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Identification of Novel Regulators of Cell Competition in Drosophila melanogaster

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Identification of Novel Regulators of Cell Competition in *Drosophila melanogaster*

by

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Abstract

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The survival and growth of cells can be influenced by the properties of adjacent cells. This reflects the ability of cells to communicate with each other and can result in either cooperation or competition for limited resources. Cell competition is an example of a situation in which cells exert a non-autonomous influence over the survival of their neighbors. The phenomenon was first discovered over forty years ago during studies of genetically mosaic *Drosophila melanogaster*. Clones of slow-growing, though viable, "*Minute*" cells were found to be eliminated from tissues in which they were surrounded by wild-type cells. It was later discovered that signaling between the two cell types at their clone borders causes the apoptosis and elimination of the *Minute* cells. Conversely, faster growing cells known as "supercompetitors", such as cells overexpressing the transcription factor, dMyc, can induce the elimination of wild-type cells. Thus, cell competition is a short-range interaction between cells with different growth rates in the same tissue and leads to the elimination of the slower-growing cells. The nature of this interaction is still unknown. Specifically, there are two important, unanswered questions regarding the mechanism of cell competition: 1) How do cells at clonal boundaries compare their properties and designate a "winner" and "loser"? 2) What are the signals that then induce the death of the designated losers?

Understanding the mechanism of cell competition is important from several standpoints. First, cell competition has been proposed to play a role in regulating organ size and tissue composition during normal development. Thus, understanding the mechanism of cell competition may advance our understanding of basic developmental biology. Second, many of the genes involved in cell competition are also misregulated in certain cancers. Thus, cell competition would be expected to occur at the borders between such tumors and their host tissue. A competitive advantage could make the tumor more malignant, while a competitive disadvantage might make the tumor more manageable. Therefore, a better understanding of the mechanisms involved in cell competition could also advance our understanding of tumor

progression and improve our ability to treat tumors more effectively. Finally, a thorough understanding of cell competition and the ability to manipulate it could eventually be useful in therapies. For example, it may become possible in regenerative medicine to give grafts the ability to replace injured or dysfunctional tissue.

The goal of my thesis research has been to identify and characterize novel regulators of cell competition in order to gain a better understanding of the underlying mechanism. Accordingly, I devised an assay that would allow me to readily identify mutations that make cells into supercompetitors. The "supercompetitor assay" described here takes advantage of established mosaic analysis techniques in *Drosophila*. Small, marked clones of cells that are heterozygous for a mutation are created in an eye primordium that is primarily composed of homozygous-mutant cells. If the mutant cells are supercompetitors, the marked heterozygous clones are eliminated and cannot be recovered in the adult eye. Using this method I was able to test candidates from a collection of mutations in tumor suppressor genes from several different pathway that regulate growth. While mutations in components of some growth-regulating pathways, notably the Hippo pathway, caused cells to become supercompetitors, mutations in other pathways did not. Thus, only a subset of the pathways that regulate growth appear to be involved in cell competition.

The supercompetitor assay was also used to conduct an unbiased genetic screen for novel mutations that make cells supercompetitors. Among the mutations found in the screen were several alleles of *crumbs* (*crb*), a gene that regulates apicobasal polarity. This gene had no previously defined role in cell competition or growth. I found that while loss of Crb causes cells to become supercompetitors, overexpression of Crb causes cells to be eliminated from wild-type epithelia. Furthermore, as expected in instances of cell competition, high levels of apoptosis were observed preferentially at boundaries between wild-type and *crb* mutant cells, as well as at boundaries between Crb-overexpressing and wild-type cells. Thus, cells that express higher levels of Crb appear to be eliminated through a mechanism that resembles cell competition when they are near cells that express lower levels of Crb.

It is still unclear how cells compare their Crb levels. Cells may compare the levels of a molecule that is downstream of Crb. One candidate is the Hippo pathway, which has been repeatedly linked to cell competition. I found that *crb* mutant cells upregulate some of the transcriptional targets of the Hippo pathway suggesting that Crb impinges upon the Hippo signaling pathway. Alternatively, cells may directly compare their levels of Crb, as it is a transmembrane protein with a large extracellular domain of unknown function. Interestingly, the extracellular domain of Crb appears to be required to elicit some of the heterotypic interactions that I observe. For example, cells that express the intracellular domain of Crb alone are not eliminated. Furthermore, I see evidence of interactions between Crb molecules on adjacent cells that could be the basis of a direct comparison mechanism. Future work aimed at testing such models may yield important insights into a mechanism of cell competition.

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Chapter 1

Introduction

Organ Size Determination

Organs are structures composed of cells with different specializations that together perform more complex functions. They often originate as small amorphous groups of progenitor cells that grow and differentiate into a characteristic and highly reproducible size and shape. While some progress has been made in understanding the mechanisms for patterning organs, our understanding of the mechanisms that establish the size of organs remains largely abstract. A molecular explanation for organ size determination must account for: (1) how the ultimate dimensions of an organ are defined, (2) how current organ size is sensed, and (3) how cell behavior, such as growth, proliferation, and death, is modulated until the appropriate dimensions are reached. The mechanism must also ensure reproducibly of organ size despite complicating circumstances. For example, organs are often able to adjust their developmental growth to compensate for damage or overgrowth of subsets of cells.

Extracellular signals are central to all coordinated cellular activities, including organ size determination. Such signals can act in three different ways. Systemic signals, or hormones, are chemical signals that are released into circulation from cells in one part of the body and exert their effects on cells in another part of the body. They can have different effects on different tissues, but typically all of the effects constitute various aspects of a coordinated response to a stimulus. For example, hormones such as Growth Hormone and Insulin drive anabolic processes in response to nutrition availability. Organ growth and patterning are also regulated by tissue intrinsic signals that are produced by a localized set of cells and diffuse through the rest of the tissue. These signals, known as morphogens, form concentration gradients across the tissue and exert different effects on cells depending on where the cells are in the concentration gradient. Finally there are also signals that have very localized effects. In these cases, membrane-bound ligands, or secreted ligands that do not have a mechanism to allow them to diffuse away from their origin, act on receptors on adjacent cells. In order to regulate organ size these extracellular signals can modulate cell growth, division, or survival of the receiving cells. An example of short-range control over cell survival is the phenomenon of cell competition.

Discovery of Cell Competition

Cell competition was discovered in the developing wing tissue of *Drosophila melanogaster* during studies of slow-growing *"Minute"* cell by Morata and Ripoll (Morata and Ripoll, 1975). *Minute* denotes the dominant phenotype typically caused by mutations in ribosomal protein genes – a dominant reduction in cellular growth rate leading to a developmental delay and thin bristles (Marygold et al., 2007; Morata and Ripoll, 1975). *Minute* heterozygous (*M* /+) flies are otherwise normal – they are viable, fertile, and have correct size and allometry. *M* /+ cells are undoubtedly viable as they can produce adult flies. Despite this fact, *M* /+ clones are rapidly eliminated from wild-type wing imaginal discs and therefore do not appear in the adult wing (Figure 1.1A, B). Morata and Ripoll speculated that there was a limited amount of space in adult structures for which developing cells must compete. They reasoned that because the *M* /+ cells grew more slowly they were less

competitive and were thus eliminated from the tissue through an active process of "cell competition" (Morata and Ripoll, 1975).

Morata and Ripoll were able to infer the existence of cell competition from observing adults, but the actual process occurs as cells proliferate during development. The adult appendages of *Drosophila*, including the wings, legs and eyes – originate as clusters of 20-50 cells that invaginate from the ectoderm in the embryo to form epithelial sacs. During the four days of the larval development, these simple epithelial tissues grow into tissues of approximately 20,000-50,000 cells. When the animal undergoes pupariation, growth and cell competition generally stop and differentiation begins.

When methods were developed to more effectively induce clones and mark them in the larvae (Golic and Lindquist, 1989; Xu and Rubin, 1993), it became possible to observe that Minute cells were being eliminated through active processes (Moreno et al., 2002). *M* /+ clones in wild-type imaginal discs appear to be progressively eliminated by apoptosis of cells at the clone boundaries. *M* /+ cells adjacent to wild-type cells display high levels of markers of apoptosis: TdT mediated Nick End Labeling (TUNEL) and activated caspase 3 (AC3). Inhibition of apoptosis by overexpression of the Baculovirus caspase-inhibitor, p35, can rescue *M* /+ cells from being eliminated (Li and Baker, 2007; Martín et al., 2009; Moreno et al., 2002). Furthermore, fragments of *M* /+ cells found in adjacent wild-type imaginal disc cells indicate that in addition to apoptosis, engulfment may play an important role in the elimination of *M* /+ cells. Furthermore, mutations in genes important for phagocytosis, such as *draper*, *wasp*, and *phosphatidylserine receptor*, in wild-type cells enhance the survival *M*/+ cells (Li and Baker, 2007; Moreno et al., 2002).

Supercompetitors

Interestingly, wild-type cells can also be eliminated by cell competition (Figure 1.1C). Wild-type cells are obviously viable. However, when wild-type cells are juxtaposed with cells overexpressing the transcription factor, dMyc, they express markers of apoptosis and are eliminated (de la Cova et al., 2004; Moreno and Basler, 2004). Loss-of-function of tumor suppressors in the Hippo pathway can also give rise to cells that can eliminate wild-type cells. Cells with this property are collectively called "supercompetitiors" (de la Cova et al., 2004; Moreno and Basler, 2004).

The existence of supercompetitors is significant because it supports the idea that cell competition is an active process. One interpretation of the elimination of *M* /+ cells could be that they are sick and poised to die in response to any form of stress. But that cannot explain the elimination of wild-type cells. Instead, there must be a more general mechanism by which all cells, including wild-type cells, can be compared and subjected to elimination. Moreover, the fact that wild-type cells can either win or lose in competition shows that the genotype of a cell does not innately make it a winner or loser. Rather, these designations depend on the context in which cells grow and how they compare to their neighbors. The

molecular mechanism for how cells might be compared and how this comparison leads to the apoptosis of loser cells is an area of active research.

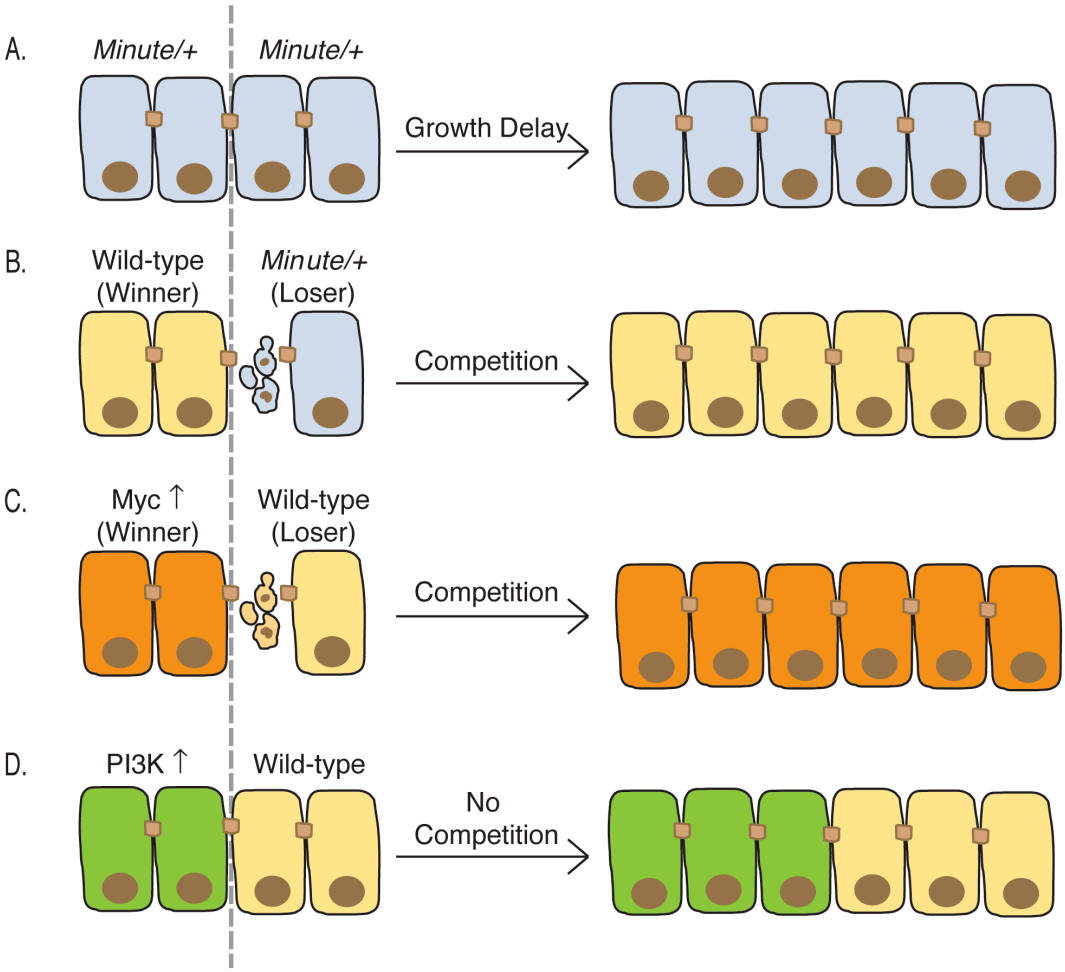

Figure 1.1 - Schematic diagram of examples and general features of cell competition
(A) *Minute/+* cells are viable in homogenous tissues, but display a slower growth rate than wild-type cells. (B) In mosaic tissues, *Minute/+* cells adjacent to wild-type cells die by apoptosis and are replaced. (C) In mosaic tissues consisting of wild-type cells and supercompetitors, such as Myc-overexpressing cells, the wild-type cells bordering the supercompetitors are eliminated by apoptosis and replaced. (D) Some types of overgrowing cells, such as PI3K-overexpressing cells, do not induce apoptosis of adjacent wild-type cells.

Related Phenomena

The elimination of *M* /+ cells by wild-type cells and the elimination of wild-type cells by Myc-overexpressing cells are considered to be the classic examples of cell competition, but there are other examples of cells that are viable in one context and eliminated in another context. Wild-type cells commonly eliminate slower-growing cells through cell competition.

Cells that are mutant for *dMyc* or certain components in the Ras and Insulin pathways appear to be viable in *Minute* tissues, but eliminated from wild-type tissues (Herranz et al., 2006; Johnston et al., 1999; Prober and Edgar, 2000). Wild-type cells also outcompete cells mutant for a set of genes that regulate apicobasal polarity, *scribbled* (*scrib*), *lethal giant larvae* (*lgl*), and *discs large* (*dlg*) (Brumby and Richardson, 2003; Igaki et al., 2009). Surprisingly, while homozygous mutant larvae of these geneotypes are developmentally delayed, the imaginal tissues in these larvae ultimately overgrow, forming large neoplastic tumors (Bilder et al., 2000). This emphasizes the importance of differences in growth rate in cell competition. However there are also examples of competition-like phenomena that do not appear to involve differences in growth rate.

Adachi-Yamada and colleagues described a phenomenon they called morphogenetic apoptosis, in which cells with altered levels of signaling of the morphogens Decapentaplegic (Dpp) and Wingless (Wg), such as from a defective receptor, are eliminated from certain regions of the wing imaginal disc (Adachi-Yamada and O'Connor, 2002; Adachi-Yamada and O'Connor, 2004) (Figure 1.2A). Morphogen gradients regulate tissue patterning and cells are thought to compare their signaling levels in order to verify that patterning is normal. A cell with aberrant signaling levels might appear to be misspecified and apoptosis could be a way to ensure that the gradient is reestablished. Morphogenetic apoptosis shows some features of cell competition but there are also notable differences. In examples of morphogenetic apoptosis, clones with perturbed signaling are not eliminated from regions of the tissue where they matched the local signaling levels. This suggests that relative rather than absolute levels of morphogen signaling are important in determining cell survival, as they are in cell competition. However, the levels of morphogen signaling rather than the rates of proliferation appear to be relevant in morphogenetic apoptosis (Moreno and Basler, 2004; Moreno et al., 2002). Furthermore, morphogenetic apoptosis is triggered on both sides of the clonal border (Adachi-Yamada and O'Connor, 2002; Adachi-Yamada and O'Connor, 2004). Because cells from both populations die, there is no clear winner or loser. The extent to which morphogenetic apoptosis and cell competition are mechanistically related is not yet clear.

Defining Cell Competition

Cell competition has been difficult to define because the mechanism is so poorly understood. In the most general view, cell competition can be thought of as a process whereby one cell-type displaces another, otherwise viable cell-type, from the epithelium in which both are growing. However, this broad definition would encompass all of the examples above, and it is not clear that they all follow the same mechanism. The competition resulting from Myc overexpression and that from *Minute* cells appear to be very closely related as they share a number of noteworthy characteristics. The strictest definition of cell competition would restrict the term to phenomena that display all of the features of *Myc* and *Minute* competition. First, as previously discussed, winning or losing is not an intrinsic property of cells, but rather depends on the context in which cells grow. For example, cells that overexpress Myc can lose if they are competing against cells that

overexpress Myc at even higher levels (Moreno and Basler, 2004). Second, as a result of cell competition apoptosis and engulfment are predominantly observed at the border between competing clones (Li and Baker, 2007; Martín et al., 2009). Therefore, cell competition is a result of localized cell interactions leading to apoptosis. Two additional observations include: competition does not occur across developmental compartment boundaries (de la Cova et al., 2004; Simpson, 1979) and organ size is maintained after competition. The latter is likely to be due to compensatory proliferation by winner cells (de la Cova et al., 2004; Morata and Ripoll, 1975).

Where Does Cell Competition Occur?

Cell competition is thought to occur in a wide range of organisms outside of *Drosophila*. The mouse mutant, Belly spot and tail (*Bst*), has a mutation in ribosomal protein L24 (Rpl24) that causes a growth defect analogous to the *Drosophila M* /+ phenotype. When wild-type embryonic stem cells were injected into *Bst* heterozygous blastocysts, chimeras were established at a higher frequency than when wild-type cells were injected into wild-type blastocysts. Furthermore, the wild-type cells injected into the *Bst*/+ blastocysts were able to contribute to a greater proportion of the adult tissue than normal (Oliver et al., 2004). Cell competition has also been observed in mammalian tissue culture cells. Madin-Darby canine kidney (MDCK) cells knocked down for an Lgl-interacing protein, Mahjong (Mahj) (Oliver et al., 2004; Tamori et al., 2010), undergo apoptosis and apical extrusion when co-cultured with wild-type MDCK cells.

Despite being widespread in different animals, cell competition appears to occur in only a subset of tissues. Wild-type cells were found to replace *cMyc* mutant cells in the mouse intestine (Muncan et al., 2006). Similarly, embryonic day 14 fetal liver cells grafted into adult rat livers were found to induce apoptosis of adjacent host liver cells (Oertel et al., 2006). In *Drosophila*, cell competition has been described in the imaginal discs and a phenomenon resembling cell competition has been described in cultured S2 cells (Senoo-Matsuda and Johnston, 2007). Furthermore, both germline stem cells and somatic stem cells in the *Drosophila* gonad were found to compete for space in the niche, a microenvironment that allows stem cells to maintain their undifferentiated and proliferative state. Losers are pushed out of the niche and therefore forced to differentiate (Jin et al., 2008; Nystul and Spradling, 2007; Rhiner et al., 2009; Sheng et al., 2009; Zhao and Xi, 2010). While there are apparent differences in the mechanism, stem cell competition and imaginal disc cell competition ultimately achieve the same effect – altering the cellular composition of an organism in favor of some cell types over others. In contrast, abdominal histoblasts do not display cell competition. Thus, *M* /+ histoblasts contribute to the adult cuticle in a way that is consistent with the observed decrease in mitotic rate of *M* /+ cells but not with their elimination. Many other tissues have not been assessed for cell competition.

When Does Cell Competition Occur?

In most of the examples above, cell competition is observed through contrived experimental conditions in which different cell types are juxtaposed in the same tissue, whereas all of the cells in an organism are normally identical. Cell competition might occur in response to natural fluctuation in signaling levels between genetically identical cells, however, very little apoptosis is observed in wild-type imaginal discs. Interestingly, a type of competition was recently discovered to occur under normal circumstances between follicle stem cells in the *Drosophila* ovary. There are two follicle stem cells located on opposite sides of each ovariole. The daughters of these follicle stem cells often migrate toward the other stem cell and on occasion, displace it from its niche (Nystul and Spradling, 2007). Thus, some types of cell competition might be a part of normal development.

An instance where cell competition certainly occurs is during aberrant development such as in the development of cancer. Cancer develops when cells in a tissue begin to accumulate genetic lesions that cause them to grow in an uncontrolled manner. Mutations in *Myc* (constitutively activating), the Hippo pathway (loss of function of the core components), and the Scrib complex (loss of function) are all common in human cancers (Humbert et al., 2003; Nesbit et al., 1999; Pan, 2007). In some cases competition caused by perturbation of these genes might give the tumor an advantage while in other cases cell competition must be overcome for a tumor to grow. Cell competition may, in fact, help to explain some puzzling features observed in cancer. First, some tumors grow undetected until very late stages because they do not cause any obvious structural changes to the tissues where they reside. This could be explained if adjacent cells are eliminated by cell competition to create room for the tumor to grow (Moreno, 2008). Second, many types of cancers have a very high rate of recurrence. Slaughter's theory of field cancerization explains this by postulating that these cancers arise from tissues that have somehow been genetically primed for transformation. Therefore, after removal of a tumor another would arise from the "cancerized field" (Rhiner and Moreno, 2009; Slaughter et al., 1953). Cell competition may explain how a cancer might spread throughout a tissue.

Why Does Cell Competition Occur?

Though cell competition appears to be a conserved mechanism, it can have negative consequences as in the case of cancer discussed above. Furthermore, there is little evidence that it occurs during normal development. Why then would such a mechanism have evolved? Generally, cell competition is thought to be a proof-reading mechanism. Stem cells and early progenitor cells proliferate to create a disproportionate amount of tissue compared to other cells. But the additional divisions these cells undergo make them more susceptible to being damaged. Since defects in these cells could have severe consequences for the fitness of an organism, a mechanism to monitor these cells and replace them if necessary would be advantageous. Along these lines, *Minutes* have been proposed to be a system for monitoring for more sinister defects. There are many genes encoding ribosomal proteins that are distributed throughout the genome. These may act as a genome surveillance mechanism.

Genomic damage or instability would very likely result in one of these genes becoming haploid. By eliminating cells with such defects, an organism may attempt to protect itself against tumor formation (Baker, 2011). Similarly, cells with polarity defects have the potential to form harmful tumors, as many of the genes that control cell polarity are neoplastic tumor suppressor genes. The elimination of cells mutant for the polarity genes has been called an "intrinsic tumor suppressor" program (Igaki, 2009; Igaki et al., 2009).

Cell competition has also been proposed to be a mechanism for maintaining organ size in case cell populations arise with different growth rates. Consistent with this idea, in situations where cell competition does occur, organ size is normal while in situations where cell competition does not occur, such as the induction of clones overexpressing PI3K or CyclinD/Cdk4, organ size is abnormal. Surprisingly, preventing cell competition from occurring by inhibiting apoptosis in wild-type wing imaginal discs did not cause a significant change in wing size, but it did result in a wider range of wing sizes (de la Cova et al., 2004). Even in cases where cell competition is induced by the presence of *M* /+ cells, blocking apoptosis does not affect the size of developmental compartments (Martín et al., 2009). Therefore, it is still unclear whether cell competition is involved in organ size determination.

The Mechanism of Cell Competition

As mentioned above the main questions in the cell competition field are how cells might be compared and how this comparison might lead to the apoptosis of loser cells. I previously mentioned morphogenetic apoptosis as one model to answer these questions (Figure 1.2A). In this section I will review some other models that have been proposed to answer these questions. However, it is always important to bear in mind that what is referred to as cell competition may actually encompass several independent mechanisms.

Differences in Growth Induce Competition

One of the first theories was that cell competition is triggered by differences in cell growth within a tissue and cells with impaired growth lose (Morata and Ripoll, 1975; Simpson, 1979). There is experimental evidence that both supports and refutes this idea. Consistent with this idea, almost all of the genes currently implicated in cell competition have a role in growth regulation. However, there are ways to alter growth that do not cause cell competition, including overexpression of PI3K or Cyclin D and CDK4 (Figure 1.1D) (de la Cova et al., 2004). This may be because cell competition is the consequence of differences in a specific type of growth, however, no consistent patterns have been observed that would implicate any specific type of growth in cell competition. By themselves, *scrib*, *lgl*, and *dlg* mutant cells grow more slowly but eventually overgrow, while, in the presence of wild-type cells they are eliminated (Grzeschik et al., 2007). Thus differences in growth rate, rather than the total amount of growth, appear to cause cell competition. However, increasing the growth rate of *lgl* mutant cells by overexpressing Yorkie or an activated form of Ras does not rescue these cells from being eliminated (Menéndez et al., 2010). Furthermore, cells that

overexpress PI3K grow faster than wild-type cells but do not induce their elimination (de la Cova et al., 2004).

Other distinctions that have been suggested to be important include: (1) “balanced” (increased growth and division rate) versus “unbalanced” (increased growth without increased division resulting in larger cells) growth and (2) whether the overgrowth of patches of cells affects overall organ size or not (de la Cova et al., 2004). However, none of these distinctions encompass all of the examples of cell competition without also including

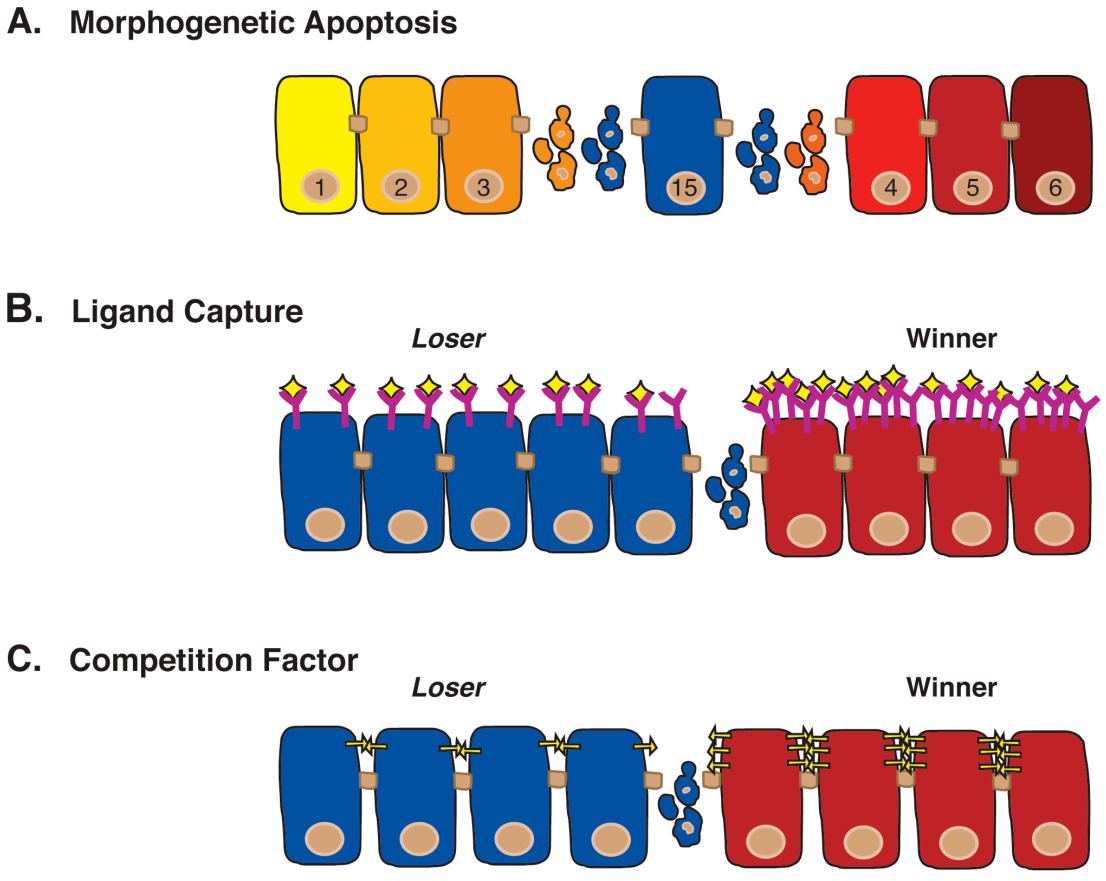

Figure 1.2 - Models for the mechanism of cell competition
(A) When neighboring cells experience significantly different levels of signaling of a morphogen (denoted by the numbers in the nucleus), apoptosis of both cells may help to reestablish a smooth morphogen gradient. (B) Cells that have a greater number of receptors (pink) for a survival factor may sequester it away from adjacent cells, thereby inducing their death. (C) Cells might compare the levels of a cell-competition-specific factor (yellow arrows). Cells with more of this factor induce the death of adjacent cells with less of it.

some instances in which cell competition does not occur. Alternatively, cell competition may be an independent process from growth.

