of the BM components is investigated.
Junctional Complexes in Epithelial Cells

Cell-cell and cell-ECM/BM contact relies primarily on two cellular features: the apical-basolateral axes and the presence of various junctional complexes. The junctional complexes also play a crucial role in defining and maintaining the cell polarity by separating the plasma membrane into apical and basolateral domains (Wang & Margolis, 2007). They are classified into three main groups: tight (TJ), anchoring, and communication junction (Alberts et al., 2002; Ramena & Ramena, 2018). Tight junctions prevent leakage of molecules between cells, anchoring junctions (adherens junctions - AJ, desmosomes - Ds, etc.) attach cell membranes of adjacent cells as well as anchor epithelial cells to ECM, and communicating junctions (gap junctions) mediate the passage of electrical and chemical signals between cells (Figure 3).

Epithelial cells are held together in a continuous sheet by specialized AJs, and have sealing junctions (TJ and SJ in vertebrates and invertebrates respectively) between apical and basolateral domains responsible for isolating the cell interior from its environment by preventing paracellular transport (Cereijido et al., 2004; Magie & Martindale, 2008; Rodriguez-Boulan & Macara, 2014; Runkle & Mu, 2013; St Johnston & Ahringer, 2010). These intercellular junctions contain transmembrane proteins capable of binding to their counterparts on neighboring cells, as well as interacting with the cytoskeleton and cytoplasmic proteins for signal relay (Giepmans & van IJzendoorn, 2009). Tight junctions in mammals are observed at the boundary between the apical and lateral domains, and adherens and other cell-cell junctions are localized further basally. In invertebrates, however, septate junctions (analogous to tight junctions) localize relatively basally, while adherens junctions are found most apically at the border between apical...
Figure 3: Apical-basolateral polarity in epithelial cells.

(A-B) Schematics representing the epithelial cells in both vertebrates and invertebrates as well as the apical-basolateral polarity. The apical domain (red) faces the external environment or the lumen, the basal domain (blue) shows attachment to the basement membrane, and the lateral domain (purple) contacts the neighboring cells. In both models, the conserved proteins coordinate with each other as well as junctional complexes to establish and maintain the apical-basolateral polarity. (A) In the vertebrate (mammalian) epithelial cell, tight junctions (TJ) prevent paracellular diffusion of small molecules, and anchoring junctions (adherens junctions – AJs, and desmosomes – Ds) adhere adjacent cells to each other to form the lateral epithelial sheets. The conserved protein show that aPKC phosphorylates and activates PAR3, and this activated PAR complex the phosphorylates and activates the Crumbs and Scribble complexes. (B) In the invertebrate (Drosophila) epithelial cell, septate junctions (SJ) block the paracellular diffusion, which AJs are localized more apically. The conserved polarity proteins show that the Par and Crumbs complex (Par6, aPKC, Baz, Cdc42, Crb, Sdt, Patj) display mutually supportive interactions with the AJ, while antagonistic interactions can be seen between the apical and basolateral polarity proteins (Lgl, Dlg, Scrib, Par1).
and basolateral domains, separating them (Giepmans & van IJzendoorn, 2009). Despite these differences, the establishment and maintenance of the AB polarity has been shown to rely on conserved biological pathways in vertebrates and invertebrates. Tight junctions are adhesion complexes that serve as a gate to control paracellular transport of solutes and ions between cells, as well as separate the apical and basolateral domains (Anderson & Van Itallie, 2009). They act as a barrier, blocking the intermixing of apical and basolateral membrane components, thus maintaining cell polarity (Hartsock & Nelson, 2008; Zihni et al., 2016). Furthermore, their importance has also been shown in controlling cell differentiation and proliferation, as loss of functional TJs has been correlated with human tumor progression and metastasis and development of hyperproliferative disorders in mice (Latorre et al., 2013; Saitou et al., 2000).

Adherens junctions in Drosophila are also thought to play a crucial role in the establishment of AB polarity (Aguilar-Aragon et al., 2019; Harris & Peifer, 2004; Nelson, 2003). Although their direct impact on the polarization is unclear, their necessity in the maintenance of the size of the plasma membrane domains has been observed (Aguilar-Aragon et al., 2019). Desmosomes, another type of anchoring junction in mammalian epithelial cells, helps resist shearing forces and forms adherent points via the interaction between their integral membrane proteins and intermediate filaments (Garrod & Chidgey, 2008; Green & Jones, 1996). The last type of junctions, communicating junction, which primarily include gap junctions, are aggregates of intercellular channels which allow for the diffusion of small molecules such as ions and metabolites between neighboring cells (Goodenough et al., 1996; Goodenough & Paul, 2009).

Due to the ability of these junctional complexes to fasten the epithelial cells as well as provide a link of communication for both inter- and intra-cellular information transfer, they play a critical role in the establishment and maintenance of AB polarity. Ergo, a compromise in the
structure and function of these complexes resulting in their dysfunction has been linked to tumorigenesis and cancer progression (Gehren et al., 2015; Jeanes et al., 2008; Kwon, 2013).

**Apical-Basal Polarity Regulators**

The regulation of plasma membrane domains, as well as the junctional complexes, is governed by certain polarity protein complexes. Table 1 shows the homologs of these conserved proteins in mammals and *Drosophila*. The interactions between these evolutionarily conserved polarity regulators are crucial for the polarization and its maintenance in epithelial cells (Figure 3) (Dow & Humbert, 2007; Tepass, 2012). Although additional cell-type specific factors are required for accurate polarity generation, in *Drosophila*, partitioning-defective (Par) protein, Crumbs (Crb), and Scribble complexes act as the core regulators, which are also known to be conserved in mammals. The Par complex consists of atypical protein kinase C (aPKC), the scaffolding protein Par6 as its regulatory subunit, the GTPase cdc42, and Bazooka (homolog of the mammalian scaffolding protein Par3)(Goldstein & Macara, 2007; Suzuki & Ohno, 2006). The Par complex works in coordination with the Crumbs complex, which includes the transmembrane protein Crumbs (Crumbs3 in mammals) that binds to scaffolding proteins Stardust (PALS1 in mammals) and dPatj (protein associated with tight junctions, PATJ, in mammals), which have been shown to play a broad role in epithelial polarity (Bulgakova & Knust, 2009; Su et al., 2013). Lastly, the Scribble complex contains the proteins Scribble (Scrib), Lethal giant larvae (Lgl), and Discs large (Dlg) (Bilder, 2004; Royer & Lu, 2011). The various mutually supportive, as well as antagonistic, interactions between these complexes result in the formation of apical-basolateral polarity domains.
The Par proteins are ubiquitously expressed, while Crumbs and Scribble show a restricted distribution, with the Scribble group—well known basolateral polarity regulators—being restricted to the epithelia and localizing to the lateral domain, and Crumbs group localizing to the apical domain (Bilder, 2004; Rodriguez-Boulan & Macara, 2014). Due to the restricted localization, Par and Crumbs complexes act in a collaborative manner to define the apical domain, while Scribble marks the basolateral domain (Cao et al., 2012). These domain markers display biologically antagonistic interactions, resulting in exclusion of each other so that they remained confined to their designated domains. This antagonism plays a significant role in the distinct separation of the apical and lateral domains (Cao et al., 2012; St Johnston & Ahringer, 2010). The scribble group of proteins suppresses the apical membrane protein identity on the basolateral domains by inhibiting the function of par proteins, while the par proteins interacts with specific domain on Crumbs protein, antagonizing the scribble activity on apical domain and subsequently promoting apical polarity (Figure 3B) (Bazellieres et al., 2009; Goldstein & Macara, 2007; Roh et al., 2003; Sherrard & Fehon, 2015; Tepass, 1996).

These polarity regulatory proteins are also involved in the formation of various junctions, cytoskeleton organization, protein signaling, and lipid metabolism via interactions with various molecules (St Johnston & Ahringer, 2010). For example, maintenance of TJs depends on the Par complex, where the recruitment of the Par complex results in the activation of Cdc42, which in turn activates aPKC, leading to the proper formation of TJ (Bazzoun et al., 2013; Ebnet et al., 2004). Furthermore, overexpression of Par3 leads to enhanced function of TJs as assessed by higher localization of TJs and development of transepithelial resistance, and induced expression of Crumbs in cells that have lost ability to form TJs show formation of TJs (Bazzoun et al., 2013; Fogg et al., 2005; Yamanaka et al., 2003). Similarly, the Scribble complex shows reciprocal
interactions with E-cadherin, where Scrib localizes to the AJs in an E-cadherin dependent manner, and Scrib and Dlg interact with E-cadherin for its proper localization to cell junctions and mediation in cell-cell adhesion (Laprise et al., 2004; Navarro et al., 2005; Qin et al., 2005). Thus, these polarity regulators work cooperatively with the junctional complexes to establish and maintain the apical-basolateral polarity. Table 1 shows the functions of homologs of these conserved proteins in *Drosophila* and mammals.

Table 1:

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<td>Par α, β, γ</td>
<td>Scaffold protein</td>
</tr>
<tr>
<td>Bazooka</td>
<td>Par3</td>
<td>Scaffold protein</td>
</tr>
<tr>
<td>Atypical PKC</td>
<td>PKC ζ, 1/π</td>
<td>Serine threonine kinase</td>
</tr>
<tr>
<td>Cdc42</td>
<td>Cdc42</td>
<td>Rho family GTPase Protein</td>
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Intracellular Trafficking and Sorting Signals in Epithelial Cell Polarity

While junctional complexes and the polarity regulators play significant roles in the establishment and maintenance of the plasma membrane domains, they also need to interact with the components of the cell trafficking pathways to accurately transport the cargo containing vesicles to their appropriate domains. The process of trafficking in epithelial cells includes many vesicular pathways for the polarized transport of protein and cargo to plasma membrane domains (Fölsch et al., 2009). In polarized cells, once translation occurs in ribosomes at the ER, the trans-Golgi network (TGN) acts as the main sorting station for these newly synthesized proteins to be delivered to plasma membrane (Griffiths & Simons, 1986). The biosynthetic trafficking routes also include apical early endosomes and basolateral early endosomes (BEE), as well as apical recycling endosomes (ARE) and common recycling endosomes (CRE), where further sorting occurs after leaving the TGN (Fölsch et al., 2009; Sheff et al., 2002). In these endosomes, the incoming proteins are categorized for recycling to the cell surface, transport to lysosome for degradation, transcytosis, or return to TGN.

Amidst this, the Rab GTPases play a significant role by marking different domains and promoting mediators of intracellular trafficking in processes such as vesicle docking and fusion. Activated by guanine nucleotide exchange factors (GEF) to turn “on” signaling via GDP to GTP exchange and deactivated by GTP-hydrolysis to turn “off” signaling via GTPase activating protein (GAP), this regulation of the Rab GTPases is a crucial component in the majority of vesicular trafficking pathways (F. A. Barr, 2009; F. Barr & Lambright, 2010; Hutagalung & Novick, 2011). Phosphoinositides also interact with the GEFs and GAPs, assuming a differential distribution in the plasma membrane domains (Devergne et al., 2014; Di Paolo & De Camilli, 2006). It is not, however, clearly understood how the Rab GTPases and GEFs localize to plasma
domains to carry out their specific functions in cargo trafficking. Since Crag is a GEF of Rab10, we are also trying to determine how Crag assumes its apico-lateral localization to fulfill its role in BM deposition.

Apical and basolateral sorting signals also play an important role in protein distribution at specific sites in the plasma membrane domains and consequently, the maintenance of the AB polarity. Well defined apical sorting signals include glycosylphosphatidylinositol (GPI) anchor, N- and O- glycans, and proteoglycan with chondroitin sulfate (Fiedler & Simons, 1995; Mayor & Riezman, 2004; Prydz & Dalen, 2000; Weisz & Rodriguez-Boulan, 2009; Yeaman et al., 1997). Well known basolateral sorting signals include proteoglycan with heparan sulfate, and tyrosine based YXXØ and NPXY motifs (where X represents any amino acid and Ø represents a bulky hydrophobic residue) located in the cytoplasmic tail of various cargo proteins (Mellman & Nelson, 2008; Wolff et al., 2010). Together, these factors work in conjunction with the junctional complexes and polarity regulators to maintain and establish the apical-basolateral polarity in epithelial cells.

II. Basement Membrane

Extracellular matrices (ECM), found nearly ubiquitously between cells, are complex mesh-like networks comprised mainly of glycoproteins and proteoglycans (Muncie & Weaver, 2018). Basement membrane (BM), also known as basal lamina, is a thin (40-120 nm) sheet of layered cell-adherent ECM vital for epithelial structure and morphogenesis (Bowman, 1840). Composed of approximately 50 proteins, Collagen IV (Coll IV), glycoproteins laminin (Lam) and nidogen, and the heparan sulfate proteoglycan (HPSG) Perlecan (Pcan) are the main components responsible for the extraordinary tensile strength of BMs (Figure 4). BMs are found
between epithelial and endothelial cells, as well as ensheathing skeletal, smooth, heart muscle cells, and adipocytes (Yurchenco et al., 2004).

**Figure 4: Basement membrane anchoring epithelial cells.**

(A) Transmission electron micrograph of basement membrane visible as a thin sheet of specialized ECM supporting the epithelial cells and overlaying the connective tissue. *Adapted from Molecular Biology of Cell.* (B) Schematic representing major components of BM, including the transmembrane receptors integrins. The epithelial cells lie above the BM, while below lies the connective tissue. *Adapted from Yurchenco et al., Matrix Biology, 2004.*

Due to the ubiquity of the BMs, they also display numerous functions. One of the main functions of the BM is to act as a barrier (Figure 5). For example, the glomerular BM acts as the kidney’s filtration barrier (Deen, 1412; Kefalides & Borel, 2005b), the alveolar and capillary BMs in the lungs allow for gas exchange between blood and air (Kefalides & Borel, 2005b; Vaccaro & Brody, 1981), and the capillary endothelial and parenchymal BM merge to form the blood brain barrier (Engelhardt et al., 2017). Another common function of the BM is to provide mechanical support via the interactions between Coll IV and Pcan (Halfter et al., 2013; Morrissey & Sherwood, 2015). For example, the BM underlying skin plays a crucial role in anchoring the skin and providing resistance against the shearing forces (Figure 5) (Miller, 2017). Although BMs are well-known for their function of separating the epithelium from underlying...
tissue, they also play a critical role in tissue physiology and development by promoting organogenesis and angiogenesis, many signaling pathways, and stem cell regulation (Lerner et al., 2013; Mak & Mei, 2017). BMs play a role in cell adhesion and migration, e.g. cell migration during the Drosophila egg morphogenesis where follicle cell migration depends on the various interactions between laminin and the receptor integrin (Díaz de la Loza et al., 2017). Moreover, the migration of neural crest cells in mice results in the establishment of the peripheral nervous system and glial cells is shown to be dependent on laminin-α5 (Coles et al., 2006).

