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The *archipelago* growth suppressor limits apoptosis via the Rb/E2f pathway and is transcriptionally regulated by the Notch receptor

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The *archipelago* growth suppressor limits apoptosis via the Rb/E2f pathway and is transcriptionally regulated by the Notch receptor

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B.S. Harding University, 2006

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Abstract

The *archipelago* growth suppressor limits apoptosis via the Rb/E2f pathway and is transcriptionally regulated by the Notch receptor

By Sarah C. Nicholson

The *Drosophila archipelago* (*ago*) growth suppressor encodes the substrate specificity component of a Skp-Cullin-F box (SCF) ubiquitin ligase that targets the Cyclin E and dMyc proteins for degradation. Its human ortholog, *Fbw7*, is commonly lost in cancers, suggesting that failure to degrade *ago/Fbw7* targets drives excess tissue growth. While much is known concerning the role of the *ago* gene in limiting growth and proliferation, there remain definite gaps in our knowledge of *ago* functions in the cell. The following questions are addressed here: (1) what is the mechanism through which the loss of *ago* reduces the size of the adult eye?, and (2) what signaling pathways are responsible for elevating *ago* expression in the morphogenetic furrow? We find that *ago* is required to restrict the apoptotic activity of the Rb/E2f pathway adjacent to the eye-specific morphogenetic furrow (MF). Thus, loss of *ago* results in elevated activity of *de2f1* (a transcription factor necessary for progression into S-phase), expression of the pro-death dE2F1 targets *hid* and *rpr*, and high rates of apoptosis, ultimately leading to a smaller adult organ. These phenotypes are modulated by *rbf1*, *de2f1*, *hid*, and the *rbf1*/*de2f1* regulators *cyclin E* and *dacapo* but are independent of *dp53*. These data show that *ago* loss requires a collaborating block in cell death to efficiently drive tissue overgrowth. In light of the apparent requirement for the Ago protein to limit cell death at the MF, we next set out to identify developmental signals that regulate *ago* expression in cells within the MF. Expression of *ago* mRNA and protein is induced by passage of the MF, and the *Hedgehog* (*Hh*) and *Notch* (*N*) pathways are required elements of this inductive mechanism. Cells mutant for *N* pathway components, or *Hh* defective cells that express reduced levels of the Notch ligand Delta, fail to upregulate *ago* transcription in the region of the MF; reciprocally, ectopic N activation in eye discs induces expression of *ago* mRNA. Furthermore, we find that the failure to upregulate *ago* in *N* pathway mutant cells correlates with accumulation of the SCF-Ago target Cyclin E in the area of the MF, and this can be rescued by re-expression of *ago*. This N/Ago/Cyclin E link represents a significant new cell-cycle regulatory mechanism in cells of the developing eye.

The *archipelago* growth suppressor limits apoptosis via the Rb/E2f pathway and is transcriptionally regulated by the Notch receptor

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being silly and making me laugh, one of life's most effective medicines. You are an amazing sister and friend, and you have always kept me a part of your own family and the lives of your sweet boys, no matter how far apart we were. Dan, thanks for supporting me in my academic endeavors and telling me that biology was for 'cool' people. You have always been such an incredible sounding board for me in my times of painful decision-making, and you are so good at focusing on someone else's problems as if they were your own. Dave, thank you for being my best friend and support in middle school and highschool and for encouraging me to pursue research. I have so often found myself trying to be like you, and your constant affirmation meant more to me than anything. To my family, it's difficult to sum up my appreciation in a few short words, but know that as youngest daughter and little sister, I have looked up to all of you more than you know, trying to follow in your footsteps in many ways, and striving to blaze my own trail in a way that would make you all proud. I will be forever grateful to God for blessing me with the support system that I have in all of you.

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Table of Contents

Chapter 1: Introduction **1**

I.A. Introduction **2**

I.B. Organ size control **5**

I.C. The fly eye as a model for growth control **10**

I.D. The morphogenetic furrow **14**

I.E. Identification of novel growth-regulatory genes in the fly eye **17**

I.F. *archipelago* **21**

I.G. *ago* patterns and functions in the eye **27**

I.H. The Hedgehog pathway **29**

I.I. The Notch pathway **33**

I.J. Objectives **38**

I.K. References **39**

Chapter 2: The *archipelago* Tumor Suppressor Gene Limits Rb/E2F-Regulated Apoptosis in Developing *Drosophila* Tissues **52**

II.A. Abstract **54**

II.B. Introduction **56**

II.C. Results **59**

II.C1. Loss of *ago* has different effects on the size of tissues in the adult head. 59

II.C2. Blocking death increases ago mutant eye size 64

II.C3. *ago* mutations elevate dE2f activity in the furrow 67

II.C4. *de2f1* and *rbf1* control apoptosis of *ago* cells 72

II.C5. Relationship between *ago* and apoptotic regulators 80

II.D. Discussion 88

II.E. Experimental Procedures 92

II.E1. Genetics 92

II.E2. Eye measurements 93

II.E3. Immunohistochemistry & Microscopy 93

II.E4. Real Time RT-PCR 93

II.E5. Cell culture 94

II.F. Acknowledgements 95

II.G. References 96

Chapter 3: *Notch*-dependent expression of the *archipelago* ubiquitin ligase subunit in the *Drosophila* eye 100

III.A. Abstract 102

III.B. Introduction 104

III.C. Results 107

III.C1. *ago* protein levels and promoter activity are elevated within the MF ..107

III.C2. Hedgehog and Notch pathway mutations block Ago induction at the MF 112

III.C3. The *ago* promoter is *N*-responsive 120

III.C4. The induction of *ago* by the *N* pathway is required to limited levels of the SCF-Ago target Cyclin E 127

III.D. Discussion 132

III.E. Experimental Procedures **137**

III.E1. Genetics 137

III.E2. Immunohistochemistry and Microscopy 138

III.E3. Real-Time Reverse Transcriptase-PCR 138

III.E4. S2R+ cell manipulations 139

III.E5. Molecular Cloning 139

III.F. Acknowledgements **140**

III.G. References **141**

Chapter 4: Future Experiments and Concluding Remarks **147**

IV.A. Summary **148**

IV.B. Screen for modifiers of *ago* 'small eye' **151**

IV.C. Small molecule testing in *Drosophila* **154**

IV.D. Defining a N/Ago feedback loop **157**

IV.E. Notch-dependent *ago* expression patterns in the eye **160**

IV.F. Ago/N studies in human cells **167**

IV.G. Concluding Remarks **169**

IV.H. References **170**

Index of Figures

Chapter 1: Introduction

Figure I.1. The cell death pathway in *Drosophila*

Figure I.2. Larval eye imaginal disc

Figure I.3. The RB/E2F pathway

Figure I.4. The *eyelessFLP/FRT* system of mitotic recombination

Figure I.5. The *ago* mosaic eye

Figure I.6. The SCF-Archipelago complex

Figure I.7. The Hedgehog pathway

Figure I.8. Hedgehog activity in the eye imaginal disc

Figure I.9. The Notch pathway

Figure I.10. Notch activity in the eye imaginal disc

Chapter 2: The *archipelago* Tumor Suppressor Gene Limits Rb/E2F-Regulated Apoptosis in Developing *Drosophila* Tissues

Figure II.1. *ago* loss reduces eye size and elevates apoptosis

Figure II.2. Blocking death of *ago* cells produces enlarged organs

Figure II.3. *ago* restricts dE2f activity

Figure II.4. *de2f1* transactivation is required for apoptosis but not the proliferative advantage of *ago* mutant cells

Figure II.5. Excess *rbf1* suppresses the cell death of *ago* mutant cells in the MF

Figure II.6. Modification of *ago* eye size by cell cycle regulators and signaling components

Figure II.7. Expression of *hid* and *reaper* is elevated in *ago* mutant tissue.

Figure II.8. Death of *ago* mutant eye cells is sensitive to extracellular signaling pathways

Figure II.S1. Patterns of cell division in *ago* mutant and *ago,H99* double-mutant MF cells

Figure II.S2. Caspase activation in *ago*^{3} mutant cells

Figure II.S3. Blocking death of *ago*^{1} mutant cells produces enlarged organs

Figure II.S4. Induction of C3 by expression of *cycE* or *dMyc*

Figure II.S5. Death of *ago*^{1} mutant eye cells is insensitive to dominant-negative p53

Chapter 3: *Notch*-dependent expression of the *archipelago* ubiquitin ligase subunit in the *Drosophila* eye

Figure III.1. Ago accumulates at the MF in a subset of Ato-positive and Elav-positive cells. *ago* promoter activity is detected several rows of cells anterior to the wave of Elav-positive clusters.

Figure III.2. Hh and N pathway mutants cause a decrease in Ago levels at the MF.

Figure III.3. N is necessary for elevated Ago expression and patterning at the MF.

Figure III.4. Elevated *Dl* restores Ago in *smo* mutant cells.

Figure III.5. Hedgehog and Notch pathway mutants cause a decrease in *ago* promoter activity at the MF.

Figure III.6. The *ago* promoter is responsive to changes in levels of *N* signaling.

Figure III.7. The decrease in Ago levels in *N* pathway mutant clones results in a build-up of the SCF-Ago substrate Cyclin E.

Figure III.S1. *Dl/Ser* mutations block the pulse of Ago expression at the MF.

Figure III.S2. The effect of *N* loss on dMyc levels at the MF.

Chapter 4: Future Experiments and Concluding Remarks

Figure IV.1. *ago* is required to restrict the apoptotic activity of the Rb/E2f pathway adjacent to the eye-specific MF

Figure IV.2. Deficiency screen for modifiers of the ‘small eye’ phenotype

Figure IV.3. Small molecule testing in *ago* mutant eyes

Figure IV.4. The N/*ago* feedback loop

Figure IV.5. Regulation of *ato* expression in the eye disc

Figure IV.6. Loss of N activity results in an increase in *ago>Gal4* reporter transcription

Figure IV.7. Ago protein expression and *ago-gal4* enhancer trap activity in the *Drosophila* eye disc

Chapter 1: Introduction

I.A. Introduction

Understanding the molecular basis for human disease is the driving force of scientific research today. With the complete sequencing of the human genome and the development of high-throughput methods of tracking genes, the genetic component of many of these diseases can be elucidated. However, uncovering the function of these novel genes and the role they play in disease and in normal development proves to be a more difficult task. To answer some of these questions scientists often use genetic model systems such as *Drosophila melanogaster*. The use of model systems allows the study of disease genes within the context of developmental and tissue-specific signaling events, and the knowledge gained from these studies leads to advances in understanding and potentially improved treatment.

Cancer is a disease caused by unchecked growth of a group of cells by one or more of the following circumstances: increased cell division, increased cell growth, decreased cell death. These conditions are the cause of cancer at the most basic level, and the combined effects of these are reflected in the size of the tumor or size of the tumorous organ. In this way, organ size serves as a model for studying cancer, where increased organ size represents unchecked, or tumor-like, growth. In essence, organisms that do not get cancer in the conventional sense can nevertheless be used to study the disease by observing changes in organ size. Because *Drosophila* have already been developed as a powerful genetic model

system, they have been used as such to make significant strides in our understanding of cancer. For example, a 1982 review article catalogued 25 *Drosophila* genes that caused tumorous growths in animals homozygous for a loss of function allele (GATEFF 1982). A second wave of discovery of tumor-suppressor genes arose from the ability to generate clones of mutant tissue in *Drosophila* imaginal discs. This technique was used to isolate mutations in two main types of tumor suppressor genes: hyperplastic, which denotes genes that, when mutant, lead to imaginal disc overgrowth without disrupting the overall architecture of the tissue, and neoplastic, which denotes genes that typically play a role in epithelial polarity and, when mutant, cause the formation of disorganized, invasive, and transplantable tumors. Some of the hyperplastic tumor-suppressor genes uncovered include *hippo* (HARVEY *et al.* 2003), *Tsc1*, and *Tsc2* (TAPON *et al.* 2001), and *archipelago* (MOBERG *et al.* 2001), along with the hyperplastic tumor-suppressor gene *tsg101* (MOBERG *et al.* 2005).

In particular, the developing fly eye is an especially good system for studying growth control. The size of the whole eye can be used as a read-out of the activity of all the growth control pathways combined, and the highly organized pattern of ommatidia is sensitive to slight changes in signaling levels, which are manifested by an easily observable "roughness" of the adult eye. Furthermore, the eye is completely dispensable for viability and fertility, allowing the isolation and recovery of mutations that produce extreme growth phenotypes. The fly eye was used in a genetic screen to uncover the novel growth control gene, *archipelago* (*ago*), which codes for the substrate specificity component for a Skp/Cullin/F-box (SCF) E3

ubiquitin ligase (SCF-Ago) and is responsible for degrading the G1/S cell-cycle regulator Cyclin E (MOBERG *et al.* 2001) and the growth regulator dMyc (MOBERG *et al.* 2004). Upon discovery in *Drosophila*, *ago* was soon found to have a human orthologue, *Fbw7*, which was subsequently implicated in a variety of tumor types such as colorectal (RAJAGOPALAN and LENGAUER 2004), endometrial (SPRUCK *et al.* 2002), and ovarian (MOBERG *et al.* 2001). Given the potential role this gene plays in tumorigenesis, a thorough knowledge of how it controls growth and how it is itself regulated is useful for understanding the pathology of *ago* mutant tumors and tumors with other lesions that affect *ago* levels. While much is known about the role of *ago* in controlling cell growth and cell division, little is known about its role in controlling cell death in flies or humans. Furthermore, the signals and pathways that regulate the transcription of the *ago* gene have not been elucidated. These questions are the subject of the work described here.

I.B. Organ size control

Highly coordinated and complex networks of signals govern the decision a cell makes to grow (accumulate mass), divide, or die. These signals act in concert to determine future organ size in developing animals and to maintain constant organ size in adult animals. While many of these signaling pathways have been studied extensively, the focus of this discussion are the proteins that mediate signals downstream of Ago, namely Cyclin E and dMyc as well as the canonical cell death pathway.

Because cell division and cell growth are tightly correlated, the signals which govern these two processes often overlap. The mammalian Cyclin/Cyclin-dependent kinase (Cdk) complexes are responsible for moving cells through the cell cycle in response to growth factors (SHERR and ROBERTS 1999), and as such are conventionally viewed as cell division proteins. Interestingly, Cyclin D, when expressed with its partner, Cdk4, increases the rate of growth in cycling and post-mitotic cells (DATAR *et al.* 2000). In contrast, overexpression of Cyclin E, which complexes with Cdk2, shortens G1 by promoting S phase entry, yet it does not cause an increase in growth (NEUFELD *et al.* 1998), making it primarily a cell division protein. Inhibition of the Cyclin E/Cdk2 complex by overexpression of either *p21*, a Cyclin-dependent kinase inhibitor (CKI), or a dominant negative form of Cdk2 results in arrest at the G1-phase of the cell cycle (HARPER *et al.* 1995; LUNDBERG and WEINBERG 1998; VAN DEN HEUVEL and HARLOW 1993; XIONG *et al.* 1993). Furthermore, genetic analysis demonstrates that these interactions are conserved in flies. For example, *Drosophila*

Cyclin E is required for S-phase entry during development (KNOBLICH *et al.* 1994), and ectopic expression of the single *Drosophila* Cyclin-dependent kinase inhibitor (CKI), *dacapo (dap)*, delays S-phase entry (DE NOOIJ *et al.* 1996). Interestingly, the phenotype of ectopic *dap* expression is enhanced by heterozygosity for *cyclin E*, but not for *cyclin A* or *B*, suggesting that *cyclin E* represents a critical target of the cell cycle inhibitory effects of *dacapo* (DE NOOIJ *et al.* 1996). These data show that *cyclin E* is critical for progression through cell division, and *dap* is sufficient to inhibit this activity.