"Ligand-Capture" – Highest Levels of Survival Factor Receptor Wins
Another model for cell competition is that cells differ in their ability to receive a limiting factor that is required for survival (Figure 1.2B). In this model, cells are eliminated when adjacent cells sequester away the factor and they are unable to receive a sufficient level.

This is known as the "ligand-capture" model. Decapentaplegic (Dpp) is a growth factor required for cell survival and has been proposed to act as the ligand that cells compete for. In support of this view, *M* /+ cells have lower levels of Dpp signaling, as indicated by levels of phosphorylated Mad, a downstream factor, while Myc overexpressing cells have elevated levels of phosphorylated Mad. Furthermore, elevated levels of Dpp appear to rescue elimination of *M* /+ cells by apoptosis. However, some features of Dpp do not fit in with this model. The supply of Dpp is not limited in imaginal discs, and the most intense competition is thought to occur in the center of the wing pouch – the region with the highest level of Dpp signaling. A ligand that has the characteristics to mediate competition in accordance with this model has not yet been identified.

Comparison of a Competition-Specific Factor
In another model, there is a pathway entirely dedicated to cell competition. Cells directly compare themselves with their neighbors for levels of some molecule or competition factor (Figure 1.2C). The levels of this factor could be very sensitive to changes in translation and thereby indicate the health or livelihood of a cell. If adjacent cells have unequal levels of the factor, then death is activated, for example, in the cell with less.

The competition factor could be a cell surface molecule, such as a cell surface receptor or adhesion molecule, or a secreted molecule, such as toxin or survival factor. Recent evidence suggests the involvement of a secreted factor in cell competition between cultured S2 cells (Senoo-Matsuda and Johnston, 2007). Cell competition was observed when wild-type S2 cells were cocultured with S2 cells that expressed elevated levels of dMyc, leading to increased apoptosis of the wild-type S2 cells. Furthermore, medium from these mixed co-cultures was sufficient to induce death of homogeneous cultured cells that expressed normal amounts of dMyc, suggesting that the effects of cell competition may be triggered by secreted factors. However, this raises the question of how compartment boundaries in imaginal discs might prevent the passage of soluble factors.

Goals and Overview of Thesis Research

Understanding the mechanism of cell competition may give us a better understanding of the mechanisms involved in producing a normal organ in development. It may also lead to a better understanding of tumor growth and ultimately aid in the development of therapies against tumors that exploit cell competition to permeate host tissues. Finally, an understanding of cell competition may lead to progress in regenerative medicine if it can be harnessed to induce repopulation of damaged organs upon transplantation of a few healthy cells.

In order to better understand the mechanism of cell competition, I sought to identify additional instances in which cell competition occurs. Poor competitors are more difficult to study because they die. Furthermore, there are many ways of causing cell death that are independent of cell competition, such as through mutations in housekeeping genes. In contrast, supercompetitors are not eliminated and, other than cell competition, not many mechanisms are known that would non-autonomously induce the death of adjacent wild-type cells. Therefore, mutations that cause cells to eliminate adjacent wild-type cells are more likely to be in a pathway that specifically regulates cell competition. However, there is currently no fast and easy method for identifying perturbations that cause cells to become supercompetitors.

I will begin by describing a method for identifying mutations that make cells supercompetitors and presenting the results of testing mutants in tumor suppressor genes as candidate supercompetitors. I then describe an unbiased screen to identify novel supercompetitors. In the screen, I identified a four-member complementation group and mapped the mutations to the gene, *crumbs (crb)*. In chapter three, I describe the characterization of *crb*. This includes confirming that *crb* cells are supercompetitors, demonstrating that Crb overexpressing clones are eliminated by competition with surrounding cells, and investigating whether Crb might be the basis of comparison in cell cell competition. Based on this work I present a few different models for how Crb might mediate cell competition.

REFERENCES

1. **Adachi-Yamada, T. and O'Connor, M. B.** (2002). Morphogenetic apoptosis: a mechanism for correcting discontinuities in morphogen gradients. *Dev Biol* **251**, 74-90.

2. **Adachi-Yamada, T. and O'Connor, M. B.** (2004). Mechanisms for removal of developmentally abnormal cells: cell competition and morphogenetic apoptosis. *J Biochem* **136**, 13-7.

3. **Baker, N. E.** (2011). Cell competition. *Curr Biol* **21**, R11-5.

4. **Brumby, A. M. and Richardson, H. E.** (2003). scribble mutants cooperate with oncogenic Ras or Notch to cause neoplastic overgrowth in Drosophila. *EMBO J* **22**, 5769-79.

5. **de la Cova, C., Abril, M., Bellosta, P., Gallant, P. and Johnston, L. A.** (2004). Drosophila myc regulates organ size by inducing cell competition. *Cell* **117**, 107-16.

6. **Golic, K. G. and Lindquist, S.** (1989). The FLP recombinase of yeast catalyzes site-specific recombination in the Drosophila genome. *Cell* **59**, 499-509.

7. **Grzeschik, N. A., Amin, N., Secombe, J., Brumby, A. M. and Richardson, H. E.** (2007). Abnormalities in cell proliferation and apico-basal cell polarity are separable in Drosophila lgl mutant clones in the developing eye. *Dev Biol* **311**, 106-23.

8. **Herranz, H., Morata, G. and Milán, M.** (2006). calderón encodes an organic cation transporter of the major facilitator superfamily required for cell growth and proliferation of Drosophila tissues. *Development* **133**, 2617-25.

9. **Humbert, P., Russell, S. and Richardson, H.** (2003). Dlg, Scribble and Lgl in cell polarity, cell proliferation and cancer. *Bioessays* **25**, 542-53.

10. **Igaki, T.** (2009). Correcting developmental errors by apoptosis: lessons from Drosophila JNK signaling. *Apoptosis* **14**, 1021-8.

11. **Igaki, T., Pastor-Pareja, J. C., Aonuma, H., Miura, M. and Xu, T.** (2009). Intrinsic tumor suppression and epithelial maintenance by endocytic activation of Eiger/TNF signaling in Drosophila. *Developmental Cell* **16**, 458-65.

12. **Jin, Z., Kirilly, D., Weng, C., Kawase, E., Song, X., Smith, S., Schwartz, J. and Xie, T.** (2008). Differentiation-defective stem cells outcompete normal stem cells for niche occupancy in the Drosophila ovary. *Cell Stem Cell* **2**, 39-49.

13. **Johnston, L. A., Prober, D. A., Edgar, B. A., Eisenman, R. N. and Gallant, P.** (1999). Drosophila myc regulates cellular growth during development. *Cell* **98**, 779-90.

14. **Li, W. and Baker, N. E.** (2007). Engulfment is required for cell competition. *Cell* **129**, 1215-25.

15. **Martín, F. A., Herrera, S. C. and Morata, G.** (2009). Cell competition, growth and size control in the Drosophila wing imaginal disc. *Development* **136**, 3747-56.

16. **Marygold, S. J., Roote, J., Reuter, G., Lambertsson, A., Ashburner, M., Millburn, G. H., Harrison, P. M., Yu, Z., Kenmochi, N., Kaufman, T. C. et al.** (2007). The ribosomal protein genes and Minute loci of Drosophila melanogaster. *Genome Biol* **8**, R216.

17. **Morata, G. and Ripoll, P.** (1975). Minutes: mutants of drosophila autonomously affecting cell division rate. *Dev Biol* **42**, 211-21.

18. **Moreno, E.** (2008). Is cell competition relevant to cancer? *Nat Rev Cancer* **8**, 141-7.

19. **Moreno, E. and Basler, K.** (2004). dMyc transforms cells into super-competitors. *Cell* **117**, 117-29.

20. **Moreno, E., Basler, K. and Morata, G.** (2002). Cells compete for decapentaplegic survival factor to prevent apoptosis in Drosophila wing development. *Nature* **416**, 755-9.

21. **Muncan, V., Sansom, O. J., Tertoolen, L., Phesse, T. J., Begthel, H., Sancho, E., Cole, A. M., Gregorieff, A., de Alboran, I. M., Clevers, H. et al.** (2006). Rapid loss of intestinal crypts upon conditional deletion of the Wnt/Tcf-4 target gene c-Myc. *Mol Cell Biol* **26**, 8418-26.

22. **Nesbit, C. E., Tersak, J. M. and Prochownik, E. V.** (1999). MYC oncogenes and human neoplastic disease. *Oncogene* **18**, 3004-16.

23. **Nystul, T. and Spradling, A.** (2007). An epithelial niche in the Drosophila ovary undergoes long-range stem cell replacement. *Cell Stem Cell* **1**, 277-85.

24. **Oertel, M., Menthena, A., Dabeva, M. D. and Shafritz, D. A.** (2006). Cell competition leads to a high level of normal liver reconstitution by transplanted fetal liver stem/progenitor cells. *Gastroenterology* **130**, 507-20; quiz 590.

25. **Oliver, E. R., Saunders, T. L., Tarlé, S. A. and Glaser, T.** (2004). Ribosomal protein L24 defect in belly spot and tail (Bst), a mouse Minute. *Development* **131**, 3907-20.

26. **Pan, D.** (2007). Hippo signaling in organ size control. *Genes Dev* **21**, 886-97.

27. **Prober, D. A. and Edgar, B. A.** (2000). Ras1 promotes cellular growth in the Drosophila wing. *Cell* **100**, 435-46.

28. **Rhiner, C., Díaz, B., Portela, M., Poyatos, J. F., Fernández-Ruiz, I., López-Gay, J. M., Gerlitz, O. and Moreno, E.** (2009). Persistent competition among stem cells and their daughters in the Drosophila ovary germline niche. *Development* **136**, 995-1006.

29. **Rhiner, C. and Moreno, E.** (2009). Super competition as a possible mechanism to pioneer precancerous fields. *Carcinogenesis* **30**, 723-8.

30. **Senoo-Matsuda, N. and Johnston, L. A.** (2007). Soluble factors mediate competitive and cooperative interactions between cells expressing different levels of Drosophila Myc. *Proc Natl Acad Sci USA* **104**, 18543-8.

31. **Sheng, X. R., Brawley, C. M. and Matunis, E. L.** (2009). Dedifferentiating spermatogonia outcompete somatic stem cells for niche occupancy in the Drosophila testis. *Cell Stem Cell* **5**, 191-203.

32. **Simpson, P.** (1979). Parameters of cell competition in the compartments of the wing disc of Drosophila. *Dev Biol* **69**, 182-93.

33. **Slaughter, D. P., Southwick, H. W. and Smejkal, W.** (1953). Field cancerization in oral stratified squamous epithelium; clinical implications of multicentric origin. *Cancer* **6**, 963-8.

34. **Tamori, Y., Bialucha, C. U., Tian, A.-G., Kajita, M., Huang, Y.-C., Norman, M., Harrison, N., Poulton, J., Ivanovitch, K., Disch, L. et al.** (2010). Involvement of Lgl and Mahjong/VprBP in cell competition. *PLoS Biol* **8**, e1000422.

35. **Xu, T. and Rubin, G. M.** (1993). Analysis of genetic mosaics in developing and adult Drosophila tissues. *Development* **117**, 1223-37.

36. **Zhao, R. and Xi, R.** (2010). Stem cell competition for niche occupancy: emerging themes and mechanisms. *Stem Cell Rev* **6**, 345-50.

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Chapter 2

An Assay for Supercompetitors

ABSTRACT

Cell competition may play an important role in the development of both normally-proliferating and cancerous tissues, but the mechanism for this phenomenon is still unknown. The most puzzling aspect of the mechanism is how cells might compare themselves to their neighbors in order to determine the winner and loser. Cell surface molecules could act as "comparison factors" that are titrated under normal circumstances. Imbalances in such molecules could trigger cell competition. Looking for additional instances of cell competition may help uncover such regulators or an entirely different mode of regulation. In this chapter I describe the development and use of a method for identifying novel genes that make cells supercompetitors.

I used the technique of mosaic analysis in *Drosophila melanogaster*, where cell competition is best understood, to devise an efficient assay for identifying supercompetitors in the eye imaginal disc. This assay allowed me to test candidate mutations, from a previous screen for negative regulators of growth, for producing supercompetitors. Surprisingly, most of the mutants that I labeled as supercompetitors fell into a single pathway, The Hippo pathway, suggesting a very important role for this pathway in cell competition. Mutations in negative regulators of the Insulin pathway, as well as *bunched*, *ark* and *tricornered,* were negative in my assay. *TSC1* and *capicua* had intermediate phenotypes suggesting that different mutants may fall into a gradient of competitive abilities. I also performed an unbiased genetic screen for novel supercompetitors, assisted at various times by Justin Bosch and Sabriya Rosemond. I recovered 35 loci that are potentially involved in competition – 6 had been previously implicated in competition while 29 appeared to be novel.

INTRODUCTION

Mosaic Analysis in *Drosophila melanogaster*

Some mutations, when homozygous, cause lethality very early in development making it difficult to analyze their role in later developmental processes. The use of mosaic assays has been instrumental in studying such essential genes. In these assays small patches or clones of homozygous mutant cells are created within a heterozygous animal.

Mosaicism is achieved through a process called mitotic recombination (Figure 2.1A). Recombination between homologous chromosomes typically occurs only during meiosis. However, X-ray irradiation causes double-stranded breaks that can lead to recombination between homologous chromosomes during mitosis. If this happens, all loci distal to the site of recombination become susceptible to incorrect allele segregation. Thus, a cell that is heterozygous for a locus distal to the site of recombination might divide to form daughter cells that are genotypically distinct from each other.

More recently, a mechanism adapted from the 2-micron plasmid of yeast has made it possible to induce mitotic recombination at high frequency, at defined sites in the genome, and in defined tissues. Flippase (FLP) is a site-specific recombinase that induces recombination at defined inverted repeat sequences known as FLP Recognition Target (FRT) sites. The enzyme and its recognition sites were introduced into *Drosophila* and used to create mosaic animals at high efficiency (Figure 2.1B) (Golic, 1991; Golic and Lindquist, 1989).

With the addition of a few more features, several research groups established an assay in the *Drosophila* eye for assessing the effects of a mutation on growth (Figure 2.2) (St Johnston, 2002; Xu and Rubin, 1993; Xu et al., 1995). In this assay, which I will denote the "growth assay," the *eyeless* promoter is used to drive high levels of Flippase early and throughout development in the tissue that represents the eye primordium. Because of the high rate of recombination, approximately half of the cells become mutant and the other half become homozygous wild-type (Figure 2.1C). Eye pigment markers are used to differentially mark the cell types making the results of the assay visible in the adult eye. Any effects that the mutation has on growth would be reflected in changes in the ratio of mutant to wild-type tissue. Generally, mutant cells are marked white (*white*-/-) and wild-type cells are marked red (P[*white*+]) (Newsome et al., 2000). Loss of function mutations in growth promoters would be expected to decrease the relative amount of mutant tissue, resulting in an eye that is more red than white. Little attention has been given to this class of mutants because there are many mutations that cause this phenotype independent of any direct role in growth regulation. Any mutations that make cells sick, such as mutations in housekeeping genes, would have this phenotype. In contrast, loss of function mutations that give cells a growth advantage, and therefore result in mosaic eyes with more white than red tissue, are more likely to have a specific role in growth control (Figure 2.2) (Hariharan and Bilder, 2006).

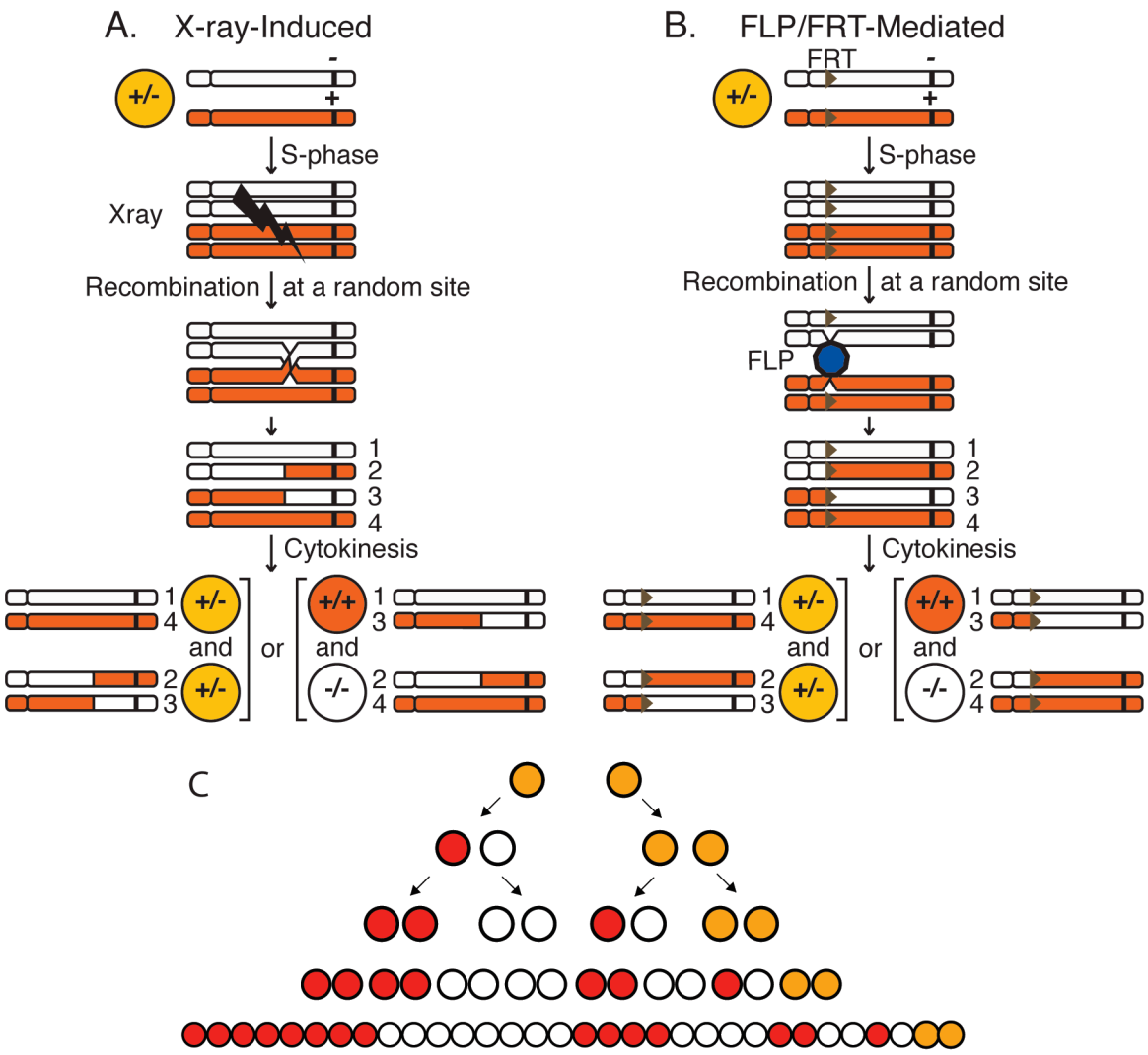


Figure 2.1 - Mitotic recombination
(A) X-rays randomly induce double-stranded breaks that can lead to mitotic recombination. In the example shown, recombination occurs between two of the homologues after S phase. The site of recombination is proximal to a locus for which the mother cell is heterozygous. The chromatid with the mutation (-) is marked white and the chromatid with the wild-type allele (+) is marked red. During cytokinesis, 50% of the time the sister chromatids will segregate in such a way (right) that one of the daughters will become homozygous wild-type (red) and the other will become homozygous mutant (white). (B) If the site of recombination is close to the centromere more genes can be affected by mitotic recombination. The FLP/FRT system can be used to direct recombination to a specific site, usually very close to the centromere. (C) A tissue that is intially composed of two heterozygous cells which undergo mitotic recombintion at every cell division cycle. Within four divisions the tissue is transformed from entirely heterozygous to predominantly homozygous mutant and homozygous wild-type.

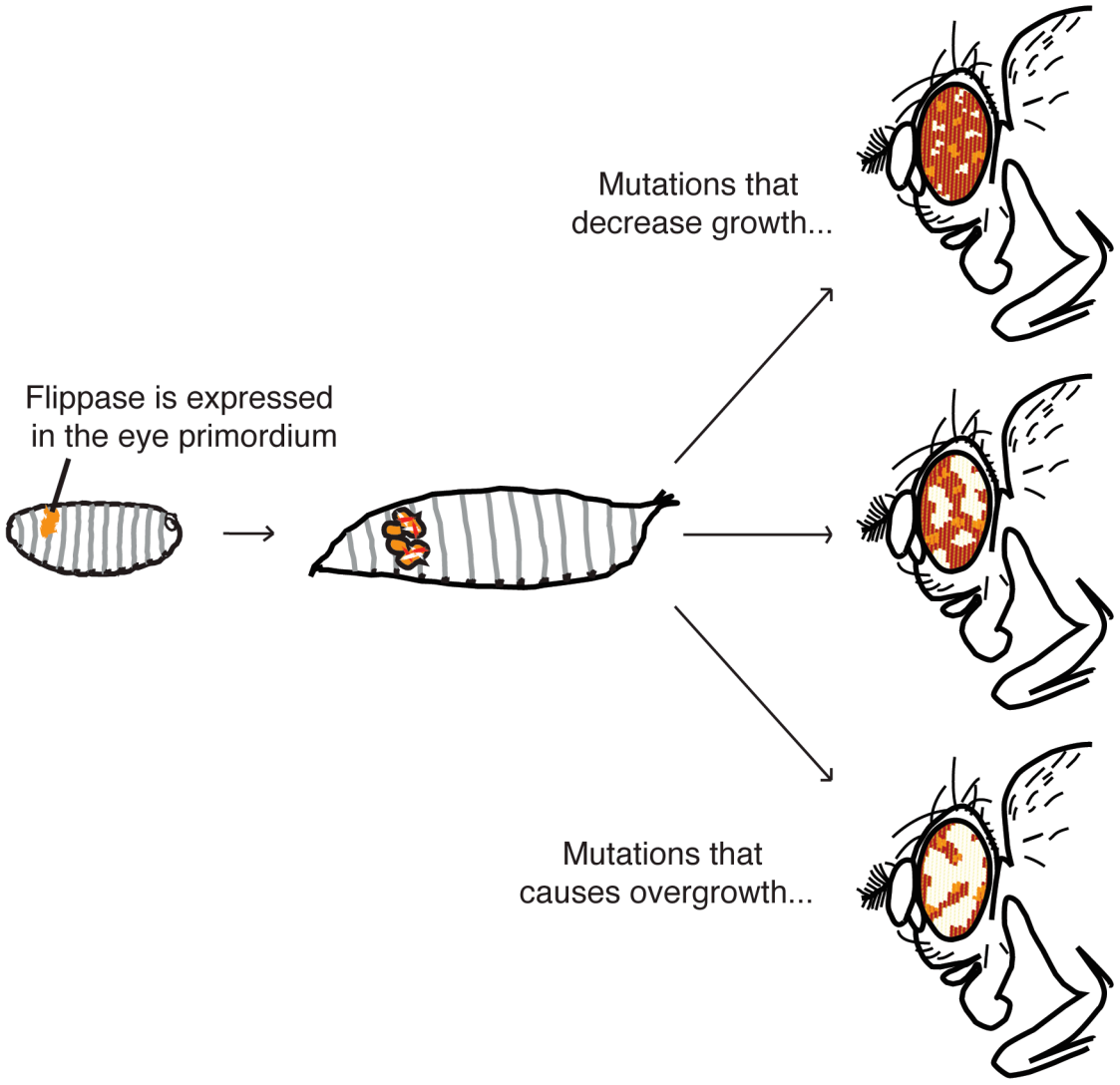


Figure 2.2 - FLP/FRT assay for identifying mutations that affect growth
Flippase is expressed through the *eyeless* promoter at high levels in the developing tissue that will become the eye. Repeated rounds of mitotic recombination causes the tissue to become predominantly composed of homozygous mutant and homozygous wild-type cells, which are marked white and red, respectively, by eye color pigments. If a mutation reduces the growth of cells, the amount of white tissue in the eye will decrease. If a mutation causes cells to overgrow, the amount of white tissue in the eye will increase.

Many research groups have conducted several large-scale genetic screens, using variations of the growth assay, for mutants with mosaic eyes that are overgrown or have more mutant (white) than non-mutant (red) tissue (St Johnston, 2002). These screens led to the identification of many new tumor suppressor genes that generally fell into one of six main pathways that directly regulate growth: the Hippo pathway, the Insulin pathway, the Tor pathway, the JAK/STAT pathway, the Myc pathway, and the Ras/MAPK pathway (Figure 2.3) (Hariharan and Bilder, 2006).

While all of the mutations identified in these overgrowth screens had a similar phenotype, there are likely to be important mechanistic differences in how the phenotype is achieved in each case. The overgrowth phenotype described above can be caused by mutations that make cells grow faster, grow for a longer period of time, or kill wild-type cells. In this dissertation research, I sought a way to specifically look for mutations that caused death of adjacent wild-type cells or supercompetitors.

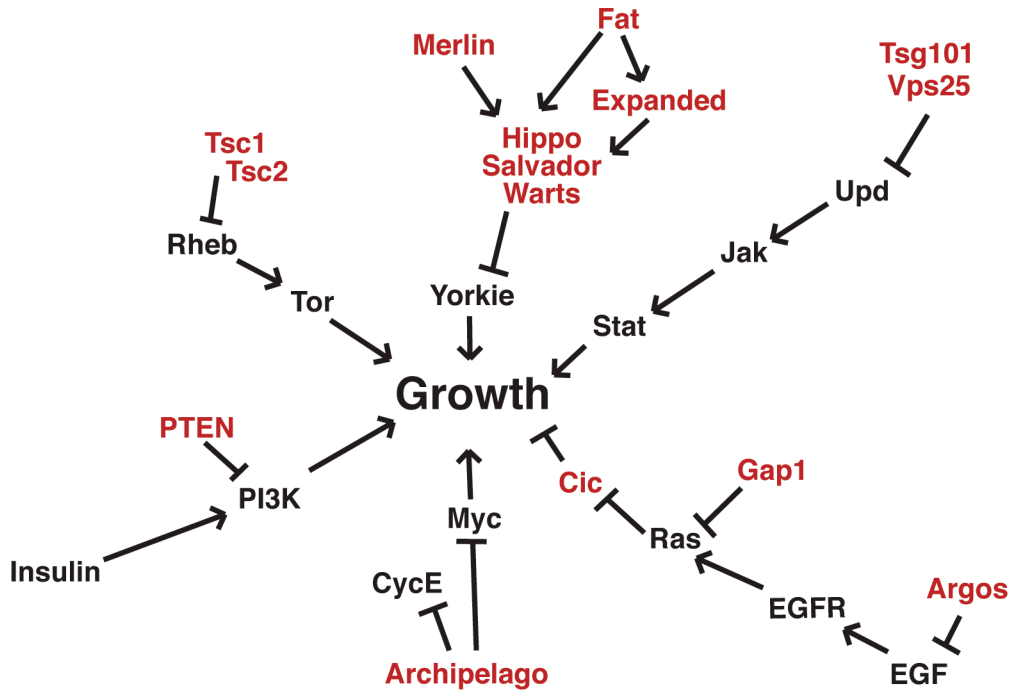


Figure 2.3 - Diagram of the six main growth pathways

RESULTS

An Assay for Supercompetitors

Supercompetitors can be identified by their ability to eliminate wild-type cells. This can occur rapidly when small patches of wild-type cells are entirely surrounded by supercompetitors (Moreno and Basler, 2004). Normally mosaic eyes generated by eyFLP/FRT-induced mitotic recombination are composed of patches of homozygous mutant (white) and wild-type (red) cells in roughly equal proportions. However, there are also small patches of heterozygous tissue (also red) due to cells that have either not undergone mitotic recombination or have segregated their sister chromatids during anaphase to preserve their heterozygosity. I took advantage of this heterozygous population that is normally ignored. I used a modification of the eyFLP/FRT system, developed by Newsome and colleagues, in which the wild-type chromosome arm also contains a recessive cell lethal mutation, and as a result, the homozygous wild-type twin spots are not viable. The homozygous mutant cells compose the majority of the tissue, and the heterozygous cells, which are still present, are forced to compete with the mutant background as desired. Mutations that make cells supercompetitors would be expected to non-autonomously decrease the size of the patches of heterozygous tissue in the eye (Figure 2.4).