BM’s role in angiogenesis and tissue morphogenesis is also evident due to its involvement in the Drosophila wing and egg chamber development and mammary duct development in mice (Ashe, 2008; Jayadev & Sherwood, 2017b; Ma et al., 2017; Williams & Daniel, 1983). By acting as a signaling platform by harboring the molecules involved, BM is responsible for many signaling pathways (Yoshizaki et al., 2013). Via its interactions with secreted signaling molecules, BM can regulate their diffusion rate as well as their interactions with surface receptors. BM is also known to directly activate signaling pathways in processes such as cell proliferation, migration, and differentiation through interactions between Laminin, Collagen IV, and Perlec an (Bonnans et al., 2014; Loscertales et al., 2016). In avian lung development, Collagen IV plays rudimentary roles in coordinating alveolar morphogenesis via moderating epithelium formation, as well as myofibroblast proliferation, differentiation, and migration (Loscertales et al., 2016). Similarly, Laminin plays a direct role in and by aiding cell proliferation, differentiation, migration, and survival by interacting with Dystroglycan and Integrin receptors (Bonnans et al., 2014). Recombinant Laminin-511 has also been shown to drive pancreatic differentiation and maintain mouse and human stem cells in pluripotent state.
Figure 5: Basement membrane functions in various tissues. 

(A) Endothelial and parenchymal merge to form the blood brain barrier surrounding the brain capillary. 
(B) Retinal pigment epithelial BM and choroid BM in the eye as a part of Bruch’s membrane which compartmentalizes the retina and choroid. 
(C) Lung endothelial and alveolar BMs combine to facilitate gas exchange. 
(D) Internal basal lamina mediates attachment of the junctional epithelium to the tooth surface and external basal lamina connects to the gingival mesenchyme. 
(E) Cutaneous BM underlies basal epithelial cells and provides mechanical support and resistance to shearing forces. 
(F) The glomerular BM as a part of kidney’s filtration unit. (Sekiguchi & Yamada, 2018).
(Domogatskaya et al., 2008; Higuchi et al., 2010; Rodin et al., 2010). BM components such as Perlecan, Agrin, and Collagen XVIII interact with heparan sulfate glycosaminoglycan chains, binding to various growth factors (i.e., FGF, VEGF, TGF-β and BMPs). Consequently, these regulate cell migration and proliferation, survival, as well as stem cell maintenance (Jayadev & Sherwood, 2017a).

Aside from these functions, the BMs are particularly important for the prevention of metastasis of in-situ carcinomas due to their barrier function and anchoring of epithelia, as well as regulating epithelial polarity. If BM integrity is lost, AB polarity will be affected and may give the epithelial cells migratory ability (Kalluri, 2003; Royer & Lu, 2011; Sekiguchi & Yamada, 2018). The epidermal BM in synchrony with its associated molecules functions as a protective skin barrier. Intact, it serves to separate tumors from the underlying tissue. However, once the basement membrane is ruptured and its barrier function is compromised by the neoplastic cells, they gain metastatic and invasive properties. Furthermore, epithelial polarity is coordinated via the organization of polarity proteins and lipids at the plasma membrane and interactions with adjacent cells and BM. For example, laminin is observed to be a crucial component in the establishment of apical polarity in teeth and polarizing the epiblast (Fukumoto et al., 2006; Jayadev & Sherwood, 2017b). Loss of Coll IV leads to instability of BM and consequently improper polarity formation in mammary epithelial cells (Plachot et al., 2009). The BM also directs the AP polarity with the apical side forming on its opposite side. Furthermore, in cell culture, such as Madin-Darby Canine Kidney (MDCK) cells which are capable of forming polarized, lumen-containing structures, upon addition of ECM proteins on the apical side of the
cells, the polarity is observed to be reversed (O’Brien et al., 2001; Wang et al., 1990a, 1990b; Yu et al., 2005; Zuk & Matlin, 1996). Such findings suggest that the apical polarity may result from interactions between the BM and the epithelial cells.

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To maintain the apical basal polarity and keep the epithelium anchored to the BM, the components that make up the basement membrane interact with various cell surface receptors. In these bidirectional interactions between the BM and the epithelial cells, basal epithelial cells (cells located in lower part of epithelia and abutting the BM) provide positional cues for spatially restricted BM formation, where the expression of Par-1b has been shown to be crucial (Gervais et al., 2016; Sekiguchi & Yamada, 2018). Post formation, the BM proteins interact with epithelial cells via basal receptors Integrin (Int) and Dystroglycan (Dg), providing basal cues and playing a crucial role in establishing apical basolateral cell polarity (Figure 6B) (Daley et al., 2012).

Figure 6: Protein localization and transmembrane membrane integrin signaling in polarity. (A) Schematic showing the distribution of proteins. Once synthesized, they are transported intracellularly to their respective domains via vesicular transport. (B) The BM proteins interact with basal receptors such as integrins. Integrin signaling is involved in the establishment of the apical-basolateral polarity.
BM in Pathological Conditions

BM disruption is also observed in many pathological conditions. Most commonly, it is known to be misregulated in tumors, allowing for loss of anchorage and barrier function, leading to metastasis (Kalluri, 2003; Sekiguchi & Yamada, 2018). However, BM has also been known to be affected in other conditions and diseases. Stroke causes loss of integrity in BM, as a reduction of BM proteins is observed. In Alzheimer’s disease, an abnormal amount of Col IV accumulation has been detected, leading to the thickening of BM. The resulting loss of proper blood brain barrier function affects its semi-permeability, obstructing drug delivery in treatment (Thomsen et al., 2017). Collagen IV mutations have been found to cause recessive dystrophic epidermolysis bullosa (Fine et al., 2014). This causes the skin to become fragile and blistering infections become extremely common, and majority of severe cases develop cutaneous squamous cell carcinoma. In children with this condition, the mortality rate is approximately 50% in the first year of life, and majority do not survive past five years of age (Fine et al., 2014; Guerra et al., 2017). Other less severe conditions also display a loss of BM components, as laminin-null mice show hypoplastic tooth germ and decreased proliferation, loss of Col IV disrupts polarity in mammary epithelial cells, and an inherited thin basement membrane results in benign familial hematuria leading to blood in urine (Chew & Lennon, 2018; S. Fukumoto et al., 2006; Y. Fukumoto et al., 2006; Plachot et al., 2009). Hence, BM is of vital importance in numerous developmental and homoeostatic processes, as its disruption can not only affect multiple body systems, but also cause lethal defects.
One of the main functions of the BM is to anchor the epithelia and establish and maintain apical-basolateral polarity. For that, however, the BM needs to be fully functional and synthesized, which is a highly time consuming and complex process due to the various of components involved. Firstly, the basal secretion of BM proteins is a key feature of homeostasis of AB polarity. There are three non-exclusive mechanisms that have been speculated to be involved in this process. The first mechanism suggests that the BM-containing vesicles are targeted directly to the basal side, second suggests that BM-containing vesicles are blocked apically, while third suggests that BM proteins are secreted on both sides but are quickly degraded or endocytosed on the apical side (Devergne et al., 2017). These mechanisms are divided into two representative models as seen in Figure 7.

![Figure 7: Models representing the mechanisms of basal secretion of BM proteins. (Model 1) Schematic representing the first mechanism of BM protein secretion, where they are directly targeted basally. (Model 2) Schematic representing the other two mechanisms, where the BM proteins are either blocked apically, or they are secreted both apically and basally, but quickly degraded or endocytosed, leading to basal accumulation of BM proteins.](image-url)
Secondly, proteins that make up the BM are not always synthesized by the epithelial cells, but rather frequently the epithelia rely on external sources for their BM protein needs (Figure 8). For example, in the *Drosophila* embryo, Lam and Coll IV have been shown to be produced primarily in hemocytes (Kusche-Gullberg et al., 1992). In humans, Coll IV secretion is observed in renal fibroblasts (Lam et al., 2004). During the late embryogenic and larval stages in *Drosophila*, laminin and collagen IV production is observed in the fat body. Fat body is an insect tissue composed of storage cells whose primary role is nutrient storage and energy utilization. Upon blocking the production of BM components in the fat body, a loss of collagen IV throughout the body is observed (Pastor-Pareja & Xu, 2011). Perlecans, another major BM protein, is synthesized in the basal cells and adjacent fibroblasts, and in vascular endothelial and smooth muscle cells (Ikarashi et al., 2004; Whitelock et al., 1996). Although these proteins are not produced directly by most epithelial cells, they are capable of traveling to distant BMs. This is speculated to be a result of the adhesive properties of basal epithelium, allowing for accurate protein accumulation. The proper diffusion is just as important as the secretion of the proteins, as improper diffusion can lead to accumulation in the wrong tissues, leading to abnormalities as well as cancerous growths (Kalluri, 2003).
Figure 8: Mechanisms of localization of ECM/BM components.

Three mechanisms have been determined for synthesis and proper accumulation of BM at target tissue. The first mechanism suggests that the BM components are synthesized locally by target tissue, while second suggests that they are synthesized locally by migrating cells. In case of both, it can happen either in a receptor dependent or receptor independent manner. For the third mechanism however, the synthesis of BM proteins distantly, but the components are captured by the target tissue via interactions with receptor proteins or another pre-localized ECM protein. (Brown, 2011).

**Polarized Trafficking in the BM**

Despite both the temporal and spatial significance of BM in developing organisms and the involvement of the BM in several pathological conditions, the factors that control the basal localization of BM components are not well understood. Of trafficking mediators such as v-SNARE cellubrevin, t-SNARE syntaxin 4, adaptor protein AP-1B, and Rab 8 and Rab 10 GTPases, only Rab8 and Rab 10 have been shown to specifically select for BM proteins (Ang et al., 2003; Devergne et al., 2017; Fields et al., 2007; Fölsch, 2005; Huber et al., 1993; Lerner et al., 2013). Furthermore, the basolateral sorting signals are located on the cytoplasmic tails of transmembrane proteins and thus remain inapplicable to the localization and trafficking of
secreted BM proteins (Hunziker & Fumey, 1994; Matter et al., 1992; Mellman & Nelson, 2008; Wolff et al., 2010).

The polarized secretion of BM proteins occurs via specialized intracellular pathways, and in the recent years, few factors that show specificity in the localized sorting of BM components have been identified. In the fruit fly, a DENN domain protein called Crag (Calmodulin binding protein related to a Rab3 GDP-GTP exchange protein) has been identified as one of these factors. Crag acts as a selective regulator of polarized BM deposition along with a serine protease-like protein called Scarface (Scaf) (Denef et al., 2008; Sorrosal et al., 2010). Loss of either Crag or Scarface has been shown to cause the BM proteins to accumulate both apically and basally, however, their effect on the cargo and mechanism of transport of BM proteins is not known (Lerner et al., 2013). Crag exhibits an apico-lateral and endosomal subcellular localization, while Scarface localizes only apically. This contradicting localization to their role suggests that instead of acting like anchors to accumulate BM proteins toward them, they prevent BM protein accumulation to the apical and lateral domains via the one or more of the three non-exclusive mechanisms stated above for basal secretion of BM proteins.

The polarized deposition of Collagen IV and Laminin begins with the targeting of their mRNA transcripts to the basal cytoplasm (Lerner et al., 2013). The intracellular Collagen IV is always located near the basal surface, and recent studies have led to the speculation that Collagen IV chains are translated directly into the basal transitional ER (tER). Laminin is also synthesized in the basal tER. While Collagen IV exits from the basal tER to travel to the basement membrane, other BM proteins including Laminin also travel with it (Lerner et al., 2013). Although mRNA of these proteins is specifically localized, the vesicles containing these proteins
are found throughout the cytoplasm, suggesting that the localization of mRNA is not critical to the localization of the protein.

In *Drosophila*, as mentioned above, Crag plays an important role in the accurate localization of the BM proteins, as Crag mutants exhibit a loss of BM integrity, with an abnormal accumulation of BM proteins on both the apical and basal sides (Denef et al., 2008). Its specificity for BM proteins is inferred, as in these Crag mutants, the apical and basolateral markers localize properly. Crag itself is localized in the apico-lateral regions of the cells. Once the BM proteins are synthesized and transported to Golgi clusters, Crag acts as a guanine nucleotide exchange factor (GEF) and targets Rab10GTPase basally, where it restricts the BM proteins to localize to the basal surface. Crag is known to act as a GTP exchange factor (GEF) for Rab10GTPase. It has been shown that in Rab10 knockdown conditions, although a portion of BM proteins still accumulates at the basal domain, the majority of the BM proteins mislocalize to the apical domain, similar to Crag mutant cells. Overexpressing Crag, however, rescues this mislocalization, and Crag and Rab10 are seen to colocalize (Lerner et al., 2013). This suggests that Crag interacts with Rab10, and that these proteins are required for robust localized secretion of the BM proteins in a specific trafficking pathway (Denef et al., 2008; Lerner et al., 2013).

Another component recently identified to be of importance in the regulation of basal localization of BM proteins is phosphatidylinositol 4,5-bisphosphate (PIP2). In the somatic follicle cells (FCs) of the FE mutant for phosphatidylinositol synthase (Pis), a member of phosphoinositide pathway, BM proteins such as collagen IV and perlecan are observed to accumulate on both the apical and basal sides. This is also the case when the production of PIP2 is decreased as a result of various components downstream of Pis being affected. PIP2 is also known to control the apico-lateral localization of Crag, as a decrease in PIP2 results in the loss of
Crag’s proper distribution on the apical side. Hence, by controlling the subcellular localization of Crag, PIP2 indirectly prevents the mislocalization of BM proteins to apical and lateral sides (Devergne et al., 2014; Isabella & Horne-Badovinac, 2015).

In addition to Crag/Rab10, another GEF/RabGTPase complex has been shown to be critical for the polarized secretion of BM proteins. Stratum is a GEF for Rab8, and together, they have been shown to play a role in controlling the basal restriction of BM proteins in epithelial cells (Devergne et al., 2017). In FCs, when Crag is knocked down, Collagen IV mislocalizes to both the apical and basal sides. Similarly, in FC conditions where Stratum expression was decreased or ceased, BM proteins Collagen IV and Perlecan were observed to be mislocalized apically. It was then identified that Stratum was indeed a GEF for Rab8. In Stratum knocked down condition, a partial rescue in BM protein localization was observed using a constitutively active form of Rab8, however, such was not the case when wild-type full-length Rab8 was used. This suggested that Rab8 indeed acts downstream of Stratum and is also activated by it. Stratum is shown to localize, and restrict Rab8 activity, basally to allow for correct localization of BM proteins. The expression of Stratum is also affected in Pis mutants, where a decrease in PIP2 leads to a decrease in Stratum levels. Similar decrease in Stratum levels is also observed in Crag mutants. Hence, this suggests that the expression and distribution of Stratum is dependent on both Crag and PIP2, and that Stratum controls Rab 8 activity, all of which are required independently for the proper localization of BM proteins. Overall, this suggests that all these factors act in the same biological pathway dedicated to the polarized secretion of BM proteins.
III. Model System: Follicular Epithelium of Drosophila melanogaster

To study the apical-basolateral polarity and the localization of BM, the model system of somatic follicular epithelium (FE) of the Drosophila melanogaster ovary was used. Ovary formation in Drosophila begins in the embryo at the posterior pole of the blastoderm where the pole cells begin to cellularize, and migrate through the posterior midgut to the mesoderm during gastrulation (Williamson & Lehmann, 1996). During the third larval instar stage, the ovary begins to differentiate, and terminal filaments start forming during metamorphosis resulting in ovariole formation (Godt & Laski, 1995). Ovarioles are long and discrete tube-like structures, of which 16-20 comprise an ovary (Figure 9A) (Horne-Badovinac & Bilder, 2005). Each ovariole can be thought of as an egg assembly line, with the age progression of egg chambers (which give rise to an egg) seen beginning at the anterior end and moving posteriorly, until the mature egg exits through the oviduct.