One of many genes that plays a role in controlling cell growth is the gene encoding the *Drosophila* homolog of the Myc family of proto-oncogenes, *dmyc* (GALLANT *et al.* 1996), which was known to biologists long before vertebrate *Myc* genes. In 1953, a mutation, *diminuitive,* was described that results in a small adult body size, disproportionally small bristles, and female sterility (BRIDGES 1935); many years later molecular cloning revealed that this gene was *dMyc* (GALLANT *et al.* 1996; SCHREIBER-AGUS *et al.* 1997). Subsequent studies showed that reduction of Myc levels decreases the size of larval diploid cells (JOHNSTON *et al.* 1999). Conversely, ectopic expression of *dmyc* in clones of the wing imaginal disc increases the size of the clones and of the cells constituting these clones, without affecting cell number (JOHNSTON *et al.* 1999). A member of the BHLHZ (basic, helix-loop-helix, leucine zipper) transcription factor famliy, Myc promotes growth by activating the expression of its targets, which include genes coding for components of the translation machinery and genes coding for ribosomal proteins (BOON *et al.* 2001;

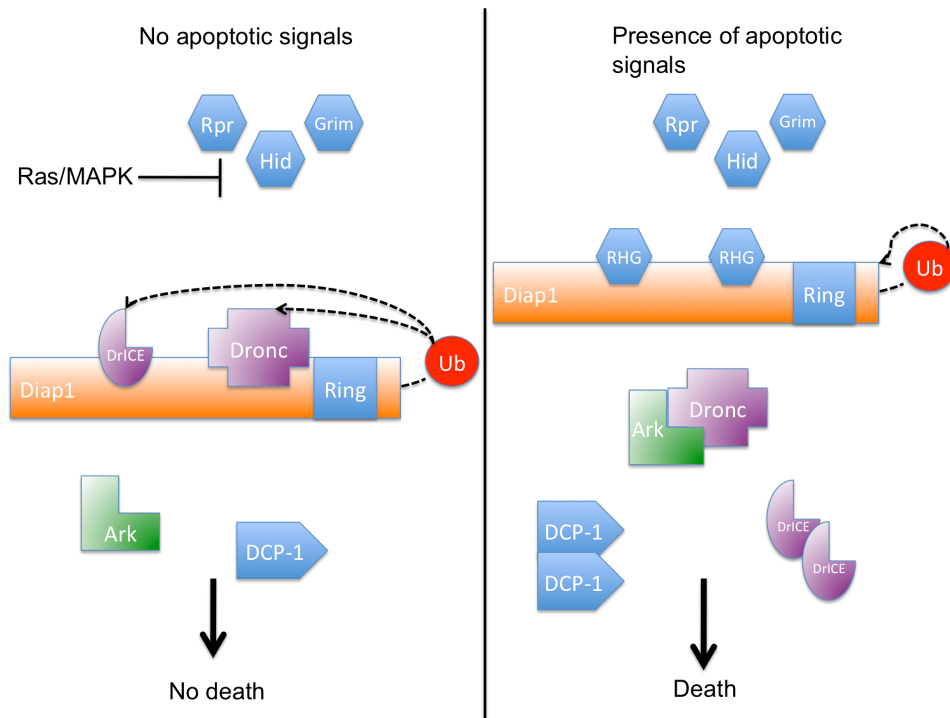
KIM *et al.* 2000). Thus, Myc activation presumably leads to increased growth through a general increase in cellular translational capacity.

A distinct set of proteins have evolved to conduct the signals needed to execute apoptosis, or programmed cell death (Figure I.1). The main players in apoptosis are a group of proteins called caspases, Cys-proteases that cleave specifically after Asp residues. The caspases critical for apoptosis in *Drosophila* are the Caspase-9 orthologue Dronc (DORSTYN *et al.* 1999), an initiator caspase, and the Caspase-3 orthologues Dcp-1 (SONG *et al.* 1997) and DrICE (FRASER and EVAN 1997; FRASER *et al.* 1997), the effector caspases. The *Drosophila* inhibitor of apoptosis protein (Diap1) (HAY *et al.* 1995) binds to caspases to inhibit their activity in the absence of pro-death signals (MEIER *et al.* 2000; ZACHARIOU *et al.* 2003). Apoptotic stimuli initiate the release of the caspases by the binding of the pro-death proteins Reaper (WHITE *et al.* 1994), Hid (GRETHER *et al.* 1995), and Grim (CHEN *et al.* 1996) (RHG proteins) to Diap1 (GOYAL *et al.* 2000; WANG *et al.* 1999; ZACHARIOU *et al.* 2003) resulting in its ubiquitin-mediated degradation (HOLLEY *et al.* 2002; RYOO *et al.* 2002; YOO *et al.* 2002). Once Dronc is released, it binds the Apaf-1 orthologue Ark, an adaptor protein, to form the apoptosome (DORSTYN *et al.* 1999; KANUKA *et al.* 1999; RODRIGUEZ *et al.* 1999; YU *et al.* 2006; ZHOU *et al.* 1999) which subsequently cleaves and activates Dcp-1 and DrICE (HAWKINS *et al.* 2000). These events result in the apoptosis and removal of the cells in which they occur.

Together, the pathways described above along with others that control cell division, cell growth, and apoptosis govern the ability of organs and organisms to

reproducibly grow to the same size. These pathways also allow different organisms to grow to different final sizes and at different growth rates. The genetically programmed developmental pathways that control rates of growth, division, and death remain elusive, and an imbalance in these signals can result in organ overgrowth and ultimately in tumorigenesis. By targeting both Cyclin E and dMyc for degradation, *ago* has the ability to simultaneously regulate both cell division and cell growth. However, no direct link has yet been made between *ago* and the cell death pathways.

Figure I.1. The cell death pathway in *Drosophila*



In the absence of apoptotic signals, Diap1 binds to the caspases Dronc and DrICE, and the RING domain of Diap1 ubiquitylates the bound caspases. Caspase ubiquitylation results in the inactivation of the caspases, however, without proteasome-mediated degradation. In the presence of apoptotic signals, RHG proteins displace caspases from Diap1. RHG proteins also stimulate auto-ubiquitylation and proteasomal degradation of Diap1. Dronc is now free to complex with Ark, forming the apoptosome and leading to activation of Dcp-1 and DrICE for cell death induction. (Adapted from (XU *et al.* 2009))

I.C. The fly eye as a model for growth control

Because of the ease of measuring organ size, the wealth of genetic tools developed for use in *Drosophila*, and the intricate signaling networks which make the finely tuned pattern of ommatidia in the adult eye sensitive to subtle genetic changes, the fly eye serves as a useful model for studying growth control. Furthermore, loss of *ago* in the fly eye results in a phenotype that is distinct from the phenotypes seen in any other tissue upon *ago* loss, suggesting that *ago* may play a unique role in eye development. The insect compound eye develops from an unpatterned simple epithelium through a series of highly ordered cell fate decisions and morphogenetic movements.

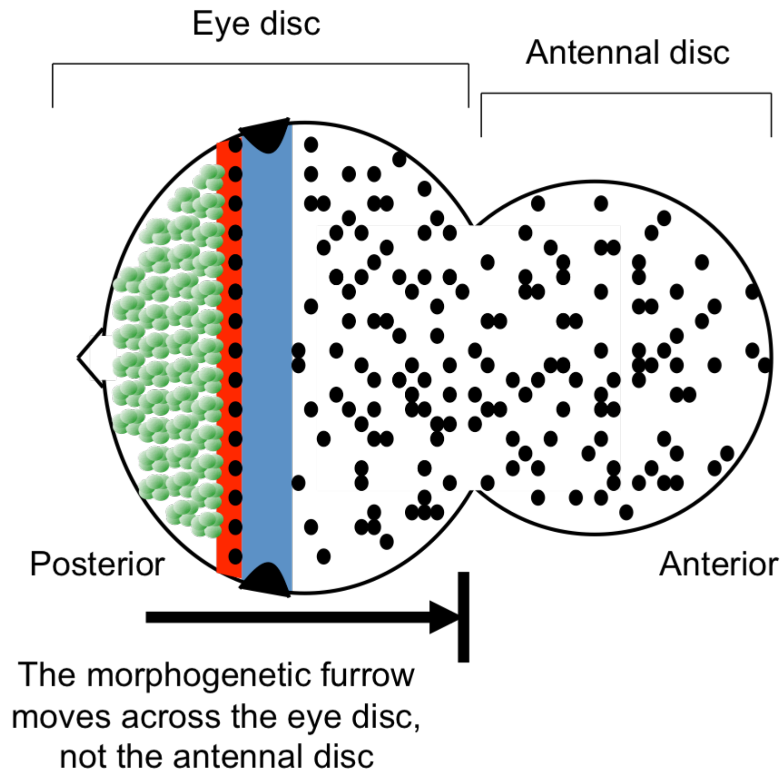
The eye, along with every other adult fly structure, is derived from a flat epithelial sac of cells called an imaginal disc. The eye disc grows by cell division until the middle of the third and final stage of larval development (third instar), when each eye contains ~10,000 cells (WOLFF T 1993). In early third instar, cells at the posterior of the eye disc begin to differentiate; this transition is accompanied by the formation of a dorsoventral groove called the morphogenetic furrow (MF), which moves anteriorly across the eye disc with post-mitotic, differentiating cells behind the MF and asynchronously dividing cells in front of it (Figure I.2) (READY *et al.* 1976; TOMLINSON and READY 1987; WOLFF and READY 1991). Differentiation occurs in a highly patterned manner beginning with the specification of the R8 photoreceptor neurons at uniformly spaced positions. The R8 is the founding cell for each

ommatidium, which is the individual unit of the fly eye and will ultimately contain 8 mature photoreceptor cells. Sequential specification of the other seven photoreceptors (R1-R7) occurs in four steps that are timed approximately four hours apart (TOMLINSON 1988). This process is coordinated with the movement of the MF. Cells in the MF arrest in early G1 (FINLEY *et al.* 1996), and a subset of these cells never re-enter the cell cycle but differentiate into the first five photoreceptor cells to be specified in each cluster (READY *et al.* 1976). The rest of the cells enter one final round of mitosis called the "second mitotic wave" (SMW), which occurs just posterior to the MF (BAKER and YU 2001; READY *et al.* 1976; WOLFF and READY 1991). These cells differentiate to provide the remaining photoreceptors and other cell types needed for each ommatidial cluster (READY *et al.* 1976). By the end of the larval stage, ~26 rows of ommatidia have formed. During pupation, the remaining rows of ommatidia are generated, and the cellular architecture of the eye is essentially complete with a total of 750 mature ommatidia (READY *et al.* 1976).

The events described above may serve as a template for genetic manipulation in studies of growth control genes, with the phenotypic outcome providing valuable information concerning the function of the gene in question. Several eye-specific promoters have been used to drive expression of a transgene in the entire eye or a specific subset of eye cells, allowing for a more thorough investigation into cell-specific gene function. Furthermore, because the eye is dispensable for survival, genetic manipulations in the eye can be done freely without altering viability. Also, the MF of the eye imaginal disc offers the unique ability to simultaneously observe populations of cells synchronized in various stages of the cell cycle and

differentiation according to their spatial location in the eye disc, making the *Drosophila* eye an ideal place to explore gene functions in growth control pathways. For this reason, we utilize the eye for our studies of *ago* function and regulation.

Figure I.2. Larval eye imaginal disc



The larval eye disc is segregated into distinct phases of retinal development. Mitotic events (black circles) occur in two phases: (1) cells in the anterior portion of the eye disc divide asynchronously, and (2) cells just behind the morphogenetic furrow (MF, blue bar) enter one final round of division, the second mitotic wave (SMW, red bar). The MF moves across the eye disc from posterior to anterior, and the cells within the MF are arrested in the G1 phase of the cell cycle. Posterior to the MF, cells begin to differentiate (green circles) and form clusters that will give rise to mature ommatidia.

I.D. The morphogenetic furrow

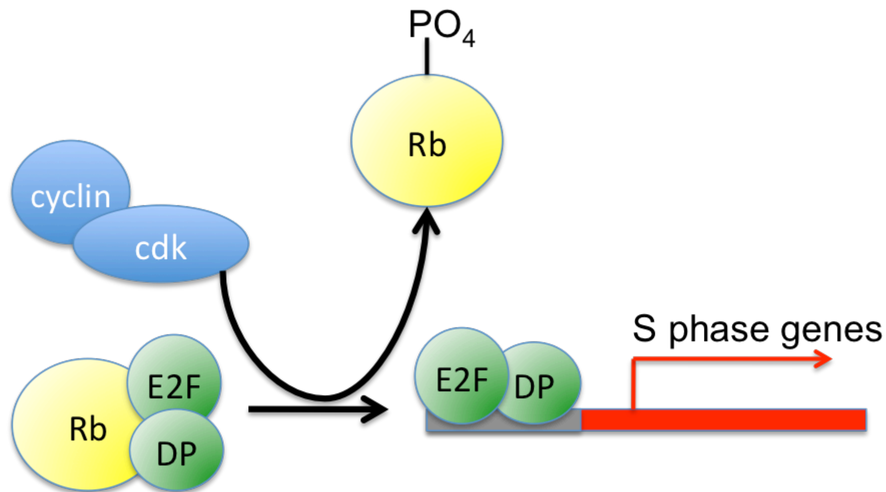
Levels of *ago* mRNA are highest at the MF (MOBERG *et al.* 2001), suggesting that *ago* plays a role in regulating the molecular events occuring within cells of the MF. These cells undergo a unique set of molecular events involving the E2F pathway that allows them to arrest in early G1. The E2F family of transcription factors is responsible for activating the transcription of many genes necessary for progression into S-phase (ISHIDA *et al.* 2001; REN *et al.* 2002). Consequently, levels of E2F-dependent transcription control the cell's passage through the cell division cycle. Interestingly, *Drosophila* E2F1 (dE2F1) is present at very high levels in the MF (MOON *et al.* 2006), although these cells do not enter S-phase, presumably due to the presence of the dE2F1 repressor, RBF1.

The *Drosophila* E2F pathway (Figure I.3) is much simpler than the mammalian network of E2F reactions, and proper control of the pathway's activity is important for G1 arrest at the MF. The *Drosophila* genome contains an activator E2F (dE2F1) (DYNLACHT *et al.* 1994; OHTANI and NEVINS 1994) and a repressor E2F (dE2F2) (SAWADO *et al.* 1998). Both dE2F1 and dE2F2 associate with the protein dDP (DYNLACHT *et al.* 1994) to form heterodimeric complexes that bind to DNA in a sequence specific manner. One well-characterized E2F antagonist is the Retinoblastoma tumor suppressor protein (pRb), which is the prototypical member of the "pocket protein" family. pRB and the other members of this family, p107 and p130, share a domain, the pocket, which is responsible for binding E2F. *Drosophila*

has two pRB-family members, RBF1 (DU *et al.* 1996a) and RBF2 (STEVAUX *et al.* 2002). While mutations in *de2f1* eliminate a program of G1-S-phase transcription (DURONIO and O'FARRELL 1994) and reduce DNA replication and cell proliferation (DURONIO *et al.* 1995; ROYZMAN *et al.* 1997), *rbf1* mutant clones show the opposite phenotype, namely deregulated expression of E2F target genes, ectopic S phases, and elevated apoptosis (DATAR *et al.* 2000; MOON *et al.* 2006). Similarly, overexpression of *de2f1* in transgenic animals induces ectopic S phases and apoptosis (ASANO *et al.* 1996; DU *et al.* 1996b). Clearly, *rbf1* and *de2f1* act in opposite directions to influence patterns of cell division in the eye.

While RBF1, which is present throughout the eye disc, associates with and antogonizes dE2F1 (DU *et al.* 1996a), this repression can be relieved through phosphorylation of RBF1 by cyclin/cdk complexes acting in a sequential manner, which leads to the progressive disassembly of the RBF1/dE2F1 complex (HARBOUR *et al.* 1999) (Figure I.3). Cyclin E activity, which is largely responsible for relieving RBF1-mediated repression of dE2F1, must be turned off for cells to remain in G1 as they do in the MF. Accordingly, levels of Cyclin E drop within the MF and spike in the second mitotic wave (SMW), where cells re-enter the cell cycle (RICHARDSON *et al.* 1995). Loss of this tight E2F regulation in the MF results in cell death in the anterior portion of the MF, where cells are particularly sensitive to E2F-induced apoptosis because of a decrease in EGFR signaling (MOON *et al.* 2006). The network of signals responsible for maintaining low levels of Cyclin E at the MF has not been fully elucidated, and the result of losing this tight regulation is not fully understood.