The assay is sensitized to detect supercompetition in two ways. First, a greater proportion of the heterozygous cells are bordered by, and therefore potentially exposed to, short-range signals from mutant cells. Second, the heterozygous tissue appears as small discrete patches, therefore any change in the size of the patches is more obvious. Hereafter, I will refer to this assay as the "supercompetitor assay". It is important to note that the assay is designed to detect recessive loss-of-function supercompetitors, because competition occurs between mutant cells and heterozygous cells. A mutation that has a dominant competition phenotype may score negative falsely in this assay. Furthermore, mutations that affect the rate of recombination or curtail developmental timing such that wild-type cells have less time to grow may also alter the amount of red tissue in the adult eye and result in a false positive. For this reason, secondary assays are necessary to confirm that mutations are indeed supercompetitors.

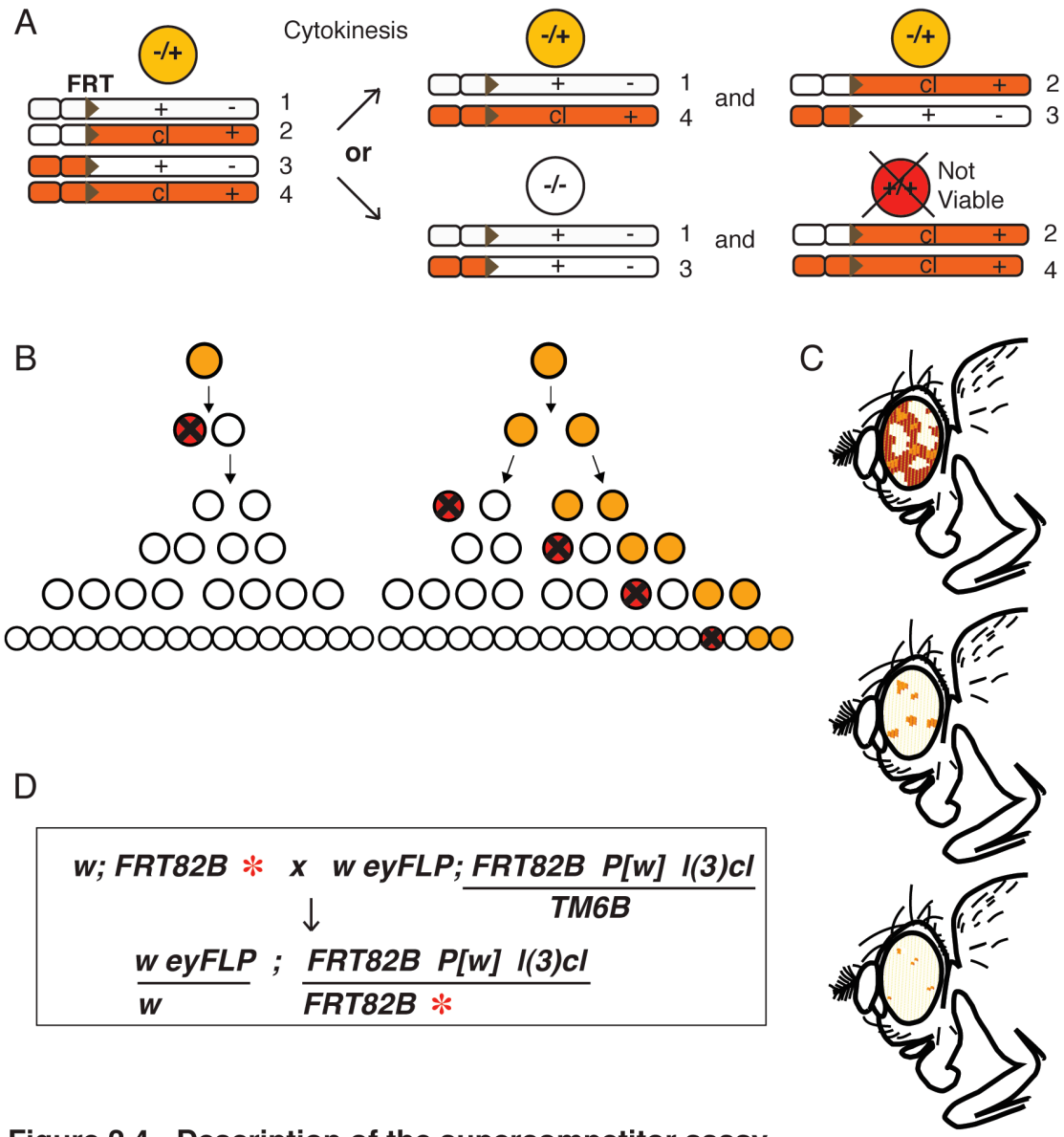


Figure 2.4 - Description of the supercompetitor assay
(A) A variation of the FLP/FRT-based mosaic assay involves the use of a recessive cell lethal mutation on the non-mutant chromosome arm. As a result, cells homozygous for this chromosome arm are no longer viable. (B) As mitotic recombination occurs through successive rounds of division, the tissue becomes predominantly mutant (compare to 2.1C). (C) Schematic representation of a control adult eye from the overgrowth assay (top) and the supercompetitor assay (middle). An adult eye from the supercompetitor assay in which the white cells bear a mutation that causes them to become supercompetitors would be expected to casue a reduction in the size of the heterozygous patches (bottom). (D) Cross scheme for identifying mutations on chromosome arm 3R that make cells supercompetitors.

To facilitate analysis of supercompetitors in developing larval tissues where cell competition is believed to occur, I recombined GFP onto the chromosome arm that contained the FRT sites, the *white+* transgene and the cell-lethal mutation. I made such GFP-containing stocks for three of the major chromosome arms: 2L, 2R, and 3R. The mosaic imaginal discs of late third instar larvae were examined for the 3R stock (Figure 2.5). While the overall morphology of the imaginal disc appeared normal, many of the GFP-positive cells were abnormal in shape and apoptotic as assessed by staining with an antibody to activated caspase 3. There was also a significant amount of GFP-positive debris associated with the discs. This indicates that cells homozygous for the cell-lethal mutation undergo apoptosis. Apoptosis is known to have non-autonomous effects on cell growth that may complicate mechanistic analysis of cell competition (Huh et al., 2004; Ryoo et al., 2004). Therefore, all characterizations beyond showing that mutations cause cells to become supercompetitors have been made in the absence of the cell-lethal mutation.

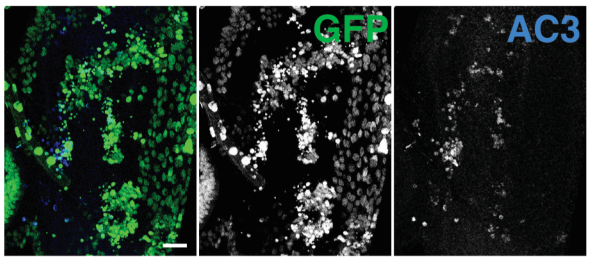
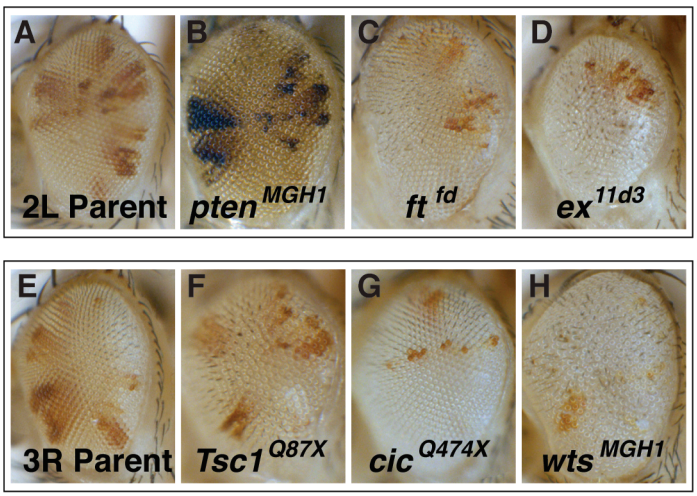


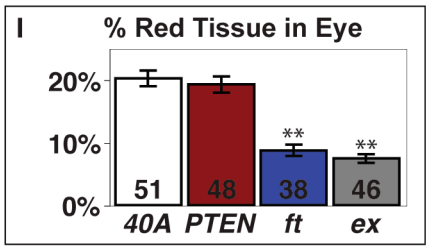
Figure 2.5 - Mosaic eye discs with recessive cell lethal mutation on 3R
Mosaic eye disc of genotype *y w eyFLP ; FRT82B / FRT82 {GFP} {w+} CL*. High levels of activated caspase 3 (AC3, blue) are associated with the GFP-positive cells (AC3, green), which are either heterozygous or homozygous for the CL mutation. Scale bar represents 10μm.

Testing Known Supercompetitors and Non-competitors

In order to determine whether the cell-lethal assay could specifically identify known supercompetitors, I tested mutations that had previously been shown to cause, or not cause, cell competition (Figure 2.6). Cells mutant for components of the Hippo pathway: *fat* (*ft*), *expanded* (*ex*), *hippo* (*hpo*), *salvador* (*sav*) and *warts* (*wts*), were previously shown to induce apoptosis of adjacent wild-type cells (Tyler et al., 2007), and similarly scored positive in the supercompetitor assay as assessed by a decrease in the amount of red tissue remaining in the eye. Additionally, a specific allele of *discs overgrown* (*dco*) that causes overgrowth, dco^3, also scored as a supercompetitor. However, I found that cells that are mutant for *pten*, a negative regulator of PI3K, do not reduce the representation of adjacent wild-type cells, consistent with a previous report that cells overexpressing Dp110, the catalytic subunit of PI3K, do not become supercompetitors (de la Cova et al., 2004). Thus, the supercompetitor assay was able to discriminate between mutations that simply caused

overgrowth and those that also made cells supercompetitors. I will, hereafter, refer to this assay as the supercompetitor assay.





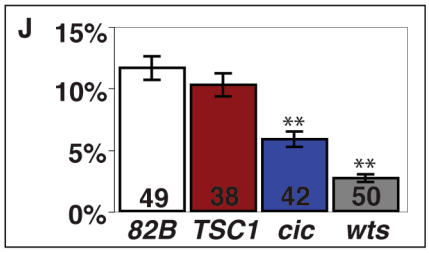


Figure 2.6 - Supercompetitor phenotypes and quantification
(A-D) Adult eyes of mutants tested using the supercompetitor assay; mutants on 2L. (E-H) Adult eyes of mutants tested using this assay; mutants on 3R. (I) Quantification of the area of red tissue in eyes mosaic for mutations on 2L. (J) Quantification of the area of red tissue in eyes mosaic for mutations on 3R. The number of eyes used to quantify each genotype is indicated. Error bars show SE. ** indicates < 0.001 significance in the Mann-Whitney Z test.

Testing Mutants from a Screen for Overgrowth as Candidates

While all mutations that cause overgrowth do not make cells supercompetitors, all of the supercompetitors identified to date do indeed have an increased growth rate. Therefore I tested several uncharacterized mutants that were recovered in a screen based on the growth assay described above. *capicua* (*cic*) (Jiménez et al., 2000) a negative regulator of growth downstream of the RTK/Ras pathway (Tseng et al., 2007) scored positive, corroborating previous findings that the Ras pathway may be involved in cell competition (Prober and Edgar, 2000). *Tsc1*, *bunched*, and *ark* were also tested and scored negative in the assay (Figure 2.6 and 2.7). Thus different growth-promoting pathways vary in their apparent ability to make cells supercompetitors, and the majority of overgrowth mutants do not act as supercompetitors.

Finally, I also tested a number of mutants from the growth screen that are homozygous viable. Because homozygous viable mutants are technically difficult to work with, they had not yet been mapped or characterized. This included *8b6* and *2a6* on 2L and *F12* and *2H270* on 3R (Figure 2.7 and 2.8).

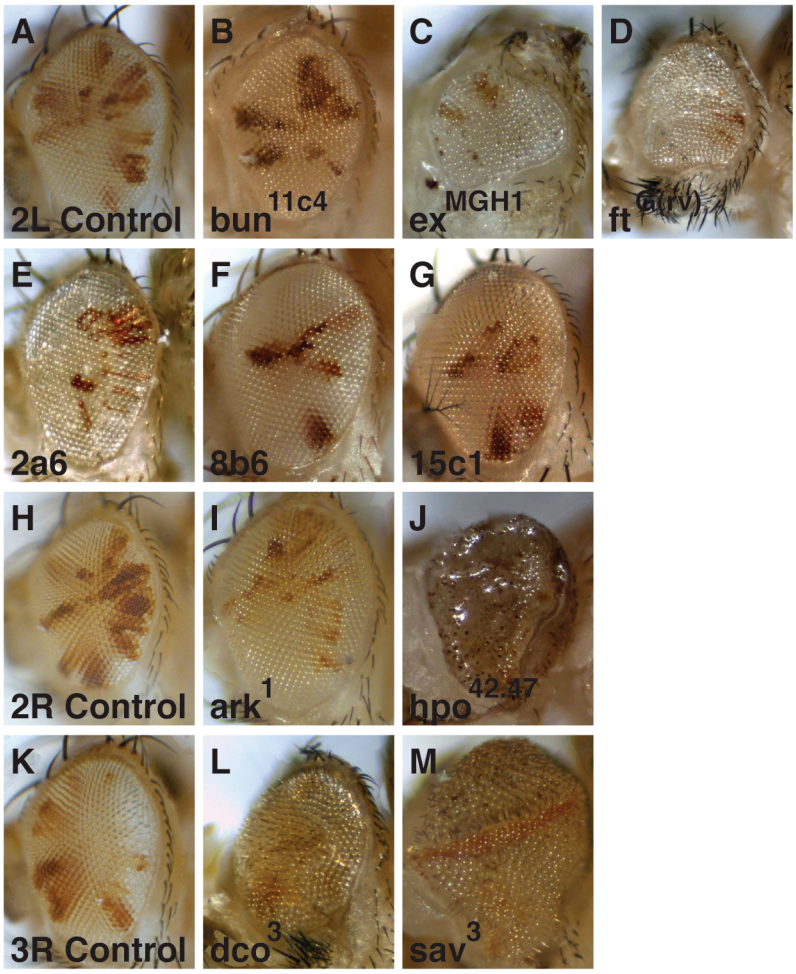


Figure 2.7 - Supercompetitor phenotypes, continued
Adult eyes of mutants tested using the supercompetitor assay. Overgrowth mutants on 2L (A-G), 2R (H-J), and 3R (K-M) were tested.

Interestingly, the strength of the growth and supercompetition phenotypes of the mutants did not always correlate. This was best exemplified by comparing two of the viable mutants – *F12* and *2H270* (Figure 2.8). I tested both mutants in the cell-lethal assay to determine whether they were supercompetitors, and in the overgrowth assay to determine the extent of overgrowth. In the absence of the recessive cell-lethal mutation, *F12* showed a very strong overgrowth phenotype while *2H270* was very weak. In contrast *2H270* had a very strong supercompetitor phenotype while *F12* did not noticeably reduce the amount of red tissue. The naïve interpretation of this result is that *F12* overgrows but is not a supercompetitor, while *2H270* is a supercompetitor but does not overtly overgrow. If generalized, this would suggest that overgrowth is neither necessary nor sufficient to cause supercompetition.

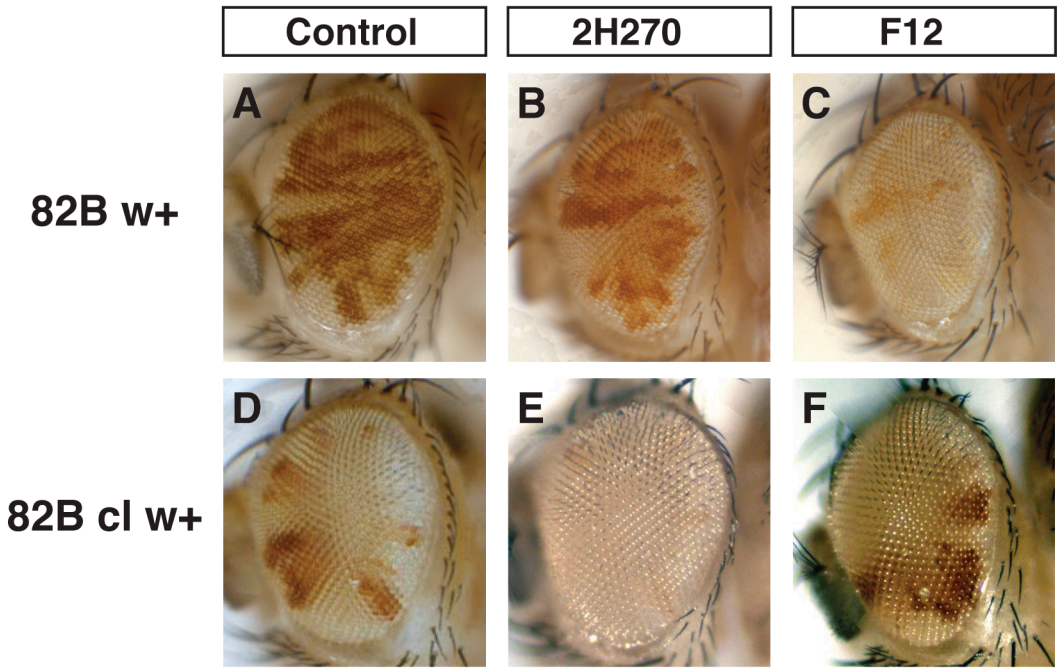


Figure 2.8 - Comparison of growth and supercompetitor phenotypes
(A) In the growth assay, the control eye is composed of approximately 50% mutant (white) and 50% wild-type (red) cells. (B) *2H270* produces a mild overgrowth phenotype in which there is slightly more mutant (white) tissue. (C) *F12* produces a very strong overgrowth phenotype, in which the ratio is greatly skewed towards more white tissue. (D) In the supercompetitor assay, the control eye is composed of mostly mutant tissue (white) but also contains small patches of non-mutant (red) tissue. (E) *2H270* produces a very strong supercompetitor phenotype in which most of the non-mutant tissue is lost from the eye. (F) *F12* does not display any significant reduction in the amount of red tissue and, therefore, scores negatively as a supercompetitor.

Mapping a Viable Supercompetitor

2H270 appeared to be a strong competitor but did not appear to overgrow in the mosaic growth assay. Preliminary characterization of the mutant showed that there was an increase in the incidence of apoptotic wild-type cells at the borders of *2H270* clones (data not shown), consistent with it being a supercompetitor. Moreover, initial mapping data suggested that the mutation in *2H270* was in a region with no previously characterized regulators of growth or competition. Because of its novelty and remarkable phenotype I assumed the task of identifying the gene responsible for this mutation.

Initial mapping of *2H270* indicated that it was in a 2.3 Mbp region, between two P element markers (R.Smith-Bolton, personal communication). *2H270* was homozygous viable and complemented a series of deficiencies that spanned this region. Therefore, I could not map it by the traditional method of complementation. Instead I sought to further narrow the region by recombination with dominantly marked transposon insertions and single nucleotide polymorphisms (SNPs). Surprisingly, the results of the different recombination tests appeared to contradict one another. This was explained by the SNP mapping which indicated that there was an abnormally high rate of multiple recombination events. Multiple recombination events can strongly bias the results of recombination mapping. Eventually I found that the mutation was distal to the interval where I had been looking and found that the mutant failed to complement a null allele of *wts*. Upon sequencing the mutant, I found a point mutation in the open reading frame of *warts* (Figure 2.9).

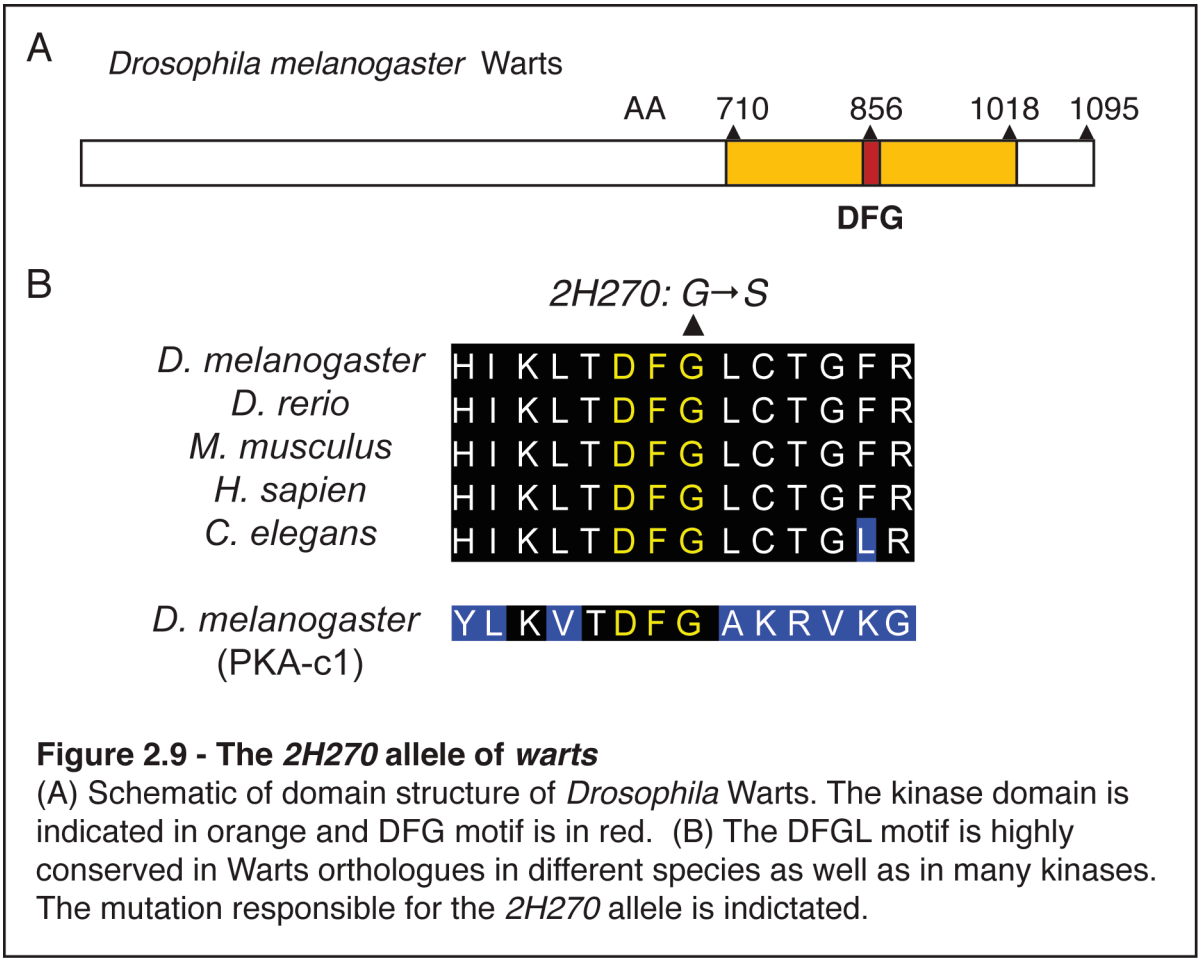


Figure 2.9 - The *2H270* allele of *warts*
(A) Schematic of domain structure of *Drosophila* Warts. The kinase domain is indicated in orange and DFG motif is in red. (B) The DFGL motif is highly conserved in Warts orthologues in different species as well as in many kinases. The mutation responsible for the *2H270* allele is indictated.

The mutation resulted in the conversion of a glycine to a serine in the highly conserved DFG motif. These residues normally orient the gamma phosphate of MgATP for transfer to the substrate. The mutation would be predicted to cause a ten to one hundred-fold reduction in the kinase activity of Warts (M. Seeliger and J. Kuriyan, personal communication). It is very

surprising that a mutant with such a drastic reduction in the kinase activity of Warts can be homozygous viable. This may indicate that the essential functions Warts may be independent of its kinase activity, and the *2H270* allele may be very useful in future studies aimed at understanding these functions.

Unbiased Screen for Novel Supercompetitors

One of the most valuable aspects of *Drosophila* genetics is the ability to conduct large-scale genetic screens, which can implicate unexpected genes in a process of interest. One concern, before doing such a screen for novel supercompetitors, was that I would recover some fraction of the mutants from the overgrowth screen, but not find any new mutants. However, based on *2H270*'s very subtle overgrowth phenotype and very strong supercompetitor phenotype, it seemed likely that there would be mutants that would have been missed in the original growth screen and could be detected in a supercompetitor screen.

With the help of Justin Bosch and Sabriya Rosemond, I screened approximately 18,000 flies for mutations on the right arm of the third chromosome that gave a supercompetitor phenotype. We retained 36 mutants where the red patches appeared smaller than those in the eyes of control flies. These included 3 alleles of *wts* and 3 alleles of *sav*. Recovery of these mutants in the screen was further validation that the assay could identify known supercompetitors in an unbiased way. We also found 2 alleles of *TSC1*, although these were most likely retained for their obvious big eye phenotype. No alleles were found of *cic* suggesting that the screen may have not reached saturation. We kept 28 additional alleles, four of which composed a lethal complementation group that did not correspond to any previously described regulators of cell competition on 3R. I mapped the responsible gene for this complementation group to the *crumbs* (*crb*) locus (see Chapter 3). The remainder of this thesis will be focused on this mutant and the characterization of its role in cell competition. The remaining 24 mutants have not yet been characterized. Descriptions and pictures of the mutants and the results of complementation tests between the mutants can be found in Tables 2.1 and 2.2, respectively.

Table 2.1 - Uncharacterized mutants from supercompetitor screen on 3R
List of the uncharacterized mutants from the unbiased screen on 3R. Notable characteristics and pictures are included if available.