Each ovariole is divided into three regions: a terminal filament, a germanium, and a vitellarium (Ogienko et al., 2007). The terminal filament is attached to the anterior tip of the germanium and is also the anterior-most portion of an ovariole. The germanium is where the basic structure of egg chamber is created. The germanium is characterized by three distinct regions.
Figure 9: Drosophila ovary and oogenesis.

(A) Schematic of *Drosophila* ovary. Each ovary contains 16-20 ovarioles where the egg chambers will travel posteriorly as they grow. (B) The posterior region of the ovariole includes the terminal filament and germarium region. Cystoblast is born here, and egg chambers are formed. (C) Shows the progression of stages through ovariole, where once the egg chamber leaves the germarium and enters vitellarium, it goes through 14 stages before maturing as an egg (Horne-Badovinac & Bilder, 2005). (D) Immunostained image of an ovariole stained for DNA (blue), F-actin (red), and PcanGFP (green) representing BM. (E) Schematic of FE cell showing the apical domain that faces the germline cells, basal domain facing the BM, and lateral domain facing the neighboring cells.
These are known as region 1, region 2 (2A and 2B), and region 3 (Figure 9B). Region 1 is the starting point for oogenesis which begins with the one of the two to three stem cells present dividing asymmetrically, resulting in a daughter stem cells and a daughter cell called cystoblast (Robinson & Cooley, 1997). The cystoblast undergoes four rounds of mitotic division with incomplete cytokinesis to produce a syncytium (a germline cyst) consisting of 16 cystocytes interconnected via cytoplasmic bridges (E. H. Brown & King, 1964; Koch & King, 1966). Only one of the 16 cystocytes will be fated to become an oocyte, while the remaining 15 will become nurse cells, providing the oocyte with necessary proteins (Roth, 2001). This cyst then proceeds to region 2, where it further develops. At the border of region 2A and 2B, the 16-cell cystoblast gets encapsulated with FCs, which serve to separate the cyst. The cysts present in region 2B contain three types of differentiated FCs: polar cells located at opposite poles of the follicle, interfollicular cells which form interfollicular stalk that separates an older egg chamber from the neighboring younger egg chamber, and epithelial FC which encapsulate the cyst (Ogienko et al., 2007; Waghmare & Page-McCaw, 2018). The third region of the germarium thus contains a single cyst, with the oocyte located posteriorly. This follicle then buds off to the vitellarium and undergoes 14 stages of morphological development (Figure 9C). However, it has been frequently debated whether the egg chamber that enters the vitellarium from germarium is stage 1 or stage 2 (King et al., 1956; Ogienko et al., 2007; Wieschaus & Szabad, 1979).

The germline cyst is enveloped by approximately 80 FCs at the time it buds off as a stage 1 egg chamber. These FCs actively proliferate during stages 1-6 of oogenesis, retaining their cuboidal shape and reaching 650-1000 in number (Horne-Badovinac & Bilder, 2005; Ogienko et al., 2007; Roth, 2001). Once the proliferation ceases, the FCs undergo various morphological and morphogenetic events in the following stages. These events include the migration of FCs towards
the posteriorly developing oocyte, becoming cylindrical in shape, and undergoing three rounds of endoreplication that result in cells possessing 16 copies of genomic DNA. The DNA is selectively replicated to amplify genes necessary for eggshell production and axes patterning and development of the oocyte (Botchan & Levine, 2004; Calvi et al., 1998; Calvi & Spradling, 1999; Lilly & Sptadling, 1996).

Entering the vitellarium, the egg chambers move posteriorly along the ovariole until they oviduct. It takes approximately 10 days for a newly formed cystoblast to reach stage 1 of egg chamber, and around 3 more days to progress through the 14 stages and develop into a mature egg (Figure 9C) (Lin & Spradling, 1993; Wieschaus & Szabad, 1979). A normal ovariole is known to produce 2 mature eggs per day, resulting in up to 80 oviposited eggs daily. This is possible due to the fact that the there is a constant formation of 16-cell cysts, which progress through the stages in an assembly line manner down the ovariole towards its posterior end. As a result, the vitellarium is composed of six to seven egg chambers which can be observed simultaneously in an ovariole, with older egg chambers seen posteriorly (Horne-Badovinac & Bilder, 2005; Ogienko et al., 2007).

Each oocyte in the Drosophila ovary develops within an egg chamber consisting of 16 germline cells surrounded by a layer of somatic follicle cells (FCs). These FCs are a monolayered simple epithelium (follicular epithelium - FE) which, just like classic epithelial cells, displays epithelial characteristics including apical-basolateral polarity. The apical side faces the germline cells, lateral sides contact the neighboring cells, and the basal side faces the external environment of the ovary, where the deposition of BM occurs. Localization of BM components can be difficult to investigate as not all epithelia produce their own BM components, and many components migrate quite a distance before localizing accurately. The FE of
Drosophila is one of the few known model systems known to synthesize and secrete all its BM components, making this one of the prime reasons for its suitability as a model system in this investigation.

IV. Crag and Polarized Secretion of BM Proteins

Crag is an evolutionarily conserved gene through vertebrates and invertebrates, and is also the focus of this thesis (Semova et al., 2003). Studies in model organisms as well as cultured cells have shown BM to play a key role in the maintenance of epithelial polarity and tissue organization. Crag, which plays a critical role in the regulation of polarized secretion of BM proteins, is thus crucial in the AB polarity. In FC mutants for Crag, BM components Pcan, Lam, and Col IV have been observed to accumulate aberrantly on both the basal and the apical domain, while the distribution of transmembrane proteins such as integrins remains unaffected (Figure 10C, C’) (Denef et al., 2008). This mislocalization of BM proteins causes a loss of AB polarity and causes the epithelial cells to gain motility and invasive abilities, which is known to be a hallmark of cancer (Denef et al., 2008; Hanahan & Weinberg, 2000, 2011). The specificity of Crag’s role in BM accumulation pathway as a novel regulator was speculated when transmembrane proteins of the apical, basolateral, and junctional cortices were observed to be correctly localized but BM proteins were mislocalized to the apical side in Crag mutant cells, and when mislocalization of BM components was not observed in the cells mutant for other proteins that led to the loss of epithelia architecture. The localization of Crag to cell cortices and to recycling and early endosome membranes is further indicative of its role in membrane trafficking, where its apical and lateral localization and its absence at the basal domain across all
stages of oogenesis suggests a mechanism in inhibiting the localization of BM proteins to these cortices and restricting them to the basal domain (Figure 10B) (Denef et al., 2008).

The Crag locus encodes for two isoforms, Crag-PA (1671 aa) and Crag-PB (1644 aa), making it a relatively large multi-domain protein (Figure 10A). Crag consists of a DENN domains at the amino terminal, followed by a CaM domain responsible for Crag’s calmodulin binding activity (also called calmodulin binding site - CBS), and a conserved domain found in Crag homologs at the carboxyl terminal (Denef et al., 2008; Levivier et al., 2001; Warr & Kelly, 1996; Xu et al., 1998). The DENN domains include a DENN (differentially expressed in normal and neoplastic cells), uDENN (upstream of DENN), and dDENN (downstream of DENN) domain. Although the function of these DENN domains in various proteins has not been determined, their role in membrane trafficking due to their presence in regulators of membrane trafficking such as Rab GTPases has been speculated (Ishida et al., 2016; Levivier et al., 2001). For example, connedenn/DENND1A DENN domain has been shown to function as a GEF or Rab35 in regulating endosomal trafficking, and mutations in these DENN domains has been known to cause developmental defects in plants and animals (Allaire et al., 2010; Yoshimura et al., 2010).

The presence of the DENN domains in Crag is consistent with its role as a GEF. GEFs are known to be the primary regulators of intracellular trafficking through their control on localization and activation of Rab-GTPases, which activate effectors downstream, mediating majority of intracellular events (Grosshans et al., 2006; Hutagalung & Novick, 2011; Novick, 2016). The DENND4 domain of Crag is predicted to be responsible for its GEF activity for activating Rab10. Crag and Rab10 have been shown to colocalize and coimmunoprecipitate, and these interactions between them suggest that Crag and Rab10 form a complex that plays a critical
Figure 10: Crag structure, localization, and mutant phenotype.  
(A) Schematic of Crag showing its domains with DENN domains occupying the N-terminus and the CBS and conserved domain occupying the C-terminus. (B-B’) Longitudinal section through FE, immunostained with Hoechst (DNA, blue) and endogenous Crag (red). Lateral localization of Crag is seen. (C-C’) Longitudinal section through FE, immunostained with Hoechst (DNA, blue) and PcanGFP (green). Show the mutant phenotype of Crag (Crag^{C1101}) where strong apical mislocalization of BM component PcanGFP was observed.
role in controlling the polarized trafficking of BM proteins (Isabella & Horne-Badovinac, 2016; Lerner et al., 2013). Activation of Rab10 GTPase by Crag results in proper placement of BM proteins, and the loss of one of these components leads to mislocalization of BM proteins to both sides of the epithelial cells. Crag has also been shown to colocalize with Rab5 and Rab11 in presence of intact DENN domain and shown to act as their GEF. However, Rab5 depleted cells retain their polarized BM secretion (Denef et al., 2008; Lerner et al., 2013). In contrast, Rab11, which plays a direct role in rhodopsin (Rh) trafficking and maintenance of adult photoreceptor cells, was shown to be indirectly required in BM polarization, as in absence of Crag and Rab10, BM proteins undergo a Rab11-dependant route to localize apically (Denef et al., 2008; Lerner et al., 2013; Xiong et al., 2012). The homology and conserved function of *Drosophila* Crag was also determined when Crag mutant defects were rescued in *Drosophila* photoreceptor cells upon using the Crag human homolog DENND4A (Xiong et al., 2012). Additionally, in certain cancer cells, DENND4A, B, and C, Crag human homologs have been shown to be mutated and misregulated. (GDC Data Portal, NCI and COSMIC database, Sanger Institute).

In this thesis, the importance of each domain in the localization of Crag and its activity in restricting BM components basally was assessed. *Crag*<sup>CJ101</sup> is an early nonsense mutation of *Crag*. This suggests that *Crag*<sup>CJ101</sup> is a los of function allele of *Crag*. *Crag*<sup>CJ101</sup> mutant background in FC was used to express various truncated versions of Crag using the GAL4/UAS system. These truncated versions of Crag, which lacked single or multiple domains, were tagged with HA epitope to locate Crag using anti-HA (antibody for HA). The full-length Crag transgene HA-Crag has been shown to rescue the defects in *Crag*<sup>CJ101</sup> mutants by reestablishing the polarized accumulation of BM proteins to the basal side. Hence, the next steps are to determine
which of the domain(s) is responsible for the localization of Crag to the apical side, resulting in, and as well as its function to block the apical accumulation of BM proteins.

V. Project Overview and Aims

Crag has been shown to be a novel regulator in the specialized pathway leading to polarized deposition of BM proteins. As it a relatively large multidomain protein, it is essential to determine which of the domain(s) are responsible for the localization of Crag. As Crag assumes a polarized localization and accumulates apically and laterally (Figure 10B, B’), it has been speculated that via certain non-exclusive mechanisms, it blocks the accumulation of BM proteins to the apical and lateral domains and instead forces the BM proteins to localize basally. The goal of this thesis is the characterization of Crag in the control of polarized secretion of BM proteins by performing a structure-function analysis. Determining how the cellular distribution and activity of Crag are regulated and the domain(s) responsible will provide critical information on the biological pathway dedicated to the polarized deposition of BM proteins and for the better understanding of cancer progression.

Aim 1: Determining the Domain(s) Responsible for the Apical-Lateral Localization of Crag

The purpose of this aim is to assess which domain(s) is/are responsible for Crag’s proper localization, which potentially has an impact on its activity of BM protein localization. Crag localizes apically and laterally (Figure 10B, B’), suggesting that Crag blocks the apical secretion of BM proteins, localizing them basally. To determine which of the Crag domain(s) is/are required for the proper cellular localization of Crag, we performed a structure-function analysis of Crag by creating various truncated forms of Crag with single or multiple domains deleted.
These different forms were then expressed in FE under the control of GAL4/UAS system in transgenic *Drosophila* lines. Each construct was tagged with HA epitope and expressed in FE, which allowed us to observe the localization of the truncated form of Crag using fluorescent microscopy. We hypothesized that the CaM domain was required in the localization of Crag to the plasma membrane, while the DENN and C-terminal domains themselves are insufficient. That is because the DENN domains primarily act as GEF, and hence, they play a role in Crag’s activity of restricting BM protein deposition basally, but not its subcellular localization.

**Aim 2: Determining the Domain(s) Responsible for Crag’s Activity in Polarized BM Deposition**

The purpose of this aim was to assess the activity of Crag – the necessity and impact each domain has on Crag’s function in the localization of BM proteins. To assess the role of the different domains in Crag activity responsible for the proper localization of BM, different truncated forms of Crag (see aim 1) were expressed in transgenic *Drosophila* lines under the control of UAS/Gal4 drivers. To test our hypothesis of the necessity of the CBS domain in Crag’s activity/function as well, rescue experiment was performed on Crag mutant phenotype.

*Rescue Experiment Approach:* The loss of Crag in FE resulted in strong BM secretion to the apical side, indicating the loss of polarized BM secretion (Denef et al., 2008). However, when we ectopically expressed of full-length Crag in these *Crag* mutant cells, the polarized BM localization was restored, rescuing the mutant phenotype. Similar rescue experiments were performed using two constructs of Crag, one containing just the N-terminal and other containing just the C-terminal. This will allow us to determine which Crag domain is required for Crag functions in the polarized secretion of BM proteins.
Materials and Methods

I. Fly Stocks and Genetics

Fly Husbandry

Stocks were maintained in plastic vials which had pre-allotted food. Food used was a cornmeal-based Bloomington Formulation. The stocks were maintained at 18°C or 24°C. Stock flies were observed at RT. The stocks were maintained by transferring ("flipping") the flies from old vial to a new vial with fresh food (2 days to 2-week-old) every 18-21 days. For generating genetic crosses, virgin females of the desired genotype were picked after anesthetizing flies using CO₂. The presence of meconium dictated the fly virginity, while those lacking it were counted as sexually mature flies. The collected virgins were kept in a separate new vial until a sufficient number was obtained, usually between 12-18 virgins. Adult males of desired genotype, usually between 8-12, were then introduced to the vial containing virgins, constituting the parental (P) generation of the cross. The crosses were placed and kept at 25°C, which after 3-4 days, were flipped to a fresh vial. This periodic flipping was performed several times, until the parental generation was no longer alive. Resulting progeny possessing desired traits was used for further analysis.