Figure I.3. The RB/E2F pathway



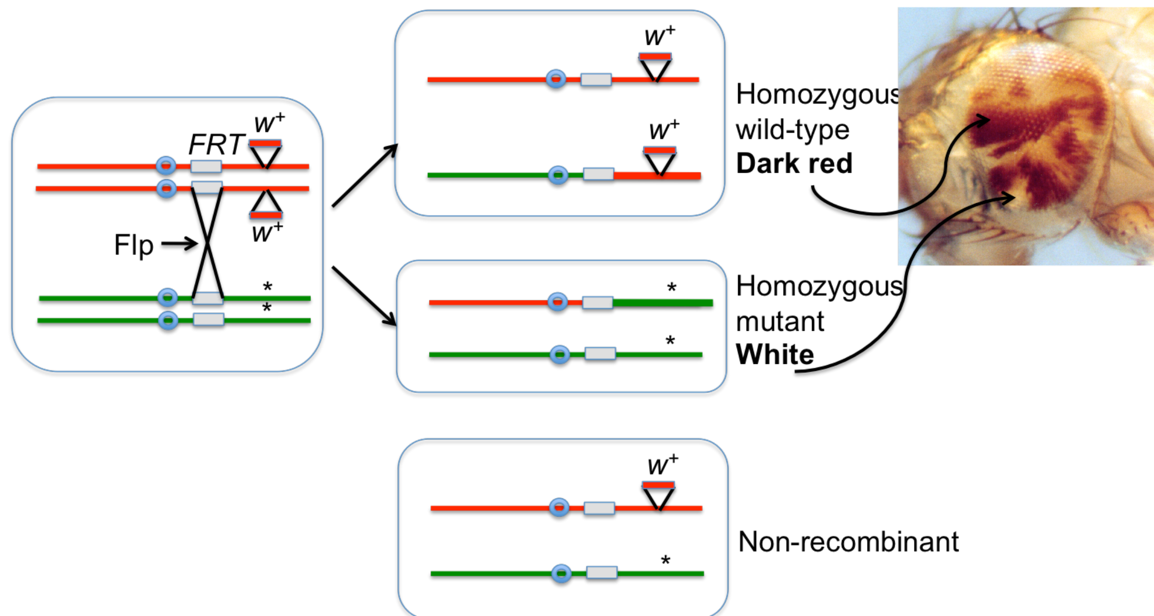
The E2F/DP complex binds the promoters of its target genes, activating these genes and initiating entry into S phase. When Rb is bound to E2F/DP, the complex acts as a repressor for its target genes. Rb phosphorylation by cyclin/cdk complexes releases E2F/DP, reversing the repression and activating S phase genes.

I.E. Identification of novel growth-regulatory genes in the fly eye

A screen was previously performed in the fly eye to uncover novel regulators of growth (MOBERG *et al.* 2001). Recessive mutations which conferred a growth advantage on mutant tissue (marked 'white' by the lack of eye pigment) over wild-type tissue (marked 'red' by the presence of pigment) were further characterized. The screen utilized ethyl methanesulfonate (EMS) chemical mutagenesis and the *eyelessFLP/FRT* system of mitotic recombination to generate eyes "mosaic" for homozygous wild-type and homozygous mutant tissue in an otherwise heterozygous fly (GOLIC and LINDQUIST 1989). 'FLPing' occurs when *FRT* sites on homologous chromosomes undergo mitotic recombination driven by the FLP recombinase protein which is expressed in a tissue specific manner. The wild-type and mutant chromosomes are thus segregated during mitosis into different daughter cells, each of which then give rise to a clone of cells of homogenous genotype (Figure I.4). Competition between 'red' wild-type cells and 'white' mutant cells results in the final ratio of red-to-white tissue in the adult eye. While wild-type chromosomes result in a 50/50 ratio of red-to-white tissue, mutations in growth suppressor genes on the 'white' chromosome cause the 'white' tissue to overgrow and produce an eye with more 'white' tissue than 'red'. Mutations conferring this phenotype, referred to as a 'white-over-red', were placed into complementation groups, and each was mapped to a gene in the *Drosophila* genome.

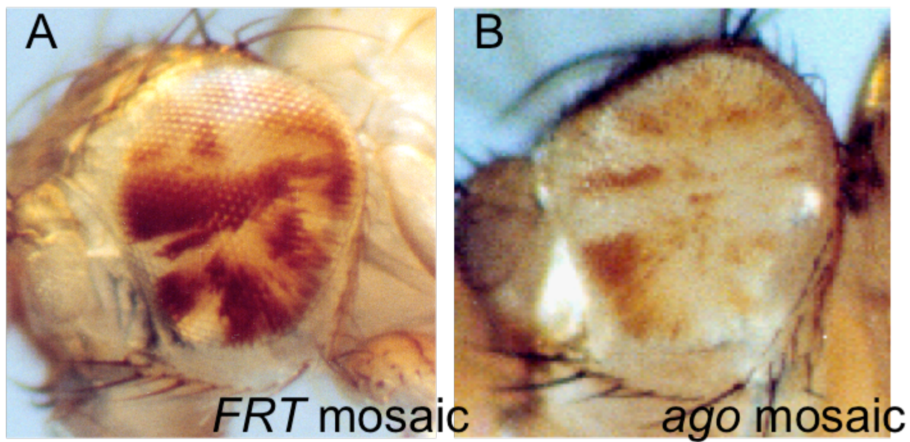
Several of the genes uncovered in this manner were further characterized and have contributed significantly to the field of growth regulation and disease models. One such gene, *hippo* (*hpo*), encodes a protein kinase most related to mammalian Mst1 and Mst2, that is necessary to prevent accumulation of Cyclin E and DIAP1. Hpo interacts physically and functionally with a serine/threonine kinase Warts (Wts) and a WW domain containing protein Salvador (Sav) to regulate growth, proliferation, and apoptosis in *Drosophila* (HARVEY *et al.* 2003). Another study using this screening technique isolated mutations in *Drosophila Tsc1* and *Tsc2*, the homologues for the mammalian genes (TSC1 and TSC2) mutated in the inherited human disease tuberous sclerosis. As negative regulators of mammalian target of rapamycin (mTOR), *Tsc1* or *Tsc2* cause an identical mutant phenotype characterized by enhanced growth and increased cell size with no change in ploidy (TAPON *et al.* 2001). The HMG-box protein Capicua (Cic), which restricts cell growth in *Drosophila* imaginal discs and is downregulated by Ras signaling, was also discovered using the *eyelessFLP/FRT* screening method. The characterization of Cic provided an important insight into how growth is promoted by the Ras pathway, which is increased in many cancers (TSENG *et al.* 2007). Lastly, one complementation group from the screen mapped to the *archipelago* gene (MOBERG *et al.* 2001) (Figure I.5), which has led to the elucidation of several significant growth phenomena in the fly eye and is the focus of this study.

Figure I.4. The *eyelessFLP/FRT* system of mitotic recombination



By placing *FLP* recombinase under the control of the *eyeless* enhancer, *FLP/FRT*-mediated recombination can be used to generate homozygous mutant clones in the eyes of flies that are otherwise heterozygous. The non-mutant chromosome (red) is marked by a *mini-white* transgene (w^+); therefore, clones of cells that are homozygous wild-type appear dark red in the adult eye due to the presence of two w^+ copies. The asterisk indicates a mutation; homozygous mutant tissue appears white in the adult eye due to lack of w^+ transgene. (Adapted from (ST JOHNSTON 2002))

Figure I.5. The *ago* mosaic eye



Adult eyes mosaic for the wild-type *FRT* chromosome (A) or mutant *ago* (B). Relative to the *FRT* control, the *ago* mosaic eye has an over-representation of white, mutant tissue (MOBERG *et al.* 2001).

I.F. *archipelago*

The eukaryotic protein degradation pathway involves ubiquitin (Ub) modification of substrates targeted for proteasome-dependent degradation. The E1 ubiquitin-activating enzyme, E2 ubiquitin-conjugating enzyme, and E3 ubiquitin ligase comprise the enzymes responsible for transferring Ub to targeted substrates in a process called ubiquitination. The multi-subunit complex of Skp, Cullin, and F-box proteins (SCF) is a class of E3 ubiquitin ligases which was later found to contain a fourth core-component, the RING box protein (Figure I.6, reviewed in (WELCKER and CLURMAN 2008)). The *archipelago* gene (*ago*, also known as *Fbw7*, *sel-10*, *cdc4*) codes for the F-box component, which contains a conserved Skp interaction domain named the F-box. Ago substrate recognition is mediated by a WD domain consisting of eight tandem WD(tryptophan/aspartic acid)-repeats. The substrates of Ago are recruited to the SCF complex where they are polyubiquitinated and targeted for proteosomal degradation (reviewed in (WELCKER and CLURMAN 2008)). The degradation of Cyclin E and dMyc by Ago contributes to its ability to limit mitotic activity, but the diverse developmental roles of Ago are the consequence of additional targets. For example, *ago* is required for the post-mitotic shaping of the *Drosophila* embryonic tracheal system, and Ago acts in this tissue by restricting levels of the bHLH-PAS domain transcription factor Trachealess (Trh) (MORTIMER and MOBERG 2007). Also, depletion of both Ago and another F-box protein, Supernumery limbs (Slimb), results in the ectopic accumulation of the glial

regulatory factor, glial cell missing/glial cell deficient (Gcm/Glide), resulting in increased glial cell numbers due to failure of certain glial cell lineages to exit the cell cycle (HO *et al.* 2009). These substrates comprise the proteins known to be targeted for degradation by Ago in flies, but additional targets have been uncovered in vertebrates, with the focus of vertebrate studies being discovery of pro-growth factors limited by mammalian *ago*, *Fbw7*.

Studies of *Fbw7* in multiple genetic model systems have contributed to the collective knowledge of its function. The first member of the *Fbw7* gene family was identified in a classic genetic screen for cell division cycle mutants in budding yeast, and named *Cdc4* (HARTWELL *et al.* 1973). *Cdc4* was found to function in the regulation of the disposal of a Cyclin-dependent kinase inhibitor (CKI) in a phosphorylation-dependent manner (FELDMAN *et al.* 1997; SCHWOB *et al.* 1994; SKOWYRA *et al.* 1997; VERMA *et al.* 1997a; VERMA *et al.* 1997b). Several additional substrates of Cdc4 were soon reported (BENITO *et al.* 1998; DRURY *et al.* 1997; HENCHOZ *et al.* 1997; MEIMOUN *et al.* 2000; PERKINS *et al.* 2001), and it became apparent that Cdc4 binds these substrates through a conserved phospho-epitope, which was later described as the Cdc4 phospho-degron (CPD) (KOEPP *et al.* 2001; NASH *et al.* 2001; PERKINS *et al.* 2001). The *Cdc4* human orthologue, *Fbw7*, and nematode orthologue, *sel-10*, were subsequently identified and found to function in a similar manner. Studies in flies showed that the *Drosophila* orthologue, *ago*, is a negative regulator of Cyclin E (MOBERG *et al.* 2001), and degradation of Cyclin E in vertebrates was found to depend on its phosphorylation by either Cdk2 or Glycogen synthase kinase 3 (GSK3)

(Clurman *et al.* 1996; Won and Reed 1996). As of yet, Ago-dependent degradation of Cyclin E in *Drosophila* has not been shown to require phosphorylation of Cyclin E.

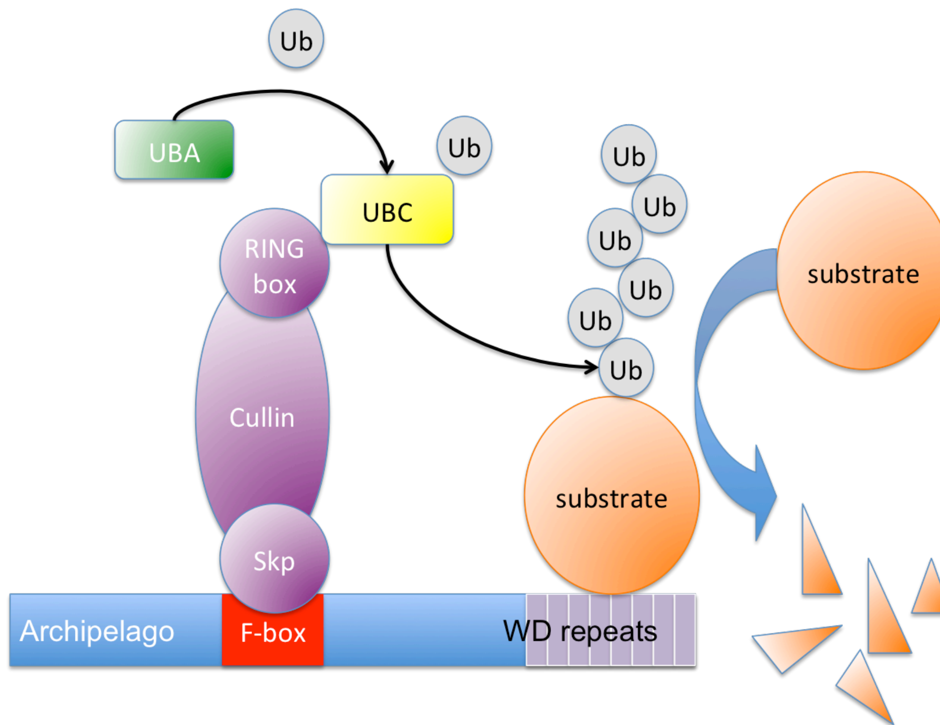
The studies in model systems laid a valuable foundation for further work on the function of mammalian *Fbw7*, which has been implicated in tumors of various origins. Human *Fbw7* acts in a similar manner to its fly counterpart, promoting ubiquitination and degradation of Cyclin E and c-Myc along with other proteins, all of which are products of proto-oncogenes. Accumulation of Cyclin E is associated with many different types of human malignancies including breast, colorectal, endometrial, and ovarian cancers (Donnellan and Chetty 1999), and c-Myc is also commonly deregulated in cancers (Grandori *et al.* 2000). Two other Fbw7 mammalian targets, the Notch1 (Gupta-Rossi *et al.* 2001; Oberg *et al.* 2001; Wu *et al.* 2001) and Notch4 (Tetzlaff *et al.* 2004; Tsunematsu *et al.* 2004) intracellular domains, play a part in the activation of transcriptional programs that stimulate cell division and prevent differentiation. Additionally, the Fbw7 target, c-Jun (Nateri *et al.* 2004; Wei *et al.* 2005), is commonly induced by mitogens and acts as a transcription factor to exert a positive effect on cell proliferation. Sterol regulatory element-binding protein (SREBP) (Sundqvist *et al.* 2005) is degraded by mammalian Fbw7, and it is a member of a family of transcription factors necessary for membrane synthesis and lipid metabolism, which are essential aspects of cell proliferation. Finally, growth factors and nutrients promote protein synthesis, cell growth, cell proliferation, and cell metabolism through another Fbw7 target, mammalian target of rapamycin (mTor) kinase (Fu *et al.* 2009; Dunlop and Tee 2009; Ma and Blenis 2009).

Because loss of Fbw7 results in the accumulation of the products of the proto-oncogenes described above, the fact that mutations in *Fbw7* are associated with many different types of cancer is not surprising. In fact, it is biallelically lost in primary tumor samples of colorectal (RAJAGOPALAN and LENGAUER 2004), endometrial (SPRUCK *et al.* 2002), and ovarian (MOBERG *et al.* 2001) origin, and in breast cancer cell lines (STROHMAIER *et al.* 2001). In addition, *Fbw7* mutations are the most frequent type of lesion found to date in T-cell acute lymphoblastic leukemia (T-ALL) (O'NEIL *et al.* 2007; THOMPSON *et al.* 2007). Furthermore, one study shows that loss of a single copy of *Fbw7* synergizes with *p53* heterozygosity to accelerate murine tumorigenesis and broaden the spectrum of tumors to include those of epithelial origin (MAO *et al.* 2004). This work places *Fbw7* among the few haplo-insufficient tumor suppressor genes identified to date and highlights a homeostatic role for Fbw7, particularly in epithelial cells. Also, the gene is located in region 4q23, which is deleted in ~30% of all human tumors (KNUUTILA *et al.* 1999). In light of this conspicuous location, *Fbw7* mutations may promote carcinogenic progression in an even wider spectrum of human tissue types than those identified so far.

Because *Fbw7* is firmly established as a tumor suppressor, the precise mechanisms that link *Fbw7* loss to the development of cancer continue to be the subject of many studies. One of the most surprising aspects of Fbw7 activity is that it targets many proto-oncogenes with essential roles in pathways that control cell division and growth. As a result, a single mutation in *Fbw7* can result in the simultaneous misregulation of multiple growth control pathways. In fact, all the outcomes of *Fbw7* loss on the cell or tissue are still not fully known. Given the role *ago* plays in

development and tumorigenesis, a more comprehensive understanding of its function and the regulation thereof may provide insights into both of these processes.