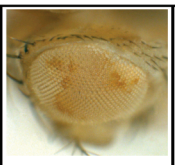
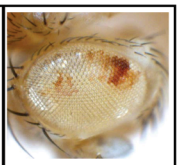
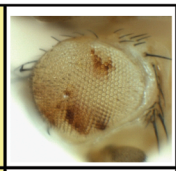
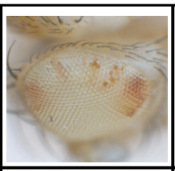
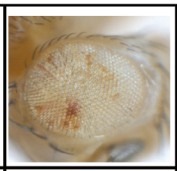
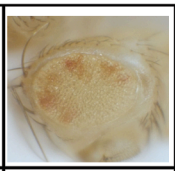
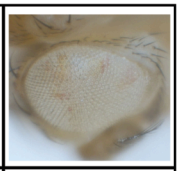
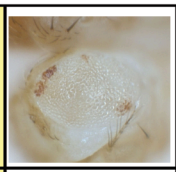
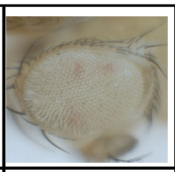
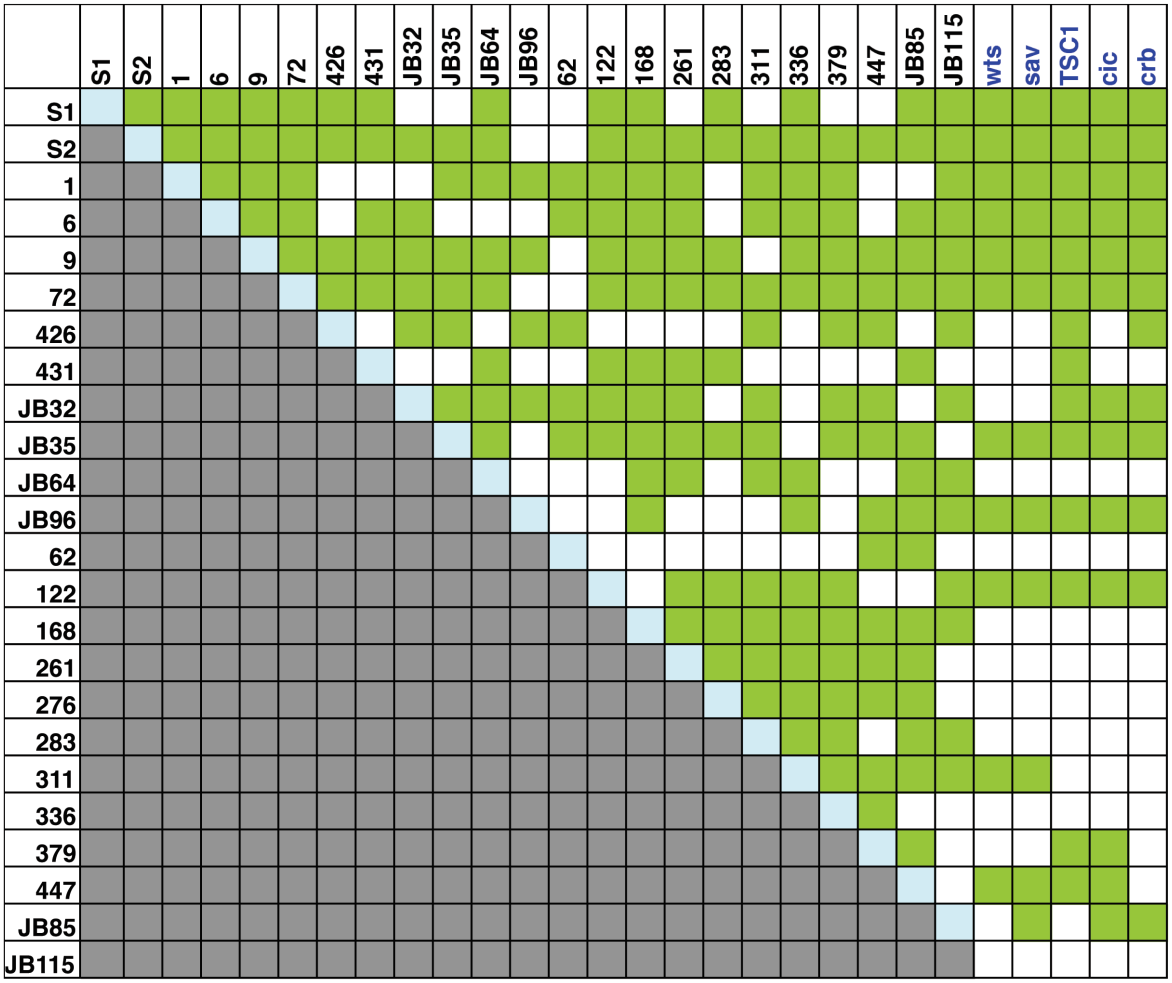
Mutant	Eye Size	Patterning	Picture
S1	-	-	
S2	-	-	
1	-	-	
6	-	-	
9	-	-	
72	variable/ bigger	rough	
426	big	rough	
431	smaller	rough	
JB32	smaller	rough	
JB35	-	-	
JB64	smaller	rough	
JB96	-	-	
62	-	-	
122	-	-	
168	-	-	
261	-	-	
276	variable	rough	
283	-	-	
311	-	-	
336	-	rough	
379	-	-	
447	-	-	
JB85	-	-	
JB115	smaller	rough	

Table 2.2 - Complementation analysis of uncharacterized mutants
Green boxes indicate that two mutants complemented, which implies that the mutations are likely to be in different genes. White boxes indicate that complementation has not yet been determined. None of the mutants tested thus far failed to complement each other.



DISCUSSION

I devised a mosaic assay in the *Drosophila* eye for identifying mutations that cause a nonautonomous reduction in the final representation of non-mutant cells. The assay could distinguish mutations that were known to cause cells to become supercompetitors from mutations that were known to cause only overgrowth. To learn something about the mechanism of cell competition I used the supercompetitor assay to test candidate overgrowth mutations in different pathways to see which ones were involved in cell competition. I also conducted an unbiased screen for supercompetitors.

The Hippo pathway appears to play a very important role in cell competition as all of the mutants tested from this pathway caused cells to become supercompetitors. Mutations in the Hippo pathway were also repeatedly recovered though unbiased testing with the supercompetitor assay. A homozygous viable mutant, called *2H270,* was found in a previous screen as a result of its subtle overgrowth phenotype. Surprisingly, despite the weak overgrowth phenotype, *2H270* had a very strong supercompetitor phenotype. I found the responsible mutation to be in *warts*, a component of the Hippo pathway. The unbiased screen I performed also uncovered at least 6 new alleles of components in this pathway. The pathway has previously been implicated in organ size control and contact inhibition. Whether cell competition is related to these processes is unclear.

Testing several different mutants in the assay and quantifying the amount of red tissue remaining in the eye revealed that the supercompetitor phenotype varied significantly between mutants. Not all mutations that caused overgrowth reduced the amount of red tissue. Furthermore, in mutants that did score as supercompetitors, the amount of red tissue was decreased by various amounts. Therefore, cell competition does not appear to occur at a constant rate, rather different mutants can cause elimination to various degrees. Furthermore, the strength of the supercompetitor phenotype did not always correlate with the amount of overgrowth displayed by a mutant. Thus overgrowth appears to be neither necessary nor sufficient to produce supercompetition.

The mechanism of cell competition is poorly understood. Cells might compare the levels of a cell surface molecule, reception levels of a survival factor, or signaling levels of a morphogen. The identification of the responsible genes for the twenty-nine novel mutants recovered in this work may implicate one of these mechanisms. A four-member complementation group that I found among these mutants mapped to the gene, *crumbs* (*crb*). Crb is a transmembrane protein with a large extracellular domain and a small cytoplasmic domain (Figure 3.2) that functions to specify the apical domain of the plasma membrane in epithelial cells of the embryo. As a transmembrane protein, Crb is in a position to mediate cell competition. In chapter three, I describe experiments aimed at determining whether Crumbs might be a basis of comparison in cell competition.

METHODS

Fly Stocks & Husbandry

y w eyFLP2 ;; FRT82B w+ cl3R3/TM6B,y+ (Newsome et al., 2000)
y w eyFLP2 ; cl2L3 w+ FRT40A /CyO, y+ (Newsome et al., 2000)
w;;FRT82B
w;;FRT82B Tsc1[Q87X] (Tapon et al., 2001)
w;;FRT82B cic[Q474X] (Tseng et al., 2007)
w;;FRT82B wts [MGH1]
w;; FRT40A
w;; pten [MGH1] FRT40A/CyO
w;; ft [fd] FRT40A/CyO (Bryant et al., 1988)
w;; ex [11d3] FRT40A/CyO
w;;FRT82B wts[2H270]
w;;FRT82B F12
y w eyFLP ;; FRT82B P[mini-w] P[Ubi-GFP]
3R Deficiency Collection from Bloomington Stock Center

Generally, experimental crosses were raised at 25º on food prepared according to the recipe from the Bloomington Stock Center.

Quantification of Red Tissue in Adult Eyes

Pictures of adult eyes were taken on a Leica Z16 APO system with Montage software from Synoptics Ltd. Total eye and red tissue area were measured in pixels by using the 'Histogram' dialogue for selected regions in Photoshop. The eye was selected by hand using the polygonal lasso. To select red pixels, the image was converted to 'grayscale' and 'shadows' were selected. The ratio of the red pixels to total pixels in the eye was calculated.

EMS Mutagenesis

3-4 day old *w;;FRT82B* males were starved, fed 25mM ethyl methanesulfonate in 1% sucrose, and then crossed to "tester" virgins, *y w eyFLP2 ;; FRT82B w+ cl3R3/TM6B,y+*. Mutagenized males were removed from the cross on Day 5. F1 flies were screened for a visual reduction in the amount of red tissue. Single males with this phenotype were re-crossed to virgins from the tester line. If the phenotype was recovered, the F2 flies were used to establish a line. F1 females with desired phenotype were crossed to males from the tester line, single F2 males were retested, and lines were established from the F3.

Immunohistochemistry

Discs were dissected in PBS, fixed in 4% paraformaldehyde in PBS, washed in 0.1% Triton in PBS, and mounted in Slow Fade Gold. The following primary antibodies were used:
AC3 (Rabbit, 1:200) from Cell Signaling
Secondary Alexa-Fluor antibodies from Invitrogen were used at 1:1000.
Fluorescent images were taken on a Leica TCS SL confocal microscope.

REFERENCES

1. **Bryant, P. J., Huettner, B., Held, L. I., Ryerse, J. and Szidonya, J.** (1988). Mutations at the fat locus interfere with cell proliferation control and epithelial morphogenesis in Drosophila. *Dev Biol* **129**, 541-54.

2. **de la Cova, C., Abril, M., Bellosta, P., Gallant, P. and Johnston, L. A.** (2004). Drosophila myc regulates organ size by inducing cell competition. *Cell* **117**, 107-16.

3. **Golic, K. G.** (1991). Site-specific recombination between homologous chromosomes in Drosophila. *Science* **252**, 958-61.

4. **Golic, K. G. and Lindquist, S.** (1989). The FLP recombinase of yeast catalyzes site-specific recombination in the Drosophila genome. *Cell* **59**, 499-509.

5. **Hariharan, I. K. and Bilder, D.** (2006). Regulation of imaginal disc growth by tumor-suppressor genes in Drosophila. *Annu Rev Genet* **40**, 335-61.

6. **Huh, J. R., Guo, M. and Hay, B. A.** (2004). Compensatory proliferation induced by cell death in the Drosophila wing disc requires activity of the apical cell death caspase Dronc in a nonapoptotic role. *Curr Biol* **14**, 1262-6.

7. **Jiménez, G., Guichet, A., Ephrussi, A. and Casanova, J.** (2000). Relief of gene repression by torso RTK signaling: role of capicua in Drosophila terminal and dorsoventral patterning. *Genes Dev* **14**, 224-31.

8. **Moreno, E. and Basler, K.** (2004). dMyc transforms cells into super-competitors. *Cell* **117**, 117-29.

9. **Newsome, T. P., Asling, B. and Dickson, B. J.** (2000). Analysis of Drosophila photoreceptor axon guidance in eye-specific mosaics. *Development* **127**, 851-60.

10. **Prober, D. A. and Edgar, B. A.** (2000). Ras1 promotes cellular growth in the Drosophila wing. *Cell* **100**, 435-46.

11. **Ryoo, H. D., Gorenc, T. and Steller, H.** (2004). Apoptotic cells can induce compensatory cell proliferation through the JNK and the Wingless signaling pathways. *Developmental Cell* **7**, 491-501.

12. **St Johnston, D.** (2002). The art and design of genetic screens: Drosophila melanogaster. *Nat Rev Genet* **3**, 176-88.

13. **Tapon, N., Ito, N., Dickson, B. J., Treisman, J. E. and Hariharan, I. K.** (2001). The Drosophila tuberous sclerosis complex gene homologs restrict cell growth and cell proliferation. *Cell* **105**, 345-55.

14. **Tseng, A.-S. K., Tapon, N., Kanda, H., Cigizoglu, S., Edelmann, L., Pellock, B., White, K. and Hariharan, I. K.** (2007). Capicua regulates cell proliferation downstream of the receptor tyrosine kinase/ras signaling pathway. *Curr Biol* **17**, 728-33.

15. **Tyler, D. M., Li, W., Zhuo, N., Pellock, B. and Baker, N. E.** (2007). Genes affecting cell competition in Drosophila. *Genetics* **175**, 643-57.

16. **Xu, T. and Rubin, G. M.** (1993). Analysis of genetic mosaics in developing and adult Drosophila tissues. *Development* **117**, 1223-37.

17. **Xu, T., Wang, W., Zhang, S., Stewart, R. A. and Yu, W.** (1995). Identifying tumor suppressors in genetic mosaics: the Drosophila lats gene encodes a putative protein kinase. *Development* **121**, 1053-63.

Chapter 3
The Role of Crumbs in Cell Competition

ABSTRACT

Several alleles of *crumbs (crb)*, a gene encoding a transmembrane protein previously characterized as a regulator of apicobasal polarity, were identified in a screen for mutations that cause cells to become supercompetitors. In this Chapter, we describe the characterization of the role of Crb in cell competition. In accordance with being supercompetitors, *crb* mutant cells significantly increase the amount of apoptosis in adjacent wild-type cells. Conversely, Crb-overexpressing cells that border wild-type cells display significantly higher levels of apoptosis than Crb-overexpressing cells that are bordered by other Crb-overexpressing cells. Thus, there is a relationship between the levels of Crb and the competitiveness of cells – those with less Crb are better competitors while cells with more Crb are worse competitors. In further characterizing the competition between Crb-overexpressing and wild-type cells, we found two notable differences to the classic examples of cell competition involving *Minutes* or Myc-overexpressing cells. First, in addition to the increased cell death that we observed in the Crb-overexpressing or loser cells, we also observed increased cell death in the wild-type or winner cells. Second, extrusion, in addition to apoptosis, appears to play an important part in the elimination of Crb-overexpressing cells.

Consistent with recent reports, we found that in *crb* mutant clones, the strict apical localization of Expanded is disrupted and some Hippo pathway target genes are upregulated. The Hippo pathway has an established role in cell competition. However, whether Crb acts through Hippo to mediate cell competition is still unclear.

We also characterized the roles of the intra- (ICD) and extra- (ECD) cellular domains in the competition between wild-type and Crb-overexpressing cells. While neither was sufficient to induce the elimination that we observed upon overexpression of full-length Crb, overexpression of each domain separately recapitulated some aspects of the phenotype. The overexpression of the ECD caused changes in the localization of Crb and Ex in adjacent cells and autonomously caused the epithelium to bend upward, suggestive of apical expansion. Overexpression of the ICD induced overgrowth and caused the epithelium to bend downward, suggestive of apical constriction.

While the basis of Crb-mediated cell competition is still not clear, we make significant progress toward defining the mechanism. Based on our work, we present two plausible models for how cells might directly compare Crb levels – one based on asymmetries in protein localization of Crb and its downstream binding partners and one based on the presence of molecules of Crb that are not incorporated into homophilic dimers. Future work may validate one of these models or reveal that cells compare the levels of a molecule downstream of Crb.

INTRODUCTION

crumbs (crb) was originally identified in 1984 in *Drosophila* as a mutant in which the embryonic epithelium loses integrity, or "crumbles" (Jurgens et al., 1984). *crb* encodes a large transmembrane protein that is required for apicobasal polarity and stabilization of intercellular junctions (Tepass et al., 1990). The specific role of the different domains of Crb, the interactions of Crb with other proteins, and, hence, the mechanism by which Crb acts in this context, are relatively well characterized (Bulgakova and Knust, 2009; Médina et al., 2002). However, more recently, new roles have been discovered for Crb and there is evidence that different mechanisms are employed by Crb for these new roles. In the last decade, Crb has been implicated in retinal cell degeneration (Izaddoost et al., 2002; Pellikka et al., 2002), and in this last year, Crb was found to control the growth of imaginal disc cells (Chen et al., 2010; Grzeschik et al., 2010; Ling et al., 2010; Richardson and Pichaud, 2010; Robinson et al., 2010). The work in this dissertation research shows that Crb may also have a role in regulating cell competition and begins to characterize the mechanism of this new function. As discussed in Chapter 1, there is a close connection between growth and cell competition, suggesting that these two functions of Crb may be related.

Orthologues of *crb* exist in a wide range of organisms from *Drosophila* and *C.elegans* to fish, mice, and humans (Bulgakova and Knust, 2009; Médina et al., 2002). In humans, perturbations of Crb are thought to be responsible for retinal dystrophies and cancer (Bulgakova and Knust, 2009; Karp et al., 2008; Knust, 2007). Therefore, a mechanistic understanding of Crb function has important biomedical implications.

The Role of Crb in the Embryonic Epithelium

Despite their apparent simplicity, epithelial cells exhibit significant complexity along their apicobasal axis. The cells of an epithelium are organized into continuous sheets and connected by specialized junctions through their lateral surfaces (Tepass et al., 2001). These junctions are important for the overall cohesiveness of the epithelium, but they also divide the plasma membrane into separate domains that are distinct in both structure (lipid and protein composition) and function (Figure 3.1) and act as barriers to prevent the diffusion of proteins between the domains. In addition to the junctions, separate pathways exist that help to delineate the different domains by trafficking proteins to either one or the other (Rodriguez-Boulan et al., 2005). The apical domain is often external or luminal and may be specialized to mediate secretion or absorption, while the basal domain is usually internal and mediates exchanges between the cell and the interstitial fluids. Meanwhile, the lateral membrane appears to be further subdivided into domains that can be identified by the localization of certain proteins, including Crb. In *Drosophila,* these include, from apical to basal, the marginal zone, zonula adherens, and basolateral domains (Tepass et al., 2001).

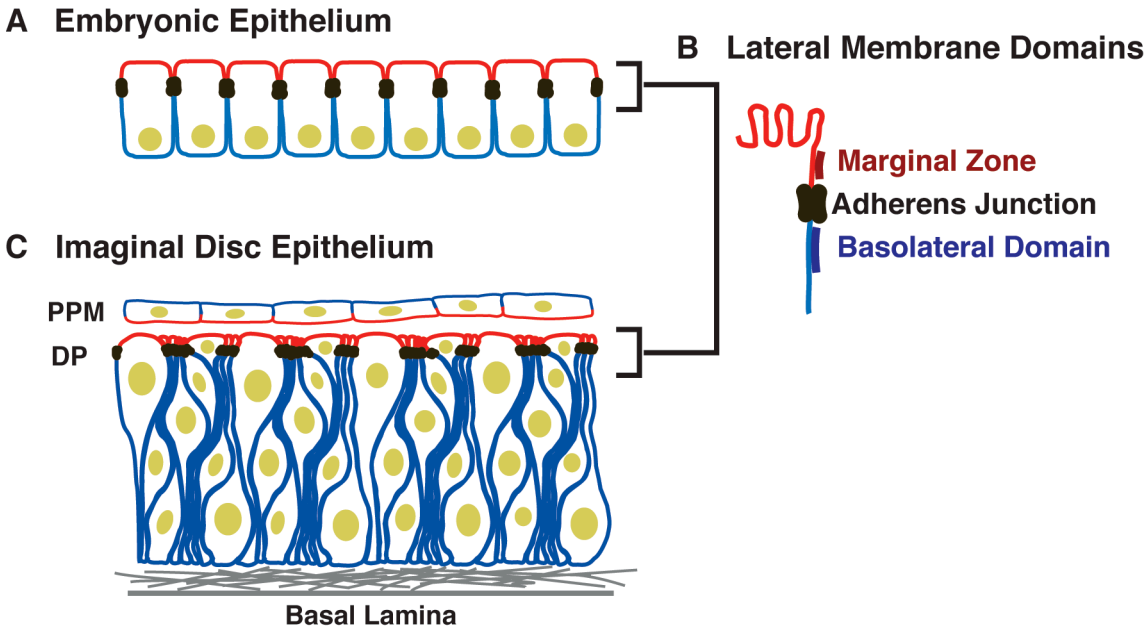


Figure 3.1 - Apicobasal polarity of epithlial tissues in *Drosophila*
(A) *Drosophila* epithelia, such as the embryonic ectoderm, have distinct apical (red) and basal (blue) domains that are separated by adherens junctions. (B) The lateral membrane has additional subdivisions including the marginal zone, which is immediately apical to the adherens junction, and the basolateral domain, which is immediately basal to the adherens junction. These domains are specialized in their lipid and protein composition and function. (C) The epithelium of the imaginal disc is pseudostratified, but also contains the membrane distinctions of other *Drosophila* epithelia.

Crb is critical for establishing and maintaining apicobasal polarity, the separation of the different membrane domains, and the integrity of the cell-cell junctions in ectodermally-derived epithelial cells of gastrulating *Drosophila* embryos (Tepass et al., 1996; Tepass and Knust, 1990; Tepass et al., 1990). Thus, in epithelia from *crb* mutant embryos, the zonula adherens is destabilized and cells have a diminished apical domain as determined by decreased expression of proteins that normally localize to the apical domain (Tepass et al., 1996). Most cells undergo apoptosis while the remainder form small vesicles, into which they secrete cuticle. Thus, a continuous epithelial sheet is not formed (Tanentzapf and Tepass, 2003; Tepass and Knust, 1990). Conversely, overexpression of Crb causes an expansion of the apical domain at the expense of the basolateral domain and multilayering of the epithelium (Bilder et al., 2000; Bilder and Perrimon, 2000; Bilder et al., 2003; Tanentzapf and Tepass, 2003; Wodarz et al., 1995). Therefore, the Crb complex appears to be necessary and sufficient for specifying the apical domain. Mammals express an orthologue of *Drosophila* Crb in epithelial tissues, CRB 3, that plays an analogous role in

stabilizing apical cell junctions and promoting specification of the apical membrane (Makarova et al., 2003).

Crb is a type-I transmembrane protein with 1,994 amino acids in its extracellular domain (ECD), which consists of several EGF and Laminin AG–like repeats, and a relatively small intracellular domain (ICD) consisting of 37 amino acids that form a FERM-binding motif (FBM) and a PDZ-binding motif (PBM) (Tepass et al., 1990). The ICD is particularly important for the control of apicobasal polarity by Crb in the embryonic epithelium. Thus, the phenotype of the *8F105* allele, in which the last 21 amino acids of the ICD (including the entire PBM and two out of fourteen amino acids of the FBM) are removed and most of the extracellular domain is left intact, recapitulates the polarity phenotype of the *crb* null allele, *11A22* (Jurgens et al., 1984; Tepass and Knust, 1990; Tepass et al., 1990). Furthermore, overexpression of a membrane-bound version of the intracellular domain is sufficient to rescue the defects of the null allele (Wodarz et al., 1995). CRB 3, the orthologue that is thought to fulfill this role in mammals has a highly conserved intracellular domain with *Drosophila* Crb and a divergent extracellular domain (Lemmers et al., 2004).

Crb forms a core complex through its ICD with two other proteins – the membrane-associated guanylate kinase, Stardust (Sdt), and the PALS1-associated tight junction protein (PATJ) (Bulgakova and Knust, 2009; Médina et al., 2002). Both *sdt* and *PatJ* mutants phenocopy the polarity phenotype of *crb* mutants. The Crb complex is antagonized by the Scribble (Scrib) complex, which includes Scrib, Discs large (Dlg) and Lethal giant larva (Lgl) (Bilder et al., 2000; Bilder and Perrimon, 2000; Bilder et al., 2003; Tanentzapf and Tepass, 2003; Wodarz et al., 1995; Woods and Bryant, 1991). This Scrib complex localizes to and is required for specifying the basolateral domain. In cells mutant for components of the Scrib complex, Crb is no longer restricted to the apical membrane domain, thus the apical domain is expanded at the expense of the basolateral domain. Furthermore, adherens junctions are disrupted and cells become rounded and form multilayered and disorganized tissues (Bilder et al., 2000; Bilder and Perrimon, 2000). The phenotype of Scrib complex mutant cells is very similar to that of cells overexpressing Crb (Bilder et al., 2000; Bilder and Perrimon, 2000; Wodarz et al., 1995). Furthermore, the basal complex components are fully epistatic to the *crb* complex components as double mutants exhibit the polarity phenotype of *scrib* single mutants (Bilder et al., 2003; Tanentzapf and Tepass, 2003). This indicates that normal function of the Scrib complex allows the differentiation of the basolateral domain by prohibiting the spreading of apical determinants, including Crb as well as at least one other apical determinant, to that region.

There appears to be a temporal aspect to the role of Crb. While loss of Crb can have drastic effects on epithelial polarity and integrity, these severe phenotypes are only observed when the epithelia are undergoing morphogenetic movement or remodeling. In the development of the embryonic epithelium, for example, Crb is only required during a specific time window beginning with stage 6 when gastrulation begins (Tanentzapf and Tepass, 2003; Tepass and Knust, 1990; Tepass et al., 1990). As mentioned, loss of Crb after this stage causes loss of polarity and widespread apoptosis. However, if apoptosis is genetically inhibited in *crb* mutant cells, they are able to reestablish polarity by the end of embryogenesis (Bilder et al.,

2003; Tanentzapf and Tepass, 2003). The specific requirement of Crb during morphogenesis is also apparent in the renal tubules. In these tissues polarity is established at stage 10 when they bud out from the hindgut primordium. However, the *crb* phenotype manifests from Stage 13 to 16 as the tubes dramatically elongate by convergence-extension. If the morphogenetic movements are delayed, then the requirement for Crb is also delayed (Campbell et al., 2009). This may imply that there are redundant or more important mechanisms for establishing polarity in "static" epithelia.

The Role of Crb in Photoreceptor Cells

Crb was also found to be important for the morphogenesis and maintenance of photoreceptor cells (Izaddoost et al., 2002; Pellikka et al., 2002). In *Drosophila*, photoreceptors begin to differentiate in the pupal stages. At this time the apical membrane undergoes a dramatic extension and ninety-degree tilt to form the important light-sensing structure, known as the rhabdomere. The stalk membrane connects the rhabdomere to the cell body of the photoreceptor cell. Crb is required for rhabdomere length, zonula adherens integrity, and formation of the stalk membrane. In addition Crb is required for the maintenance of photoreceptor cells and loss of Crb causes the photoreceptors to degenerate upon exposure to light (Johnson et al., 2002). In mammals an analogous function is fulfilled by CRB 1, which is expressed in the brain and retina. Mutations in CRB 1 in humans are associated with degenerative retinal diseases including retinitis pigmentosa 12 and Leber congenital amaurosis (den Hollander et al., 1999). While *Drosophila* Crb plays an important role in the morphogenesis of photoreceptor cells during pupal development, its role in the eye imaginal disc is unclear. Similar to the malphigian tubules, where Crb is mainly necessary to maintain polarity during morphogenesis and tissue remodeling (Campbell et al., 2009), Crb may only be necessary for regulating polarity during specific periods in the development of these tissues.

The Role of Crb in the Imaginal Discs

We obtained four alleles of *crumbs* (*crb*) in the screen for mutations that cause cells to become supercompetitors, described in Chapter 2. At the time, no role had been reported for *crb* in regulating either growth or cell competition. While *scrib*, *dlg*, and *lgl* mutant clones had been known to act as neoplastic tumor suppressors and to be eliminated by cell competition from wild-type imaginal discs, it was generally assumed that this was a result of abnormal polarity or growth (Brumby and Richardson, 2003; Igaki et al., 2009). That a perturbation of polarity might improve the growth or competitiveness of cells was therefore initially very surprising.

In the past year, a series of papers have emerged that link Crb to the Hippo pathway and growth regulation. The exact nature of the relationship between Crb and the Hippo pathway or growth control is not yet clear as both overexpression and loss of Crb appear to activate

the Hippo pathway[1] and cause overgrowth (Chen et al., 2010; Grzeschik et al., 2010; Ling et al., 2010; Richardson and Pichaud, 2010; Robinson et al., 2010). Robinson and colleagues suggest a model in which Crb regulates both the degradation and activity of Expanded (Ex), a negative upstream regulator of the Hippo pathway. According to this model Ex function is lost in Crb-overexpressing cells because the protein is degraded whereas Ex function is lost in *crb* mutant cells because the protein is mislocalized and therefore incapacitated. A close link between activity and degradation has been previously observed for other proteins, including Myc (Salghetti et al., 2000), and is thought to be a sign of stringent regulation of that protein. Recent work has generally focused on the role of the intracellular domain, which binds to Ex, in growth regulation. In contrast, little attention has been given to the extracellular domain in the context of growth regulation, though a role has been suggested for this domain (Richardson and Pichaud, 2010).

In addition to Crb acting upstream of the Hippo pathway, Hippo pathway mutant clones upregulate Crb and become apicalized (Genevet et al., 2009; Hamaratoglu et al., 2009). This is most likely to be a negative feedback mechanism, which is a commonly observed feature of the Hippo pathway. Nevertheless, it emphasizes the degree of interconnection between Crb, the Hippo pathway, polarity, and growth.

An intimate relationship between the regulation of growth and polarity is becoming increasingly clear as more and more perturbations are found to affect both processes. A basis for this may be that cells regulate growth control genes indirectly by modulating their polarity. Indeed many regulators of growth, including the EGFR, Notch and components of the JAK/Stat and Hippo pathways, are detected exclusively at the apical domain of epithelial cells. Furthermore, the activity of these genes can depend on their localization (Badouel and McNeill, 2009; Sotillos et al., 2008). Alternatively regulators that affect both processes may be simply playing two independent roles. A better mechanistic understanding of these dual regulators of growth and polarity, including Crb, may help distinguish between these possibilities.