For the crosses containing flies with heatshock flippase (hsFLP), each flipped vial (the vial that did not contain P generation anymore) was heat shocked for 2 hours at a time at 37°C for three consecutive days to activate the expression of flippase enzyme to allow for mitotic
recombination and clone generation. The crosses for both the localization and rescue experiments were made between a driver and a reporter line. Table 2 and Table 3 list the stocks and sex used to make crosses for localization and rescue experiments, respectively.

Table 2:
*Drosophila* stocks used for Crag's localization experiments

<table>
<thead>
<tr>
<th>Line category</th>
<th>Stock genotype and sex used for experimental cross (♀ x ♂)</th>
</tr>
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<tr>
<td>Follicle Cell Driver Lines</td>
<td>PcanGFP, tj-Gal4</td>
</tr>
<tr>
<td>UAS Reporter Lines</td>
<td>w; UASp-HA-CragA/CyO</td>
</tr>
<tr>
<td></td>
<td>UAS-HA-CragN/CyO</td>
</tr>
<tr>
<td></td>
<td>UAS-HA-CragC/CyO</td>
</tr>
<tr>
<td></td>
<td>w; s/CyO; UASp-HA-CragΔCBS/TM6B</td>
</tr>
<tr>
<td></td>
<td>UAS-HA-CragΔNΔCBS/CyO</td>
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<tr>
<td></td>
<td>UAS-HA-CragΔCterm/FM7c</td>
</tr>
<tr>
<td></td>
<td>w; UAS-HA-CragNCBS/CyO</td>
</tr>
</tbody>
</table>

Table 3:
*Drosophila* stocks used for Crag's rescue experiments

<table>
<thead>
<tr>
<th>Line category</th>
<th>Stock genotype and sex used for experimental cross (♀ x ♂)</th>
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</thead>
<tbody>
<tr>
<td>Follicle Cell Driver Lines</td>
<td>ubiGFP, FRT19A; e22c-Gal4 UAS-Flp/CyO</td>
</tr>
<tr>
<td></td>
<td>ubi-nls-mRFP, hsFlp122 Frt19A/(FM7); tj-Gal4/CyO</td>
</tr>
<tr>
<td>UAS Reporter Lines</td>
<td>PcanGFP, Crag\textsuperscript{CJ101}, FRT19A/FM7 #25</td>
</tr>
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<td>PcanGFP, Crag\textsuperscript{CJ101}, FRT19A/FM7; UAS-HA-CragA/CyO</td>
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<td>PcanGFP, Crag\textsuperscript{CJ101}, FRT19A/FM7; UAS-HA-CragN/CyO</td>
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<td>PcanGFP, Crag\textsuperscript{CJ101}, FRT19A/FM7; UAS-HA-CragC/CyO</td>
</tr>
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Table 4:

Genotypes of egg chambers imaged and their corresponding figure numbers

<table>
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<th>Figures</th>
<th>Genotypes</th>
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<tr>
<td>10B-B'; 15A-A'</td>
<td>VkgGFP line stained for endogenous Crag</td>
</tr>
<tr>
<td>10C-C'; 22A-A'</td>
<td>PcanGFP, Crag\textsuperscript{CJ101}, FRT19A #25 /ubiGFP, FRT19A</td>
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<tr>
<td>15B-B'; 16A-A'; 17A-A'; 18A-A'</td>
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</tr>
<tr>
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<td>16C-C'</td>
<td>PcanGFP/w; UAS-HA-CragC/tj-Gal4</td>
</tr>
<tr>
<td>17B-B'</td>
<td>PcanGFP; s/tj-Gal4; UAS-HA-Crag\textDelta CBS</td>
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<tr>
<td>17C-C'</td>
<td>PcanGFP/w; UAS-HA-Crag\textDelta N\textDelta CBS /tj-Gal4</td>
</tr>
<tr>
<td>18B-B'</td>
<td>PcanGFP/UAS-HA-Crag\textDelta Cterm; tj-Gal4</td>
</tr>
<tr>
<td>18C-C'</td>
<td>PcanGFP/w; UAS-HA-CragNCBS/tj-Gal4</td>
</tr>
<tr>
<td>20A-A'</td>
<td>PcanGFP, Crag\textsuperscript{CJ101}, FRT19A/ubi-nls-mRFP, hsFlp122 Frt19A</td>
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<tr>
<td>20B-B'</td>
<td>PcanGFP, Crag\textsuperscript{CJ101}, FRT19A/ubi-nls-mRFP, hsFlp122 Frt19A; UAS-HA-CragC/tj-Gal4</td>
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<tr>
<td>20C-C'</td>
<td>PcanGFP, Crag\textsuperscript{CJ101}, FRT19A/ubiGFP, FRT19A/ubi-nls-mRFP, hsFlp122 Frt19A; UAS-HA-CragA/tj-Gal4</td>
</tr>
<tr>
<td>22B-B'</td>
<td>PcanGFP, Crag\textsuperscript{CJ101}, FRT19A/ubiGFP, FRT19A; UAS-HA-CragN/e22c-Gal4 UAS-Flp</td>
</tr>
<tr>
<td>22C-C'</td>
<td>PcanGFP, Crag\textsuperscript{CJ101}, FRT19A/ubiGFP, FRT19A; UAS-HA-CragA/e22c-Gal4 UAS-Flp</td>
</tr>
</tbody>
</table>
The FRT/FLP System

Many loss-of-function mutations involved in development of an organism are lethal, which is also the case with Crag mutation CJ101. As it is recessive and homozygous lethal, it is not possible to study it on the entire organism as it would not survive. Hence, the FRT/FLP site-directed recombination system is utilized, allowing for the generation of homozygous mutant cells in specific parts of the heterozygous organism (Golic & Lindquist, 1989; St Johnston, 2002). The resulting mosaic tissues (in our case the FE) contain cells that are homozygous mutant, homozygous wildtype, and heterozygous, making the analysis and comparison of the mutation less challenging (Figure 11B).

FRT/FLP system contains two components: the flippase recognition target (FLP) site flanking the gene of interest and flippase recombinase (FLP) derived from yeast *Saccharomyces cerevisiae* (Gronostajski & Sadowski, 1985). When expressed, FLP recognizes identical FRT sites on homologous chromosomes, binds to them, and carries out mitotic recombination (Figure 11A). The expression of FLP can be controlled temporally, spatially, and with cell-type specificity using heat-shock (hsFLP) or tissue specific promoters such as and e22c-Gal4 (Muñoz-Jiménez et al., 2017; Park et al., 2011). Using these inducible promoters will not hinder the overall development of the organism and will allows us to compare cells lacking the gene of interest (mutant cells) with neighboring wild type cells in the same environment.
Figure 11: FRT/FLP system.

(A) To study homozygous lethal mutations, the FRT/FLP recombinase system was utilized to generate homozygous mutant clones in a heterozygous background. The resulting mosaic tissue was a product of via mitotic recombination at the FRT sites via the Flippase enzyme. UbiGFP acts as a clonal marker of the wildtype chromosome. Adapted from St Johnston, Nature Reviews Genetics, 2002. (B) Longitudinal section of a mosaic egg chamber stained for F-actin (red), DNA (blue), and (B') ubiGFP (green). Lack of ubiGFP as a nuclear marker represent homozygous mutant clone. 1=homozygous mutant cells (no GFP fluorescence), 2=heterozygous cells (dim fluorescence of GFP), 3=homozygous wildtype cells (bright fluorescence of GFP).
The Gal4/UAS System

The Gal4/ Upstream activation sequence (UAS) is a powerful tool that allows for targeted expression of a gene. This yeast derived transcription activation system consists of two parts: the transcription factor Gal4 and its target enhancer UAS. As such, the *Drosophila* line that contains the Gal4 gene is called the driver line, while the line containing the UAS sequence is called the reporter line. The expression of Gal4 can be controlled using tissue specific or ubiquitous drivers, in turn controlling the expression of reporter genes downstream of UAS (Figure 12) (Frickenhaus et al., 2015; Hudson & Cooley, 2014).

The GAL4/UAS system can be used in conjunction with the FRT/FLP system to control the expression of a gene both temporally and spatially, allowing for the generation of homozygous mutant and wildtype cells in a tissue. To express the truncated Crag constructs in FE for both the localization and rescue experiments, tj-Gal4, a FE specific driver was used. The constructs were cloned downstream to a UAS sequence, which controlled their expression. Another FE specific driver, e22c-Gal4 was also utilized, which, controlled the expression of both FLP and various Crag constructs in rescue experiments. As these drivers were only expressed in FE, FLP expression and mitotic recombination made FE the only location to have homozygous mutant cells in the heterozygous fly (Duffy et al., 1998).
Figure 12: UAS/GAL4 system.
The yeast transcriptional activator Gal4 is used to activate the upstream activating sequence (UAS). Once Gla4 is expressed under the control of a tissue specific genomic enhancer, it binds to the UAS sequence present upstream of the gene of interest (gene X). Thus, the UAS sequence regulates the transcription and expression of gene X (St Johnston, 2002).

Balancer Chromosomes

_Crag_ C1101 is a homozygous lethal mutation for _crag_, meaning that two copies of the mutation in a fly is lethal. To maintain such mutant lines, they need to be kept as heterozygous stocks using balancer chromosomes. There are three unique characteristics of balancer chromosomes that make them an indispensable tool in genetics. First, they contain recessive deleterious mutations, meaning that two copies of the gene will result in a lethal or sterile phenotype in the fly. Thus, flies containing two chromosome balancer will be lethal or sterile (Miller et al., 2019; Muller, 1928). Second, they contain multiple inversions which prevent meiotic recombination between the deleterious mutation, eliminating the chance to produce a chromosome containing no copies of the mutation (Crown et al., 2018; Muller, 1928). Third, they contain convenient features that allow for the phenotypic characterization of flies. For
example, balancer chromosomes can contain recessive lethal dominant markers, where one copy of the balancer will result in a distinct phenotype which will allow us to characterize the flies based on whether they inherited the balancer chromosome, and in turn, the mutation being studied (Muller, 1928). CyO is one such example, where one copy of the balancer will result in curly wings in the flies, but two copies will result in lethality. Examples of dominant mutations on balancer chromosomes include: Bar (B) eye phenotype on first chromosome balancer included FM7; Curly (Cy) which results in curly wings on the second chromosome balancer CyO; and Tubby (Tb1) on the third chromosome balancer TM6B.

II. Ovary Dissection and Immunofluorescent Staining

Fly Dissections

Once the first filial (F1) generation emerged in a vial, females (1-2 days old) with desired genotypes (determined by phenotypic markers present on the balancer chromosome) were collected. To obtain F1 females of desired genotype, flies were first separated using a dissecting microscope after being anesthetized with CO2. Once more than 10 females (1-2 days old) were obtained, they were nourished with baker’s yeast (which was placed atop food in a fresh vial) and left at 25°C for another 2 days. Flies resulting from crosses containing hsFLP in genotype were transferred to 29°C instead. The presence of yeast boosts the egg production in the flies, fattening the ovaries, easing the process of dissection. After the waiting period, the flies were obtained and anesthetized. Using a dissecting microscope, the flies were then dissected individually by placing them in phosphate-buffered saline (PBS) contained in a glass dissection well using forceps. The ovaries were separated from other organs and excised out of the fly.
Immunofluorescence Staining

Immunostaining was performed post dissection, in order to visualize the follicular epithelium, specifically protein localization, in the dissected ovaries. Ovaries dissected in PBS were fixed in 1 mL of 4% PFA (200 µL of 20% paraformaldehyde in 800 µL PBS) for 15 minutes on a nutator. Next, the PFA solution was removed, and the ovaries were washed with 1 mL of 0.1% Triton in PBS (PBST). Two quick washes, followed by three long washes (15 min each), were performed using a nutator.

For DNA (Hoechst) and F-actin staining (Phalloidin), after the wash steps, ovaries were stained simultaneously with Hoechst (1:1000, 10 µg/mL, Life Technologies) and either Alexa Fluor 546 phalloidin (1:500, Life Technologies) or Alexa Fluor 647 phalloidin (1:100, Life Technologies) in PBST for 15 minutes on a nutator. Next, two quick washes, followed by three long washes (15 min each), were performed using a nutator.

For antibody staining, the step that followed the fixation and post-fixation washes was blocking the ovaries using 5% bovine serum albumin (BSA diluted in PBS) for 1 hour on nutator. Next, BSA was removed, and ovaries were stained with 500 µL primary antibody solution (1:50 of anti-HA in 5% BSA) overnight at 4°C on a nutator, washed with PBST (two quick and three long washes), and stained with secondary antibody. Starting with the secondary antibody staining, the vial containing ovaries was wrapped in aluminum foil to keep them in the dark. The secondary antibody solution contained a fluorescent secondary antibody, Hoechst (1:1000) for DNA staining, and Alexa Fluor 647 phalloidin for F-actin staining. The ovaries were nutated for 2 hours for secondary staining, followed by two quick and three long washes.
After the last wash in either of the staining methods, the egg chambers were separated by triturating the ovaries using P1000. After the chambers settled to the bottom of the vial, PBST from the last wash was discarded. One drop of either Aqua-Poly/Mount (Polysciences) or ProLong™ Glass Antifade Mountant (Invitrogen) was used as mounting medium to transfer the sample to a glass slide, which was stored at room temperature overnight to allow drying before being used for microscopy.

**Immunofluorescence Microscopy**

Slides were imaged using various microscopes. For rescue experiments and for clonal analysis of various constructs of Crag, ThermoFisher Scientific EVOS M600 Imaging System equipped with 10x, 20x, and 40x objectives and 40x coverslip corrective and 60x oil emersion objectives was utilized. Imaging for localization of Crag (full length and truncated versions) as well as higher quality microscopy, including super resolution, for rescue experiments was performed using the Zeiss LSM 900 with Airyscan 2 Confocal Microscope acquired via a NSF MRI grant #2018748 (Yasui, PI). The objectives used were 40x (water) and 63x (oil), and images were acquired and processed using the Zeiss Zen software.