Figure I.6. The SCF-Archipelago complex



The components of the SCF-Archipelago complex include a Cullin protein, which provides a rigid scaffold connecting Skp and the RING box protein, a Skp protein, which recruits Archipelago by binding to the F-box domain, and a RING box protein, which serves as interface for E2 ubiquitin-conjugating enzymes. The RING box protein binds to the E2 ubiquitin-conjugating enzyme (UBC) that was previously charged with ubiquitin (Ub) by an E1 ubiquitin-activating enzyme (UBA). The Ub is then transferred to the substrate bound to the WD repeats of Archipelago and targeted for destruction by the proteasome. (Adapted from (WELCKER and CLURMAN 2008))

I.G. *ago* patterns and functions in the eye

Although much is known about how FBW7 acts to restrict human tumor formation in various tissues, little is known about how it is regulated. Using *Drosophila* to uncover novel mechanisms of Ago regulation may lead to significant advances in our understanding of its regulation in the mammalian system. *ago* transcript is present throughout the eye disc, but its levels are highest within the MF (MOBERG *et al.* 2001). This pattern of expression leads to two questions: (1) What signals cause *ago* transcript to build up at the MF?, and (2) What is the biological significance of this mechanism?

Predictions can be made in answer to both of these questions. Concerning the first question, the specific upregulation of *ago* transcript in the MF makes the *Drosophila* eye imaginal disc an ideal place to look for potential *ago* regulators. Essentially, the spatial distribution of *ago* transcript suggests that the signals involved in initiation and progression of the MF may also serve as *ago* regulatory signals, providing several candidate pathways to test for their ability to regulate *ago* transcription. These pathways include the Hedgehog, Ras, Notch, Wingless, and Dpp pathways, all of which are important for coordinating developmental events in and around the MF. The Hedgehog and the Notch pathways will be discussed here in more detail due to their involvement in *ago* regulation.

In regard to the second question, the fact that Ago binds and degrades Cyclin E suggests that Ago is needed in the MF to keep levels of Cyclin E down and allow cells

to arrest in G1. Considering that a mutation in *ago* would result in abnormally high Cyclin E activity, the outcome of *ago* loss is likely to drive ectopic RBF1 phosphorylation and subsequent inactivation. Thus, loss of *ago* should phenocopy loss of *rbf1*. *rbf1* mutant eye discs have a stripe of cells anterior to the MF that stain positive for the apoptotic epitope, cleaved caspase-3 (C3) (MOON *et al.* 2006). If *ago* mutant cells are also more sensitive to apoptosis in this region, the pathway through which *ago*-dependent death occurs may intersect with the RB/E2F pathway. Furthermore, the signals which render the cells in the anterior portion of the MF sensitive to apoptosis when mutant for *ago* may provide clues concerning potential drug targets for treating *ago*-deficient tumors.

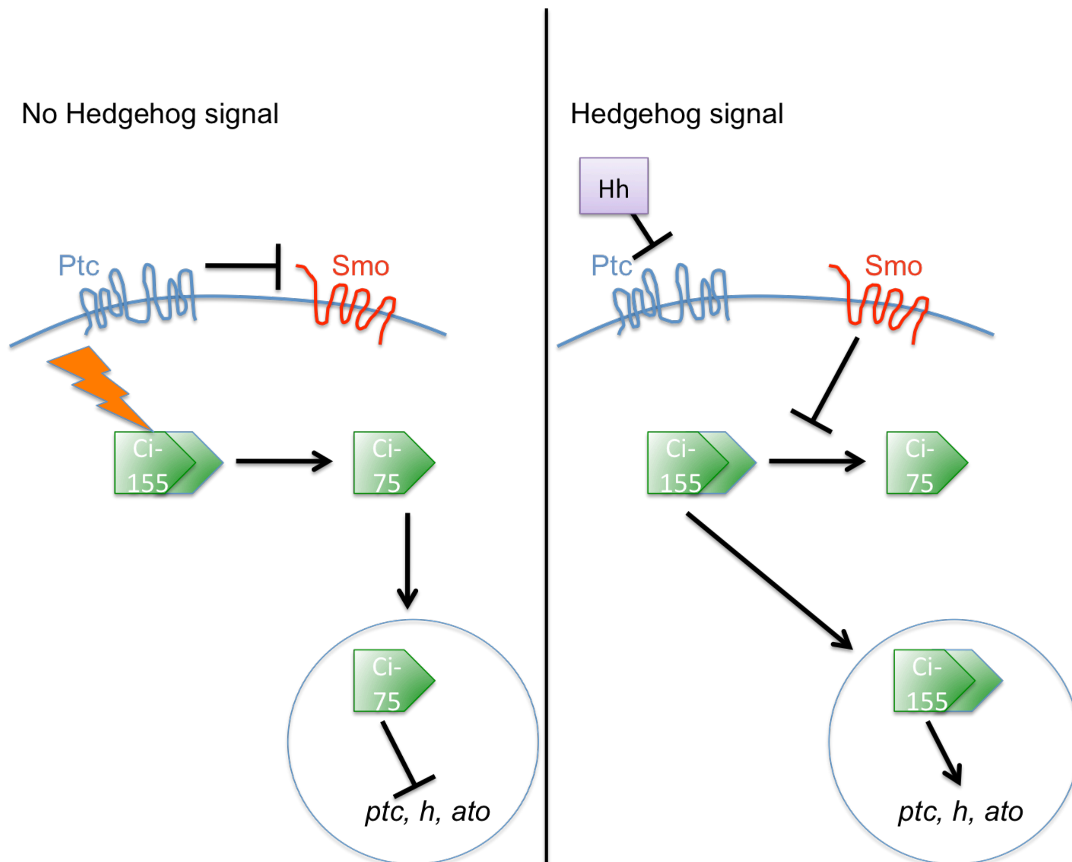
I.H. The Hedgehog pathway

A relatively small number of intercellular signaling pathways are used repeatedly throughout development to control many different biological processes. The signals involved in retinal patterning are understood in detail, and they begin in cells behind the MF, which express Decapentaplegic (DPP; a TGF-β superfamily member), Hedgehog (Hh, Figure I.7), and ligands for Notch and Receptor Tyrosine Kinases (RTKs) in intricate patterns. Hh and Dpp diffuse anteriorly to initiate differentiation in progressively more anterior regions of the eye disc, which allows the movement of the MF across the eye disc. Hh in particular is necessary for proper MF initiation (BOROD and HEBERLEIN 1998; CHEN *et al.* 1999b; CURTISS and MLODZIK 2000; DOMINGUEZ and HAFEN 1997; PAPPU *et al.* 2003) and progression (HEBERLEIN *et al.* 1993; LEE *et al.* 1992; LI *et al.* 1995; MA *et al.* 1993). Cells anterior to the furrow respond to the Hh signal via the Smoothened (Smo) transmembrane receptor and ultimately through the transcription factor Cubitus interuptus (Ci) (FU and BAKER 2003; LUM and BEACHY 2004; PAPPU *et al.* 2003; STRUTT and MLODZIK 1996; STRUTT *et al.* 1997). In the absence of Hh, another transmembrane receptor, Patched (Ptc) inhibits Smo, resulting in the cleavage of full-length Ci (Ci-155) to produce the transcriptional repressor Ci-75. When Hh is present, Smo is de-repressed, preventing the cleavage of Ci-155 (Figure I.7). The accumulation of Ci-155 in the nucleus results in transcriptional activation of its targets (AZA-BLANC *et al.* 1997; MOTZNY and HOLMGREN 1995). Targets of Ci-155 include *patched (ptc)*, which negatively feeds back on the Hh pathway, the bHLH transcription factor, *hairy (h)*,

which negatively regulates neurogenesis, and *atonal*, a neurogenic bHLH transcription factor (BAKER and YU 1997; DOMINGUEZ 1999; HEBERLEIN *et al.* 1993; MA and MOSES 1995; MA *et al.* 1993; PAPPU *et al.* 2003; SHYAMALA and BHAT 2002).

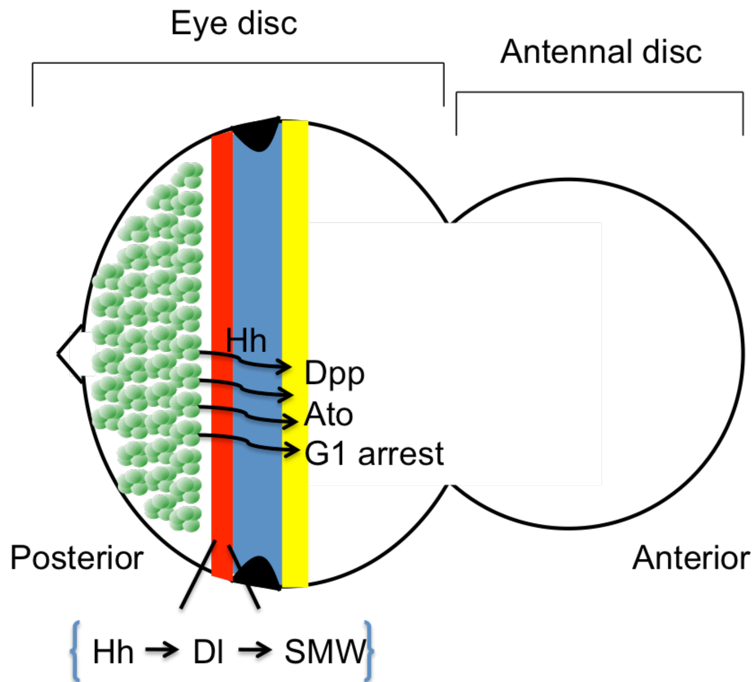
The Hh pathway plays several important roles in eye development (Figure I.8). Specifically, Hh promotes G1 arrest ahead of the furrow in conjunction with Dpp, and it arrests cells that cannot respond to Dpp. Furthermore, *smo* mutants show a delay in the second mitotic wave (SMW) nonautonomously through Notch (N), and expression of the N ligand Delta (Dl) is delayed in *smo* mutant clones (FIRTH and BAKER 2005). Within the MF, Hh activates transcription of the basic helix-loop-helix (bHLH) transcription factor Atonal (Ato), which is required for the specification of R8 photoreceptor cells, the first photoreceptors to differentiate in each cluster (BROWN *et al.* 1995; JARMAN *et al.* 1994; JARMAN *et al.* 1995; WHITE and JARMAN 2000). Clearly, the Hh pathway is active in cells in and around the MF, suggesting the pathway as one of several logical candidates for *ago* regulation at the furrow.

Figure I.7. The Hedgehog pathway



Hh signal is transduced through the Smo receptor. In the absence of Hh signaling, the transmembrane protein Ptc suppresses Smo activity and the Ci transcription factor is cleaved, producing the Ci-75 transcriptional repressor. In the presence of Hh ligand, Ptc is unable to affect Smo signaling, and Smo inhibits the cleavage of Ci, allowing the accumulation of the Ci-155 transcriptional activator. (Adapted from (Xie 2008))

Figure I.8. Hedgehog activity in the eye imaginal disc



The differentiating cells within the developing ommatidial clusters (green circles) behind the MF release Hh ligand which diffuses towards the anterior of the disc, activating the transcription of Dpp and Ato in cells just anterior to the MF (MF denoted by the blue bar, region anterior to the MF denoted by the yellow bar). Hh and Dpp together initiate G1-arrest, and Hh is responsible for initiation and progression of the MF. Also, timely entry into the SMW (red bar) is the indirect result of Hh signaling through the N pathway.

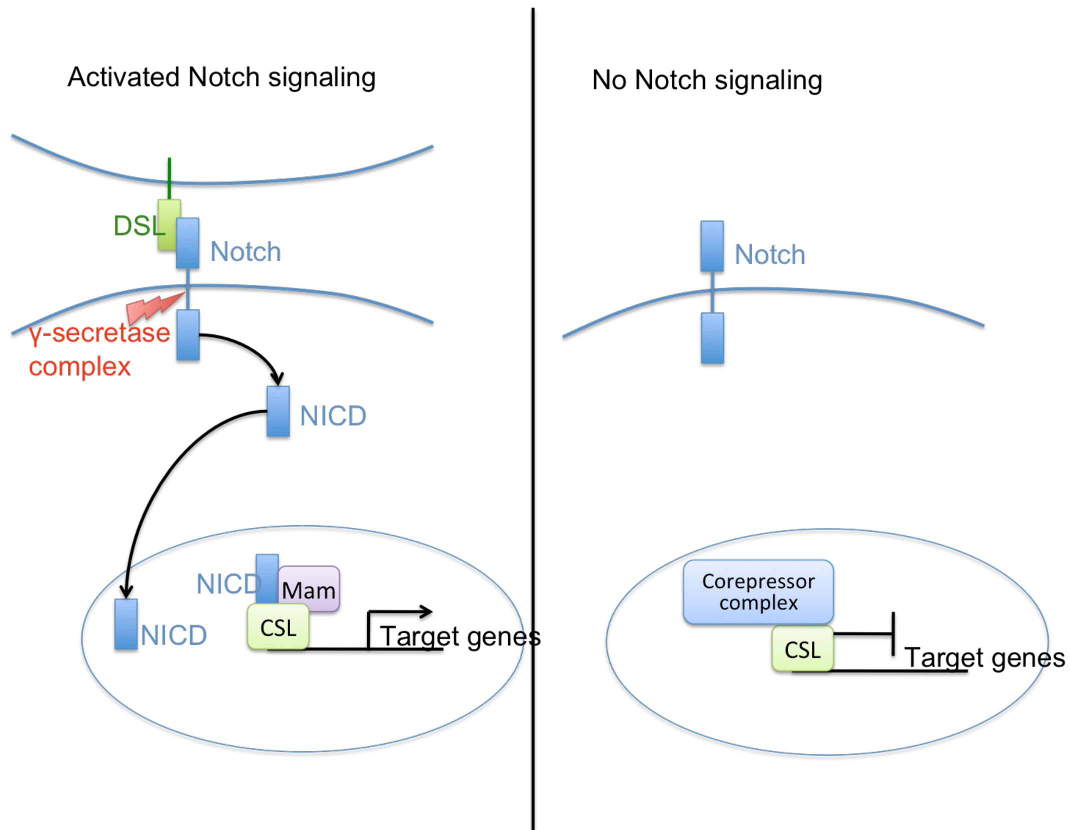
I.I. The Notch pathway

The pathways important for coordinating the cellular events that lead to eye development tend to intersect to influence common subsets of cells and common processes. For example, another pathway important for eye development in *Drosophila* is the Notch (N) pathway, which has been shown to act downstream of the Hedgehog pathway (VRAILAS and MOSES 2006). The N ligands are proteins of the DSL (Delta/Serrate/LAG-2) family, most of which are transmembrane proteins. Upon binding one of its ligands, the N receptor is proteolytically cleaved by the γ-secretase complex, releasing intracellular N (NICD) (DE STROOPER *et al.* 1999; REBAY *et al.* 1991), which translocates to the nucleus to promote transcription of its target genes (SCHROETER *et al.* 1998). Transcription factors of the CSL (CBF/Suppressor of Hairless/LAG-1) family mediate Notch signaling in mammals, worms, and flies (LAI 2002). Similar to the Hh pathway, in the absence of N signaling, CSL proteins associate with a corepressor complex and repress target gene transcription, commonly through the action of histone deacetylases (CHEN *et al.* 1999a; LAI 2002). When N signaling is active, the NICD replaces the corepressor complex and recruits the coactivator complex, which contains Mastermind/LAG-3/Sel-8 proteins (DOYLE *et al.* 2000; KITAGAWA *et al.* 2001; PETCHERSKI and KIMBLE 2000; WU *et al.* 2000) (Figure I.9). In this way, N acts as a switch from transcriptional repression to transcriptional activation of CSL target genes. While the CSL transcription factors control the expression of many different target genes, the best understood targets in

Drosophila are the basic helix-loop-helix (bHLH) proteins of the *Hairy/Enhancer of split* (HES) family (BAILEY and POSAKONY 1995; ISO *et al.* 2003), which are often used as a read-out of N activity.