[1] In previous reports, "activation" of the Hippo pathway has had contradictory meanings in different contexts. Here we use "activation" of the Hippo pathway to describe a decrease in the function of the core components, Hpo, Sav, and Wts, leading to increased Yki activity.

RESULTS

Loss of Crb Results in Cells that are Supercompetitors

We recovered a four-member lethal complementation group, that we initially called *phelps*, in a genetic screen based on the supercompetitor assay (see Chapter 2). The mosaic eye phenotype is similar in all four alleles – the whole eye is slightly larger and rougher and the non-mutant red patches appear smaller and more punctated (Figure 3.2B,C). Quantification of the size of these red patches confirmed that they were significantly smaller (Figure 3.2D).

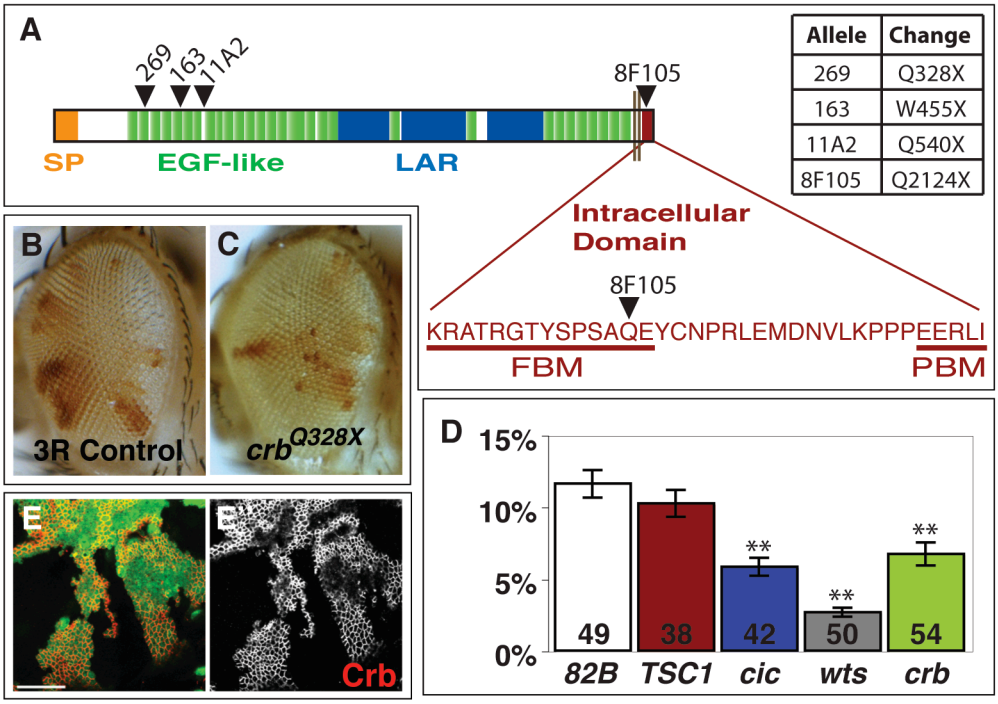


Figure 3.2 - Mutations in *crb* were found in a screen for supercompetitors
(A) Cartoon showing the domains of Crb and the location of point mutations responsible for the previously published alleles, *11A22* and *8F105*, and the alleles recovered in the supercompetitor screen, *269* and *163*. Signal peptide (SP), EGF-like Repeat (ELR), Transmembrane Domain (TM), FERM Domain-binding Motif (FDB), and PDZ-binding Motif (PBM) are shown. Adult eye phenotype of control (B) and crb^{269} (C) in the supercompetitor assay.
(D) Quantification of the area of red tissue in eyes mosaic for mutations on 3R, including *crb*. The number of eyes used to quantify each genotype is indicated. Error bars show SE. ** indicates < 0.001 significance in the Mann-Whitney Z test.
(E, E') Anti-Crumbs antibody stains wild-type (GFP-positive) but not *crb* mutant (GFP- negative) clones in wing imaginal discs.
Scale bar: 20μm

We took two approaches to identify the mutation responsible for the *phelps* phenotype. We mapped the lethality of *phelps* by crossing one of the alleles to a set of deficiency stocks that covered the majority of 3R. The allele failed to complement four non-overlapping deficiencies at the following cytological locations: 81F6-82D7, 87D1-88D6, 95D7-95F15, 98E1-98F5. This indicates that the chromosome contained a number of mutations in addition to the one responsible for the supercompetitor phenotype. These might include hypomorphic mutations from the parental stock that are homozygous viable, permitting the viability of the parental stock, but become lethal in trans to a deficiency. They may also represent additional mutations induced by the EMS. Although the EMS dose was calibrated to induce on average one to two recessive lethal mutations in each fly, there can be significant variability in the actual number of mutations induced in each fly within the same round of EMS mutagenesis (Abby Gerhold, unpublished). Our second approach was to calculate recombination distances between *phelps* and other mutations on 3R that cause various growth phenotypes. We found that *phelps* was closely linked to the *TSC1* locus, which is at 95E1. Therefore, we focused on the 95D7-95F15 region. Using smaller deficiencies we narrowed the search to a region containing seven large genes, including *crb*.

Several lines of evidence suggest that *crb* is the gene responsible for the supercompetitor phenotype of these mutants. First, the independently derived crb^{11A22} allele, which is reported to be null, produced a similar phenotype (Figure 3.3C) and failed to complement the lethality of our four alleles (Tepass et al., 1990). Second, sequencing the coding region of the *crb* gene in two of the four alleles revealed nonsense mutations that would result in highly truncated proteins that would most likely be nonfunctional (Figure 3.2A). Finally, an anti-Crb antibody raised against the ECD (Pellikka et al., 2002) fails to detect any protein in mutant clones in the imaginal discs (Figure 3.2E). Taken together, these findings indicate that *crb* clones reduce the amount of heterozygous tissue in the adult eye.

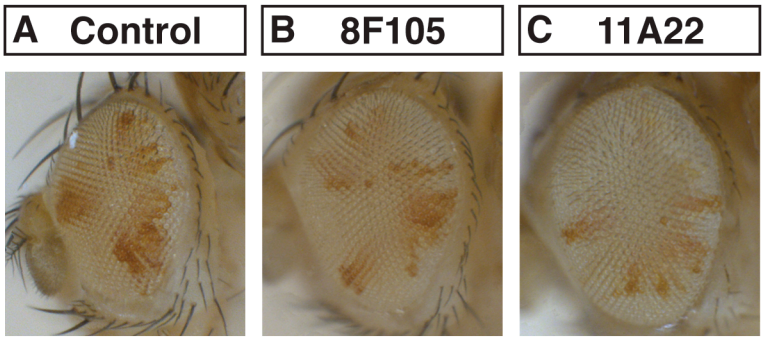
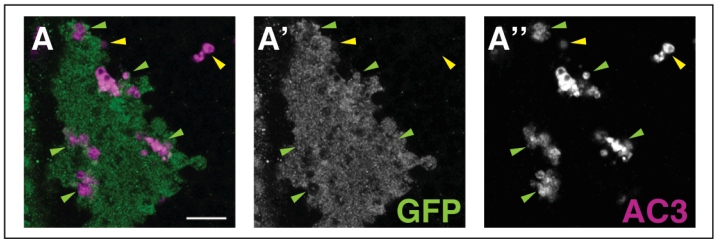


Figure 3.3 - Supercompetitor phenotype of published *crb* alleles
(A) Adult eye phenotype of 3R parental control in the supercompetitor assay. (B) Adult eye phenotype of *8F105*, a hypomorphic allele of *crb*, in the supercompetitor assay. (C) Adult eye phenotype of *11A22*, a null allele of *crb*, in the supercompetitor assay.

We also sought to determine whether loss of the ICD of Crb was sufficient to induce the supercompetition phenotype. We tested an independently derived allele, *8F105*, in the supercompetitor assay. In this allele the ECD of Crb is intact, but 21 out of 37 amino acids of the ICD are omitted (Tepass and Knust, 1990; Tepass et al., 1990). Unlike crb^{11A22} and the other alleles that came out of our screen, the crb^{8F105} mutant cells did not clearly score as supercompetitors in our assay (Figure 3.3B). There did appear to be some subtle differences in the ratio of mutant and non-mutant tissues, however, it is difficult to interpret these subtle differences in the absence of a true isogenic control for the line.

The reduction in the size of the patches of heterozygous tissue adjacent to *crb* mutant clones might be caused by a mechanism other than cell competition. For example, it may be caused by an increased rate of growth of *crb* cells in conjunction with the maintenance of overall tissue size, which would result in a premature termination of the growth of the heterozygous tissue. However, since the overall size of the eye is slightly increased this possibility seems unlikely. Moreover, while *pten* cells cause a greater increase in the overall size of the eye than *crb* cells (compare Figure 2.6B to 3.2C), indicating a stronger overgrowth phenotype, they do not cause a comparable reduction in size of the patches of heterozygous tissue (compare Figure 2.6I to 3.2D).

Alternatively, *crb* cells may eliminate their wild-type neighbors through apoptosis, as occurs during cell competition. To test this hypothesis, we examined eye-imaginal discs from third instar larvae using an antibody to activated caspase 3 (AC3) (Figure 3.4). Twelve discs of each genotype were examined and the discs were scored without the experimenter being aware of the genotype, so as to prevent any scoring bias. The total number of AC3-positive cells in 12 discs containing *crb* clones (total = 273 AC3-positive cells) was much higher than in 12 discs containing clones of a FRT82B chromosome (total = 70 AC3-positive cells). Of the 273 AC3-positive cells in discs containing *crb* clones, 188 (69%) were also GFP-positive indicating that they were either wild-type or heterozygous cells. Of these 188 AC3/GFP-positive wild-type cells, 150 (80%) were immediately adjacent to *crb* mutant cells (Figure 3.4B). By comparison, Li and Baker found that in discs containing *Minute* Rps18/+ clones, 94% of AC3-positive cells were *M*/+ cells and of those, 71% were adjacent to wild-type cells (Li and Baker, 2007). Thus, the pattern of cell death in mosaic discs containing *crb* clones indicates that *crb* cells can function in a non-autonomous manner to promote the death of wild-type cells at the clonal boundaries and suggests that cell competition is occurring at those boundaries.



B	FRT 82B	FRT82B crb
Cells that are…	(n=12 discs)	(n=12 discs)
AC3 +	70	273
AC3 +, GFP +	23	188
AC3 +, GFP +, next to GFP – cells	20	150
AC3 +, GFP –	47	85
AC3 +, GFP –, next to GFP + cells	22	19

3.4 - Increased apoptosis of wild-type cells adjacent to *crb* mutant cells
(A) Clusters of AC3-positive wild-type cells (green arrowheads) are found near the border of *crb* clones in mosaic eye discs. Occasional AC3-positive mutant cells are also seen (yellow arrowheads). Scale bar: 10μm.
(B) Total numbers of AC3-positive (AC3 +) cells from 12 discs containing clones homozygous for the FRT 82B chromosome and 12 discs containing clones homozygous for the FRT82B *crb* chromosome. Mutant cells are GFP-negative (GFP –) and wild-type cells are GFP-positive (GFP +).

Does Crb Regulate Polarity in the Imaginal Discs?

Crb was previously characterized as part of a complex that regulates apicobasal polarity in the embryonic epithelium. In that context, loss of Crb diminishes the apical domain of cells, whereas loss of basal polarity determinants, such as Scrib, diminishes the basolateral domain(Bilder and Perrimon, 2000; Tanentzapf and Tepass, 2003; Tepass and Knust, 1990; Tepass et al., 1990). Whether Crb also regulates polarity in the larval imaginal discs is currently under dispute (Genevet et al., 2009; Hamaratoglu et al., 2009; Pellikka et al., 2002). We wanted to resolve this issue because if Crb regulates both polarity and competition in the same tissue, then the two processes may be functionally related. For example, the ratio of apical-to-basolateral domain of cells might determine their competitiveness. Alternatively, the regulation of cell competition might be independent of the regulation of polarity, and Crb and Scrib might instead act as part of a signaling module that regulates many different processes. Indeed, findings in the malphigian tubules suggest

that Crb is only required for polarity during tissue morphogenesis (Campbell et al., 2009), thus it may not be required in the imaginal tissues until the pupal stages.

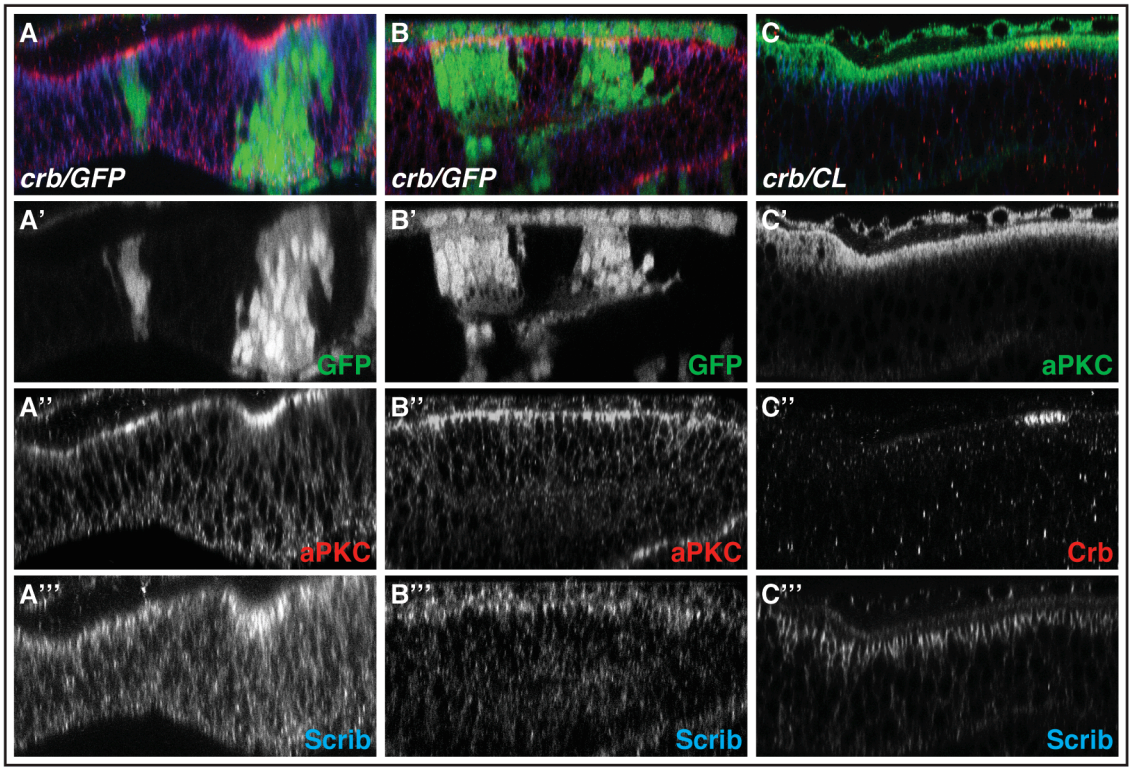


Figure 3.5 - Polarity of *crb* mutant cells
XZ images of mosaic eye imaginal discs. (A-B) Mosaic discs with control (GFP-positive) and *crb* mutant (GFP-negative) clones stained for aPKC (red) and Scrib (blue). (C) Mosaic eye imaginal disc stained for aPKC (green) and Scrib (blue) containing a control clone, indicated by Crb (red) staining, in a *crb* mutant background.
Apical is up, and basal is down. Anterior is to the left, and posterior is to the right.

In order to determine whether Crb regulates polarity in the imaginal disc, we examined *crb* mutant clones for various markers of apicobasal polarity. *crb* mutant clones appeared to express slightly less of the apical marker, aPKC, than wild-type clones (Figure 3.5A,B) (Genevet et al., 2009; Hamaratoglu et al., 2009). This phenotype had low penetrance, so that in many discs aPKC levels did not appear to differ between *crb* mutant and wild-type clones (Figure3.5C). The levels of Scrib, which is a marker for the basolateral domain, were also unaffected in *crb* mutant clones. Moreover, cell shape and the structure of the epithelial sheet were normal, the zonula adherens was intact, and apical and basal markers indicated that the domains remain separated (Figure 3.5). Therefore, Crb may have, at most, a very

subtle role in regulating polarity in the larval imaginal discs under normal circumstances. Subtle differences may nevertheless be sufficient to mediate cell competition. Examination of the effects on cell competition of other perturbations of apicobasal polarity may help answer this question.

Crb-Overexpressing Cells Are Eliminated from Wild-type Imaginal Discs

If *crb* clones can influence the survival of adjacent wild-type cells, then similar interactions might occur between wild-type cells and cells that overexpress Crb. Using the FLP-out method (Struhl and Basler, 1993), we generated clones of cells overexpressing the full-length Crb protein in wild-type wing imaginal discs at different times of development and compared them at 114 hours after egg deposition (hr AED) to control clones that

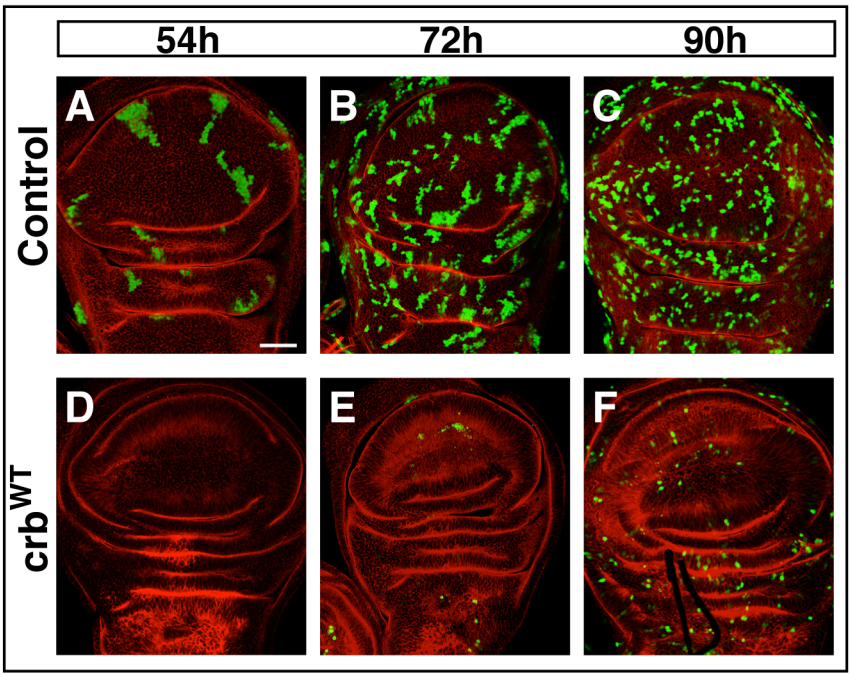


Figure 3.6 - Cells overexpressing Crb are eliminated from wild-type imaginal discs
Wing imaginal discs with GFP-positive flip-out clones (green) stained with phalloidin (red). Clones were induced at one of three times during larval development and dissected at 114hAED. (A, D) 54hr AED induction of control clones (A) and Crb-overexpressing clones (D). (B, E) 72hr AED induction of control clones (B) and Crb-overexpressing clones (E). (C, F) 90hr AED induction of control clones (C) and Crb-overexpressing clones (F). Anterior is left, and posterior is right. Scale bar: 50μm

overexpressed GFP alone (Figure 3.6). As expected, control clones induced at earlier times (54 hr AED) were larger than those induced at later times (72 hr or 90 hr AED) (Figure 3.6A-C). In marked contrast, no Crb-overexpressing clones were observed at 114 hr AED when they were induced at 54 hr AED, and only a few clones were observed when induced at 72 hr AED. Crb-overexpressing clones were most consistently observed when they were induced at 90 hr AED, however, they were significantly smaller than control clones induced at the same time (Figure 3.6D-F). Thus, Crb-overexpressing clones are eliminated from wild-type imaginal discs over time.

Importantly, while Crb-overexpressing cells are rapidly eliminated from wild-type imaginal discs, the overexpression of Crb alone is not sufficient to cause cells to die. When Crb was previously overexpressed in the entire posterior compartment of the wing disc, the cells were viable and neoplastic overgrowth of the tissue was observed (Lu and Bilder, 2005). Therefore, a heterotypic interaction with the wild-type cells appears to be required for the elimination of Crb-overexpressing cells.

To better understand how Crb-overexpressing cells are eliminated from wild-type imaginal discs and whether cell competition is involved, we stained discs containing Crb-overexpressing clones with the AC3 antibody. Only small clones could be recovered, and most of these few surviving Crb-overexpressing cells were positive for AC3, indicating that they were undergoing apoptosis (Figure 3.7A-C).

If Crb-overexpressing cells undergo apoptosis in response to heterotypic interactions with adjacent wild-type cells, as occurs in cell competition, then Crb-overexpressing cells in contact with wild-type cells should be more susceptible to apoptosis, while Crb-overexpressing cells surrounded by other Crb-overexpressing cells should not. We created large Crb-overexpressing clones by using with a temperature-sensitive form of Gal80 (Gal80-TS) in conjunction with the flip-out method of clone induction. The Gal80-TS allowed us to inhibit Crb expression until the clones had grown to a large size (Figure 3.7D,E). Clones were induced at 48 hr AED, but Crb expression was kept off (through incubation at 18˚C) until 24hr before dissection when the culture was shifted to 30˚C. Upon staining these discs for AC3, we observed higher levels of AC3 at the borders of these large clones, while AC3 was rarely observed in the cells in the center of the clones. This supports the idea that a heterotypic interaction with wild-type cells causes Crb-overexpressing cells to undergo apoptosis.

The elimination of Crb-overexpressing cells resembles cell competition in another way as well. In *Minute*- and Myc-induced cell competition, losers are eliminated first from the pouch region of the wing imaginal disc where the rate of proliferation is the highest (Moreno and Basler, 2004; Moreno et al., 2002). Similarly, Crb-overexpressing cells in the wing imaginal disc were eliminated first from the pouch (Figure 3.6D-F). An analogous pattern was observed in the eye-imaginal disc (Figure 3.7B). Anterior to the morphogenetic furrow, where cells are mitotically active, there were fewer Crb-overexpressing cells, and these cells

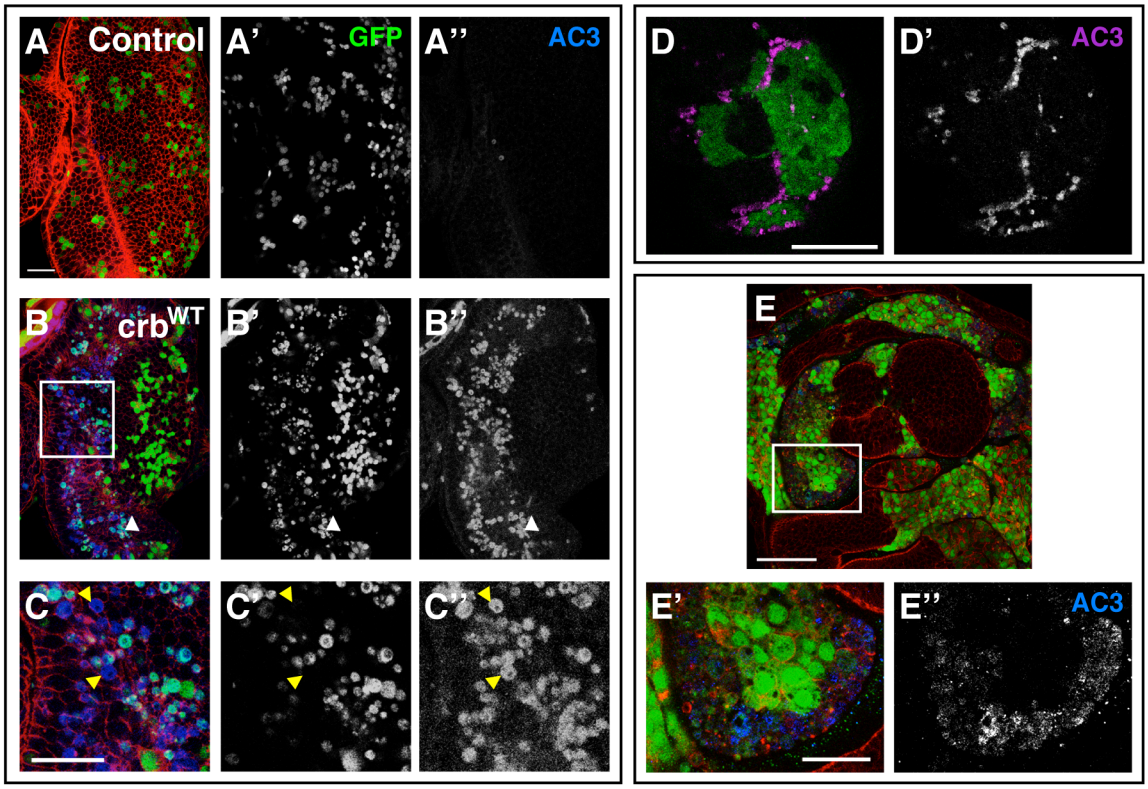


Figure 3.7 - Apoptosis is observed at the borders of Crb-overexpressing clones

Imaginal discs with GFP-positive flip-out clones (green) (A-C) Phalloidin (red) and AC3 staining (blue) of eye imaginal discs with control (A) or Crb-overexpressing clones (B). White arrowhead in (B) indicates position of morphogenetic furrow. (C) High-magnification image of boxed region in (B). Yellow arrowheads point to AC3-positive, GFP-negative cells. (D-E) Large flip-out clones were made using a temperature-sensitive Gal80 (see Methods). (D) Eye imaginal discs stained for AC3 (magenta). (E) Wing imaginal discs stained for phalloidin (red) and AC3 (blue). (E', E'') High-magnification images of boxed region in (E) showing an extruded clone with intact cells in the center that do not stain for AC3 surrounded by AC3 positive debris. Anterior is left, and posterior is right.
Scale bars: 20μm in A-C, E', E'' and 50μm D, E

were more frequently AC3-positive. In contrast, Crb-overexpressing cells were more likely to survive and less likely to be AC3-positive posterior to the morphogenetic furrow, where cells are mostly post-mitotic. This suggests that the mechanisms that induce the apoptosis of Crb-overexpressing clones operate mainly during the proliferative phase of disc development, as has been observed in other instances of cell competition.

We also discovered a number of features in the competition between Crb-overexpressing cells and wild-type cells that have not been previously reported in other instances of cell competition. Crb-overexpressing cells have an irregular shape and polarity (Figure 3.8). Whereas normally cells in the wing imaginal disc have an elongated columnar shape and have an accumulation of Actin near their apical membrane, Crb-overexpressing cells are more rounded and have high levels of Actin near the outline of the entire cell. Perhaps as a result of their irregular structure, Crb-overexpressing cells do not form normal epithelial connections with neighboring wild-type cells. Upon examining imaginal discs in which we had created large Crb-overexpressing clones, we found that several of the clones were no longer a part of the disc proper but had been extruded apically (Figure 3.8B,C). Cells in these extruded clones continued to express GFP that localized to seemingly-intact nuclei, but large numbers of AC3 positive cells and cell debris surrounded the intact cells (Figure 3.7E, 3.8B). This extrusion may contribute to the elimination of Crb-overexpressing cells. Indeed, expression of the anti-apoptotic protein p35 (Hay et al., 1994; Moreno and Basler, 2004; Moreno et al., 2002), did not significantly increase the size of Crb-overexpressing clones, implicating caspase-independent mechanisms in the elimination of these cells.