**III. Molecular Cloning and Generation of Transgenic Lines**

To assess the role of different domains in Crag’s localization and activity, various truncated versions of Crag that lacked single or multiple domains were required. Although some were generated in the Schüpbach lab (see part I of the materials and methods section), two Crag constructs (UAS-HA-Crag-NCBS and UAS-HA-Crag-ΔCBS) needed to be created to establish the importance and necessity of each domain. To generate these constructs, molecular techniques...
that included PCR, agarose gel electrophoresis, restriction digest and ligation, bacterial transformation, and plasmid isolation were utilized. The construct amplified using PCR was first inserted into the plasmid vector pTiger_5’HA. This plasmid contained genes (features/sequences) necessary for the expression of construct in latter steps. These included the AmpR gene for bacterial transformation, attB site for insertion into fly genome, mini w+ gene to allow for fly selection, the UAS/Gal4 binding site for expression of the construct in the flies, as well as an HA epitope for immunostaining (Figure 13). Once the constructs were inserted into the plasmid, they were then injected in fly embryos to generate transgenic lines.

For UAS-HA-CragNCBS generation, traditional molecular cloning techniques were successfully used. However, for UAS-HA-Crag∆CBS, traditional cloning did not yield successful results. Trying to insert the two fragments flanking CBS domain (Strategy 1) together resulted in the bacteria not able to take up the fragments properly. We used Gibson assembly as an alternate approach trying to insert the fragments in pTiger_5’HA both together (Strategy 2) as well as individually (Strategy 3). Since pTiger_5’HA is a relatively large plasmid (10338 bp), we also used a smaller plasmid (pBluescript KS, 2985 bp) to try to generate UAS-HA-Crag∆CBS (Strategy 4).
Figure 13: pTiger-5’HA plasmid map.
Map of the plasmid pTiger-5’HA, which was used to insert the various truncated forms of Crag. The various features required for future steps of insertion and expression of Crag protein in the flies are shown.
Polymerase Chain Reaction (PCR)

Full length cDNA clone for Crag A was used as the template for PCR to amplify the DNA fragment encoding for CragNCBS and CragΔCBS. Different DNA polymerases used included illustra™ puReTaq Ready-To-Go PCR beads (GE Healthcare), LongAmp® Hot Start Taq 2X Master Mix (NEB), and Q5® Hot Start High-Fidelity 2x Master Mix (NEB). Each PCR reaction contained basic components of dNTPs, DNA polymerase, template DNA, and primers. The primers used for cloning contained a palindromic endonuclease recognition site for future restriction digests. The reaction mixes underwent PCR run, which consisted of initial denaturation, 35 repeated cycles (each of which included denaturation, annealing, and elongation steps), and final elongation step. The temperatures for each step varied based on the requirements of the protocol used. The primers used for various strategies for generation of UAS-HA-CragΔCBS are listed in Table 5, and the primers used for generation of UAS-HA-CragNCBS are listed in Table 6.
Table 5:

Primers used to generate UAS-HA-CragΔCBS

<table>
<thead>
<tr>
<th><strong>Fragment Length (bp)</strong></th>
<th><strong>Strategy 1 (Traditional cloning- pTiger_5'HA+F1+F2)</strong></th>
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<tbody>
<tr>
<td><strong>F1 (1-2292)</strong></td>
<td>5KpnICrag 2 CCGGGGTACCATGGAGGAAAAGCGCATCGCC (68.8°C)</td>
</tr>
<tr>
<td></td>
<td>3EcoRI-CragdCam2 CCGGAATTCACAGGTGGCCAACAAGTA (62.7°C)</td>
</tr>
<tr>
<td><strong>F2 (2803-5016)</strong></td>
<td>5EcoRI-CragdCam CCGGAATTCGATTTTGCCCGCTTCGAT (63.3°C)</td>
</tr>
<tr>
<td></td>
<td>3XbaI-CragdCam GCTCTAGATCAGGGCAGCAGAAGCGG (64.6°C)</td>
</tr>
</tbody>
</table>

| **Strategy 2 (Gibson Assembly - pTiger_5'HA+F1+F2)** |
|--------------------------|--------------------------------------------------------|
| **F1 (1-2292)**          | CdC_F1_F wd_Gib GTTCCAGATTACGCTGGTACATGGAGGAAAAGCGCATCGC (67.8°C) |
|                         | CdC_F1_R ev_Gib CGATCGAAGGCGGCAAATCACAGGTGGCCAACAAGTGATT (68°C) |
| **F2 (2803-5016)**      | CdC_F2_F wd_Gib AATACCTTGTTGGCCACCTGTGATTTTGCCCGCTTCGATCGC (69.7°C) |
|                         | CdC_F2_R ev_Gib TCTGATCCCCGGCAGGTTACAGGGCAGCAGAAGCGG (75.8°C) |

| **Strategy 3A (Gibson Assembly - pTiger_5'HA+F1)** |
|--------------------------|--------------------------------------------------------|
| **F1 (1-2292)**          | CdC_pT5'_F1_F_Gib CAGGATCCATATCCATATGACGTTCCAGATTACGCTGGTACCATGGAGGAAAAGCGCATCGC (70.3°C) |
|                         | CdC_F1_R _Gib CGATCGAAGGCGGCAAATCACAGGTGGCCAACAAGTGATT (68.8°C) |

| **Strategy 3B (Gibson Assembly - pTiger_5'HA+F2)** |
|--------------------------|--------------------------------------------------------|
| **F2 (2803-5016)**      | CdC_pT5'_F2_R_Gib TCTAGATGCGTCCGCTCAGCTTGCTCTTGGCGGCCTTCGATCGC (73.8°C) |

| **Strategy 4A (Gibson Assembly - pBluescript KS+F1)** |
|--------------------------|--------------------------------------------------------|
| **F1 (1-2292)**          | CdC_pBS_F1_F_Gib GGGCGGCCGCTCTAGAAGGTTGACGCTCCCCGGCGTCAAGGTGGATGAGGGAAAGCGCATCGC (74.8°C) |
|                         | CdC_F1_R _Gib CGATCGAAGGCGGCAAATCACAGGTGGCCAACAAGTGATT (68.6°C) |

| **Strategy 4B (Gibson Assembly - pBluescript KS+F2)** |
|--------------------------|--------------------------------------------------------|
| **F2 (2803-5016)**      | CdC_pBS_F2_F_Gib GGGCGGCCGCTCTAGAAGGTTGACGCTCCCCGGCGTCAAGGTGGATGAGGGAAAGCGCATCGC (68°C) |
|                         | CdC_pBS_F2_R_Gib TCGAAATTAACCCCTACAAGGGGAAACAAAAGCTGGG TACCTCAGGGGCAAGCAGG (71.1°C) |
Table 6:

Primers used to generate UAS-HA-CragNCBS

<table>
<thead>
<tr>
<th>Fragment Length</th>
<th>Crag-NCBS Strategy (PCR)</th>
</tr>
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<tr>
<td>1-2082nt</td>
<td>5’KpnI_Crag CGGGGTACCATGGAGGAAAAGCGCATCGCC (71.1°C)</td>
</tr>
<tr>
<td></td>
<td>3EcoRI_Crag-EndCBS_Stop CCGGAATTCTCAGAGCAGTCCATGGCCACCGCC (70.1°C)</td>
</tr>
</tbody>
</table>

**Agarose Gel Electrophoresis**

For agarose gel electrophoresis, the DNA was electrophoresed through a 1% gel made using UltraPure™ Agarose (Invitrogen) and Tris-borate-EDTA (TBE) buffer (RPI). This mixture was first heated in a microwave until agarose was fully dissolved, 5µL of SYBR safe DNA gel stain (Invitrogen) was added and mixed by swirling, and then poured in gel cast to solidify. Once solidified, the DNA was run through its length via electrophoresis, and observed using blue light or UV light filters for imaging using Biorad Gel Documentation System.

Gel electrophoresis was performed several times during the cloning procedure, and it served two purposes. Electrophoresis aided in the determination of the size of the DNA fragment based on a reference molecular weight ladder, and in turn helped determine whether the amplification of DNA or digestion of DNA was successful. Furthermore, it helped identify the different DNA fragments, and select those needed for future steps by extracting them.

**DNA Purification Using Gel Extraction or PCR Clean-up**

To clean up the DNA that had run through an agarose gel, DNA bands of correct length were excised from the gel using a razor blade and extracted using the QIAquick® Gel Extraction
Kit (Qiagen) and its provided protocol. To clean up the DNA that had not been run through gel but rather was a product of only PCR or restriction digest, PCR clean-up was performed using QIAquick® PCR Purification Kit (Qiagen). The purpose of these techniques was to isolate the DNA from other components to determine its concentration.

**Concentration Measurement Using Spectrophotometry**

Concentration of DNA (ng/µg) was measured multiple times during the molecular cloning process using a NanoDrop™ Spectrophotometer. That is because many techniques require a minimum concentration of DNA to proceed, while some require both a minimum and a maximum. For example, concentration of DNA is required for PCR, gel electrophoresis, DNA transformation, and sequencing. Since all these steps were performed during the molecular cloning process, knowing the DNA concentration was important.

**Restriction Digest, CIP Treatment, and Ligation**

The amplified and purified DNA, along with the circular vector (plasmid) in which it was to be inserted, were double digested using restriction enzymes and CutSmart® Buffer (NEB) for 3 hours at 37°C. The vector (pTiger-5’HA) and DNA fragment of (amplified region of Crag) were cut at identical specific restriction sites using restriction enzyme (Table 7). The vector contained a multiple cloning site (MCS) which contained sites for restriction enzymes to cleave, which would become the location DNA fragment insertion. It also contained ampicillin resistance gene AmpR and a mini-w+ gene which would result red eyes in flies. Following restriction digest, the vector was treated with calf intestinal alkaline phosphatase (Invitrogen) for 10 minutes at 37°C to dephosphorylate the 5’ end of the vector, preventing self-ligation. The
DNA fragment and vector were then ligated using Rapid DNA Ligation Kit and the protocol provided (Thermo Scientific).

Table 7:
Restriction enzymes used during the generation of truncated forms of Crag

<table>
<thead>
<tr>
<th>Construct</th>
<th>Restriction Enzymes</th>
</tr>
</thead>
<tbody>
<tr>
<td>UAS-HA-Crag∆CBS</td>
<td>Strategy 1 (KpnI, EcoRI, and XbaI)</td>
</tr>
<tr>
<td></td>
<td>Strategy 2, 3, and 4 (KpnI and EcoRI)</td>
</tr>
<tr>
<td>UAS-HA-CragNCBS</td>
<td>KpnI and EcoRI</td>
</tr>
</tbody>
</table>

Bacterial Transformation

Once the DNA and vector were ligated, with DNA properly inserted in the vector, the next step was to amplify the whole vector, as that would be the used to create transgenic *Drosophila* lines. This large-scale amplification was performed using transformed bacteria, where DH5α strain of *E. coli* was transformed using the MAX Efficiency® DH5α™ Chemically Competent Cells kit and protocol provided (Invitrogen). pTiger-5’HA containing the desired Crag construct was inserted into the bacteria which, following transformation, were plated on LB+100µg/mL ampicillin (Amp) plates and incubated overnight at 37°C.

Miniprep, Restriction mapping, and Sequencing

Obtaining bacterial growth on plates, ~10 individual colonies (for replicates, as well as to ensure multiple colonies containing inserted plasmid) were picked with a pipette tip, streaked on a plate, as well as incubated individually in 5 mL of LB + Amp broth overnight at 37°C. The plasmid DNA from the bacterial culture was isolated using QIAprep® Spin Miniprep Kit, and as
performed before, electrophoresed, and extracted through 1% agarose gel for purification. Restriction mapping was also performed on an aliquot of the purified plasmid DNA in order to determine the presence of insert. Once confirmed, the DNA along with necessary primers was sent for Sanger Sequencing (Eurofins) to confirm that the insert present was as expected, and no mutations or errors occurred during any steps. Table 8 below lists the primers that were used for sequencing of CragNCBS.

### Table 8:

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Primer Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>UASP-F</td>
<td>GGTGGACATAGGAGTCCTGTA</td>
</tr>
<tr>
<td>UASP-R</td>
<td>CGGAACTGTTGCAGAGCAC</td>
</tr>
<tr>
<td>3'BglII_CragdCam</td>
<td>ACAGGTGGCCAACACAGTA</td>
</tr>
<tr>
<td>UpCamMidSeq</td>
<td>TACAAGCCGGAGATCCT</td>
</tr>
<tr>
<td>CragupDENN290</td>
<td>GGTGGACATAGGAGTCCTGTA</td>
</tr>
</tbody>
</table>

Midiprep for Isolation of Larger Quantity of DNA

Once the sequence of inserted DNA fragment was determined to match 100% to the known sequence, a higher quantity of DNA was required. Using the streaked bacteria representing the colony for which the sequence of insert was confirmed, a 3 mL LB + Amp broth culture was incubated at 37°C for ~5-7 hours. 100µL from this culture was then pipetted into a fresh 100 mL LB + Amp broth, and incubated overnight at 37°C. As in miniprep, a larger scale plasmid purification was performed using QIAfilter Plasmid Midi Kit. The resulting purified
DNA was then sent, in the required amount, to Rainbow Transgenic Flies, Inc. for insertion in the *Drosophila* genome.

**Gibson Assembly**

Compared to traditional cloning method which requires restriction digest and multiple restriction sites to be present, as well as addition of unwanted sequences at times which leave a scar, Gibson assembly works more efficiently when inserting multiple fragments simultaneously. Hence, this method was also utilized when trying to create Crag∆CBS, for which it was required to remove a segment inside of Crag and join the two regions flanking it. Gibson assembly works with homology between neighboring DNA segments to be inserted. When creating PCR primers, they are designed specifically with 15-20 bp overhangs as the overlapping complementary sequence of neighboring DNA segment, which ensures correct order of inserts upon ligation. This homology based technique results in a seamless assembly of multiple fragments of DNA (Gibson et al., 2009).

To create Crag∆CBS, Gibson Assembly was performed using GeneArt™ Gibson Assembly® EX Master Mix (Invitrogen) was used. As its protocol suggested, One Shot® TOP10 Chemically Competent *E. coli* (Invitrogen) were used for transformation.

**Embryo Injection and AttP-attB Recombination**

Once the plasmid containing the insert was obtained in desired amount, it was sent to Rainbow Transgenic Flies, Inc. for integration into the fly genome. The plasmid used (pTiger-5′HA) contained multiple genes which served different purposes throughout our experimental steps, and included the newly inserted truncated version of Crag. It also contained attB site,
while the genome of the fly contained an attP site on the desired chromosome (second chromosome). The fly stock contains with attP site contains a transgene that codes for phiC31 integrase, which recombines the attP and attB sites, allowing for site specific integration of our plasmid into the fly genome (Gao et al., 2008; Groth et al., 2004). Once the attB and attP sites have recombined, new hybrid sites are created which the phiC31 integrase can no longer bind to as its substrate, resulting in a unidirectional reaction.

**Transgenic Line Generation**

This insertion during the attP/attB recombination happens in the pole cells of flies, which become the future germline cells. Hence, once the fly with the integrated genome laid fertilized eggs, the progeny containing the inserted plasmid were obtained, which were selected for based on their eye color (red due to the presence of mini w+ gene in the pTiger plasmid) (Figure 14). Below is the cross scheme for the creation a line to determine localization of the HA-Crag-NCBS construct.