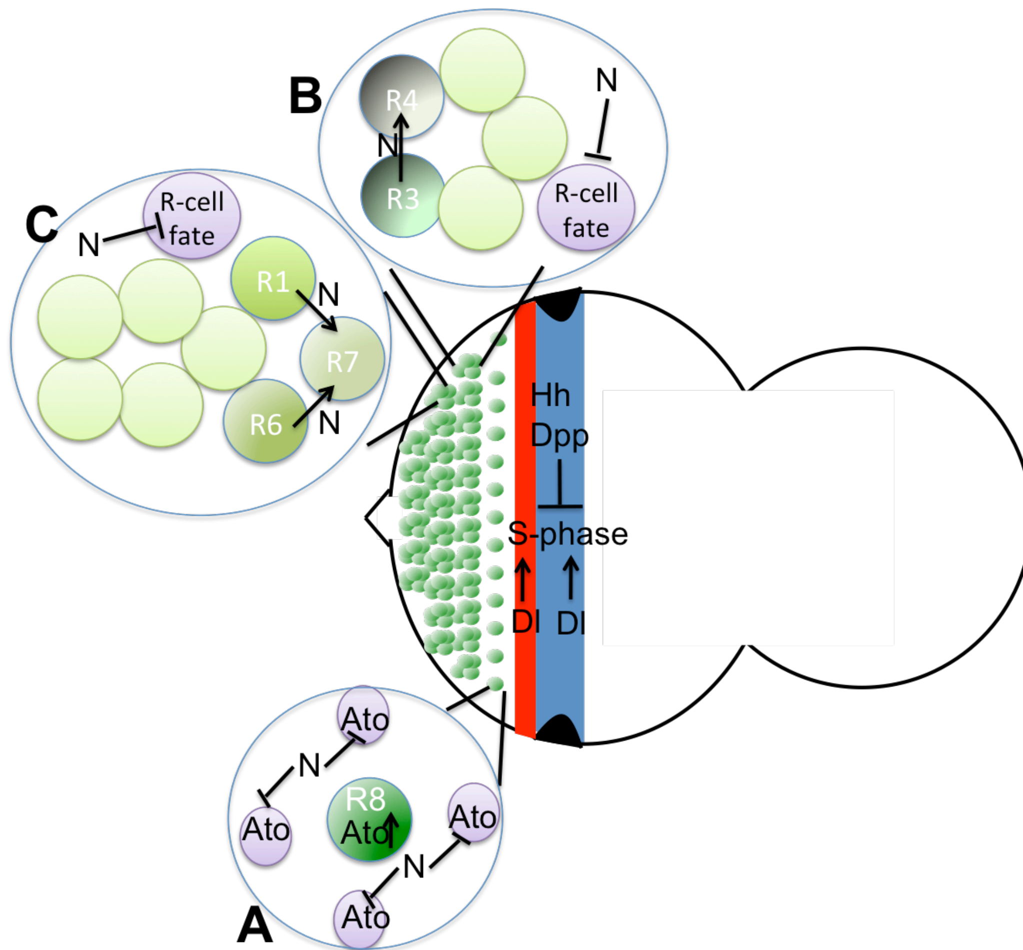
N signaling takes part in many developmental events crucial to eye formation (Figure I.10). First, N is necessary and sufficient for S phase entry into the SMW (FIRTH and BAKER 2005; BAONZA and FREEMAN 2005). Expression of the N ligand Dl begins at the anterior portion of the morphogenetic furrow (BAKER and YU 1998), but S-phases begin 6-8 cell diameters more posteriorly (behind the MF) (BAKER and YU 2001). This delay is because Hh and Dpp signals antagonize the S-phase promoting activity of Dl within the MF (FIRTH and BAKER 2005). Also, expression of the bHLH transcription factor Ato is gradually refined to single R8 cells within the MF in a process requiring lateral inhibition mediated by the Notch receptor (BAKER and ZITRON 1995; DOKUCU *et al.* 1996; JARMAN *et al.* 1994; JARMAN *et al.* 1995). R8 cells release signals to recruit subsequent phororeceptor cells, the cone cells, and the primary pigment cells to the ommatidial cluster. Dl acts as a critical secondary signal for several of the later differentiating cell types (FLORES *et al.* 2000; NAGARAJ and BANERJEE 2007; TOMLINSON and STRUHL 2001; TSUDA *et al.* 2002). The founder role of the R8 cell makes their specification and spacing, which is dependent on N signaling, crucial to proper eye formation. The N pathway plays a part in a wide range of developmental phenomena, even within the context of the fly eye, and it is likely that novel functions of N will be discovered in the future.

Figure I.9. The Notch pathway



Notch interacts with the DSL (Delta, Serrate, LAG-2) ligands resulting in proteolytic cleavage of Notch by the γ-secretase complex, which allows for NICD nuclear translocation. In the nucleus, NICD recruits the co-activator Mam and binds to CSL (CBF1/Su(H)/LAG-1) to activate transcription of target genes. In the absence of Notch signaling, CSL is bound by a corepressor complex and transcription of target genes is repressed.

Figure I.10. Notch activity in the eye imaginal disc



The Notch pathway plays many roles in *Drosophila* eye development. The N ligand Dl is expressed throughout the MF (blue) and the SMW (red) where it promotes entry into S phase. However within the MF, this signal is antagonized by Hh and Dpp, such that cells do not exit G1 until they enter the SMW. In differentiating cells (green), proper spacing of the *ato*-expressing R8 photoreceptor is achieved by lateral inhibition through the N pathway (A). Thus, N is active in cells surrounding the R8, repressing *ato* transcription in unspecified cells (purple). Epidermal growth factor (EGF) is used to recruit the rest of the photoreceptors (R1-R7) after the ommatidium founder cell R8 has been specified. EGF is the recruitment signal, and

the newly recruited cells (green) express Dl to signal to the surrounding undifferentiated progenitor cells (purple) to inhibit them from adopting R-cell fate. In addition, N signaling from R3 to the R4 (B) and from R1 and R6 to the R7 (C) is required for proper specification of these fates.

I.J. Objectives

The goals of this work involve developing a better understanding of signals both upstream and downstream of *ago* in eye development. Concerning downstream signals, much is already known about targets of Ago-dependent degradation in both flies and mammals, with most of this knowledge being focused on the role of Ago in antagonizing cell *growth* by controlling levels of pro-growth proteins. However, the following observation has been made in flies which cannot be explained by our present knowledge of Ago function: a fly eye completely deficient for Ago protein is smaller than wild-type. We have hypothesized that this observation suggests a novel role for *ago* in antagonizing cell *death* during eye development. This hypothesis will be experimentally tested by looking for markers of cell death in *ago* mutant eyes. The pathway leading to the death of *ago* mutant cells will then be elucidated in the *Drosophila* eye.

The signals upstream of *ago* will be uncovered by making mutations in pathway components of MF signals and looking for those that disrupt *ago* promoter activity. In this way, the specific signal responsible for initiating *ago* transcription can be determined. The new information gained by these studies is an important contribution to the fields of eye development and growth control and may lead to significant advances in the understanding and treatment of certain types of tumors.

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Chapter 2: The *archipelago* Tumor Suppressor Gene Limits Rb/E2F-Regulated Apoptosis in Developing *Drosophila* Tissues

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The *archipelago* tumor suppressor gene limits Rb/E2F-regulated apoptosis in developing *Drosophila* tissues

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Running title: *ago* limits eye apoptosis

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II.A. Abstract

Background: The *Drosophila archipelago* gene (*ago*) encodes the specificity component of a ubiquitin-ligase that targets the Cyclin E and dMyc proteins for degradation. Its human ortholog *Fbw7* is commonly lost in many cancers, suggesting that failure to degrade *ago/Fbw7* targets leads to excess tissue growth.

Results: Here we show that although loss of *ago* induces hyperplasia of some organs, it paradoxically shrinks the size of the adult eye. We find that this reflects a requirement for *ago* to restrict apoptotic activity of the *rbf1/e2f1* pathway adjacent to the eye-specific morphogenetic furrow: *ago* mutant cells display elevated *de2f1* activity, express the pro-death dE2f1 targets *hid* and *rpr*, and undergo high rates of apoptosis. This death and the resulting small-eye phenotype are dependent on *rbf1*, *de2f1*, *hid*, and the *rbf1/de2f1* regulators *cyclin E* and *dacapo*, but are independent of *dp53*. A transactivation-deficient *de2f1* allele blocks MF-associated apoptosis of *ago* mutant cells but does not retard their clonal overgrowth, indicating that intact *de2f1* function is required for the death but not overproliferation of *ago* cells. Alleles of *EGFR* and *wg* pathway components further modulate the *ago* apoptotic and eye size phenotypes, suggesting these pathways control rates of *de2f1*-driven apoptosis among *ago* mutant cells.

Conclusions: These data show that *ago* loss requires a collaborating block in cell death to efficiently drive tissue overgrowth and that this conditional growth-suppressor phenotype reflects a role for the gene in restricting apoptotic output of

the *rbf1/de2f1* pathway. Moreover, the susceptibility of *ago* mutant cells to succumb to this apoptotic program appears to depend on local variations in extracellular signaling that could thus determine tissue-specific fates of *ago* mutant cells.

II.B. Introduction

Genetic screens in the fruit fly *Drosophila melanogaster* have identified many genes that restrict growth of developing tissues (HARIHARAN and BILDER 2006; PAN 2007). In some cases, orthologs of these *Drosophila* growth suppressor genes have subsequently been implicated as vertebrate tumor suppressors. One example is the *archipelago* (*ago*) gene, which was identified because *ago* mutations confer a growth advantage to imaginal disc cells (MOBERG *et al.* 2001). *ago* encodes an F-box/WD (tryptophan/aspartic acid) protein (Ago) that acts as the substrate-adaptor for an Skp/Cullin/F-box (SCF) E3 ubiquitin ligase. SCF-Ago targets the G1/S cell cycle regulator Cyclin E (CycE) and dMyc, the fly ortholog of the c-Myc proto-oncogene, for degradation in vivo (MOBERG *et al.* 2001; MOBERG *et al.* 2004). These proteins hyper-accumulate in *ago* mutant cells and drive balanced increase in rates of division and growth, producing enlarged clones composed of normally sized cells (MOBERG *et al.* 2004). In addition to this mitotic role, *ago* also regulates hypoxia-sensitivity and post-mitotic morphogenesis of the tracheal system via degradation of the Trachealess transcription factor (MORTIMER and MOBERG 2007; MORTIMER and MOBERG 2009).

Fbw7, the human *ago* ortholog, is frequently mutated in a wide array of human tumor types, including those of endometrial, colorectal and hematopoetic origin (WELCKER and CLURMAN 2008). Moreover, deletion of murine *Fbw7* increases cancer incidence and collaborates with *p53* mutations to promote epithelial carcinogenesis (MAO *et al.* 2004). Growth suppression by *Fbw7* is linked to defective degradation of

SCF substrates including CycE, c-Myc, the Notch intracellular domain, c-Jun, SREBP, and mTor kinase (reviewed in (WELCKER and CLURMAN 2008), (MAO *et al.* 2008)). Thus, *ago* and *Fbw7* both behave as anti-growth genes *in vivo*, and this property derives in part from their role in the timely destruction of common oncogenic substrates like CycE and Myc.

Many *Drosophila* mutations that accelerate the rate of cell proliferation also increase size of the corresponding adult organ. Such mutations affect various regulatory networks, including the Tsc/Tor, IGF/PI3K, Sav/Wts/Hpo, ras/EGFR, and Notch pathways (HARIHARAN and BILDER 2006; KOCH and RADTKE 2007). Notably, ectopic expression of pro-growth factors such as *dMyc* in larval discs also increases adult organ size (JOHNSTON *et al.* 1999). It is therefore somewhat surprising that adult eyes composed mainly of *ago* mutant cells expressing very high levels of dMyc are not obviously enlarged (MOBERG *et al.* 2001; MOBERG *et al.* 2004). One explanation of this may be that excess dMyc causes *ago* cells to behave 'super competitors' that kill adjacent normal cells (DE LA COVA *et al.* 2004; MORENO and BASLER 2004), thus balancing the overall rate of growth of a mosaic organ. Alternately, an as yet unrecognized cell autonomous mechanism may limit the ability of *ago* mutant cells to give rise to an enlarged organ.

Here we uncover a requirement for *ago* to maintain eye size that reflects a cell-autonomous role for *ago* upstream of the *rbf1/e2f1* pathway in cells just anterior to the eye-specific morphogenetic furrow (MF). *ago* mutant cells fail to down-regulate *de2f1*-dependent transcription, express elevated levels of pro-death dE2f1 target genes, and undergo high rates of apoptosis. Blocking this death causes *ago* mutant

discs to grow into enlarged adults eyes, indicating that *ago* loss requires a collaborating anti-apoptotic event to drive eye hypertrophy. *ago* apoptotic phenotypes are also sensitive to *EGFR* and *Wg* signaling, indicating that extracellular pathways can alter the threshold for *e2f1*-driven death of *ago* mutant cells. These data identify *ago* as a required upstream regulator of the *rbf1/de2f1* pathway in eye disc cells, and show that apoptosis mediated by this pathway can act as a significant brake to the growth of developing tissues lacking the *ago* tumor suppressor.

II.C. Results

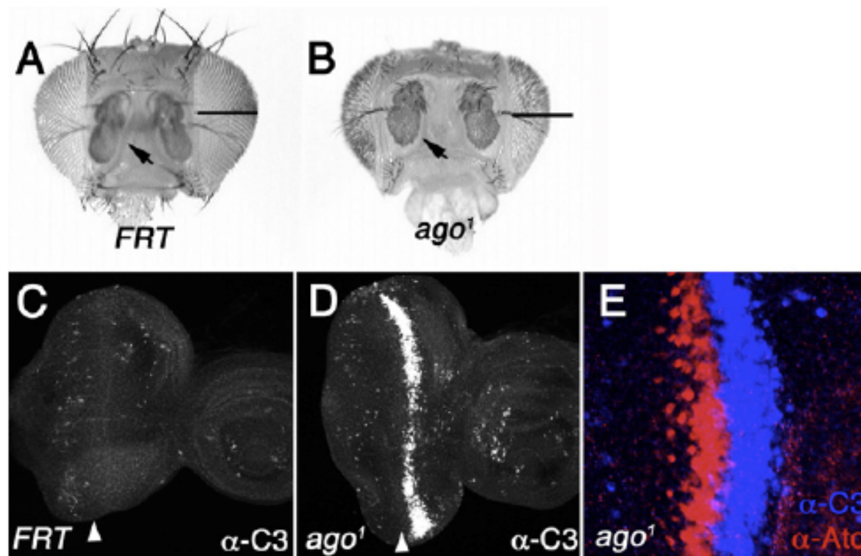
II.C1. Loss of *ago* has different effects on the size of tissues in the adult head

Drosophila with eyes/heads composed almost entirely of *ago* mutant tissue were generated using the *ago*^{1} allele and the 3L *Minute* (*M*) allele *RpL14*^{1} (SAEBOE-LARSSEN *et al.* 1997) as a recessive 'cell-lethal' mutation to kill *M/M* cells, allowing *ago*^{1}*/ago*^{1} cells to populate the disc and adult structures derived from it. *ago*^{1} encodes a prematurely truncated protein that cannot bind CycE and dMyc and increases levels of these proteins in vivo (MOBERG *et al.* 2001; MOBERG *et al.* 2004). Thus the '*ago*^{1}*/M(3)*' genotype provides a model of organ development in the absence of normal *ago* activity. Adult *ago*^{1}*/M(3)* eyes are smaller than control eyes (Figs. 1A, B). This phenotype is specific to the eye: other organs in *ago*^{1}*/M(3)* heads, such as the antennae (arrows, Fig. 1) and interocular cuticle, grow larger. A similar effect is observed with other *ago* alleles (data not shown). Thus, while *ago* behaves as a growth suppressor in some tissues, it is required for the developing eye to reach its normal size.