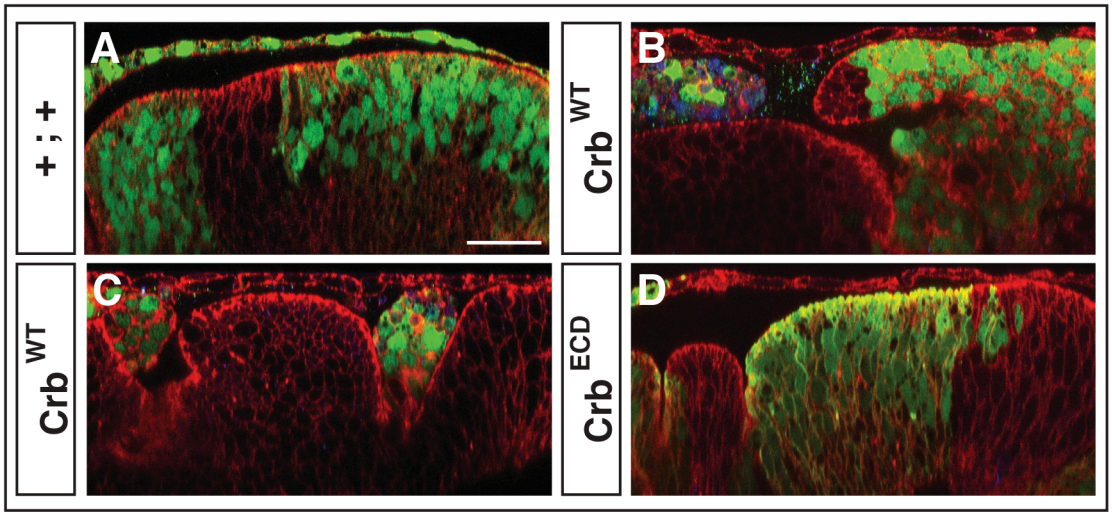


Figure 3.8 - Extrusion of Crb-overexpressing cells
(A-D) XZ images of wing imaginal discs with GFP-positive flip-out clones (green) stained with AC3 (blue) and phalloidin (red). In (B) an entirely extruded clone can be seen on the left and on the right the wild-type epithelium can be seen folding basally as another clone is apparently being extruded.

Interestingly, the Crb-overexpressing cells also appeared to have some nonautonomous effects on the surrounding wild-type cells. Wild-type epithelium surrounding intact Crb-overexpressing clones was often folded basally in what appeared to be a precursory step to the extrusion of the Crb-overexpressing cells. Furthermore, in addition to the GFP-positive Crb-overexpressing cells, we clearly observed some GFP-negative (wild-type) cells that stained with the anti-AC3 antibody (arrow in Figure 3.7C). While we cannot exclude the possibility that some dying cells cease to express GFP, a more likely explanation is that some of the wild-type cells adjacent to Crb-overexpressing cells also undergo apoptosis. Currently cell competition is thought to involve the elimination of one cell-type by another. However, our findings may indicate that both cell-types experience damage as a result of cell competition. This may have important implications for the mechanism of cell competition.

In conclusion, the levels of Crb appear to correlate with the competitive ability of cells – the more Crb a cell has the less competitive it is. Therefore, cells may compare their levels of Crb with that of their neighbors and if there are differences in these levels cell competition is triggered. We decided to further characterize the competition mediated by Crb by trying to determine the basis of the cell-cell comparison and how the death of the loser is signaled in this case of cell competition. We first asked whether Crb might signal the death of the loser through a known supercompetitor pathway. We then investigated whether, as a transmembrane protein, Crb itself might be the basis of the cell-cell comparison that triggers competition.

Interactions Between Crb and Other Supercompetitor Pathways

The ability of cells with increased growth rate to induce the death of adjacent wild-type cells was first described for cells overexpressing Myc (de la Cova et al., 2004; Moreno and Basler, 2004), and later for cells mutant for components of the Hippo pathway (Tyler et al., 2007). Therefore we examined mosaic imaginal discs containing *crb* clones for reporters of Myc and Hippo pathway activity. Myc activity can be inferred from (1) its protein levels, as it is regulated by transcription and degradation, and (2) by the size of the nucleolus, as Myc activation upregulates ribosomal biogenesis (Grewal et al., 2005). There was no change in levels of Myc protein or in the Myc reporter, Fibrillarin, between *crb* and wild-type clones (Figure 3.9A-D) suggesting that the apoptosis that is observed in mosaic discs does not arise from non-uniform Myc activity.

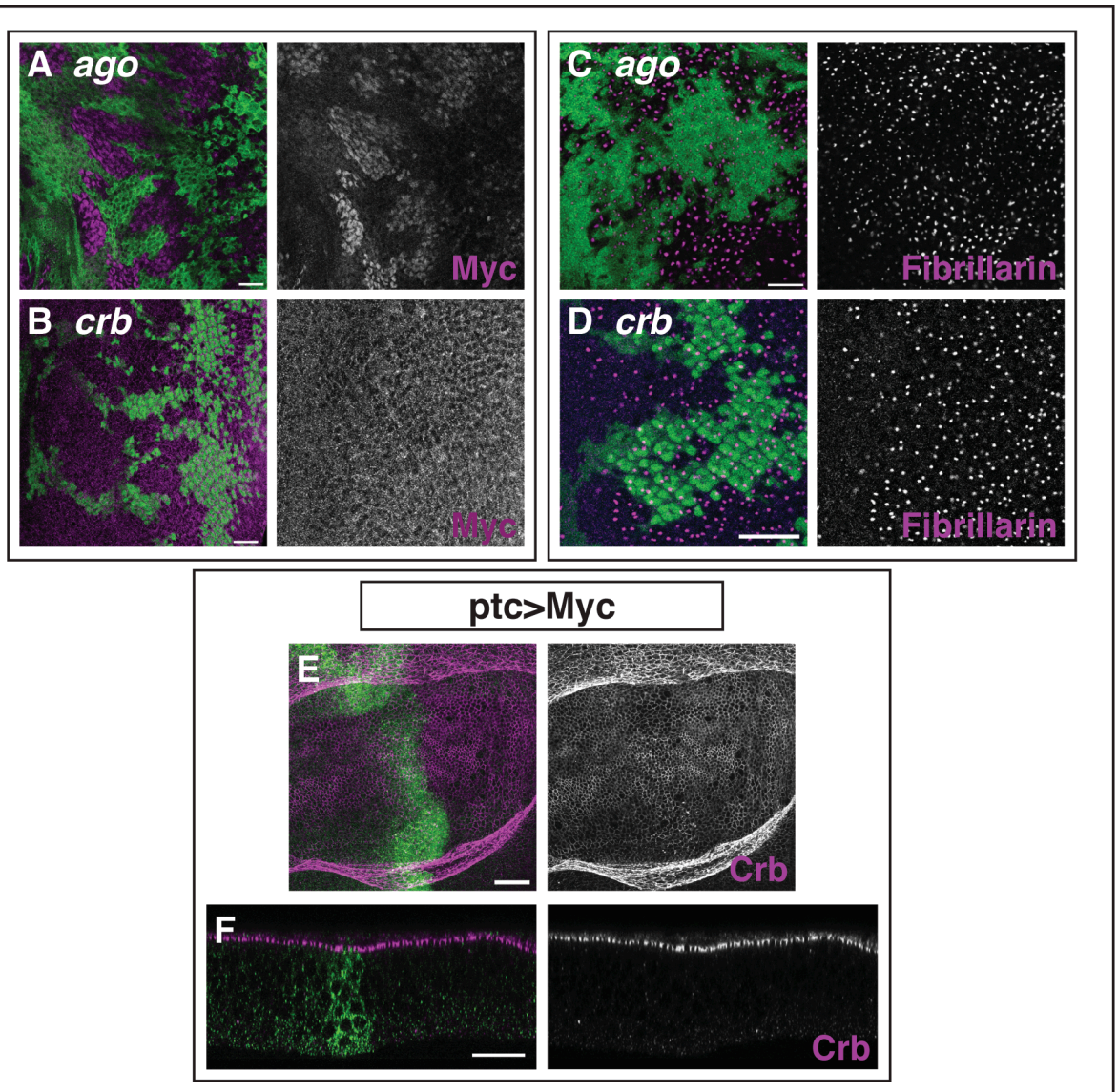


Figure 3.9 - Investigating interactions between Myc and Crb
(A-D) Mosaic eye imaginal discs with control (GFP-positive) and mutant (GFP-negative) clones. (A) *ago* mutant clones stained for anti-Myc (magenta). (B) *crb* mutant clones stained for anti-Myc antibody (magenta). (C) *ago* mutant clones stained for anti-Fibrillarin (magenta). (D) *crb* mutant clones stained for anti-Fibrillarin (magenta). (E,F) Wing imaginal disc in which the *patched* promoter is driving expression of Myc stained with anti-Crumbs. (E) Merged z-stack of 30 2μm XYsections. (F) XZ section through the disc in E to show apical localization of Crb. Anterior is left, and posterior is right. In (F) also apical is up and basal is down. Scale bars: 20μm

The Hippo pathway was originally characterized through mutants identified in mosaic screens in *Drosophila* for overgrowth (Harvey et al., 2003; Tapon et al., 2002; Udan et al., 2003; Wu et al., 2003; Xu et al., 1995). The core pathway components include three tumor suppressor genes: Hippo, an MST kinase, Salvador, a scaffold, and Warts, an NDR kinase. These components negatively regulate the transcription factor Yorkie (Yki) which activates a number of genes including, *Diap 1*, *cyclin E*, *bantam*, and *expanded*, and thus promotes growth and cell survival. The function of the Hippo pathway in regulating growth is highly conserved in vertebrates. Furthermore, mutations in the Hippo pathway have been identified in a number of mammalian tumor lines, implicating the pathway in the development of certain cancers (Pan, 2010). Mutations that activate the Hippo pathway (i.e. resulting in increased Yki-mediated transcription) were recently found to cause cells to become potent supercompetitors (Tyler et al., 2007; Ziosi et al., 2010).

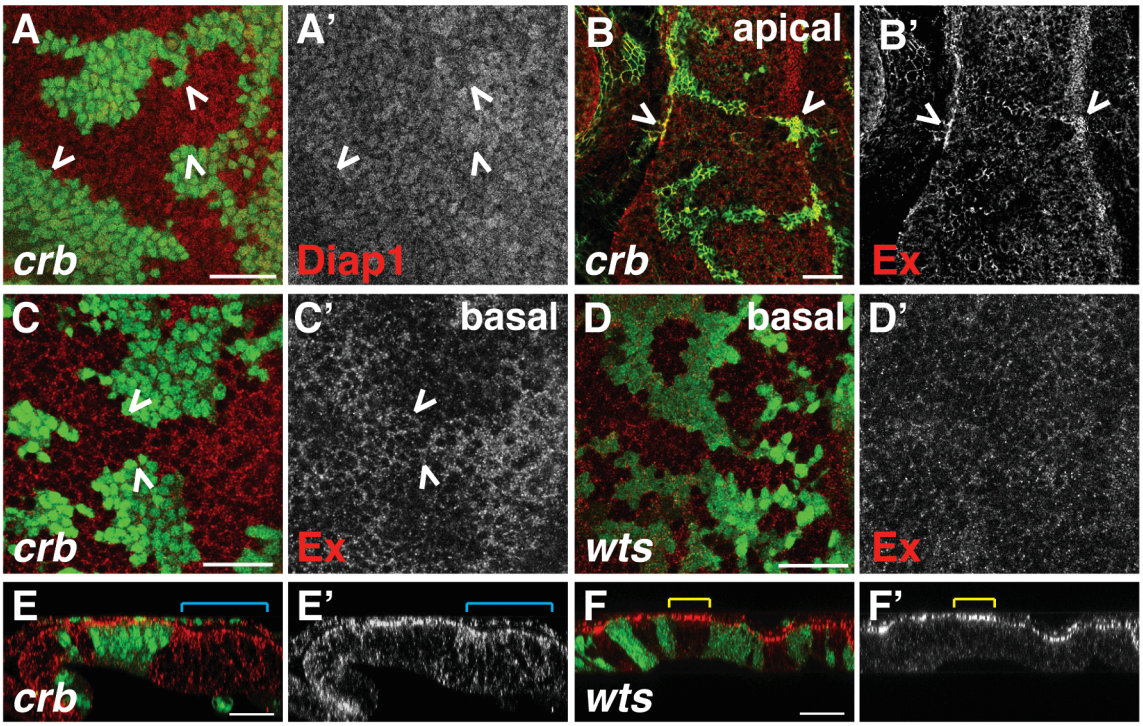


Figure 3.10 Cell-autonomous activation of the Hippo pathway in *crb* cells
Third instar mosaic imaginal discs stained with reporters of the Hippo pathway. Mutant clones are GFP-negative. (A) Anti-DIAP1 (red) staining of *crb* mosaic disc. (B) Apical section of *crb* mosaic disc stained with Crb (green) and Ex (red) antibodies. (C,D) Basal section of *crb* mosaic disc (C) or *wts* mosaic disc (D) stained with Ex antibody (red). (E,F) XZ section of *crb* mosaic disc (E) or *wts* mosaic disc (F) stained with Ex antibody (red). Brackets denote *crb* (blue) or *wts* (yellow) mutant clones. Scale bars: 20μm

To look for changes in the activity of the Hippo pathway, we stained *crb* mosaic discs with antibodies to DIAP1, since DIAP1 levels are increased when signaling via this pathway is reduced (Tapon et al., 2002). We observed a slight increase in the levels of the DIAP1 protein (Figure 3.10A) as has also been reported recently by others (Grzeschik et al., 2010; Ling et al., 2010; Richardson and Pichaud, 2010). However, the levels of two other Hippo pathway reporters, Merlin and Cyclin E were unchanged (data not shown). This might mean that DIAP1 is a more sensitive reporter of Hippo pathway activity or that Crb only regulates a subset of Hippo target genes.

We observed more complex changes in Ex, which is also negatively regulated by Hippo signaling. In wild-type cells Ex is localized apically, while in *crb* mutant cells we found Ex in puncta that were not restricted to the apical domain (Figure 3.10B-E). This mislocalization of Ex in *crb* clones has also been described recently by several other groups (Grzeschik et al., 2010; Ling et al., 2010; Robinson et al., 2010). This, together with the observation by one group that Ex binds directly to the ICD of Crumbs (Ling et al., 2010) suggests that the localization of Ex to the apical membrane is dependent upon Crb and that mislocalization of Ex results in increased expression of genes that are normally repressed by Hippo signaling. In contrast, even though *wts* clones express elevated levels of Ex, the apical localization of Ex is maintained (Figure 3.10F).

The Hippo pathway regulates both growth and cell survival, and loss of Hippo pathway activity is associated with reduced growth as well as death. Lower levels of Hippo signaling in wild-type cells in comparison to *crb* mutant cells is consistent with their death. However, the reduction of Hippo pathway levels appears to be uniform outside of the mutant clones. There was no specific decrease in wild-type cells immediately adjacent to the clone boundary (Figure 3.10F). In contrast, a signal responsible for the death of losers would be expected to occur exclusively in loser cells that are in contact with winner cells as these are the cells in which apoptosis is observed. Thus, the Hippo pathway may not be the signal that leads to the death of the wild-type cells adjacent to *crb* mutant cells.

Increased activity of the Hippo pathway by loss of negative regulators of the pathway was able to rescue *Minute* cells from being eliminated by wild-type cells (Tyler et al., 2007). To see whether the Hippo pathway is similarly involved in the elimination of Crb-overexpressing cells, we tested whether they could be rescued by activation of Hippo signaling. We used the Mosaic analysis with a repressible cell marker (MARCM) technique (Lee and Luo, 1999) to overexpress Crb in cells that were homozygous for a null allele of *wts, wts*^{X1}. The *wts*^{X1} mutant, Crb-overexpressing clones were significantly larger, but they still had disrupted polarity and showed signs of extrusion and apoptosis at the clone borders (Figure 3.11). Thus, being mutant for *wts* did not completely rescue Crb-overexpressing cells from loss of polarity, extrusion, or increased apoptosis. This may imply that these effects are induced independently of the Hippo pathway or through a non-canonical Hippo pathway that does not include *wts*.

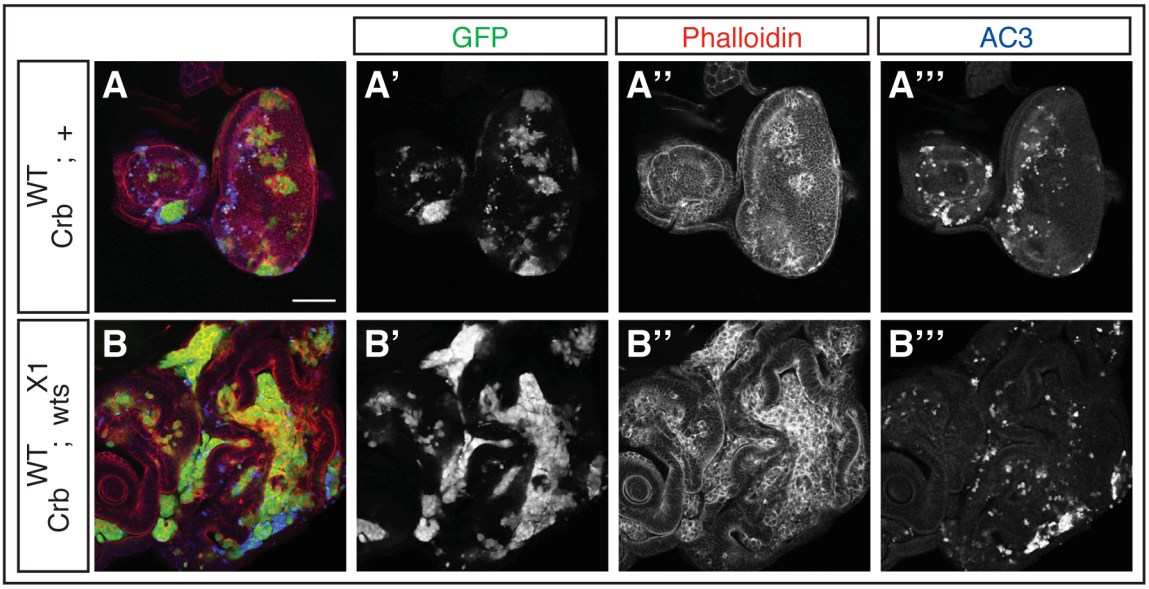
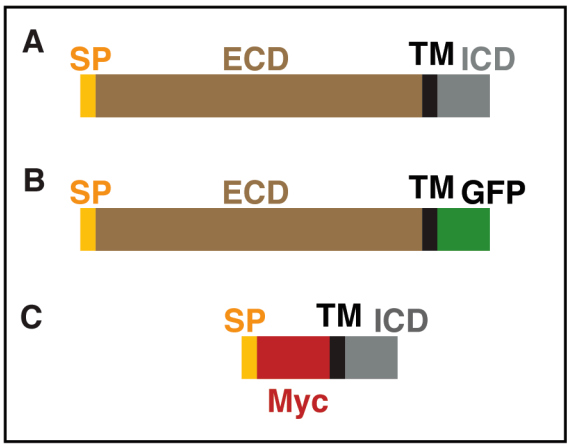


Figure 3.11 - Wts is not required for apoptosis or extrusion of Crb-overexpressing cells
Imaginal discs with MARCM clones (GFP-positive) stained with AC3 (blue) and phalloidin (red). (A) GFP-positive clones express full-length Crb. (B) GFP-positive clones express full-length Crb and are homozygous mutant for *wts*^{X1}.

Crb ECD is Required for the Elimination of Crb-Overexpressing Cells

Crb has a very large ECD with several EGF repeats that can mediate protein-protein interactions, however, no binding partners have been conclusively shown to interact with the ECD. Moreover, little or no role has been identified for the ECD in many contexts, including polarity and growth regulation.

In order to determine the region of the Crb protein that was required for the elimination of Crb-overexpressing cells, we made clones that overexpressed either a membrane-bound, GFP-tagged ECD or a membrane-bound, Myc-tagged ICD (Figure 3.12A-C). The size and frequency of clones expressing the ECD were indistinguishable from GFP-expressing control clones (Figure 3.12F). Similarly clones overexpressing the ICD were not reduced in size or eliminated (Figure 3.12G). Instead the ICD-expressing clones were larger and had smoother outlines than control clones, and this effect was exaggerated in the notum. This is consistent with the activation of the Hippo pathway and growth that has been ascribed to this domain (Chen et al., 2010; Grzeschik et al., 2010; Ling et al., 2010; Robinson et al., 2010). In conclusion, both the ECD and ICD appear to be required for the elimination of cells that overexpress Crb. Our results suggest that there is a functional distinction between the ICD and the full-length protein, in contrast to previous studies in embryos suggesting that the two are functionally similar (Wodarz et al., 1995).



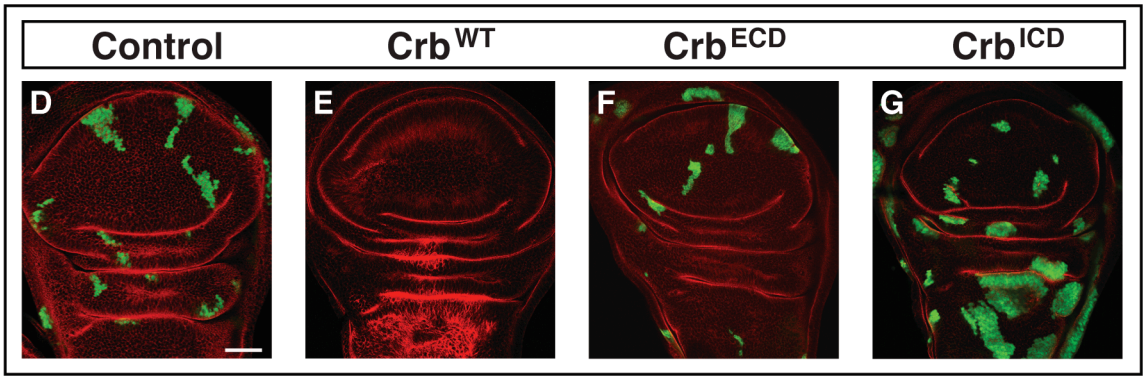


Figure 3.12 - ICD and ECD are both required for the elimination of Crb-overexpressing cells
Schematic diagram showing the components of the full-length Crb (A) Crb-ECD (B) and Crb-ICD (C) constructs. Includes signal peptide (SP), extracellular domain (ECD), transmembrane domain (TM), intracellular domain (ICD), GFP-, and Myc- tags. (D-G) Wing imaginal discs with GFP-positive flip-out clones (green) that were induced at 54 hr AED. Discs were dissected and stained with phalloidin (red) at 114 hr AED. (D) Control GFP-expressing clones (E) full-length Crb–overexpressing clones (F) Crb-ECD-overexpressing (G) Crb-ICD-overexpressing clones. Scale bar: 20μm

We also looked at the morphology of these discs to see if any extrusion was occurring. While we did not observe any overt extrusion, subtle morphological differences were observed upon overexpression of the ECD or ICD (Figure 3.13). In many of the samples there appears to be a "bend" in the disc in the region of highest Ptc expression. Overexpression of Crb-ECD caused an upward bend while Crb-ICD caused a downward bend. This may indicate subtle polarity changes or be related to the extrusion phenotype of cells overexpressing full length Crb. Thus, the ICD and ECD produce opposite phenotypes with respect to the curvature of the epithelium, indicating that one of them may have a dominant-negative effect.

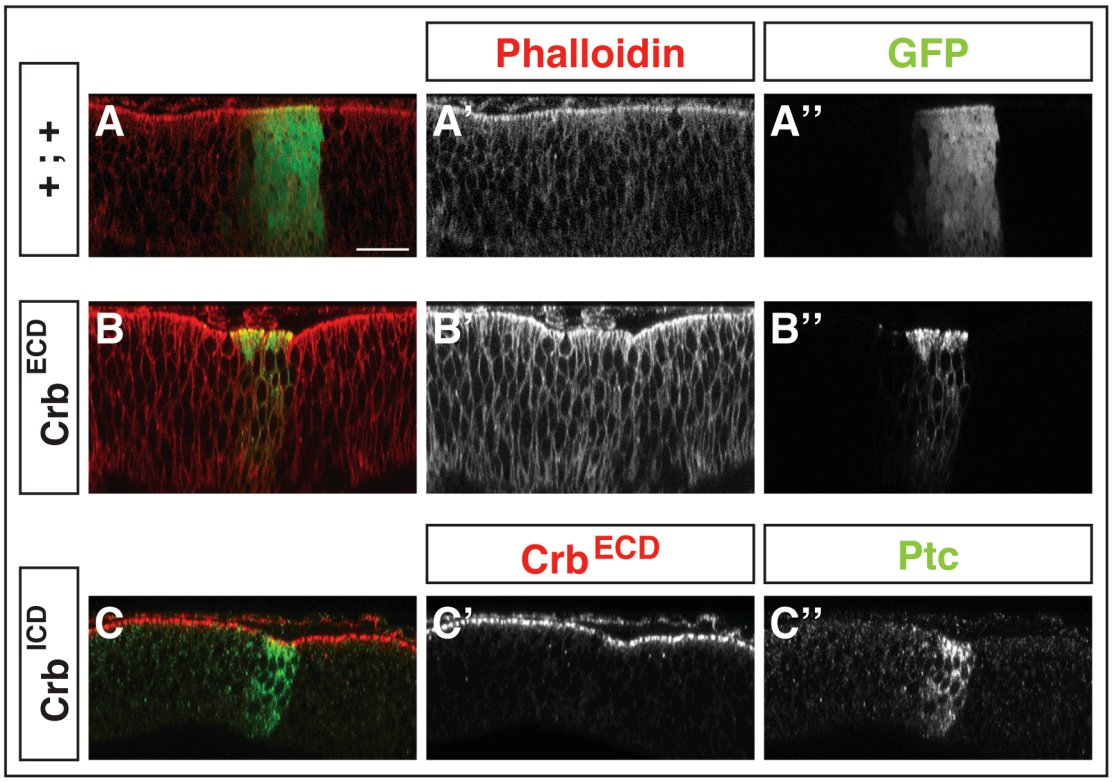


Figure 3.13 - Morphological changes induced by expression of Crb constructs
XZ images of wing imaginal discs expressing (A) GFP, (B) Crb ECD, or (C) Crb ICD construct driven by *ptc-Gal4*. Expression domain is delineated by GFP (A'', B'') or *ptc* (C'') expression. Morphology visualized through Phalloidin (A', B') or anti-Crb ECD antibody staining. Scale bar: 20μm.

Non-autonomous Effects of Crb on Protein Localization in Adjacent Cells

We next investigated whether the ECD could mediate any non-autonomous effects by binding to molecules on adjacent cells. Consistent with a previous report, we observed that the localization of Crb protein in wild-type cells adjacent to *crb* clones is altered (Figure 3.14A) (Pellikka et al., 2002). Specifically, the level of Crb protein is greatly reduced on the surface of the wild-type cells that is immediately adjacent to mutant cells. This results in an asymmetric distribution of Crb in these wild-type cells, and suggests that, either directly or indirectly, Crb interacts with Crb molecules on adjacent cells.

Non-autonomous effects on localization are also seen in downstream molecules that interact with Crb. Ex protein levels are greatly reduced in the portion of the membrane where wild-type cells abut *crb* mutant cells (Figure 3.14B). This is consistent with the recent finding that

Ex physically interacts with the ICD of Crb (Ling et al., 2010). In contrast, we observed no differences in the level of Merlin at the interface between wild-type cells and *crb* cells, pairs of *crb* cells, or pairs of wild-type cells (Figure 3.14C). E-cadherin and Armadillo protein levels are similarly unaltered at these junctions (Figure 3.14D,E). Thus, there is not a general lack of adhesion between cells at the boundaries of *crb* clones.

Our results show that the localization of Crb on a particular cell membrane requires that the neighboring cell also express Crb at the shared border. This suggests that Crb molecules on adjacent cells interact. We wondered whether this interaction between Crb molecules could also cause additional Crb molecules to be drawn to a border where the adjacent cell expressed higher levels of Crb. To test this, we overexpressed the Crb-ECD fused to GFP in the anterior compartment of the wing imaginal disc using the *patched (ptc)* promoter and Gal4/UAS (Chen and Struhl, 1996). In these discs, denoted *ptc*>Crb^{ECD}, a Crb-ECD-overexpressing cell on the anterior side of the A-P compartment boundary experiences higher levels of Crb at its anterior border, which is shared with other Crb-ECD-overexpressing cells, than at its posterior border, which is shared with wild-type cells (Figure 3.14F). As expected the Crb-ECD, visualized through its GFP tag, was mostly found at the anterior of the border. Only very low levels of GFP-tagged ECD were found on the posterior border shared with wild-type cells. While the level of *ptc* expression steadily decreases anterior to the A-P border, the decrease appears to be gradual enough that we did not observe any striking differences in protein localization at borders between Crb-ECD overexpressing cells.