**Step 1:**

\[
\frac{w- \text{ UAS-HA-Crag-NCBS}}{w-^{5} \text{ CyO}} \times \frac{w-^{sco} \text{ Y}^{7} \text{ CyO}}{}
\]

**Step 2** (red eye male from F1 of Step 1):

\[
\frac{w- \text{ UAS-HA-Crag-NCBS}}{Y \text{ CyO}} \times \frac{w-^{sco} \text{ w-}^{\text{CyO}}}{w-^{\text{CyO}}}
\]

**Step 3** (self-cross for stock generation):

\[
\frac{w- \text{ UAS-HA-Crag-NCBS}}{w-^{5} \text{ CyO}} \times \frac{w- \text{ UAS-HA-Crag-NCBS}}{Y \text{ CyO}}
\]
Figure 14: Drosophila transgenesis.
Plasmid carrying white+ transgene DNA (red) is injected into the embryos of generation zero (G0) that are obtained from the parental (P) generation. For germline transmission to occur, the inserted DNA must be taken up into the pole cells that will become germ cells. The pole cells (red pole cell) that have the transgenic DNA integrated into the can transit from one generation to the next (G1 progeny). If flies that are mutant for the white strains (have white eyes) are used, the presence of white+ gene will result in darker eye color (red). Hence, white+ will act as a marker and allow for easy selection of flies with desired genotype and inserted transgene. (Venken & Bellen, 2007).
For the clones analyzed during the rescue experiments, statistical analysis was performed. The clones were counted as either those showing any level of mislocalization, or those showing no mislocalization for determining whether the results were significant. Fisher’s exact test was performed between $Crag^{CJI01}$ line where no Crag was ectopically expressed and between the other crosses where different truncated versions of Crag were expressed. The percentage rescue for each cross was analyzed.
Results

Part 1: Generation of Truncated Crag Constructs

To visualize the localization and assess the activity of the different truncated versions of Crag, a series of steps was performed (see materials and methods for details). First, I generated the DNA constructs using molecular cloning techniques that allow the different truncated forms of Crag to be express under the control of the Gal4/UAS system (pTiger vector). Second, in order to generate transgenic Drosophila lines in which the pTiger construct is inserted in their genome, these DNA constructs (e.g., pTiger_5’HA_CragNCBS) were injected in the germline cells of Drosophila embryos. Third, I established the transgenic fly stocks containing the different constructs that allow the expression of the truncated forms of Crag. Fourth, I expressed the different construct in the FE using the UAS/Gal4 system and observe the localization (Part 2) and activity (Part 3) of the different truncated forms of Crag using fluorescent microscopy (i.e., widefield and confocal microscopy. All the constructs (in their respective transgenic lines) except HA-CragNCBS and HA-CragΔCaM were generated and provided as a gift from Schüpbach Lab. In the following paragraphs, I briefly describe the approach (strategy) I used to generate the DNA constructs pTiger_5’HA_CragNCBS (UAS-HA-CragNCBS) and pTiger_5’HA_CragΔCaM (UAS-HA-CragΔCaM).
UAS-HA-CragNCBS Generation

To generate the DNA construct pTiger_5’HA_CragNCBS (UAS-HA-CragNCBS) that allow the expression of HA-CragNCBS under the control of the UAS/Gal4 system, I used traditional cloning method (i.e, PCR, restriction enzymes and ligase, see materials and methods for details). Briefly, a full-length cDNA clone for Crag was used as the template for PCR to amplify the DNA fragment encoding for CragNCBS. Then, these PCR fragments were digested with restriction enzymes (KpnI and EcoRI) along with pTiger-5’HA plasmid and were ligated using DNA ligase. DH5α E. coli were transformed with a DNA mixture containing the ligated plasmid (pTiger-5HA-CragNCBS) and incubated overnight to obtain colonies containing the plasmid. Next, to determine if the bacteria colonies contain pTiger-5HA-CragNCBS plasmid, purification was performed followed by restriction mapping using KpnI and EcoRI. The plasmids purified from the different bacteria colonies were digested and agarose gel electrophoresis was performed. If the DNA fragment encoding for CragNCBS was successfully inserted in the pTiger-5’HA plasmid, two bands were expected, one corresponding to the DNA fragment (2802 bp) and one corresponding to the pTiger-5’HA plasmid (10338 bp). Figure 15A shows that CragNCBS has migrated correctly, as it is 2802 bp long, and is located on the gel between the 3000 and 2000 reference bands on the ladder. A band representing the 10338 bp vector is also seen above the 10000bp band (topmost band) on the ladder. Some of the wells show missing bands, which may be due to the bacteria taking up a plasmid that had ligated prior to CragNCBS insertion, or the bacteria not taking up the plasmid at all. The inserted DNA was sequenced, compared to the known sequence of Crag, and correct insertion was determined as
shown in Figure 15B. This construct was used to generate a transgenic line and localization experiment was performed.

Figure 15: Cloning of CragNCBS in the pTiger-5’HA plasmid. (A) Restriction mapping: Agarose gel readout of pTiger-5’HA+ CragNCBS digestion with KpnI and EcoRI restriction enzymes. It shows that CragNCBS has been inserted into the plasmid properly (e.g., clone #2). The wells missing either pTiger-5’HA or CragNCBS band represent the bacteria colonies that transformed with the plasmid without the CragNCBS construct or did not uptake the plasmid (e.g., clone #1,8). (B) Shows the insertion (red arrow) of CragNCBS in the MCS, which is preceded by 5’HA epitope and the UAS sequence. This will result in the expression of HA-CragNCBS in transgenic flies under the control of the UAS/Gal 4 system.

UAS-HA-CragΔCaM Generation

Similar molecular techniques were applied toward the generation of HA-CragΔCaM construct, however, the results were not as expected. Using traditional cloning which included PCR, restriction digest, and ligation, the bacteria was not able to transform with the Crag construct (as described above). As CragΔCaM is the truncated version of Crag which has the CBS domain deleted, the two flanking regions need to be ligated together. However, the ligation was unsuccessful. Upon trying to insert just one flanking region at a time, we were able to insert the left flanking region containing the DENN domains (Fragment 1) into the pTiger5’HA.
however, the right flanking region (Fragment 2) which contained the DNA sequence of Crag gene downstream of CBS domain, was not inserted successfully. Gibson assembly was also used to try to generate this construct, however, this attempt was also unsuccessful, as the bacteria was not transformed, and no colonies were observed.

**Part 2: Determination of the Crag’s Domain(s) Responsible for the Proper Cellular Localization of Crag**

To determine the Crag’s domain(s) required for the proper cellular localization of Crag (i.e., apical and lateral localization), a structure function analysis was performed. To do so, once the transgenic Drosophila lines were obtained, they were used to assess the localization of the different truncated forms in the FE. Using the Gal4/UAS system, we were able to express the different HA-Crag constructs as they were under the control of the UAS. Hence, the follicular epithelium drive traffic jam was used to express the Gal4 protein, which would bind to the UAS sequence and drive the expression of HA-Crag constructs specifically in the FE. Then, the subcellular localization of the different truncated forms of Crag were assessed using antibodies against the HA tag.

**Comparison of the Subcellular Localization of Crag and HA-Crag**

Before performing the localization experiments with the truncated versions of Crag, we wanted to determine whether the addition of an HA epitope to Crag affected its localization. To do so, immunostaining was performed on the progeny of the following cross: PcanGFP; tj-Gal4 males and w; UASp-HA-CragA/CyO females. For this cross, I selected the progeny with the
genotype PcanGFP/w; UASp-HA-CragA/tj-Gal4 because these flies express HA-Crag (full length) in the FE. The fly ovaries were dissected and immunostained with anti-HA antibodies. In wildtype FE, endogenous Crag accumulate at the apical and lateral plasma membrane (Figure 16A, A’) and localize in intracellular punctate (not shown). Similarly, HA- Crag localize at the apical and lateral plasma membrane indicating that HA-Crag assumes the same localization that endogenous Crag (Figure 16 B, B). Although the amount of HA-Crag at the lateral domain of the cells seemed to be higher compare to endogenous Crag, we speculated that the increase of Crag is the result of overexpression due to the Gal4/UAS system. Thus, the addition of an HA-tag at the N-terminal part of Crag does not affect the cellular localization of Crag.

Determination of the Importance of the N-terminus and the C-terminus Domains in the Subcellular Localization of Crag.

After determining that the presence of HA epitope does not interfere with the localization of Crag, the next step was to determine which of its domains are responsible for the apical-lateral localization of Crag. Hence, we first determined the importance of both termini of Crag. We expressed constructs with just the N-terminus and just the C-terminus and observed their localization. HA-CragN was the truncated form of Crag which only included the DENN domains (the N-terminus), and HA-CragC was the truncated form of Crag which included only the CBS and the conserved C-terminus domains (C-terminus). The localization of both constructs was compared to that HA-CragA (full length Crag).
Figure 16: HA-Crag assumes an identical localization to endogenous Crag in FE.  
(A-A’) Longitudinal section through FE of egg chamber immunostained for endogenous Crag (red) and stained with Hoechst (DNA, blue). (A’) The localization of Crag can be seen at the lateral domains of the cells (arrows).  
(B-B’) Longitudinal section through FE of egg chamber. Egg chamber is immunostained for HA (red) and stained with Hoechst (DNA, blue). The localization of Crag can be seen at the lateral domains of the cells (arrows). (Bars, 10 μm)
For HA-CragN, immunostaining was performed on the progeny of the following cross: PcanGFP; tj-Gal4 males and UAS-HA-CragN/CyO females. The progeny I selected had the genotype PcanGFP/w; UAS-HA-CragN/tj-Gal4. For HA-CragC localization, the progeny I selected had the genotype PcanGFP/w; UAS-HA-CragC/tj-Gal4. This progeny resulted from the cross between PcanGFP; tj-Gal4 males and UAS-HA-CragC/CyO. For progeny of both crosses, their ovaries were immunostained for HA. Figure 17 shows the localization of these truncated versions of HA-Crag. When HA-CragN is expressed (Figure 17 B, B’), its mislocalization to the plasma membrane is apparent. Instead of accumulating at the plasma membrane like HA-Crag, HA-CragN is localized in the cytoplasm. Contrary to this, HA-CragC localizes at the lateral domains (Figure 17 C C’), similar to HA-Crag and endogenous Crag. These results indicate that the DENN domains (N-terminal domains) are not sufficient to localize Crag at the plasma membrane, and that the C-terminal part of Crag is required for its localization.

**Determination of the Importance of C-terminus Domains of Crag in its Localization**

After observing the analogous localization of HA-Crag and HA-CragC, we proceeded to the next step to determine which of the domains of the C-terminus play a role in the Crag’s cellular localization. First, we wanted to see whether the CBS domain plays a role in the localization of Crag. Hence, localization of HA-Crag∆CBS and HA-Crag∆N∆CBS was observed. HA-Crag∆CBS consisted of all of the domains but the CBS domain, although it was not completely removed and part of it remained (Figure 18B). Immunostaining for this construct was performed on the progeny of the following cross: PcanGFP; tj-Gal4 males and w; s/CyO; UASp-HA-Crag∆CBS/TM6B females. The progeny selected for ovarian staining had the genotype PcanGFP; s/tj-Gal4; UAS-HA-Crag∆CBS (Figure 18B, B’).
Figure 17: C-terminus part of Crag is required for the apico-lateral localization of Crag. 
(A-A’) Longitudinal section through FE of egg chamber immunostained for HA (red) and stained with Hoechst (DNA, blue). The localization of HA-CragA can be seen at the lateral domains of the cells. 
(B-B’) Longitudinal section through FE of egg chamber. Egg chamber is immunostained for HA (red) and stained with Hoechst (DNA, blue). Mislocalization of HA-CragN can be seen throughout the cytoplasm. 
(C-C’) Longitudinal section through FE of egg chamber. Egg chamber is immunostained for HA (red) and stained with Hoechst (DNA, blue). HA-CragC is localized to the lateral domains of the cells, similar to HA-CragA (A-A’). (Bars, 10 μm).
HA-CragΔNΔCBS was also used to determine the importance of CBS domain. This construct had the N-terminus DENN domains and the CBS domain removed, and it contained the sequence only downstream of CBS domain. This helped in determining the importance of CBS domain in absence of the N-terminus of Crag. This construct would also help determine whether the conserved C-terminus is sufficient to localize Crag correctly. Immunostaining for this construct was performed on the progeny of the following cross: PcanGFP; tj-Gal4 males and UAS-HA-CragΔNΔCBS/CyO females. The progeny selected had the genotype PcanGFP/w; UAS-HA-CragΔNΔCBS /tj-Gal4 (Figure 18C, C’). We observed that the lack of CBS domain causes a significant disruption in the localization of HA-Crag, which results in a dispersed localization through the cytoplasm (Figure 18 B,B’). On the other hand, the absence of CBS domain when the N-terminus is not present displays a less severe impact on Crag’s localization, as it still aggregates, although less distinctly, to the lateral domains (Figure 18 C,C’). Hence, although CBS domain plays an important role in the localization of Crag to the apical and lateral domains, it may not be sufficient.

Although the results we found for HA-CragΔCBS and HA-CragΔNΔCBS were different due to the mislocalization of Crag in absence of CBS domain from the entire protein and almost-accurate localization of Crag when CBS domain is missing along with the N-terminus, they still suggest that CBS domains play an important role in the localization of Crag. Hence, next, we wanted to determine the whether the absence of conserved domain has an impact on Crag’s apico-lateral localization. With that purpose, I observed the localization of HA-CragΔCterm and HA-CragNCBS. HA-CragΔCterm construct lacked just the conserved domain of the C-terminus, meaning, the construct contained the region of DNA between the CBS domain and the conserved C-terminus domain.
Figure 18: CBS domain is required for the proper localization of Crag.

(A–A’) Longitudinal section through FE of egg chamber immunostained for HA (red) and stained with Hoechst (DNA, blue). The localization of HA-CragA can be seen at the lateral domains of the cells. (B–B’) Longitudinal section through FE of egg chamber. Egg chamber is immunostained for HA (red) and stained with Hoechst (DNA, blue). HA-Crag∆CBS does not localize at the lateral domains as it does with HA-Crag (A, A’). Instead, it is dispersed throughout the cytoplasm. (C–C’) Longitudinal section through FE of egg chamber immunostained for HA (red) and stained with Hoechst (DNA, blue). Crag is observed accumulating at the lateral domains, although not as distinctly as with endogenous Crag or HA-Crag (A, A’). (Bars, 10 μm).
For this, progeny of the following cross was selected: PcanGFP; tj-Gal4 males and UAS-HA-CragΔCterm/FM7c females. The progeny selected for ovarian immunostaining of HA had the genotype PcanGFP/UAS-HA-CragΔCterm; tj-Gal4. Experiment to determine the apico-lateral localization on HA-CragNCBS was also performed. This construct was terminated downstream of CBS domain. The termination was performed to confirm whether the region in between CBS domain and the conserved C-terminus domain also played any role in Crag’s localization. The cross made for this construct was between PcanGFP; tj-Gal4 males and and w; UAS-HA-CragNCBS/CyO females. The progeny immunostained for HA-CragNCBS had genotype progeny PcanGFP/w; UAS-HA-CragNCBS/tj-Gal4. In the FE, HA-CragΔCterm localizes to the lateral domains (Figure 19B,B’) similar to HA-CragA or endogenous Crag (Figure 19A,A’). However, HA-CragNCBS, where the truncated protein stopped just after the CBS domain, mislocalized throughout the cytoplasm (Figure 19 C,C’), suggesting that although the conserved C-terminus domain was not important in the localization of Crag, the DNA sequence between the CBS domain and the conserved domain may play a role in localization of Crag to the apical and lateral domains along with CBS domain.