Eye development is uniquely dependent the morphogenetic furrow (MF), a moving compartment boundary which sweeps posterior-to-anterior across the larval disc and separates it into areas: asynchronously dividing cells anterior to the MF, G1-phase arrested cells within the MF, and largely post-mitotic cells posterior to the MF (WOLFF and READY 1991). *ago* loss does not substantially alter the pattern of S-phase entry in the vicinity of the MF (Fig. S1), but it does produce an intense 'stripe' of apoptosis, as detected with an antibody to the cleaved form of Caspase-3

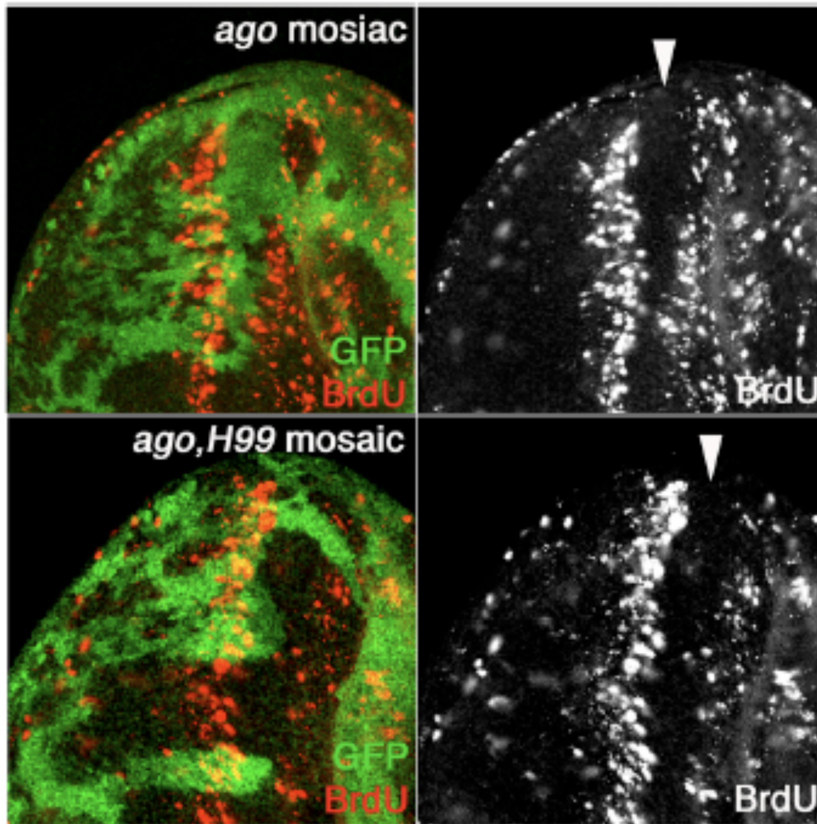
(C3), that extends laterally across the entire 3rd instar eye disc (Figs. 1C,D). A second *ago* allele, ago^3 (MOBERG *et al.* 2001), produces a similar pattern of apoptosis (Fig. S2). Co-staining for C3 and Atonal protein, which marks cells within the MF (BAKER *et al.* 1996; JARMAN *et al.* 1994; JARMAN *et al.* 1995), shows that this death extends ~5-10 cell diameters from the anterior edge of the MF into the anterior portion of the disc (Fig. 1E). This pattern is strongest at the lateral margins of $ago^1/M(3)$ eye discs, but is still penetrant enough to appear as a contiguous stripe in medial areas. Notably, C3 is also detected in ago^1 clones that cross the MF (see Fig. 4A), indicating that this apoptosis is autonomous to *ago* cells and not a product of the 'cell-lethal' technique used to generate $ago^1/M(3)$ discs. Thus, in addition to its growth suppressor function, *ago* is also required to protect cells from death in a region just anterior to the MF.

Figure II.1. *ago* loss reduces eye size and elevates apoptosis



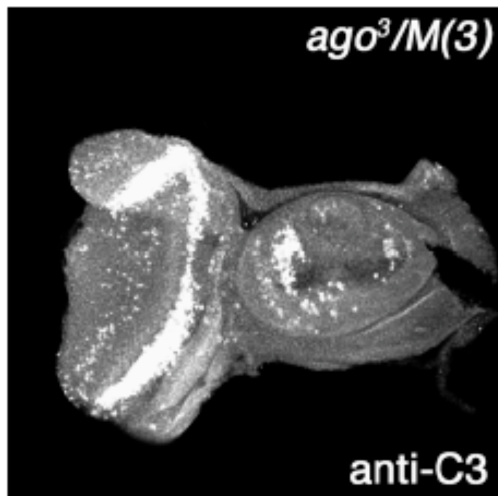
Images of (A) *FRT80B/M(3)* and (B) *ago¹/M(3)* adult female heads. Black bar denotes depth of *FRT80B/M(3)* eyes. Arrows indicate increased antennal size in *ago¹/M(3)* heads. (C) Merged confocal sections of *FRT80B/M(3)* and (D,E) *ago¹/M(3)* larval eye-antennal discs stained for C3, Senseless, or Atonal as indicated. (C-E) Arrowheads mark position of the MF. In this and all following images, posterior is to the left.

Figure II.S1. Patterns of cell division in *ago* mutant and *ago,H99* double-mutant MF cells



BrdU (red) incorporation to mark S-phase cells in 3rd instar larval eye discs carrying clones of *ago*[1] (top) or *ago*[1]*,H99* (bottom) mutant cells marked by the absence of GFP (green). Arrowheads indicate position of the MF.

Figure II.S2. Caspase activation in *ago³* mutant cells

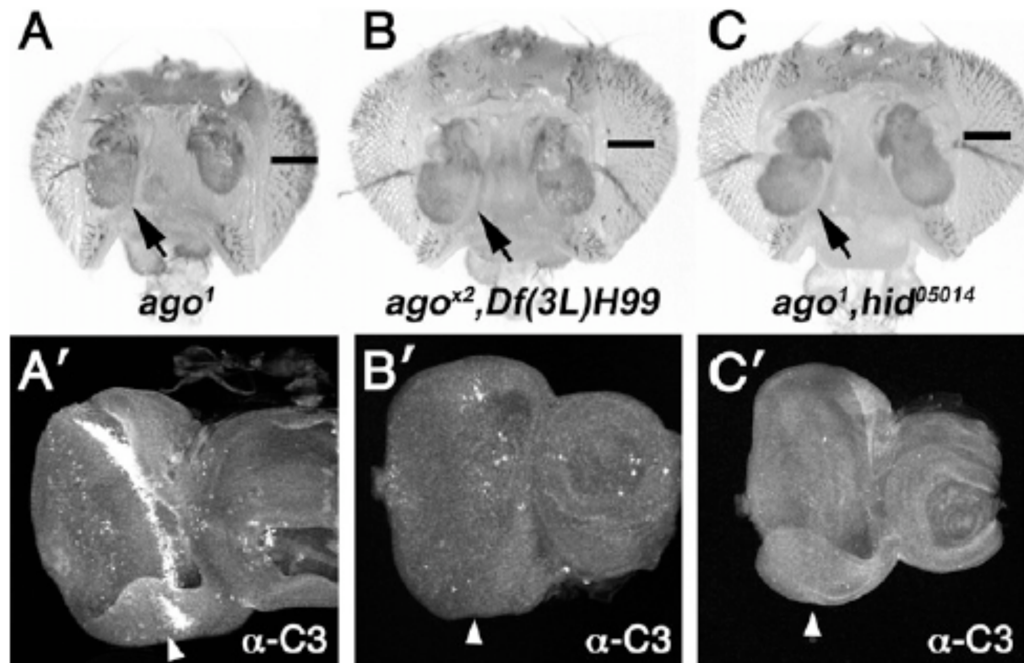


Merged confocal sections of C3 expression in an *ago³* mutant larval eye disc generated using the Min cell lethal technique.

II.C2. Blocking death increases ago mutant eye size

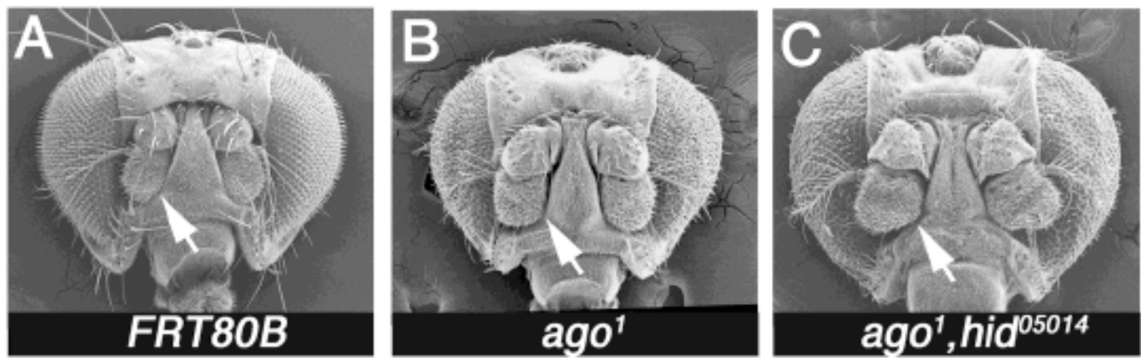
The C3 data suggests the MF is preceded by an intense wave of cell death that culls many cells from developing *ago*[1]*/M(3)* eyes. To test whether blocking this could reverse the *ago* small-eye phenotype, the *ago*[1] allele was combined with the *Df(3)H99* genomic deletion, which removes the pro-apoptotic genes *rpr*, *grim* and *hid* (WHITE *et al.* 1994). This completely blocks the C3 'stripe' (Figs. 2A' vs. 2B') and produces adult heads that are much larger than *ago*[1]*/M(3)* or *H99/M(3)* heads (Figs. 2A vs. B). *H99/M(3)* heads are similar in size to control *FRT80B/M(3)* heads (Fig. 6C and data not shown). Thus, the *ago*[1] and *H99* mutations cooperatively increase organ size. This effect is apparent in other head organs as well (e.g. antennae in Figs. 2 and S3). Combining *ago*[1] and the *hid*[05014] allele (SPRADLING *et al.* 1999) is also sufficient to block C3 accumulation and increase organ size (Figs. 2C-C' and S3). Together these data indicate that *hid*- and *H99*-dependent apoptosis of *ago*[1] mutant cells restrains the oncogenic effect of *ago* loss in multiple tissues, and that in the larval eye this death is concentrated in an area just anterior to the MF.

Figure II.2. Blocking death of *ago* cells produces enlarged organs



(A) *ago*^{1}*/M(3)*, (B) *ago*^{x2}*,Df(3L)H99/M(3)*, or (C) *ago*^{1}*,hid*^{05014}*/M(3)* adult female heads, and merged confocal sections of α-C3 staining in corresponding larval eye discs (A'-C'). Bar is standardized to the depth of *ago*^{1}*/M(3)* eyes*;* arrows indicate increased antennal size. Arrowheads indicate position of the MF.

Figure II.S3. Blocking death of *ago^1* mutant cells produces enlarged organs



Scanning electron micrographs of female heads of the indicated genotypes. Arrows indicate progressively increased antennal size.

II.C3. *ago* mutations elevate dE2f activity in the furrow

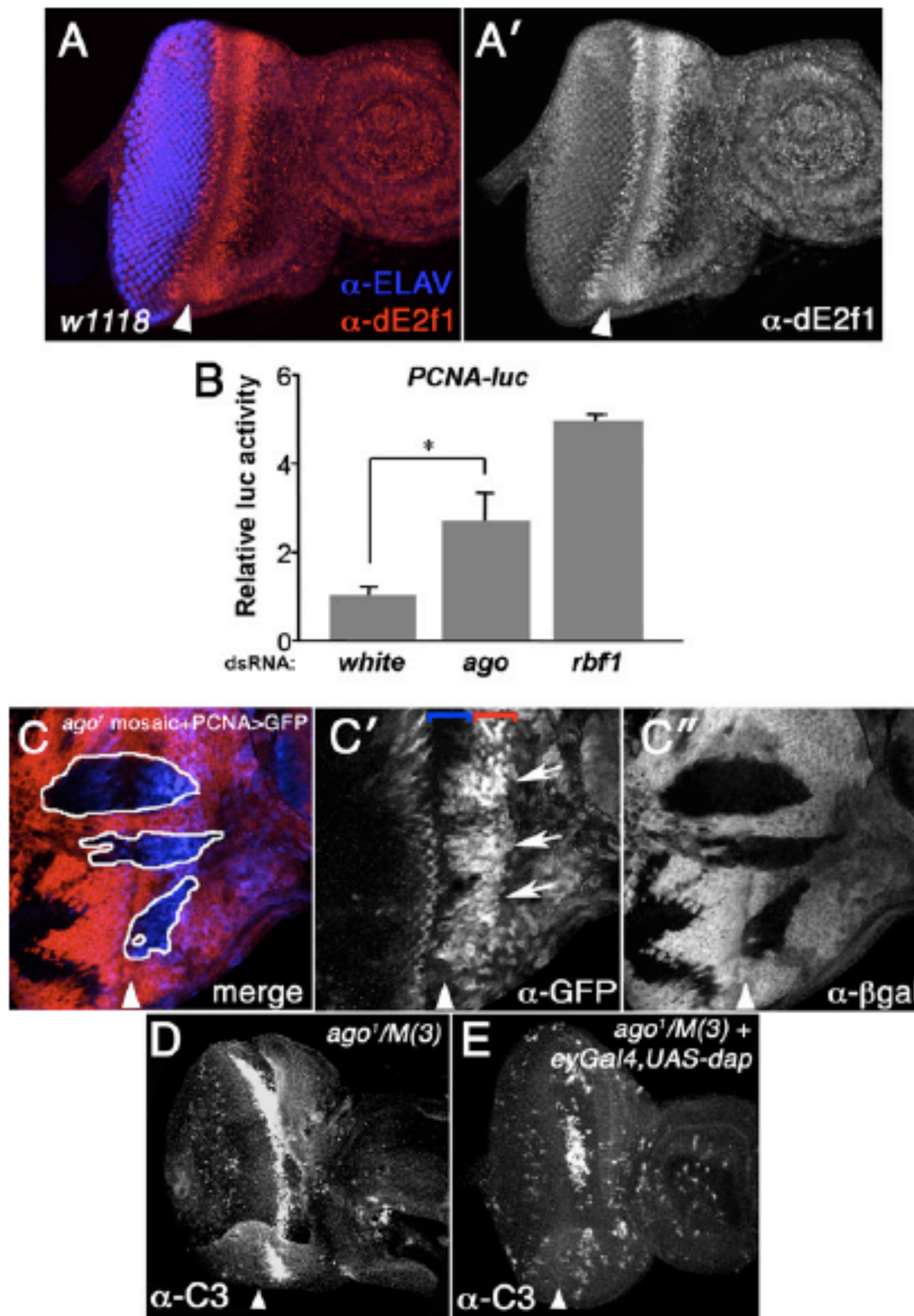
ago MF-associated apoptosis resembles the pattern of death among cells lacking the *Drosophila* retinoblastoma (Rb) gene homolog *rbf1* (MOON *et al.* 2006), suggesting these genes function within a common anti-apoptotic pathway in cells just anterior to the MF. Like mammalian Rb, Rbf1 binds to the dE2f1 transcription factor and inhibits the expression of dE2f1 target genes (VAN DEN HEUVEL and DYSON 2008). This inhibition is reversed by G1 cyclin-dependent kinases that phosphorylate Rbf1 and dissociate it from dE2f1, allowing dE2f1 to transactivate target-gene promoters. *rbf1* mutations thus up-regulate expression of dE2f1 targets, including *PCNA* (DU 2000) and the pro-apoptotic genes *hid* (TANAKA-MATAKATSU *et al.* 2009) and *rpr* (ASANO *et al.* 1996), which leads to MF-associated death of *rbf1* mutant cells (MOON *et al.* 2006).

To test whether *ago* controls *de2f* activity in the eye, the dE2f-reporter transgene *PCNA-GFP* (THACKER *et al.* 2003) was placed into the background of ago^1 mosaic eye discs (Fig. 3C). dE2f1 protein levels normally rise in cells just anterior to the MF (Figs. 3A-A'), presumably due to a requirement for dE2f1 in Rbf1-mediated transcriptional repression. Accordingly, expression of *PCNA-GFP* within the MF is low in wild type areas of mosaic eye discs (Figs. 3C-C";blue bracket denotes MF). By contrast, *PCNA-GFP* expression in ago^1 clones is elevated in the region just anterior to the MF (denoted by red bracket) and within the MF (Fig. 3C'). The elevated *PCNA-GFP* expression just anterior to the MF appears to correlate spatially with the

location of C3 expression in *ago¹* cells. RNAi depletion of *ago* also activates the dE2f-responsive reporter *PCNA-luc* in S2 cells (Fig. 3B), although this is not associated with high rates of apoptosis (data not shown). The magnitude of the effect on *PCNA-luc* expression is less than that produced by directly targeting *rbf1* with dsRNA, consistent with the idea that *ago* loss inactivates Rbf1 indirectly through intermediate factors. As *ago* mutations do not change patterns of dE2f1 protein expression in discs (SCN et al., unpublished), they appear to de-repress the existing pool of dE2f1 protein. Together these observations show that *ago* mutations deprive cells of an important check on dE2f1, and suggest that this may activate a MF-associated apoptotic program similar to that elicited by loss of *rbf1*.