We were also able to assess the localization of endogenous Crb in the *ptc*>Crb^{ECD} discs with an antibody that recognizes the ICD of Crb (Figure 3.14G). In doing so we observed a striking increase in the levels of endogenous Crb along the border between the wild-type and Crb-overexpressing cells (Figure 3.14G'). This is consistent with the endogenous Crb in the wild-type cells being drawn toward the Crb-ECD-overexpressing cell. Ex levels were also higher at this border, mimicking the pattern of Crb localization (Figure 3.14G''). In contrast, Scrib localization was not changed, demonstrating that there is no general defect at that border (Figure 3.14H). Changes in localization of Crb-ECD, endogenous Crb, and Ex were similarly observed at the border of FLP-out clones, eliminating the possibility that the changes in localization of these proteins adjacent to the Patched-expressing domain were due to unusual properties of the compartment boundary (data not shown). Together these results suggest that Crb and its intracellular binding partners are drawn to borders where the adjacent cell expresses higher levels of Crb.

Thus, the localization of Crb protein and its intracellular binding partners within a cell can be influenced by the amount of Crb expressed on adjacent cells. Specifically Crb protein is preferentially found on membranes where there is more Crb available from the adjacent cell. This pattern of Crb localization is consistent with the ECD of Crb binding, either directly or indirectly, to Crb molecules on adjacent cells. If the level of Crb in a cell can influence the localization of individual proteins in neighboring cells, then it may also influence their biological properties.

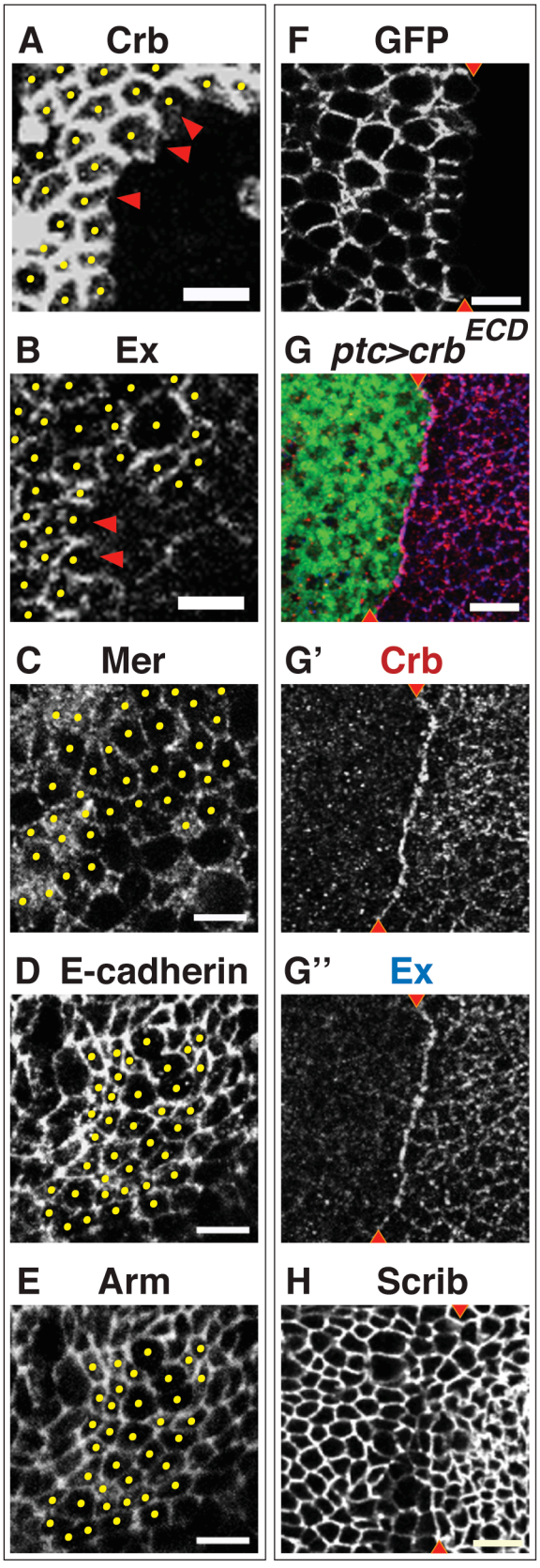


Figure 3.14 - Changes in levels of Crb affect adjacent cells
(A-E) Regions where *crb* mutant clones abut wild-type clones in mosaic imaginal dics. Wild-type cells are marked with dots. Wild-type cells are marked with yellow dots. Stains shown are Crumbs (A), Expanded (B), Merlin (C), E-cadherin (D), and Armadillo (E) (D and E show the same clone). Crb and Ex are depleted from the membrane of wild-type cells that abuts the mutant cells (red arrowheads). (F-H) *ptc-Gal4* used to overexpress Crb ECD tagged with GFP. (F) The recombinant protein, indicated by GFP, preferentially localizes to borders where it is also being overexpressed in the adjacent cell and is depleted from borders with wild-type cells (blue arrowheads). (G) Endogenous Crb, detected by an antibody to the ICD (G', red), and Ex (G'', blue) are concentrated along the border between wild-type cells and Crb ECD overexpressing cells. (H) Scrib distribution does not appear to be affected along this border.
Scale bars: 5μm.

Mechanism of Crb-Mediated Cell Competition

We wondered if the changes in protein localization that we were observing could influence cell survival and thus mediate cell competition. If so we would expect to see the elimination of wild-type cells adjacent to Crb-ECD-overexpressing cells, but wild-type cells adjacent to Crb-ICD-overexpressing cells should not be eliminated. We drove expression of the Crb-ECD in flip-out clones and using the *ptc*-Gal4 driver and stained wing imaginal discs with the AC3 antibody (Figure 3.15). Surprisingly we saw no increase in AC3-positive cells as a result of overexpression of the ECD (Figure 3.15B). Thus, asymmetry of Crb and Ex protein localization is not sufficient to induce elimination of cells.

In contrast, overexpression of the ICD using the *ptc*-Gal4 driver, significantly increased the number of AC3-positive cells on both sides of the compartment boundary, including at a significant distance from the border on the posterior side of the wing disc (Figure 3.15C). It was surprising that the death caused by Crb-ICD overexpression was so widespread in the wing disc. Previously, induction of apoptosis in the anterior compartment was found to induce nonautonmous apoptosis in the adjacent compartment boundary and this was suggested to be part of a mechanism for coordinating the size of the adjacent compartments. However, the induction of apoptosis in a compartment as a result of overgrowth in the adjacent compartment would set apart rather coordinate compartment size. Furthermore, it is generally assumed that compartment boundaries protect cells from cell competition (de la Cova et al., 2004; Simpson, 1979). Myc-overexpression, for example, did not produce any death in the posterior compartment (Figure 3.15D). Thus, expression of the Crb ICD appears to be sufficient to induce both autonomous and non-autonomous cell death, and the nonautonomous death is qualitatively different from that observed in Myc-induced supercompetition.

Generality of the Role for Crumbs in Cell Competition

The results above suggest that the levels of Crb are inversely proportional to the competitiveness of cells. Does this trend apply to other examples of cell competition? Crb levels were increased in Hippo pathway mutant cells (Genevet et al., 2009; Hamaratoglu et al., 2009). This appears to be a paradox as upregulation of Crb should cause cells to be outcompeted by wild-type cells while Hippo pathway mutant cells are, in fact, supercompetitors. A model in which asymmetries cause cell death, however, could explain this observation (see discussion).

In order to determine if Crb might play a role in Myc-induced cell competition, we expressed Myc using *ptc*-Gal4 and dissected and stained wing imaginal discs with antibodies to Crb and Scrib (Figure 3.9E,F). We saw no obvious difference in levels of either molecule between the Myc-overexpressing cells and the wild-type cells. This suggests that Myc and Crb might regulate cell competition independently. However, as will be discussed, the Myc and Hippo pathways were recently found to influence each others' levels.

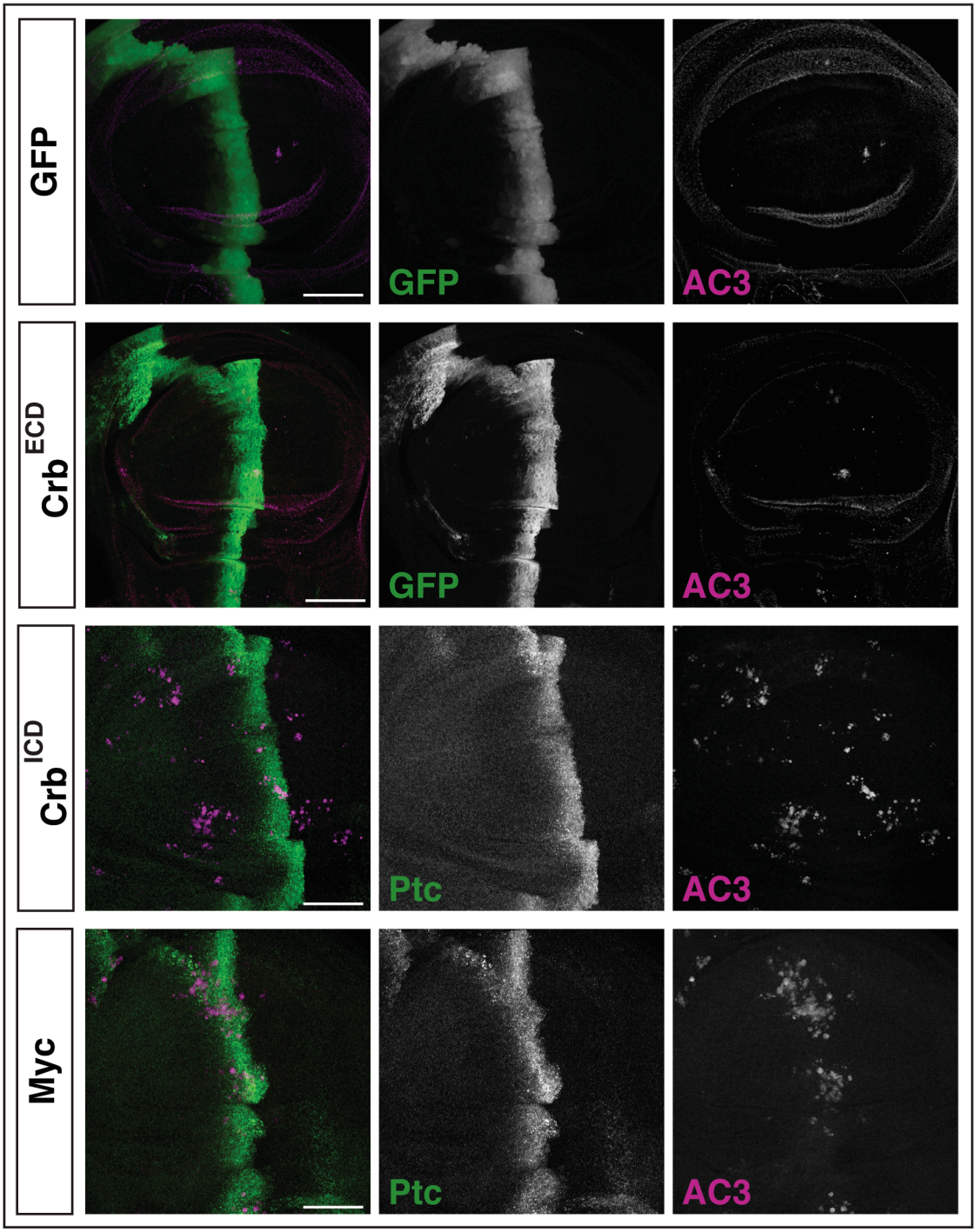


Figure 3.15 - Apoptosis induced by expression of Crb constructs
Wing imaginal discs expressing (A) GFP, (B) Crb ECD, (C) Crb ICD, (D) Myc construct driven by ptc-Gal4 and stained with AC3 (blue). Expression domain is delineated by GFP (A') or Ptc (B', C', D') expression. Morphology visualized through Scrib (A) or Phalloidin (B-D) staining. Scale bar: 50μm.

DISCUSSION

Crumbs is Involved in Cell Competition

We found several alleles of *crb* in a screen for mutations that cause cells to become supercompetitors and showed that wild-type cells adjacent to *crb* mutant cells undergo apoptosis at a high rate. *crb* is the first example, to our knowledge, of a perturbation in a polarity regulator that increases rather than decreases the competitive fitness of cells. The discovery that *crb* cells are supercompetitiors further suggests that cell polarity could have significance beyond simply being required for normal cell function. The ratio of apical-to-basal domain, for example, could be a mode for regulating growth-related processes. Furthermore, as a transmembrane protein, Crb is in a position to mediate a cell-cell comparison that might distinguish winners and losers in cell competition. For these reasons, we further characterized the involvement of Crb in cell competition.

Overexpression of Crb in a large domain or an entire compartment of the wing disc was previously shown to cause neoplastic overgrowth (Lu and Bilder, 2005). In contrast, we found that small clones overexpressing Crb in wild-type imaginal discs are eliminated over time. Thus, Crb-overexpressing cells are eliminated as a result of certain heterotypic interactions, presumably through cell competition. This correlation between the levels of Crb and the competitive fitness of cells opens the possibility that the levels of Crb might determine the competitive ability of cells.

The elimination of Crb-overexpressing clones might be expected to resemble other instances of cell competition, particularly the elimination of *scrib, lgl* or *dlg* mutant clones because their polarity phenotypes are so similar. However, two features of competition between Crb-overexpressing and wild-type cells have not yet been reported for cell competition involving Myc, *Mintues*, or the basal polarity complex components. First, although Crb-overexpressing clones are eliminated by cell competition, they also appear to induce death of adjacent wild-type cells. This observation is not entirely unprecedented. Dpp signaling-deficient clones are thought to trigger a homeostatic response by causing disruptions in the slope of the Dpp morphogen gradient that also leads to their elimination from wild-type imaginal discs. In this process, known as “morphogenetic apoptosis,” death on both sides of the border is thought to re-establish the slope of the gradient (Adachi-Yamada and O'Connor, 2002; Adachi-Yamada and O'Connor, 2004). The apoptosis in the wild-type cells in both of these examples may indicate that cell competition involves a bidirectional signal.

Second, a considerable amount of apical extrusion is observed of large clones overexpressing Crb in wild-type imaginal discs. The significance of this extrusion warrants further investigation. It may be a byproduct of the increased size of the apical domain similar to the formation of vesicles upon overexpression of Crb in the embryonic ectoderm (Wodarz et al., 1995). Alternatively it may be the major mechanism by which Crb overexpressing cells are eliminated. Consistent with this idea, inhibition of apoptosis does not appear to rescue the elimination of Crb-overexpressing cells. Extrusion was also previously found to play an important role in the elimination of Dpp signaling-deficient cells

(Gibson and Perrimon, 2005; Shen and Dahmann, 2005). The similarities between Crb- and Dpp- mediated cell competition are consistent with a recent report that mammalian Crb can impinge upon TGF-ß signaling (Varelas et al., 2010).

The extrusion of Crb-overexpressing cells also offers an alternate explanation for the death seen in the adjacent wild-type cells. During the extrusion some wild-type cells may be displaced physically from the epithelium and extruded as well. Such cells would be expected to undergo apoptosis as a result of losing contact with the epithelium through a process known as anoikis (Frisch and Francis, 1994).

Surprisingly, death of winners and extrusion of losers was not observed in the case of competition between *crb* mutant and wild-type cells. These effects may only occur in a limited time window or when winners and losers are present at a specific ratio. However, this may also indicate that the mechanism by which *crb* mutant cells act as supercompetitors is distinct from the mechanism by which Crb-overexpressing cells are eliminated.

Which Signaling Pathways Cooperate With Crb In Cell Competition?

We investigated candidate supercompetitor pathways that might signal downstream of Crb during cell competition. Recently, the Hippo pathway was reported to be downstream of Crb in regulating growth. Consistent with this we observed upregulation of the Hippo target gene, Diap 1, and mislocalization of the apically localized upstream regulator and target gene, Ex, in *crb* mutant clones in imaginal discs. Thus, there seems to be a subtle activation of the Hippo pathway in *crb* mutant cells. This activation of the Hippo pathway has been used to explain the overgrowth phenotype of *crb* mutant tissue. As the Hippo pathway is a known supercompetitor pathway we wondered whether its activation might also explain the supercompetitor phenotype of *crb* cells. For example, cells might compare their levels of Crb directly, then signal through the Hippo pathway to induce the death of the losers. Two lines of evidence suggest that this is not the case. First, there were uniform differences in Hippo pathway levels between control and crb mutant clones. If Hippo were signaling the death of losers then the pathway would be especially repressed in loser cells that border winners. Furthermore, we found that loss of *wts* did not rescue the elimination of Crb-overexpressing cells. Thus, Crb either signals the elimination of losers through a noncanonical Wts-independent branch of the Hippo pathway, perhaps through Ex, or another pathway.

We did not detect any differences in levels of Myc in *crb* mutant clones nor did we detect any differences in Crb levels in Myc overexpressing cells. Furthermore, we find a mechanistic difference to the competition induced by Myc. Myc-overexpressing cells are supercompetitors but cannot cause death across compartment boundaries. In contrast we found that expression of Crb^{ICD} causes nonautonmous apoptosis across the A-P compartment boundary. We suggest that there may be at least two independent mechanisms of cell competition.

Homophilic Binding of Crb

We performed a series of experiments to address a previous observation from another group suggesting that Crb molecules on adjacent cells might interact with each other. Crb is normally distributed evenly around the border of a cell, however, in many different examples examined Crb concentrated along one part of the border where the adjacent cell sharing that part of border was expressing higher levels of Crb. Molecules that bind to Crb similarly display asymmetric localization at borders where there are differences in Crb levels. While this demonstrates an interaction between Crb molecules on adjacent cells, it is unclear whether this interaction is direct or indirect. Testing for the interaction in heterologous cells where any intermediate molecules would not be present may help distinguish between these possibilities.

The interaction of Crb molecules across cell borders has at least two consequences. When there is a change in the localization of Crb, an identical change is seen in the localization of Ex, reflecting the binding of Ex to the ICD of Crb. Thus, first, Crb can be used by a cell to nonautonmously influence the properties of an adjacent cell, specifically the localization of at least two, but potentially many, proteins in that cell. Second, the interaction between Crb molecules results in the stabilization of Crb at a border, either by preventing it from being internalized or diffusing away laterally.

Roles of the ICD and ECD of Crb in Cell Competition

In a number of recent studies, the ICD of Crb was found to regulate both the localization and stability of Ex by directly binding with it. Thus, the function of Crb in regulating growth was ascribed to this small part of the protein, while the ECD was largely ignored (Chen et al., 2010; Grzeschik et al., 2010; Ling et al., 2010; Robinson et al., 2010). In another study expression of a membrane-bound ECD was sufficient to rescue the overgrowth phenotype of *crb* mutant cells (Richardson and Pichaud, 2010). Thus, there is some ambiguity over the role of the different domains of Crb in regulating growth.

We investigated the contribution of the ICD and ECD to the elimination of Crb-overexpressing cells by examining effect of overexpression of membrane-bound constructs of each alone. The juxtaposition of Crb-overexpressing cells and wild-type cells leads to a number of phenotypes (we note that some of these phenotypes can only be observed upon the creation of large Crb-overexpressing clones through expression of a temperature-sensitive Gal80): (1) apoptosis of Crb-overexpressing cells bordering wild-type cells (2) apical extrusion of Crb-overexpressing clones (3) apoptosis of wild-type cells (4) changes in Crb and Ex localization in both cells.

The extrusion and apoptosis both appear to contribute to the rapid elimination of Crb-overexpressing cells from wild-type epithelia, and neither expression of the ICD nor ECD alone was sufficient to induce this elimination. Some interaction mediated by the ECD, potentially homophilic binding with Crb molecules on an adjacent wild-type cell, is required

for the elimination of Crb-overexpressing cells. The extrusion of Crb-overexpressing cells is also not the result of preferential adhesion of these cells to each other and their consequent loss of adhesion with the surrounding epithelium, as overexpression of the ECD alone was not sufficient to cause the elimination of cells from wild-type imaginal discs. Crb overexpression may still increase cell adhesion, but increased adhesion is not sufficient to induce extrusion. Thus, the elimination of Crb-overexpressing cells appears to require an intracellular signal through the ICD, as well as an interaction with the adjacent cell through the ECD.

We were able to replicate certain aspects of the phentoype through expression of the domains independently. Overexpression of the ICD was sufficient to cause nonautonomous apoptosis of wild-type cells when expressed in the *ptc* domain. Overexpression of the ECD was sufficient to cause the changes in protein localization. It also caused a slight upward bend of the epithelium reminiscent of apical extrusion. In constrast overexpression of the ICD caused a slight downward bend of the epithelium, suggesting that it may have a dominant negative effect on epithelial morphogenesis in comparison to full length Crb.

One explanation for why both domains appear to be required for the phenotypes is if each recruits a required component of an active complex that induces the dramatic changes observed upon overexpression of full-length Crb. If so then, the two domains must be connected to be able to colocalize the different components. Thus it will be interesting to see whether overexpression of the ECD and ICD in the same cell is sufficient to induce elimination or whether the two domains must be adjoined as part of the same molecule to induce this effect.

What is the Basis of Comparison in Cell Competition?

Because Crb interacts with itself across cell borders, we considered whether a direct comparison of Crb levels might lead to cell competition. A direct comparison mechanism could be based on one of at least two features that distinguish borders at which Crb levels are uneven. First, as shown above, cells at borders where there are differences in Crb levels may have asymmetric localization of Crb and its downstream interacting molecules. Second, there will be Crb molecules that are not in homophilic protein complexes on the side of the border where there is more Crb. Alternatively, the cell-cell comparison may occur downstream of Crb. We next present three models for Crb-mediated competition based on our observations. Each model explains some but not all aspects of cell competition. This may be because different mechanisms are involved in the different instances of cell competition. For example, different mechanisms might cause *crb* mutant cells to act as winner and Crb-overexpressing cells to act as losers.

Asymmetry Model for Crb-mediated Cell Competition
The first model is based upon our observation that when two populations of cells that express different levels of Crb abut each other, the cells at the boundary are exposed to a different level of Crb at their homotypic border than at their heterotypic border. This results

in a planar asymmetry of both Crb and the proteins that it binds to, such as Ex, within those cells. We propose that the asymmetric distribution of one of these Crb binding molecules promotes apoptosis.

An open question is how such subtle differences as asymmetric distribution of proteins along a cell membrane can generate signals that alter cell growth or survival. We speculate that the levels of certain proteins may be tightly balanced with the levels of negative regulators of its function. The effect of redistribution might be to produce high enough levels of the protein in a small, localized area to activate signaling. Indeed, work by other groups has indicated that asymmetries in components of the Hippo pathway such as changes in the cadherins, Ft and Dachsous (Ds) [30], might regulate growth and proliferation [31, 32]. Therefore, Ex, may be a good candidate for such a molecule.

This model explains why death occurs preferentially in wild-type cells adjacent to *crb* clones (*crb* cells have a slight increase in DIAP1 levels) but on both sides of clones that overexpress full-length Crb, albeit at different levels. A role for Crb in this comparison mechanism can also explain the apparent paradox that Hippo-activated cells, including *ex*, *hpo*, *sav* and *wts* mutant cells, behave as supercompetitors even though they express higher levels of Crb. In these mosaic tissues the higher levels of Crb in the Hippo-activated cells would, through asymmetries, activate apoptosis of both Hippo-activated and wild-type cells. However, the Hippo-activated cells also express the anti-apoptotic protein DIAP1. Therefore, the apoptosis and elimination would only occur in the wild-type cells.

Also consistent with this model is the elimination by cell competition of clones of cells that are mutant for *scrib* or *dlg* [35, 36] which express higher levels of Crb [37]. Indeed scrib clones have been shown to induce JNK activity in mutant cells as well as in a nonautonomous manner [38].

The main observation that argues against this model is that overexpression of the Crb ECD alone, which we showed to induce planar asymmetry of Crb and Ex in adjacent wild-type cells, does not promote apoptosis of those wild-type cells. One explanation may be that other proteins that associate specifically with full-length Crb are necessary for the elimination of the wild-type cells or that Crb may participate in “inside-out” signaling as has been observed for Integrins [39].

The death observed as a result of overexpression of the Crb ICD is also inconsistent with this model. One explanation for this is if it causes changes in levels of endogenous Crb. Indeed there appears to be a general decrease in the levels of endogenous Crb in all ECD overexpressing cells. This may indicate a feedback mechanism for regulating Crb levels of cells – thus cells may recognize the overabundance of Crb ECD and compensate by lowering expression of endogenous Crb.

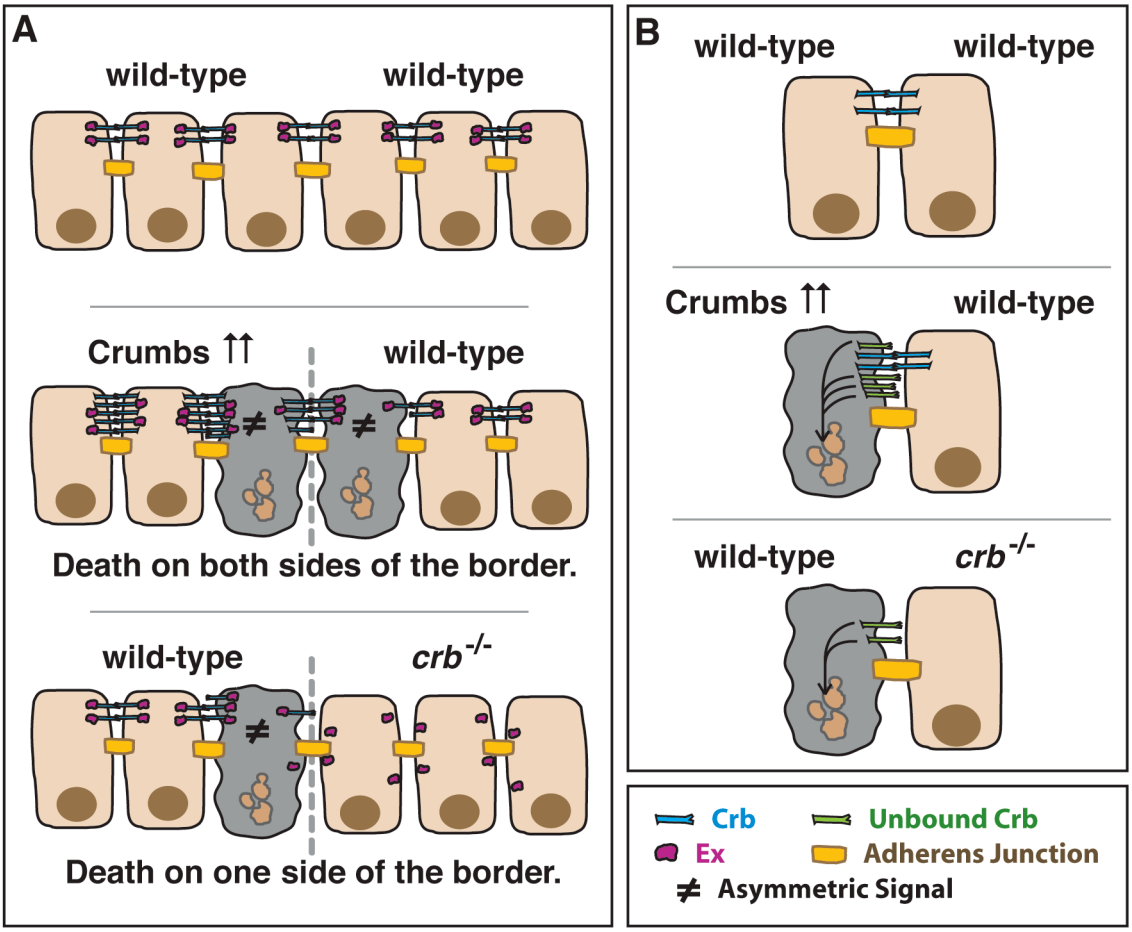


Figure 3.16 - Models for Crb-induced cell competition
(A) A model for cell competition induced by differences in levels of Crb at a border (see discussion). (B) A model for cell competition induced by undimerized Crb molecules (see discussion).

Unbound Crumbs Signals Death Model for Cell Competition
A second model is that cells with more Crb adjacent to cells with less Crb are likely to have unbound Crb molecules, and that this signals death in the cells with more Crb. This model explains the death of wild-type cells adjacent to Crb mutant cells and the death of Crb-overexpressing cells adjacent to wild-type cells. However, it does not explain the apoptosis seen in wild-type cells adjacent to Crb-overexpressing cells. It also does not explain why Hippo-activated cells, which have more Crb, are supercompetitors.