**Part 3: Role of Crag**

**Crag’s Rescue Experiments**

In parallel to determining the domain(s) responsible for Crag’s localization, we also wanted to know which of the domain(s) of Crag is/are responsible for its activity as a regulator of polarized deposition of BM proteins. To do this, we performed rescue experiments (Figure 20). We used a *Drosophila* line mutant for *Crag* (mutant background of *Crag*^CJ101^) and used
Figure 19: CBS domain is not sufficient for the proper localization of Crag. (A-A’) Longitudinal section through FE of egg chamber immunostained for HA (red) and stained with Hoechst (DNA, blue). The localization of HA-CragA can be seen at the lateral domains of the cells. (B-B’) Longitudinal section through FE of egg chamber. Egg chamber is immunostained for HA (red) and stained with Hoechst (DNA, blue). Although not as distinctly, HA-Crag∆Cterm localizes to the lateral domains in a similar manner to HA-Crag (A, A’). (C-C’) Longitudinal section through FE of egg chamber immunostained for HA (red) and stained with Hoechst (DNA, blue). HA-CragNCBS accumulates aberrantly throughout the cytoplasm and is not only restricted to the lateral domains. (Bars, 10 μm)
endogenous BM component Pcan which was tagged with GFP (PcanGFP) as a readout for BM localization. CJ101 is a nonsense mutation of Crag which ceases Crag’s activity in polarized BM protein deposition, which leads to the mislocalization of BM components to the apical side. In the mutant background, as Crag is crucial for proper BM deposition, PcanGFP accumulated aberrantly to the apical side. To determine which domains of Crag play a role in its activity, we ectopically expressed different constructs of Crag, and analyzed the mislocalization of PcanGFP in clones. Clones represented the cells of FE that were homozygous mutant for the mutation (marked by the absence of the clonal marker, i.e., GFP or RFP). For clonal analysis, each was divided into one of the three categories based on the strength of the signal produced by mislocalized PcanGFP. These categories included clones with strong mislocalization, partial mislocalization, and no mislocalization (rescue) of PcanGFP to the apical side.

Using the tj-Gal4 Driver to Ectopically Express Crag Constructs

For the rescue experiments, we first needed cells that are homozygous for the mutation. As it is a lethal mutation, the FRT/FLP system was used to generate mosaic egg chambers with cells homozygous for wild type as well as mutation, and cells that are heterozygous. We also needed to ectopically express different constructs of Crag, which was done using UAS-Gal4 system. Our first rescue approach consisted of the driver line w; ubi-nls-mRFP, hsFlp122 Frt19A/(FM7); tj-Gal4/CyO and reporter lines that contained either just the Crag\textsuperscript{CJ101} background, HA-Crag, HA-CragN, or HA-CragC constructs. The traffic jam-Gal4 (tj-Gal4) is a very strong FE specific driver. The reporter line without ectopically expressed Crag was PcanGFP, Crag\textsuperscript{CJ101}, FRT19A/FM7 #25. To determine whether the full length of Crag was able
**Figure 20: Rescue experiment approach.**
Schematic of the rescue approach used to determine the domain(s) important for Crag’s activity. The image shows three types of cells. The left cell is a wildtype cell, meaning a cell that does not have any copy of the $\text{Crag}^{\text{CJ101}}$ mutant. As such, it displays a proper accumulation of BM on the basal side. The cell in the middle is homozygous mutant for $\text{Crag}^{\text{CJ101}}$, and hence is counted as a clone. As it does not have any functional copy of Crag, while BM is seen to localize at the basal side, it also accumulates at the apical side. The cell on the right is also a clone, however, here, Crag is ectopically expressed. As there is no mislocalization of BM on the apical side, this is called a rescue of the mutant phenotype.
the mislocalization of BM components, we used the transgenic line PcanGFP, Crag^{CJ101}, FRT19A/FM7; UAS-HA-CragA/CyO. Upon crossing it with the driver line, we can generate mutant clones in the FE and express full-length HA-Crag. The next step, just as in Crag’s localization experiments, was to determine the importance of the N- and C-terminus domains. Hence, for the N-terminus, PcanGFP, Crag^{CJ101}, FRT19A/FM7; UAS-HA-CragN/CyO females were used as reporter line, and for C-terminus, PcanGFP, Crag^{CJ101}, FRT19A/FM7; UAS-HA-CragC/CyO females were used. When crossed with driver line, the progeny will contain mutant clones in the FE and express the respective truncated version of HA-Crag. Figure 21 shows the different categories of clones for the tj-Gal4 driver condition. In the mutant cells for Crag, the expressed BM components mislocalized to the apical domain either evenly, which represented strong mislocalization (Figure 21A,A’), or unevenly, which represented partial mislocalization (Figure 21B,B’). When different constructs of Crag are ectopically expressed, we also observe a third phenotype, where in mutant cells, no mislocalization of PcanGFP (Figure 21C,C’) is seen. This is referred to as a rescue. For each construct, the number of clones was recorded, and statistical analysis was performed using Fisher’s exact test to determine the statistical significance of the data obtained. The values for strong and partial mislocalization were grouped together as mislocalization, and no mislocalization was counted as rescue.

For each category, the number of clones for each condition were counted to calculate the percentage of rescue. Here, the category represented the phenotype observed (Figure 21) and the condition represented the different constructs of Crag. The raw data for clonal analysis is shown in Table 9.
Figure 21: Representative images for Crag^{CJ101} clones for rescue crosses made using tj-Gal4 driver line

(A-A') Longitudinal section of FE of egg chambers stained for DNA (blue) and endogenous fluorescence of ubiRFP (red), and PcanGFP (green). Strong mislocalization of BM component PcanGFP is on the apical side (arrows). 

(B-B') Longitudinal section of FE of egg chambers stained for DNA (blue) and endogenous fluorescence of ubiRFP (red), and PcanGFP (green). Partial mislocalization of PcanGFP on the apical side is seen in Crag^{CJ101} clones (arrows). 

(C-C') Longitudinal section of FE of egg chambers stained for DNA (blue) and endogenous fluorescence of ubiRFP (red), and PcanGFP (green). Crag^{CJ101} clone show no localization of PcanGFP on the apical side (rescue). (Bars, 10 μm)
Table 9:
Number of clones counted and categorized for rescue crosses with tj-Gal4 driver line

<table>
<thead>
<tr>
<th>Category</th>
<th>Crag$^{CJ101}$#25</th>
<th>HA-CragA</th>
<th>HA-Crag-N</th>
<th>HA-Crag-C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Strong Mislocalization</td>
<td>96</td>
<td>0</td>
<td>16</td>
<td>112</td>
</tr>
<tr>
<td>Partial Mislocalization</td>
<td>51</td>
<td>0</td>
<td>106</td>
<td>49</td>
</tr>
<tr>
<td>No Mislocalization (Rescue)</td>
<td>0</td>
<td>206</td>
<td>86</td>
<td>34</td>
</tr>
<tr>
<td><strong>Total Clones</strong></td>
<td><strong>147</strong></td>
<td><strong>206</strong></td>
<td><strong>208</strong></td>
<td><strong>195</strong></td>
</tr>
</tbody>
</table>

The number of clones for each condition was taken and percentage was calculated for each category of PcanGFP localization. Figure 22 shows a graph representing each category for each of the crosses. The cross with line Crag$^{CJ101}$#25 showed zero percent of rescue, which was expected as there was no ectopic expression of any of the Crag constructs in the mutant background. For full length crag (HA-CragA) ectopic expression, a 100% rescue was seen. For HA-CragN (Crag containing N-terminus DENN domains) and for HA-CragC (Crag containing the C-terminus CBS and conserved domains), a rescue of 41% and 18% was observed, respectively (Figure 22). Upon performing the Fisher’s exact test for statistical significance, the p-value for each of these conditions was found to be p<0.0001. This meant that all these conditions provided results that were statistically significant. Hence, although the N-terminus plays a greater role than C-terminus in the activity of Crag, the C-terminus is also required for proper activity of Crag. However, the 0% of strongly and partially mislocalized clones and 100% rescue in the HA-CragA suggested that the tj-Gal4 was an extremely strong driver, and the excessive production of Crag under its control may be the cause of this high rescue. If so, then it
may also have resulted in a higher percentage of partial localization and rescue clones in HA-CragN and HA-CragC conditions. Hence, the next step was to use a weaker drive.

![Figure 22: Clone analysis for rescue crosses between reporter lines and the driver line containing tj-Gal4 driver.](image)

The graph shows the percentage of clones categorized into strong mislocalization, partial mislocalization, and no mislocalization (rescue) based on PcanGFP accumulation at the apical side of the FE cells. This classification can be seen for each of the crosses (condition) made for rescue approach using the tj-Gal4 driver and respective Crag constructs. **** = p<0.0001.

Using the e22c-Gal4 Diver to Ectopically Express Crag Constructs

After observing the high rescue percentages when using the tj-Gal4 driver, we decided that a comparatively weaker driver will be used to determine whether there are any differences in these results. Hence, using the same reporter lines, crosses were made for rescue experiments using ubiGFP, FRT19A; e22c-Gal4 UAS-Flp/CyO, which contained the e22c-Gal4 as its driver (Figure 23).
Figure 23: Representative images for Crag<sup>CJ101</sup> clones for rescue crosses made using e22c-Gal4 driver line.

A-A’) Longitudinal section of FE of egg chambers stained for DNA (blue) and endogenous fluorescence of ubiGFP (green), and PcanGFP (green). Strong mislocalization of BM component PcanGFP is on the apical side (arrows). (B-B’) Longitudinal section of FE of egg chambers stained for DNA (blue) and endogenous fluorescence of ubiFFP (green), and PcanGFP (green). Partial mislocalization of PcanGFP on the apical side is seen in Crag<sup>CJ101</sup> clones (arrows). (C-C’) Longitudinal section of FE of egg chambers stained for DNA (blue) and endogenous fluorescence of ubiGFP (green), and PcanGFP (green). Crag<sup>CJ101</sup> clone show no localization of PcanGFP on the apical side (rescue). (Bars, 10 μm)
E22c is expressed early during oogenesis and in lower quantities, unlike traffic-jam. Hence, when crossed with e22c-Gal4 driver line, the results may be different compared to tj-Gal4. Figure 23 shows the different categories of clones for the e22c-Gal4 driver condition. In the mutant cells for Crag, BM mislocalizes to the apical domain either very strongly (Figure 23A,A’), and partially (Figure 23B,B’), and no mislocalization of PcanGFP (Figure 23C,C’) is also seen. For each construct, statistical analysis was performed in the same manner as earlier using Fisher’s exact test.

For each category, the number of clones were counted to calculate the percentage of rescue. The raw data for clonal analysis is shown in below. Here, the category represented the phenotype observed (Figure 23) and the condition represented the different constructs of Crag. The raw data for clonal analysis is shown in Table 10.

Table 10:

Number of clones counted and categorized for rescue crosses with e22c-Gal4 driver line

<table>
<thead>
<tr>
<th>Reporter Line</th>
<th>Crag&lt;sub&gt;CJ101#25&lt;/sub&gt;</th>
<th>HA-CragA</th>
<th>HA-Crag-N</th>
<th>HA-Crag-C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Strong Mislocalization</td>
<td>112</td>
<td>103</td>
<td>140</td>
<td>147</td>
</tr>
<tr>
<td>Partial Mislocalization</td>
<td>66</td>
<td>102</td>
<td>74</td>
<td>70</td>
</tr>
<tr>
<td>No Mislocalization (Rescue)</td>
<td>2</td>
<td>139</td>
<td>70</td>
<td>43</td>
</tr>
<tr>
<td><strong>Total Clones</strong></td>
<td><strong>180</strong></td>
<td><strong>344</strong></td>
<td><strong>284</strong></td>
<td><strong>260</strong></td>
</tr>
</tbody>
</table>
The number of clones for each condition was taken and percentage was calculated for each category of PcanGFP localization. Figure 24 shows a graph representing each category for each of the crosses. The cross with line Crag\(^{CJ101\#25}\) showed 1% of no mislocalization, however, that was not considered a rescue, as it was not statistically significant, and may simply have been a result of the mutant phenotype not fully penetrating the FE. For full length crag (HA-CragA) ectopic expression, a 40% rescue is seen. For HA-CragN (Crag containing N-terminus DENN domains) and for HA-CragC (Crag containing the C-terminus CBS and conserved domains), a rescue of 24.6% and 16.5% is observed, respectively. Upon performing the Fisher’s exact test for statistical significance, the p-value for each of these conditions was found to be p<0.0001. This means that all these conditions provided results that were statistically significant. Hence, it shows that both the N- and C-terminus domains play a role in Crag’s activity, however, the role of the DENN domains (N-terminus) was greater than the C-terminus.

Based on the data we obtained from the rescue experiments for both drivers (tj-Gal4 and e22c-Gal4), we speculated that the DENN domains (N-terminus) are important for the activity of Crag in regulating polarized deposition of BM proteins, however, they are not sufficient. HA-CragC showed a lower rescue than HA-CragN, however, as it was a significant result, we speculated that the C-terminus also plays a role in Crag’s activity. Although its role is much less compared to the DENN domains, it may still be required for full activity of Crag in BM protein deposition.
**Figure 24:** Clone analysis for rescue crosses between reporter lines and the driver line containing e22c-Gal4 driver.