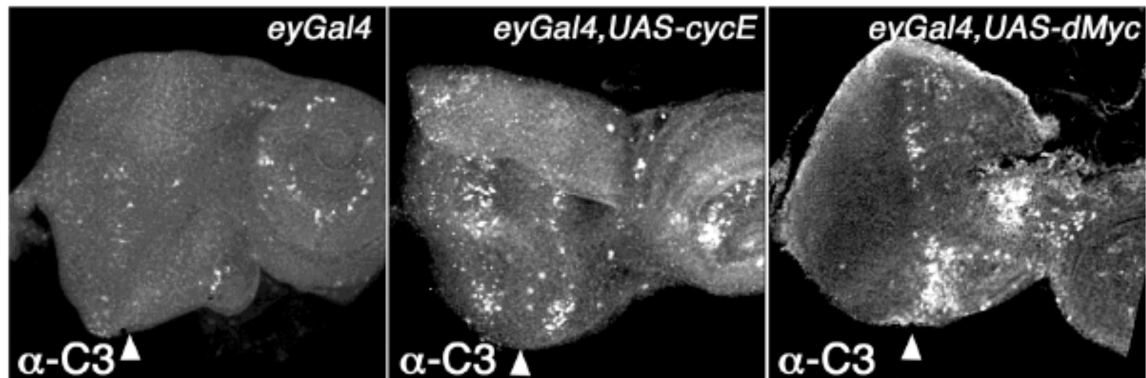
As CycE hyper-accumulates in *ago* mutant cells throughout the eye disc (MOBERG *et al.* 2001; MOBERG *et al.* 2004) and acts upstream of *de2f1* [reviewed in 21], CycE-associated kinase activity may contribute to *ago¹* MF-associated apoptosis. To test this hypothesis, the CycE/cdk2 inhibitor *dacapo* was expressed ubiquitously in *ago¹/M(3)* discs. This reduces C3 staining (Fig. 3D,E), particularly toward the lateral edges of the disc. Reciprocally, expression of either *cycE* or *dMyc* in otherwise normal larval eye discs was able to induce ectopic apoptosis anterior to the MF (Fig. S4). Thus, elevated expression of two SCF-Ago targets is sufficient to elevate rates of apoptosis just anterior to the MF; moreover, the activity of one of these proteins, CycE, appears to necessary for the *ago* MF-associated apoptotic phenotype.

Figure II.3. *ago* restricts dE2f activity



(A-A') Merged confocal sections of an eye disc stained for Elav (blue) and dE2f1 (red) showing rise in dE2f1 levels anterior to and within the MF (arrowhead). (B) Relative luciferase activity in S2 cells transfected with *PCNA-luc* and dsRNAs corresponding to the *white* (lane 1), *ago* (lane 2), or *rbf1* (lane 3) genes (* $p<0.05$). (C-C") *PCNA-GFP* expression (blue) in *ago*^{1} mutant clones marked by absence of β-galactosidase (red). GFP expression in *ago* clones (outlined in C) anterior to the MF is indicated with arrows in C'. Blue bracket denotes MF; red bracket denotes area just anterior to MF. (D-E) Expression of *dap* reduces MF-associated death in *ago/M(3)* discs. Arrowheads mark position of the MF.

Figure II.S4. Induction of C3 by expression of *cycE* or *dMyc*



C3 accumulation in *eyGal4*; *eyGal4*,*UAS*-*cycE*; and *eyGal4,UAS*-*dMyc* discs. *cycE* overexpression drives C3 accumulation in three areas: (1) posterior cells behind the MF; (2) cells in a region just anterior to the MF; and (3) a patch of cells within the antennal disc that also appears in *ago* mutant discs (see also Figures 1D and 2A'). *dMyc* expression also increased cell death anterior to the MF, and at the junction of the eye and antennal disc.

II.C4. *de2f1* and *rbf1* control apoptosis of *ago* cells

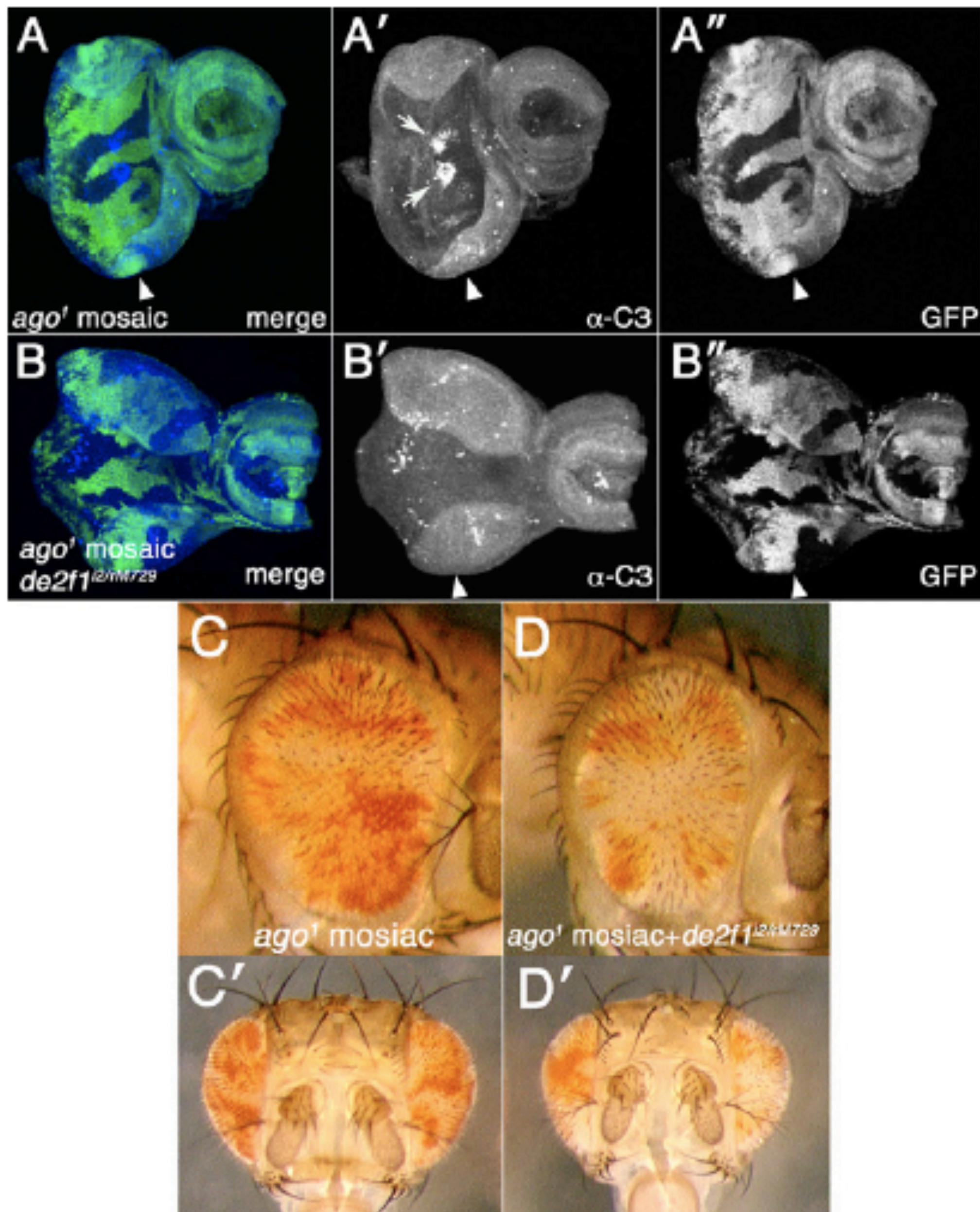
To directly test whether *de2f1* is required for the MF-associated death of *ago* cells, the *de2f1*^{i2}/^{rM729} viable allele combination was used to reduce *de2f1* activity in the background of *ago* mosaic eye discs (Fig. 4). *de2f1*^{rM729} is an amorph <Duronio, 1995 #327>, while *de2f1*^{i2} is a hypomorph that lacks the activation domain and Rbf1-binding motif (Royzman *et al.* 1999). The *de2f1*^{i2}/^{rM729} allele combination has no residual dE2f1 *trans*-activation function but supports normal patterns of division and MF progression in the eye disc (Moon *et al.* 2006). MF-spanning *ago* clones accumulate high levels of C3 (Figs. 4A-A") (as noted previously [3], the ratio of mutant:normal tissue in *ago* mosaic larval eye is less than that observed in the adult eye, indicating that the *ago* growth advantage arises progressively during eye development), but C3 is absent from *ago*^{1} clones in the *de2f1*$^{i2/rM729}$ background (Figs. 4B-B"). Residual C3 is sometimes detected in lateral areas of these discs (compare Figs. 2B' and 4B'), suggesting that death of *ago*^{1} cells in this area is not as dependent on *de2f1* as in more central regions. Expression of *rbf1* in *ago*^{1}*/M(3)* discs is also able to substantially reduce the MF-associated C3 'stripe' (Fig. 5). Thus reducing *de2f1* activity, by either elevating levels of *rbf1* or removing the *de2f1* *trans*-activation function, blocks death of *ago*^{1} cells. Interestingly although *de2f1*$^{i2/rM729}$ animals are proportionately smaller than *de2f1*$^{+}$ animals, *ago*^{1} mosaic eyes generated in the *de2f1*$^{i2/rM729}$ background show a strong white:red ratio that is at least comparable to the white:red ratio of *ago*^{1} mosaic eyes (Figs. 4C-C" and D'-D"). Thus transactivation by dE2f1 is not required for the clonal growth advantage

of *ago*^{1} cells. Rather the evidence suggests that main effect of elevated *de2f1* activity in *ago*^{1} cells is to promote apoptosis, particularly in cells lying just anterior to the MF.

The somewhat paradoxical finding that expression of *rbf1* may increase cell number by rescuing the death of *ago*^{1} MF cells promoted a closer examination of the relationship between *ago*^{1} eye size and gene dosage of G1/S regulatory factors. Eye size was determined by normalizing the two-dimensional *en face* area of the eye to the area between the L3, L4 and posterior cross veins (Fig. 6). This technique does not take into account bulging out of the surface of the eye, and can under-represent increases in eye size (e.g. *ago*^{1}*,hid*^{05014}*/M(3)* eyes are slightly larger than *FRT80B/M(3)* controls in an *en face* view [Fig. 6C], but are much larger than *FRT80B/M(3)* in head-on views [Fig. S3]). Nonetheless, we observed that heterozygosity for *de2f1* significantly increases *ago*^{1}*/M(3)* eye size (Figs 6A-C). A similar effect is observed with a null allele of *cyclin E* (*cycE*^{AR95}; (KNOBLICH *et al.* 1994)). Thus loss of *ago* creates a situation in which G1/S factors that normally promote the growth of wild type organs become dosage-sensitive inhibitors of growth. Alleles of *cdk4* (Fig. 6C) and *dMyc* (data not shown) also dominantly increased *ago*^{1}*/M(3)* eye size but simultaneously increased adult wing size so that the final eye:wing ratio was unchanged. As *de2f1* is required for apoptosis of *ago*^{1} eye cells, these data are consistent with a model in which reducing the dose of *de2f1* and *cycE* restores their activities to levels that are permissive for proliferation but which reduce apoptosis. This phenomenon may be the invertebrate correlate of the ‘E2F threshold’ hypothesis in which the effect of E2F on cell number is postulated to

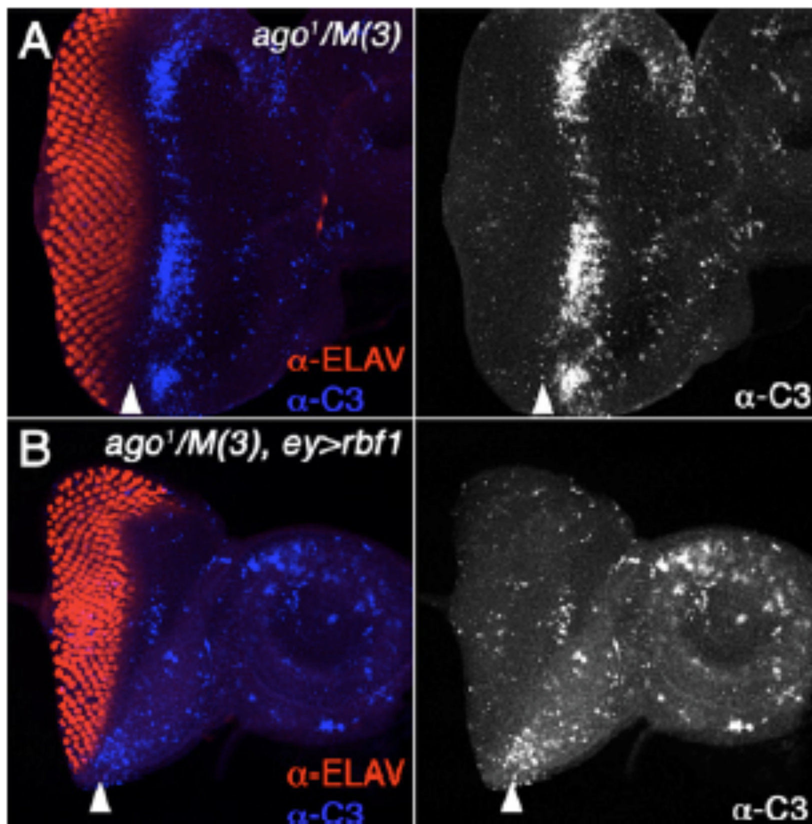
depend on a hypothetical threshold of activity below which E2F promotes cell cycling and above which it becomes pro-apoptotic (TRIMARCHI and LEES 2002).

Figure II.4. *de2f1* transactivation is required for apoptosis but not the proliferative advantage of *ago* mutant cells



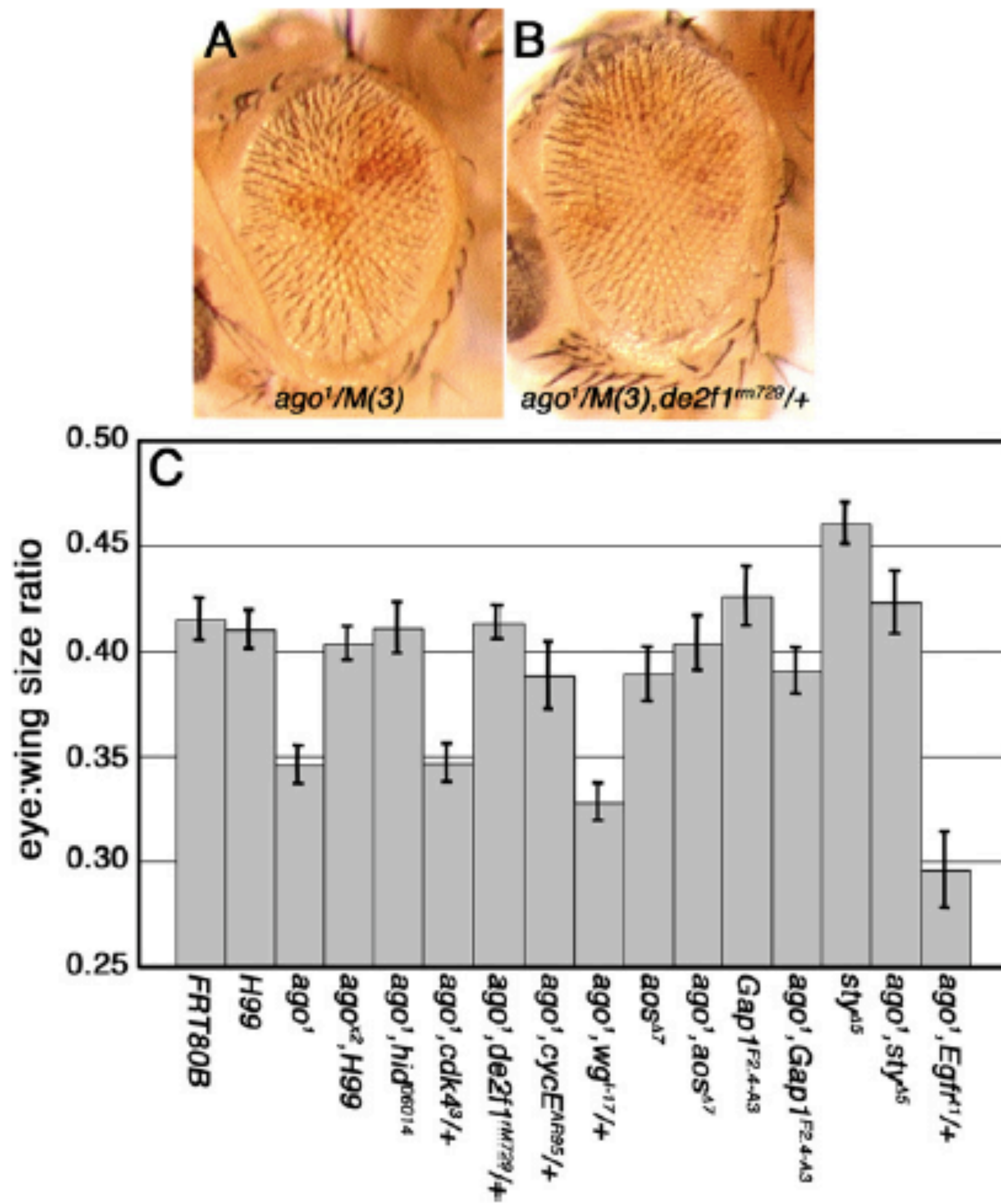
Merged confocal sections of *ago*^{1} clones marked by the absence of GFP (green) generated in (A-A") wild type or (B-B") *de2f1*$^{i2/rM729}$ mutant backgrounds and stained with α-C3 (blue). Arrowheads mark the MF; arrows indicate C3 signal in *ago* clones that span the MF. Note the absence of C3 signal in *ago* mutant clones that span the MF in *de2f1*$^{i2/rM729}$ discs. Paired views of eyes and heads from (C-C') *ago*^{1} mosaic or (D-D') *ago*^{1} mosaic+*de2f1*$^{i2/rM729}$ adult females in which *ago*^{1} mutant tissue is marked by the absence of red pigment; the white:red imabalance persists in the *de2f1*$^{i2/rM729}$ background. Reduced organ size in panels D-D' are due to the effect of the *de2f1*$^{i2/rM729}$ genotype on organism size.