Changes in Cell Polarity Induce Cell Competition
We also considered whether there might be a mechanism for adjacent cells to sense and compare their polarity. In this model, the cell-cell comparison might occur downstream of Crb. While we observed very subtle differences in polarity in *crb* mutant clones compared with adjacent wild-type tissue, the overexpression of Crb had dramatic effects on cell

polarity. Thus, while Crb may normally play only a minor role, it has the ability to significantly affect the polarity of cells in the larval imaginal discs. The degree of disruption of polarity in each case also correlates with the strength of competition observed in each case.

Consistent with this model, Crb regulates apical polarity in other tissue contexts by antagonizing basal polarity regulators, Scrib, Dlg, and Lgl. We note that this antagonistic relationship appears to be conserved in cell competition - loss of regulators of basal polarity causes cells to be eliminated from wild-type imaginal discs while loss of Crb causes cells to become supercompetitors. Based on these examples, differences in polarity might induce cell competition. However future studies will need to address how extensive the correlation is between apicobasal polarity and competitive ability and whether changes in polarity always result in cell competition. We did not see any changes in polarity in Myc-overexpressing clones indicating that polarity is not generally necessary for producing cell competition.

Conclusion

In conclusion, we show that when cells with different levels of Crb are present in the same tissue during development, the cells with more Crb are less likely to be represented in the adult tissue. We also show that Crb impinges upon the Hippo pathway and differences in Crb can nonautonomously influence protein localization and survival of adjacent cells. These characteristics make Crb an intriguing candidate for a factor that adjacent cells might compare in determining winners and losers in cell competition. Our work raises several important questions for future studies. Do Crb levels indeed play a role in other instances of cell competition such as with *Minutes*? Do cells compare Crb levels directly through the ECD of Crb or through a downstream molecule? And moreover what do Crumbs levels indicate about a cell? One hypothesis is that Crb is a read-out, as well as a regulator, of apicobasal polarity of cells. This may indicate that cell competition can act as mechanism a mechanism for monitoring the polarity of cells and eliminating cells whose polarity differs from that of the rest of the tissue. Future studies that address these questions may reveal important mechanistic features of cell competition, and ultimately lead to a better understanding of both normal development and tumor growth.

METHODS

Fly Stocks & Husbandry

y w eyFLP2 ;; FRT82B w+ cl3R3/TM6B,y+ (Newsome et al., 2000)
y w eyFLP2 ; cl2L3 w+ FRT40A /CyO, y+ (Newsome et al., 2000)
w;;FRT82B
w;;FRT82B Tsc1[Q87X] (Tapon et al., 2001)
w;;FRT82B cic[Q474X] (Tseng et al., 2007)
w;;FRT82B wts [MGH1]
UAS-CrbWT (Wodarz et al., 1995)
UAS-CrbExtraTMGFP (Pellikka et al., 2002)
UAS-CrbIntraMyc2b (Wodarz et al., 1995)
w;; Act<CD2<Gal4, UAS-GFP
tubGal80TS (McGuire et al., 2003)
y w eyFLP ;; FRT82B P[mini-w] P[Ubi-GFP]
yw ubxFLP ;; FRT82B P[mini-w] P[Ubi-GFP]
Generally, experimental crosses were raised at 25º on food prepared according to the recipe from the Bloomington Stock Center.

Quantification of Red Tissue in Adult Eyes

Pictures of adult eyes were taken on a Leica Z16 APO system with Montage software from Synoptics Ltd. Total eye and red tissue area were measured in pixels by using the 'Histogram' dialogue for selected regions in Photoshop. The eye was selected by hand using the polygonal lasso. To select red pixels, the image was converted to 'grayscale' and 'shadows' were selected. The ratio of the red pixels to total pixels in the eye was calculated.

Identification of Mutations Responsible for *crb* Alleles

The complementation group failed to complement Df(3R)BSC317 (BL24343), DF(3R)crb-F89-4 (BL4432), and Df(3R)BSC317 (BL24996). The following primers were used to identify mutations in two alleles.
Mut 269 was a c to t mutation resulting in Q328X
5F: AAGTAACCATGCCGTTCTGG
6R: CAGCTTCCGTTGTTCTGACA
Mut 163 was a g to a mutation resulting in W455X
7F: GAACAATGGAGCATGTGTGG
8R: GGGGGTAATGGAGAGGTGTT

"Flip-Out" Clone Induction Protocol

Eggs were collected at room temp for 4-6 hr on grape juice plates in the dark. Grape plates were then placed at 25º. After 24hr, L1 larvae were picked and 40 larvae were placed in each vial with a dollop of yeast paste. 7 min heat shocks were performed at the indicated time points at 37º in a circulating water bath. Imaginal discs were dissected at 114hAED. In experiments with the temperature-sensitive Gal80, vials with L1 larvae were kept at 18º. 20min heat shocks were performed on Day 2 at 37º in a circulating water bath. Vials were transferred to 30º on Day 7 and imaginal discs were dissected on Day 8.

Immunohistochemistry
Discs were dissected in PBS, fixed in 4% paraformaldehyde in PBS, washed in 0.1% Triton in PBS, and mounted in Slow Fade Gold. The following primary antibodies were used:
AC3 (Rabbit, 1:200) from Cell Signaling
phalloidin-TRITC (1:500) from Sigma
Expanded (Guinea Pig, 1:5000) from R. Fehon (Maitra et al., 2006)
Merlin (Guinea Pig, 1:7500) from R.Fehon (McCartney and Fehon, 1996)
E-Cadherin (Rat, 1:50) from DSHB
Armadillo (Mouse, 1:20) from DSHB
Diap1 (Mouse, 1:200) from B. Hay (Yoo et al., 2002)
Secondary Alexa-Fluor antibodies from Invitrogen were used at 1:1000.
Fluorescent images were taken on a Leica TCS SL confocal microscope.

REFERENCES

1. **Adachi-Yamada, T. and O'Connor, M. B.** (2002). Morphogenetic apoptosis: a mechanism for correcting discontinuities in morphogen gradients. *Dev Biol* **251**, 74-90.

2. **Adachi-Yamada, T. and O'Connor, M. B.** (2004). Mechanisms for removal of developmentally abnormal cells: cell competition and morphogenetic apoptosis. *J Biochem* **136**, 13-7.

3. **Badouel, C. and McNeill, H.** (2009). Apical junctions and growth control in Drosophila. *Biochim Biophys Acta* **1788**, 755-60.

4. **Baker, N. E.** (2011). Cell competition. *Curr Biol* **21**, R11-5.

5. **Bilder, D., Li, M. and Perrimon, N.** (2000). Cooperative regulation of cell polarity and growth by Drosophila tumor suppressors. *Science* **289**, 113-6.

6. **Bilder, D. and Perrimon, N.** (2000). Localization of apical epithelial determinants by the basolateral PDZ protein Scribble. *Nature* **403**, 676-80.

7. **Bilder, D., Schober, M. and Perrimon, N.** (2003). Integrated activity of PDZ protein complexes regulates epithelial polarity. *Nat Cell Biol* **5**, 53-8.

8. **Brumby, A. M. and Richardson, H. E.** (2003). scribble mutants cooperate with oncogenic Ras or Notch to cause neoplastic overgrowth in Drosophila. *EMBO J* **22**, 5769-79.

9. **Bryant, P. J., Huettner, B., Held, L. I., Ryerse, J. and Szidonya, J.** (1988). Mutations at the fat locus interfere with cell proliferation control and epithelial morphogenesis in Drosophila. *Dev Biol* **129**, 541-54.

10. **Bulgakova, N. A. and Knust, E.** (2009). The Crumbs complex: from epithelial-cell polarity to retinal degeneration. *J Cell Sci* **122**, 2587-96.

11. **Campbell, K., Knust, E. and Skaer, H.** (2009). Crumbs stabilises epithelial polarity during tissue remodelling. *J Cell Sci* **122**, 2604-12.

12. **Chen, C.-L., Gajewski, K. M., Hamaratoglu, F., Bossuyt, W., Sansores-Garcia, L., Tao, C. and Halder, G.** (2010). The apical-basal cell polarity determinant Crumbs regulates Hippo signaling in Drosophila. *Proc Natl Acad Sci USA* **107**, 15810-5.

13. **Chen, Y. and Struhl, G.** (1996). Dual roles for patched in sequestering and transducing Hedgehog. *Cell* **87**, 553-63.

14. **de la Cova, C., Abril, M., Bellosta, P., Gallant, P. and Johnston, L. A.** (2004). Drosophila myc regulates organ size by inducing cell competition. *Cell* **117**, 107-16.

15. **den Hollander, A. I., ten Brink, J. B., de Kok, Y. J., van Soest, S., van den Born, L. I., van Driel, M. A., van de Pol, D. J., Payne, A. M., Bhattacharya, S. S., Kellner, U. et al.** (1999). Mutations in a human homologue of Drosophila crumbs cause retinitis pigmentosa (RP12). *Nat Genet* **23**, 217-21.

16. **Frisch, S. M. and Francis, H.** (1994). Disruption of epithelial cell-matrix interactions induces apoptosis. J Cell Biol 124, 619-26.

17. Genevet, A., Polesello, C., Blight, K., Robertson, F., Collinson, L. M., Pichaud, F. and Tapon, N. (2009). The Hippo pathway regulates apical-domain size independently of its growth-control function. J Cell Sci 122, 2360-70.

18. **Gibson, M. C. and Perrimon, N.** (2005). Extrusion and death of DPP/BMP-compromised epithelial cells in the developing Drosophila wing. *Science* **307**, 1785-9.

19. **Golic, K. G.** (1991). Site-specific recombination between homologous chromosomes in Drosophila. *Science* **252**, 958-61.

20. **Golic, K. G. and Lindquist, S.** (1989). The FLP recombinase of yeast catalyzes site-specific recombination in the Drosophila genome. *Cell* **59**, 499-509.

21. **Grewal, S. S., Li, L., Orian, A., Eisenman, R. N. and Edgar, B. A.** (2005). Myc-dependent regulation of ribosomal RNA synthesis during Drosophila development. *Nat Cell Biol* **7**, 295-302.

22. **Grzeschik, N. A., Amin, N., Secombe, J., Brumby, A. M. and Richardson, H. E.** (2007). Abnormalities in cell proliferation and apico-basal cell polarity are separable in Drosophila lgl mutant clones in the developing eye. *Dev Biol* **311**, 106-23.

23. **Grzeschik, N. A., Parsons, L. M., Allott, M. L., Harvey, K. F. and Richardson, H. E.** (2010). Lgl, aPKC, and Crumbs regulate the Salvador/Warts/Hippo pathway through two distinct mechanisms. *Curr Biol* **20**, 573-81.

24. **Hamaratoglu, F., Gajewski, K., Sansores-Garcia, L., Morrison, C., Tao, C. and Halder, G.** (2009). The Hippo tumor-suppressor pathway regulates apical-domain size in parallel to tissue growth. *J Cell Sci* **122**, 2351-9.

25. **Hariharan, I. K. and Bilder, D.** (2006). Regulation of imaginal disc growth by tumor-suppressor genes in Drosophila. *Annu Rev Genet* **40**, 335-61.

26. **Harvey, K. F., Pfleger, C. M. and Hariharan, I. K.** (2003). The Drosophila Mst ortholog, hippo, restricts growth and cell proliferation and promotes apoptosis. *Cell* **114**, 457-67.

27. **Hay, B. A., Wolff, T. and Rubin, G. M.** (1994). Expression of baculovirus P35 prevents cell death in Drosophila. *Development* **120**, 2121-9.

28. **Herranz, H., Morata, G. and Milán, M.** (2006). calderón encodes an organic cation transporter of the major facilitator superfamily required for cell growth and proliferation of Drosophila tissues. *Development* **133**, 2617-25.

29. **Huh, J. R., Guo, M. and Hay, B. A.** (2004). Compensatory proliferation induced by cell death in the Drosophila wing disc requires activity of the apical cell death caspase Dronc in a nonapoptotic role. *Curr Biol* **14**, 1262-6.

30. **Humbert, P., Russell, S. and Richardson, H.** (2003). Dlg, Scribble and Lgl in cell polarity, cell proliferation and cancer. *Bioessays* **25**, 542-53.

31. **Igaki, T.** (2009). Correcting developmental errors by apoptosis: lessons from Drosophila JNK signaling. *Apoptosis* **14**, 1021-8.

32. **Igaki, T., Pastor-Pareja, J. C., Aonuma, H., Miura, M. and Xu, T.** (2009). Intrinsic tumor suppression and epithelial maintenance by endocytic activation of Eiger/TNF signaling in Drosophila. *Developmental Cell* **16**, 458-65.

33. **Izaddoost, S., Nam, S.-C., Bhat, M. A., Bellen, H. J. and Choi, K.-W.** (2002). Drosophila Crumbs is a positional cue in photoreceptor adherens junctions and rhabdomeres. *Nature* **416**, 178-83.

34. **Jiménez, G., Guichet, A., Ephrussi, A. and Casanova, J.** (2000). Relief of gene repression by torso RTK signaling: role of capicua in Drosophila terminal and dorsoventral patterning. *Genes Dev* **14**, 224-31.

35. **Jin, Z., Kirilly, D., Weng, C., Kawase, E., Song, X., Smith, S., Schwartz, J. and Xie, T.** (2008). Differentiation-defective stem cells outcompete normal stem cells for niche occupancy in the Drosophila ovary. *Cell Stem Cell* **2**, 39-49.

36. **Johnson, K., Grawe, F., Grzeschik, N. and Knust, E.** (2002). Drosophila crumbs is required to inhibit light-induced photoreceptor degeneration. *Curr Biol* **12**, 1675-80.

37. **Johnston, L. A., Prober, D. A., Edgar, B. A., Eisenman, R. N. and Gallant, P.** (1999). Drosophila myc regulates cellular growth during development. *Cell* **98**, 779-90.

38. **Jurgens, G., Wieschaus, E., Nusslein-Volhard, C. and Kluding, H.** (1984). Mutations affecting the pattern of the larval cuticle in Drosophila melanogaster. *Roux's Archives of Developmental Biology* **193**, 283-295.

39. **Karp, C. M., Tan, T. T., Mathew, R., Nelson, D., Mukherjee, C., Degenhardt, K., Karantza-Wadsworth, V. and White, E.** (2008). Role of the polarity determinant crumbs in suppressing mammalian epithelial tumor progression. *Cancer Res* **68**, 4105-15.

40. **Knust, E.** (2007). Photoreceptor morphogenesis and retinal degeneration: lessons from Drosophila. *Curr Opin Neurobiol* **17**, 541-7.

41. **Lee, T. and Luo, L.** (1999). Mosaic analysis with a repressible cell marker for studies of gene function in neuronal morphogenesis. *Neuron* **22**, 451-61.

42. **Lemmers, C., Michel, D., Lane-Guermonprez, L., Delgrossi, M.-H., Médina, E., Arsanto, J.-P. and Le Bivic, A.** (2004). CRB3 binds directly to Par6 and regulates the morphogenesis of the tight junctions in mammalian epithelial cells. *Mol Biol Cell* **15**, 1324-33.

43. **Li, W. and Baker, N. E.** (2007). Engulfment is required for cell competition. *Cell* **129**, 1215-25.

44. **Ling, C., Zheng, Y., Yin, F., Yu, J., Huang, J., Hong, Y., Wu, S. and Pan, D.** (2010). The apical transmembrane protein Crumbs functions as a tumor suppressor that regulates Hippo signaling by binding to Expanded. *Proc Natl Acad Sci USA*.

45. **Lu, H. and Bilder, D.** (2005). Endocytic control of epithelial polarity and proliferation in Drosophila. *Nat Cell Biol* **7**, 1232-9.

46. **Maitra, S., Kulikauskas, R. M., Gavilan, H. and Fehon, R. G.** (2006). The tumor suppressors Merlin and Expanded function cooperatively to modulate receptor endocytosis and signaling. *Curr Biol* **16**, 702-9.

47. **Makarova, O., Roh, M. H., Liu, C.-J., Laurinec, S. and Margolis, B.** (2003). Mammalian Crumbs3 is a small transmembrane protein linked to protein associated with Lin-7 (Pals1). *Gene* **302**, 21-9.

48. **Martín, F. A., Herrera, S. C. and Morata, G.** (2009). Cell competition, growth and size control in the Drosophila wing imaginal disc. *Development* **136**, 3747-56.

49. **Marygold, S. J., Roote, J., Reuter, G., Lambertsson, A., Ashburner, M., Millburn, G. H., Harrison, P. M., Yu, Z., Kenmochi, N., Kaufman, T. C. et al.** (2007). The ribosomal protein genes and Minute loci of Drosophila melanogaster. *Genome Biol* **8**, R216.

50. **McCartney, B. M. and Fehon, R. G.** (1996). Distinct cellular and subcellular patterns of expression imply distinct functions for the Drosophila homologues of moesin and the neurofibromatosis 2 tumor suppressor, merlin. *J Cell Biol* **133**, 843-52.

51. **McGuire, S. E., Le, P. T., Osborn, A. J., Matsumoto, K. and Davis, R. L.** (2003). Spatiotemporal rescue of memory dysfunction in Drosophila. *Science* **302**, 1765-8.

52. **Médina, E., Lemmers, C., Lane-Guermonprez, L. and Le Bivic, A.** (2002). Role of the Crumbs complex in the regulation of junction formation in Drosophila and mammalian epithelial cells. *Biol Cell* **94**, 305-13.

53. **Menéndez, J., Pérez-Garijo, A., Calleja, M. and Morata, G.** (2010). A tumor-suppressing mechanism in Drosophila involving cell competition and the Hippo pathway. *Proc Natl Acad Sci USA* **107**, 14651-6.

54. **Morata, G. and Ripoll, P.** (1975). Minutes: mutants of drosophila autonomously affecting cell division rate. *Dev Biol* **42**, 211-21.

55. **Moreno, E.** (2008). Is cell competition relevant to cancer? *Nat Rev Cancer* **8**, 141-7.

56. **Moreno, E. and Basler, K.** (2004). dMyc transforms cells into super-competitors. *Cell* **117**, 117-29.

57. **Moreno, E., Basler, K. and Morata, G.** (2002). Cells compete for decapentaplegic survival factor to prevent apoptosis in Drosophila wing development. *Nature* **416**, 755-9.

58. **Muncan, V., Sansom, O. J., Tertoolen, L., Phesse, T. J., Begthel, H., Sancho, E., Cole, A. M., Gregorieff, A., de Alboran, I. M., Clevers, H. et al.** (2006). Rapid loss of intestinal crypts upon conditional deletion of the Wnt/Tcf-4 target gene c-Myc. *Mol Cell Biol* **26**, 8418-26.

59. **Nesbit, C. E., Tersak, J. M. and Prochownik, E. V.** (1999). MYC oncogenes and human neoplastic disease. *Oncogene* **18**, 3004-16.

60. **Newsome, T. P., Asling, B. and Dickson, B. J.** (2000). Analysis of Drosophila photoreceptor axon guidance in eye-specific mosaics. *Development* **127**, 851-60.

61. **Nystul, T. and Spradling, A.** (2007). An epithelial niche in the Drosophila ovary undergoes long-range stem cell replacement. *Cell Stem Cell* **1**, 277-85.

62. **Oertel, M., Menthena, A., Dabeva, M. D. and Shafritz, D. A.** (2006). Cell competition leads to a high level of normal liver reconstitution by transplanted fetal liver stem/progenitor cells. *Gastroenterology* **130**, 507-20; quiz 590.

63. **Oliver, E. R., Saunders, T. L., Tarlé, S. A. and Glaser, T.** (2004). Ribosomal protein L24 defect in belly spot and tail (Bst), a mouse Minute. *Development* **131**, 3907-20.

64. **Pan, D.** (2007). Hippo signaling in organ size control. *Genes Dev* **21**, 886-97.

65. **Pan, D.** (2010). The hippo signaling pathway in development and cancer. *Developmental Cell* **19**, 491-505.

66. **Pellikka, M., Tanentzapf, G., Pinto, M., Smith, C., McGlade, C. J., Ready, D. F. and Tepass, U.** (2002). Crumbs, the Drosophila homologue of human CRB1/RP12, is essential for photoreceptor morphogenesis. *Nature* **416**, 143-9.

67. **Prober, D. A. and Edgar, B. A.** (2000). Ras1 promotes cellular growth in the Drosophila wing. *Cell* **100**, 435-46.

68. **Rhiner, C., Díaz, B., Portela, M., Poyatos, J. F., Fernández-Ruiz, I., López-Gay, J. M., Gerlitz, O. and Moreno, E.** (2009). Persistent competition among stem cells and their daughters in the Drosophila ovary germline niche. *Development* **136**, 995-1006.

69. **Rhiner, C. and Moreno, E.** (2009). Super competition as a possible mechanism to pioneer precancerous fields. *Carcinogenesis* **30**, 723-8.

70. **Richardson, E. C. N. and Pichaud, F.** (2010). Crumbs is required to achieve proper organ size control during Drosophila head development. *Development* **137**, 641-50.

71. **Robinson, B. S., Huang, J., Hong, Y. and Moberg, K. H.** (2010). Crumbs regulates Salvador/Warts/Hippo signaling in Drosophila via the FERM-domain protein expanded. *Curr Biol* **20**, 582-90.

72. **Rodriguez-Boulan, E., Kreitzer, G. and Müsch, A.** (2005). Organization of vesicular trafficking in epithelia. *Nat Rev Mol Cell Biol* **6**, 233-47.

73. **Ryoo, H. D., Gorenc, T. and Steller, H.** (2004). Apoptotic cells can induce compensatory cell proliferation through the JNK and the Wingless signaling pathways. *Developmental Cell* **7**, 491-501.

74. **Salghetti, S. E., Muratani, M., Wijnen, H., Futcher, B. and Tansey, W. P.** (2000). Functional overlap of sequences that activate transcription and signal ubiquitin-mediated proteolysis. *Proc Natl Acad Sci USA* **97**, 3118-23.

75. **Senoo-Matsuda, N. and Johnston, L. A.** (2007). Soluble factors mediate competitive and cooperative interactions between cells expressing different levels of Drosophila Myc. *Proc Natl Acad Sci USA* **104**, 18543-8.

76. **Shen, J. and Dahmann, C.** (2005). Extrusion of cells with inappropriate Dpp signaling from Drosophila wing disc epithelia. *Science* **307**, 1789-90.

77. **Sheng, X. R., Brawley, C. M. and Matunis, E. L.** (2009). Dedifferentiating spermatogonia outcompete somatic stem cells for niche occupancy in the Drosophila testis. *Cell Stem Cell* **5**, 191-203.

78. **Simpson, P.** (1979). Parameters of cell competition in the compartments of the wing disc of Drosophila. *Dev Biol* **69**, 182-93.

79. **Slaughter, D. P., Southwick, H. W. and Smejkal, W.** (1953). Field cancerization in oral stratified squamous epithelium; clinical implications of multicentric origin. *Cancer* **6**, 963-8.

80. **Sotillos, S., Díaz-Meco, M. T., Moscat, J. and Castelli-Gair Hombría, J.** (2008). Polarized subcellular localization of Jak/STAT components is required for efficient signaling. *Curr Biol* **18**, 624-9.

81. **St Johnston, D.** (2002). The art and design of genetic screens: Drosophila melanogaster. *Nat Rev Genet* **3**, 176-88.

82. **Struhl, G. and Basler, K.** (1993). Organizing activity of wingless protein in Drosophila. *Cell* **72**, 527-40.

83. **Tamori, Y., Bialucha, C. U., Tian, A.-G., Kajita, M., Huang, Y.-C., Norman, M., Harrison, N., Poulton, J., Ivanovitch, K., Disch, L. et al.** (2010). Involvement of Lgl and Mahjong/VprBP in cell competition. *PLoS Biol* **8**, e1000422.

84. **Tanentzapf, G. and Tepass, U.** (2003). Interactions between the crumbs, lethal giant larvae and bazooka pathways in epithelial polarization. *Nat Cell Biol* **5**, 46-52.

85. **Tapon, N., Harvey, K. F., Bell, D. W., Wahrer, D. C. R., Schiripo, T. A., Haber, D. A. and Hariharan, I. K.** (2002). salvador Promotes both cell cycle exit and apoptosis in Drosophila and is mutated in human cancer cell lines. *Cell* **110**, 467-78.

86. **Tapon, N., Ito, N., Dickson, B. J., Treisman, J. E. and Hariharan, I. K.** (2001). The Drosophila tuberous sclerosis complex gene homologs restrict cell growth and cell proliferation. *Cell* **105**, 345-55.

87. **Tepass, U., Gruszynski-DeFeo, E., Haag, T. A., Omatyar, L., Török, T. and Hartenstein, V.** (1996). shotgun encodes Drosophila E-cadherin and is preferentially required during cell rearrangement in the neurectoderm and other morphogenetically active epithelia. *Genes Dev* **10**, 672-85.

88. **Tepass, U. and Knust, E.** (1990). Phenotypic and developmental analysis of mutations at the crumbs locus, a gene required for the development of epithelia in Drosophila melanogaster. *Roux's Archives of Developmental Biology* **199**, 189-206.

89. **Tepass, U., Tanentzapf, G., Ward, R. and Fehon, R.** (2001). Epithelial cell polarity and cell junctions in Drosophila. *Annu Rev Genet* **35**, 747-84.

90. **Tepass, U., Theres, C. and Knust, E.** (1990). crumbs encodes an EGF-like protein expressed on apical membranes of Drosophila epithelial cells and required for organization of epithelia. *Cell* **61**, 787-99.

91. **Tseng, A.-S. K., Tapon, N., Kanda, H., Cigizoglu, S., Edelmann, L., Pellock, B., White, K. and Hariharan, I. K.** (2007). Capicua regulates cell proliferation downstream of the receptor tyrosine kinase/ras signaling pathway. *Curr Biol* **17**, 728-33.

92. **Tyler, D. M., Li, W., Zhuo, N., Pellock, B. and Baker, N. E.** (2007). Genes affecting cell competition in Drosophila. *Genetics* **175**, 643-57.

93. **Udan, R. S., Kango-Singh, M., Nolo, R., Tao, C. and Halder, G.** (2003). Hippo promotes proliferation arrest and apoptosis in the Salvador/Warts pathway. *Nat Cell Biol* **5**, 914-20.

94. **Varelas, X., Samavarchi-Tehrani, P., Narimatsu, M., Weiss, A., Cockburn, K., Larsen, B. G., Rossant, J. and Wrana, J. L.** (2010). The crumbs complex couples cell density sensing to Hippo-dependent control of the TGF-β-SMAD pathway. *Developmental Cell* **19**, 831-44.

95. **Wodarz, A., Hinz, U., Engelbert, M. and Knust, E.** (1995). Expression of crumbs confers apical character on plasma membrane domains of ectodermal epithelia of Drosophila. *Cell* **82**, 67-76.

96. **Woods, D. F. and Bryant, P. J.** (1991). The discs-large tumor suppressor gene of Drosophila encodes a guanylate kinase homolog localized at septate junctions. *Cell* **66**, 451-64.

97. **Wu, S., Huang, J., Dong, J. and Pan, D.** (2003). hippo encodes a Ste-20 family protein kinase that restricts cell proliferation and promotes apoptosis in conjunction with salvador and warts. *Cell* **114**, 445-56.

98. **Xu, T. and Rubin, G. M.** (1993). Analysis of genetic mosaics in developing and adult Drosophila tissues. *Development* **117**, 1223-37.

99. **Xu, T., Wang, W., Zhang, S., Stewart, R. A. and Yu, W.** (1995). Identifying tumor suppressors in genetic mosaics: the Drosophila lats gene encodes a putative protein kinase. *Development* **121**, 1053-63.

100. **Yoo, S. J., Huh, J. R., Muro, I., Yu, H., Wang, L., Wang, S. L., Feldman, R. M. R., Clem, R. J., Müller, H.-A. J. and Hay, B. A.** (2002). Hid, Rpr and Grim negatively regulate DIAP1 levels through distinct mechanisms. *Nat Cell Biol* **4**, 416-24.

101. **Zhao, R. and Xi, R.** (2010). Stem cell competition for niche occupancy: emerging themes and mechanisms. *Stem Cell Rev* **6**, 345-50.

102. **Ziosi, M., Baena-López, L. A., Grifoni, D., Froldi, F., Pession, A., Garoia, F., Trotta, V., Bellosta, P., Cavicchi, S. and Pession, A.** (2010). dMyc functions downstream of Yorkie to promote the supercompetitive behavior of hippo pathway mutant cells. *PLoS Genet* **6**.