The graph shows the percentage of clones categorized into strong mislocalization, partial mislocalization, and no mislocalization (rescue) based on PcanGFP accumulation at the apical side of the FE cells. This classification can be seen for each of the crosses (condition) made for rescue approach using the tj-Gal4 driver and respective Crag constructs. **** = p<0.0001

From the results of both the localization experiments and the rescue experiment altogether, my data indicates that the DENN domains are not required for the localization of Crag. However, they are necessary for the activity of Crag, but are not sufficient. On the other hand, the C-terminus is necessary for the localization of Crag. Specifically, the CBS domain plays a critical role in Crag’s localization, however, is not sufficient and other parts of the C-terminus may be required. Furthermore, the C-terminus (possibly CBS domain) may also be required for Crag to perform its activity in BM deposition with full accuracy.
Discussion

Epithelial cells are found abundantly throughout the body and are responsible for various functions. Their epithelial characteristics are maintained via accurate polarized trafficking of domain protein (Rodriguez-Boulan et al., 2005). One crucial aspect of epithelial cells is their apical-basolateral polarity resulting from intra- and intercellular interactions including contact with neighboring cells and protein pathways (Cao et al., 2012). Basement membrane is a specialized sheet of extracellular matrix, which is crucial to the formation and maintenance of this polarity. It is also crucial in various processes such as polarization and tissue morphogenesis and maintenance (Kefalides & Borel, 2005b, 2005a; Sekiguchi & Yamada, 2018; Yurchenco, 2011). It is found contacting the epithelial cells on the basal side, functioning as their anchor point. The regulation of functional BM is essential, as the loss of its integrity has been found in many diseases and is a hallmark of cancer (Lee & Vasioukhin, 2008; Macara & McCaffrey, 2013; Royer & Lu, 2011). Upon mislocalization of BM components, the apical-basolateral polarity is also lost, and cells gain invasive properties (Denef et al., 2008; Devergne et al., 2014, 2017; Moreno-Bueno et al., 2008). It has also been shown that accumulation of the BM proteins to the apical side result in polarity reversal (Wang et al., 1990a, 1990b; Williamson & Lehmann, 1996; Yu et al., 2005). Despite the necessity of proper localization of BM components to the basal side, not much is known about the components of biological pathways dedicated to the polarized deposition of BM proteins.
Crag is one protein known to be a major regulator of the BM proteins deposition to the basal side and is also the focus of this thesis. As a multidomain protein, it is speculated to have more than one function, however, its primary function is the regulation of polarized deposition of BM proteins (Denef et al., 2008). It acts as a GEF for the Rab10 GTPase, which work together to drive and restrict the localization of BM components to the basal side. In this thesis, we aimed to identify the domains that are responsible for the localization of Crag and its activity that control the polarized deposition of BM proteins using *Drosophila melanogaster* follicular (FE) as the model system.

**Generation of Truncated Crag Constructs**

Although majority of the constructs were provided as a gift by Schüpbach lab, we needed to create two constructs that were necessary to determine the domains responsible for Crag’s activity. CragNCBS was the first constructed that I created, which contained the DENN domains and the CBS domain, and stopping right after CBS domain. The second construct that needed to be created was Crag∆CaM which would have the CBS domain precisely deleted, unlike Crag∆CBS construct that had a portion of CBS domain remaining. These would help us determine the significance of CBS domain and the conserved C-terminus region in the localization and activity of Crag. During the generation of these two Crag constructs, HA-CragNCBS was generated with ease using molecular cloning techniques. However, the generation of HA-Crag∆CaM construct was not achieved due to some setbacks. Using both the traditional molecular cloning technique and Gibson assembly yielded unsuccessful results. Via restriction digest, we were able to insert one part of the Crag∆CaM construct in the pTiger 5’HA plasmid, but not the complete sequence. Using Gibson assembly as an alternate approach was
also unsuccessful. We speculated that this DNA construct poses a challenge for the plasmid and bacteria to accept. As for the colonies that grow without the construct, we believe it to be the result of accepting plasmids that do not contain the Crag construct. Hence, as the immediate next step, we will try to change the transformation procedure by using electroporation for the competent cells instead of heatshock to generate HA-CragΔCaM. Furthermore, to confirm the role of CBS domain in Crag’s activity in the control of polarized secretion of BM proteins, I will generate a Crag mutant where the CBS domain is deleted using CRISPR/Cas9 gene editing.

**Determination of Crag’s Domain(s) Responsible for Proper Cellular Localization of Crag**

Endogenous Crag localizes apically and laterally, and the addition of HA epitope does not affect this localization, as seen in Figure 16. The proper localization of Crag is important for its function of regulating the accumulation of BM proteins to the basal side (Denef et al., 2008). That is because Crag is thought to function via a pathway where it restricts the accumulation of BM proteins on the basal side by blocking it on the apical side (Denef et al., 2008). Thus, understanding how Crag assumes its polarized distribution is critical to our understanding of the mechanisms dedicated to the proper placement of the BM.

**The C-terminus is important for the localization of Crag**

As a multidomain protein that plays a critical role in polarized deposition of BM proteins by blocking their accumulation to the apical and lateral domains, it is important to know which domain(s) are responsible for the localization of Crag. During our experiments, it was determined that the N-terminus (HA-CragN) Crag mislocalizes throughout the cytoplasm. When only the C-terminus was present however, and the N-terminus containing the DENN domains
was deleted, Crag localized properly to lateral side of the FE (Figure 17). Previous studies show that DENN domains, as in the N-terminus part of Crag, are responsible for the GEF activity. Our results indicate that these domains are not required for the proper localization of Crag. Instead, we show that the C-terminus domain of Crag that contains the calmodulin binding domain is important for its proper localization. As the DENN domains (N-terminus) have the primary function of GEF activity in Crag, we speculated that the C-terminus, rather than the N-terminus, may be responsible and may play a role larger in the localization of Crag to the apical and lateral domains.

The CBS Domain is Necessary, but not Sufficient, for the Localization of Crag

Once we determined that the C-terminus was necessary for the localization of Crag, next step was to determine the importance of each of the domains of the C-terminus. For Crag constructs that had the CBS domain deleted from the full-length Crag and when from just the C-terminus Crag construct, Crag was strongly mislocalized in CragΔCBS, however, only partially mislocalized in CragΔNΔCBS, with a light accumulation seen at the lateral domains (Figure 18). The quasi-lateral localization of CragΔNΔCBS and the strong mislocalization of CragΔCBS was surprising, as it meant that although CBS domain is important, it is not the only domain responsible for the accumulation of Crag. It works in conjunction with another part of the protein, which makes possible its accurate apico-lateral localization.

Due to these results, we decided to see whether the conserved C-terminus domain is also important for Crag’s localization. When just the conserved domain was deleted (HA-CragΔCterm), Crag localized in a similar manner to full length Crag or HA-Crag (Figure 16). However, when both the conserved domain as well as the region following CBS domain was
deleted in the HA-CragNCBS construct, Crag was aberrantly mislocalized to the cytoplasm (Figure 19). Based on these results, we speculated that the CBS domain is not the only region of Crag responsible for its localization. The region following the CBS domain may also play a role in the localization of Crag. This may be due to the CBS domain playing a role in the tertiary structure of protein, where its deletion may result in a conformation change. Another possibility is the role of CBS domain in binding calmodulin (CaM – calcium modulating protein) in a Ca\(^{2+}\) dependent manner (Denef et al., 2008). Although both CaM and Ca\(^{2+}\) may be present, Crag may act as an effector of CaM, and without binding to CaM, may not relay signals downstream that are responsible for its localization. CaM is a highly conserved, eukaryotic, calcium-binding protein that is involved in many cellular processes in a calcium dependent manner (Clapham, 2007). The formed Ca\(^{2+}\)/CaM complex acts as an effector in fertilization, cell cycle, proliferation, apoptosis, muscle contraction, and intracellular signaling (Chin, 2005; Courtot et al., 1999; Federico et al., 2017; Takuwa et al., 1995; Walsh, 1994). If Crag is unable to bind to CaM due to the lack of CBS domain, it may not be able to localize properly, and in turn, its function may be affected as well. Hence, the CBS domain plays an important role in the localization of Crag to the apical and lateral domains.

**Crag’s Activity is Crucial in Proper Deposition of BM Proteins**

In parallel to determining the domain(s) responsible for the localization of Crag, we also wanted to determine the domain(s) responsible for the activity of Crag in regulating the BM protein accumulation to the basal domain. The cells mutant for Crag lose their basal distribution of BM components, where Perlecan, Collagen IV, Laminin, Viking, and Dystroglycan have been found to aggregate on the apical side (Denef et al., 2008). In severely perturbed epithelium in
Crag mutants, the cells form bilayers and multilayer and lose their cuboidal shape, adopting round or irregular morphologies. Furthermore, the neighboring wildtype cells are also affected and lose their epithelial integrity (Denef et al., 2008). Thus, Crag plays a crucial role in the regulation of BM components and is in turn responsible for the maintenance of apical-basolateral polarity.

**The DENN Domains Suggest a Primary Role in Crag’s Activity**

To determine which domains were responsible, we used the FLP/FRT system to generate mosaic tissue in the FE and two drivers specific to the FE, tj-Gal4 and e22c-Gal4, to perform rescue experiments by expressing Crag constructs under the control of UAS. To validate this approach, I first expressed the full length of Crag in FC mutants for Crag. Using this approach, I was able to restore the polarized secretion of BM proteins, i.e., rescue the mutant phenotype due to the loss of Crag indicating that I could use this approach to assess the role of the different domain in the crag activity involved in the polarized deposition of BM protein. While tj-Gal4 is a much stronger driver than e22c-Gal4, the results of both showed a higher rescue in the HA-CragN construct compared to the HA-CragC construct. Rescue was observed where, in homozygous mutant FE cells, BM proteins reestablished their basal domain and were no longer accumulated aberrantly to the apical side. As expected, the ectopic expression of full-length Crag showed the highest percentage of rescue. However, the comparison between HA-CragN and HA-CragC rescue experiments showed that the N-terminus rescues more clones than C-terminus as higher rescue observed in HA-CragN. Statistical analysis was performed on all the conditions for crosses with both driver lines. All the results were calculated to be statistically significant with the p-value of p<0.0001, meaning that the results obtained were not merely due to chance, and
the specific termini indeed play a role for full activity of Crag. However, based on the percentage of rescue, although both the N-terminus domains and the C-terminus domains play a role in Crag’s activity, although, the role of the N-terminus DENN domains is higher.

A higher rescue in HA-CragN was speculated to occur as the N-terminus contains the DENN domains, while the C-terminus contains the CBS domain and the conserved domain. The DENN module consisting of uDENN, DENN, and dDENN is an evolutionary conserved module that is primarily a GDP-GTP exchange factor (GEF) which turns “on” Rab GTPases (Marat et al., 2011; Zhang et al., 2012). Rab GTPases act as molecular switches to control almost all the aspects of intracellular trafficking including membrane identity, vesicular budding, docking, fusion, and transport of biological cargo throughout the cell (Hutagalung & Novick, 2011; Stenmark, 2009; Stenmark & Olkkonen, 2001).

During the process of BM secretion, Crag is shown to act as a GEF for Rab10, which is consistent with the presence of the DENN domains (Lerner et al., 2013). Crag works by activating Rab10 for the proper localization of BM proteins to the basal side. Although it also acts as a GEF for Rab11 in adult photoreceptor cells and is required there for rhodopsin transport, in the BM proteins transport, Crag and Rab10 have been speculated to work in an antagonistic manner with Rab11 in blocking the BM accumulation to the apical side (Xiong et al., 2012).

Due to Crag’s function as a GEF, if the DENN domains are absent, Rab10 cannot be activated and the rescue percentage is lower compared to when the DENN domains are present. However, the results are different from when full-length HA-Crag is ectopically expressed. If the DENN domains were sufficient by themselves, then a rescue equivalent to HA-Crag should have been observed in the HA-CragN construct. However, the drop in the percentage of rescued
clones suggests that the DENN domains are important, but not sufficient. This may be related to the localization of Crag, which we assume plays a role in blocking the BM accumulation to the apical domain. As Crag localizes apically and laterally, it drives the BM accumulation basally. Hence, when just HA-CragN is expressed ectopically, it mislocalizes throughout the cytoplasm (Figure 18B, B’) rather than assuming its normal apico-lateral localization. Although the DENN domains may play an important role in Crag’s activity, they do not play any significant role in the localization of Crag. The resulting mislocalization of Crag due to the absence of C-terminus results in the decreased function of Crag, leading to a rescue that is lower than the full-length Crag. Hence, CBS domain, indirectly and possibly directly, may also be required for the full activity of Crag. This can be determined by expressing different truncated forms, including CragNCBS and CragΔCaM, in Crag<sup>CJ101</sup> mutant background and determining the percentage of rescue for each.

**Conclusion**

Crag is a protein that plays a crucial role in the pathway regulating the polarized deposition of the BM components. In this thesis, I showed the domains that are important in the localization of Crag and its activity. Specifically, the CBS domain plays an important role in the localization of Crag. Although it is not sufficient and may need to work in conjunction with other parts of the C-terminus, without the CBS domain, Crag mislocalizes strongly to the cytoplasm rather than being restricted to apical and lateral domains. For the activity of Crag, the DENN domains play an important role due to their GEF activity for Rab GTPases, as Crag is a GEF for Rab10, which is also shown to be important in the BM deposition pathway. We speculate that the CBS domain may also be required for full activity of Crag due to its importance in Crag’s proper
localization, and because Crag’s localization is important for its activity of driving and restricting the BM protein accumulation to the basal side. In the absence of DENN domains, a lower rescue is seen than in the absence of the C-terminus domains, suggesting that the DENN domains play a key role in Crag’s activity. These results allow us to grasp the understanding of the importance of each of the domains of Crag in its proper localization and its activity that regulate the polarized deposition of BM protein. Upon performing in future steps in this structure-function study of Crag, we will be able to determine the relative importance of each domain in the both the localization, and in turn the activity, of Crag. This will allows us to further understand the role of Crag in the control of polarized secretion of BM proteins to the basal side of epithelial cells.

Future Directions

Based on our results of localization experiment, although we determined that the CBS domain may be important, it is not sufficient, and a part or the entirety of the sequence following it may also be required. However, the extent of the importance of CBS domain has not been elucidated yet, as we were unable to generate the Crag construct that is precisely deleted of the CBS domain. Hence, the next step is to first generate this construct, and determine the localization of Crag to determine whether CBS domain is essential by itself or is needed in conjunction with other parts of the sequence for the apico-lateral localization that Crag assumes. We will also perform co-immunoprecipitation experiment for Crag and Calmodulin. As CBS domain is responsible for binding to Calmodulin, the results of co-IP may link the role of CBS domain and Calmodulin in the proper localization of Crag.
The importance of each of the domains in Crag’s activity is also not yet completely determined. The next step in determining the domain(s) important for its activity would be to create transgenic lines with Crag\textsuperscript{CJ101} background and ectopically express the remaining constructs used for the localization experiments. These constructs include the truncated versions Crag\textsuperscript{ΔCBS}, Crag\textsuperscript{ΔNΔCBS}, Crag\textsuperscript{ΔCterm}, Crag\textsuperscript{NCBS}, and Crag\textsuperscript{ΔCaM}. Analyzing and comparing the rescue for each of these constructs will provide a better insight into the role of each domain in the activity of Crag, as well as the localization of Crag. It will help us determine whether the CBS domain is also important in Crag’s activity and help conclude whether the localization of Crag plays a role in its activity by driving BM components to localize on its opposite side. Performing co-IP with Calmodulin to see whether it binds to different truncated forms of Crag will also be helpful to determine whether the CBS domain is important in the activity of Crag.
References