Figure II.5. Excess *rbf1* suppresses the cell death of *ago* mutant cells in the MF



ago¹/M(3) (A) and *ago¹/M(3), eyGal4,UAS-rbf1* (B) eye discs stained for Elav (red) and C3 (blue) to mark dying cells. Position of the MF is indicated by arrowheads.

Figure II.6. Modification of *ago* eye size by cell cycle regulators and signaling components



Light microscopic images of *ago*^{1}*/M(3)* (A) and *ago*^{1}*/M(3),de2f1*$^{rM729/+}$ adult female eyes. Note the eye in B is larger. (C) Graphic summary of the effect of the indicated genotypes on *en face* adult female eye size. Error bars represent 95% confidence intervals. Wing areas between the L3,L4, and PCV veins.

II.C5. Relationship between *ago* and apoptotic regulators

E2F-driven cell death can occur by pathways that are either dependent or independent of the *p53* transcription factor (reviewed in (HARRIS and LEVINE 2005)). Expression of dominant-negative form of *Drosophila p53* (*UAS-dp53*^{R155H}; (BRODSKY *et al.* 2000)) does not block C3 accumulation in *ago*^{1} cells (Fig. S5). *e2f1*-driven death of *ago*^{1} MF cells thus proceeds by *dp53*-independent mechanism that can be reversed by elevating expression of *rbf1* or by removal of the dE2f1-target gene *hid*. *hid* and *rpr*, which is also inducible by dE2f1 (ASANO *et al.* 1996), are both overexpressed in *ago*^{1}*/M(3)* eye discs (Figs. 7A,B), and Hid and Rpr proteins accumulate in *ago*^{1} clones that overexpress the anti-apoptotic gene *p35* (Figs. 7C-D"). This effect occurs anterior to the MF, but also in posterior areas of the eye disc. The MF-associated Hid accumulation seems to be slightly more anterior relative to the MF than the C3 stripe, consistent with a delay between Hid expression and C3 build-up. Notably, not all '*ago*^{1}+*p35*' cells in the area anterior to the MF accumulate Hid and Rpr, indicating either that these proteins accumulate transiently in *ago*^{1} cells even the presence of p35, or that additional factors are limiting for their expression. Together these molecular data indicate that *ago*^{1} MF-associated apoptosis requires the *de2f1* transactivation domain and transcription of the *de2f1*-targets *hid* and *rpr*.

The pro-survival activity of the *EGFR* receptor tyrosine kinase (RTK) is linked in part to its ability to inhibit *hid* (BERGMANN *et al.* 2002; KURADA and WHITE 1998) and this function is proposed to underlie a pro-survival role for EGFR in *rbf1* mutant MF cells (MOON *et al.* 2006). Given that *ago* acts upstream of *de2f1* pathway and *hid*,

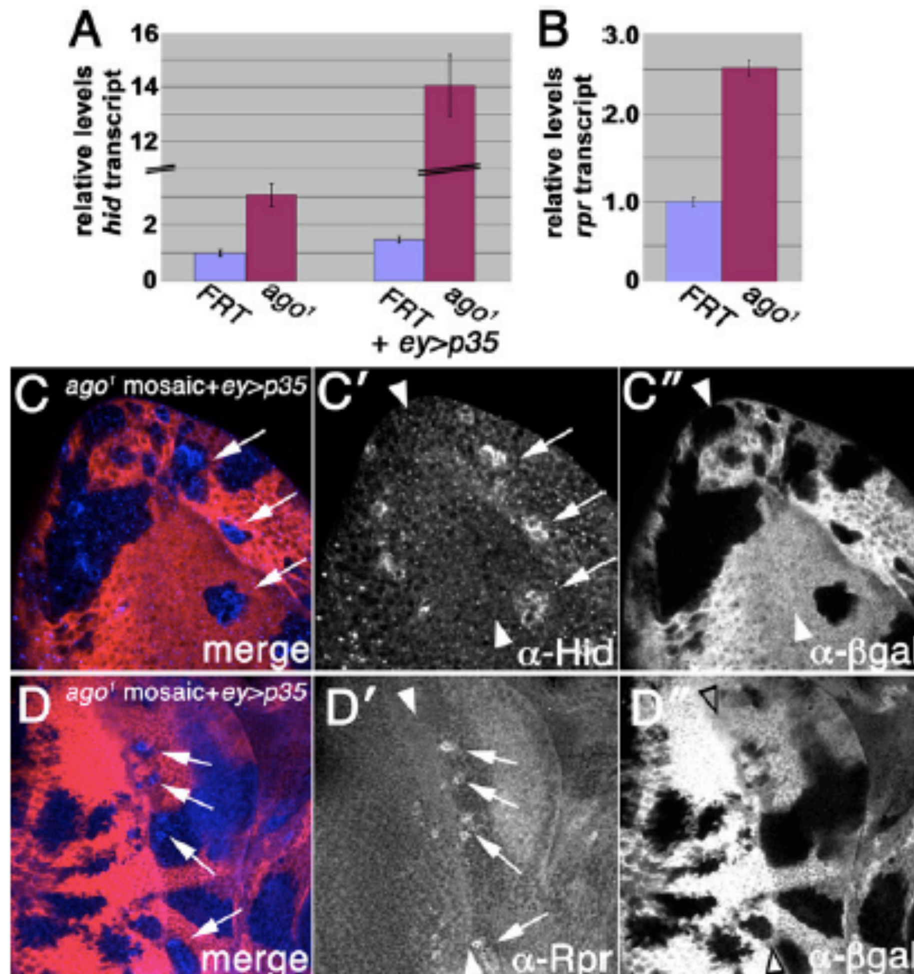
we sought to determine whether the death of *ago*^{1} cells also proceeds by an *EGFR*-sensitive mechanism. *eyGal4*-driven expression of the *EGFR*^{Ellipse} gain-of-function allele (*Elp*) (Baker and Rubin 1989) blocks MF-associated C3 in *ago*^{1}*/M(3)* discs (Figs. 8A-A') and synergizes with *ago*^{1}*/M(3)* to increase disc size (Fig. 8B). Elav expression is normal in this genotype, indicating that C3 loss is not due indirectly to MF loss. C3 signal in posterior areas of *ago*^{1}*/M(3)+ey>Elp* discs is also observed in *ey>Elp* discs (data not shown), indicating it occurs independent of *ago*^{1}. In the reciprocal experiment, *EGFR* activity was reduced in *ago*^{1}*/M(3)* discs with the *EGFR*^{t1} hypomorphic allele (Schupbach 1987). This dramatically shrank *ago*^{1}*/M(3)* adult eyes (Fig. 6C). A similar effect was observed with a second allele *EGFR*^{f24} (data not shown). These data indicate that *ago*^{1} sensitizes eye cells to reduced EGFR signaling. To further test the interaction between EGFR/RTK signals and *ago*, C3 expression was tested in eye discs doubly mutant for *ago* and the EGFR inhibitors *Gap1* (*Gap1*$^{F2.4\text{-}A3}$; K. Moberg and I.K. Hariharan, unpub.) or *argos* (*aos*$^{\Delta7}$; (Freeman *et al.* 1992)), or the RTK inhibitor *sprouty* (*sty*$^{\Delta5}$; (Hacohen *et al.* 1998)). Whereas each of these alleles is able to rescue *rbf1* cell death in the MF (Moon *et al.* 2006), they are not equally capable of rescuing the death of *ago*^{1} MF cells (Figs. 8C-E): *sty*$^{\Delta5}$ largely blocks C3 accumulation, with two areas of C3 remaining laterally (Fig. 8C), whereas *Gap1*$^{F2.4\text{-}A3}$ or *aos*$^{\Delta7}$ do not detectably alter the pattern of C3 accumulation (Figs. 8D,E). As *aos*$^{\Delta7}$ rescues the death of *rbf1* mutant MF cells (Moon *et al.* 2006) these data argue that although MF-associated apoptosis of *ago* and *rbf1* cells is similar in that they both require *de2f1*, *ago*^{1} cells require stronger EGFR/RTK signals (i.e. *sty*$^{\Delta5}$ and *ey>Elp*) to survive than do *rbf1* mutant cells. In support of this, *sty*$^{\Delta5}$*/M(3)* eyes

are larger than *Gap1*$^{F2.4\text{-}A3}$*/M(3)* and *aos*$^{\Delta 7}$*/M(3)* (Fig. 6C) indicating that the *sty*$^{\Delta 5}$ allele elicits stronger or more varied effects on downstream growth and survival pathways. Interestingly compound loss of *ago* and *sty*, *Gap1*, or *aos* does not lead to a readily detectable increase in adult eye size beyond that observed with *sty*, *Gap1*, or *aos* alone (Fig. 6C). Synergy in *ago,Gap1* and *ago,aos* eyes might be obscured by MF-associated cell death. However the lack of a synergistic effect between *ago*^{1} and the *sty*$^{\Delta 5}$ allele may indicate that *ago* and *sty* regulate growth via overlapping mechanisms or that simultaneous loss of both genes activates other compensatory mechanisms that reduce final organ size.

C3 expression at the lateral margins of *ago*^{1}*/M(3),ey>Elp* eye discs resembles the expression pattern of the *wingless* (*wg*) morphogen, which plays a pro-apoptotic role at the margins of the late pupal eye disc (CORDERO *et al.* 2004; LIN *et al.* 2004). To test whether this lateral C3 might require *wg*, one copy of the *wg*$^{l\text{-}17}$ loss-of-function allele (BAKER 1987) was placed in the *ago*^{1}*/M(3)* background. This strongly reduced adult eye size (Fig. 6C) and elevated rates of apoptosis among cells behind the MF (bracket in Fig. 8F). Thus rather than engaging a Wg-dependent death pathway, *ago*^{1} appears to sensitize cells to a Wg survival signal that operates among differentiated cells behind the MF. Together these data argue that MF-associated death of *ago*^{1} cells is driven by an autonomous mechanism requiring *de2f1*, but that the strength of this signal can be modified by signals transduced through the EGFR and RTK pathways. Moreover, a second pathway involving *wg* appears to modulate the sensitivity of *ago*^{1} cells to death at a subsequent stage of development behind the MF. The net growth effects of *ago* mutations are thus a product of intrinsic

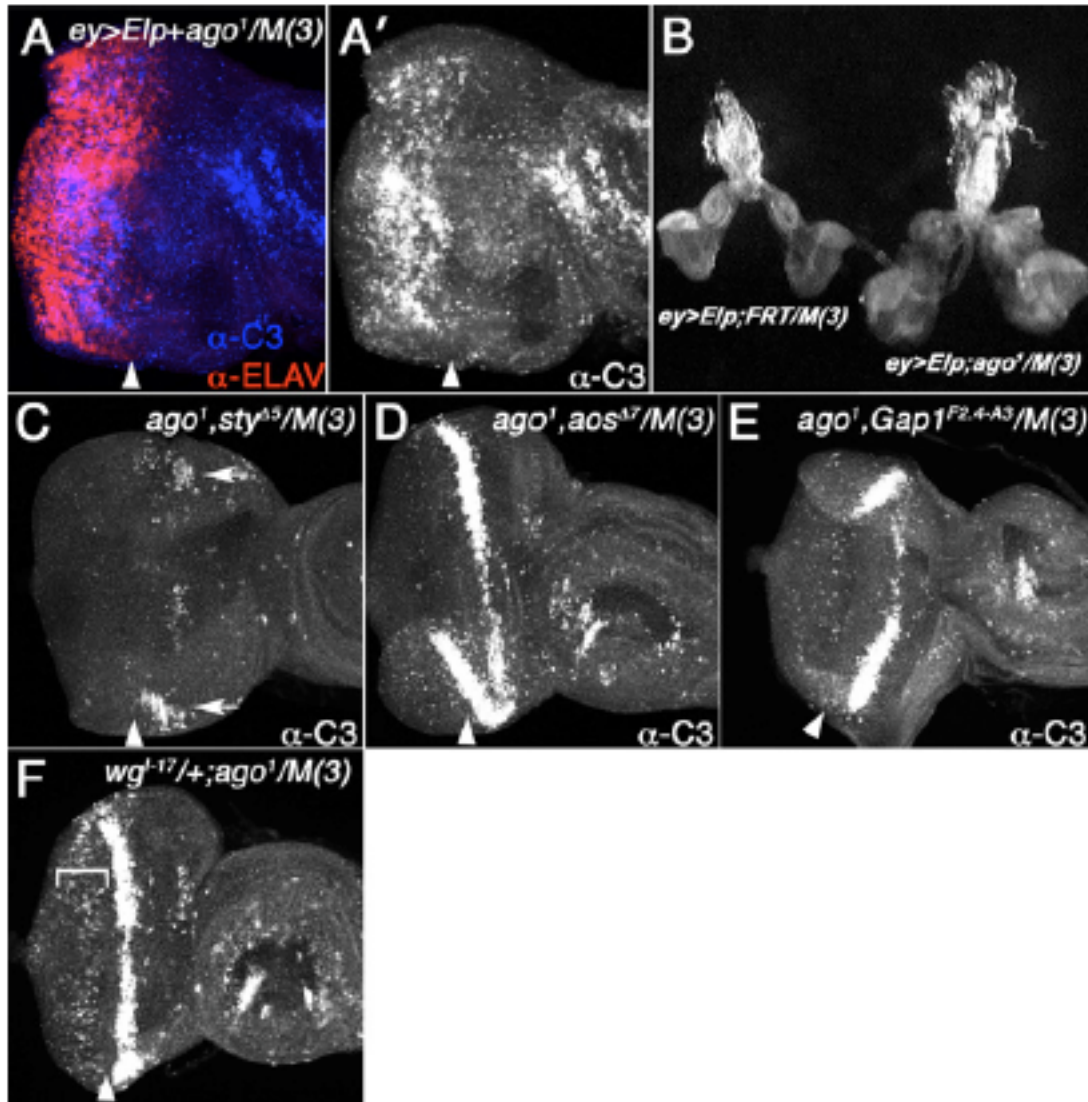
proliferative properties and extrinsic signals that modulate rates of apoptosis among these cells.

Figure II.7. Expression of *hid* and *reaper* is elevated in *ago* mutant tissue



Quantitative real-time PCR analysis of the expression of (A) *hid* and (B) *rpr* mRNAs in *FRT80B/M(3)* control or *ago¹/M(3)* eye antennal discs. *hid* mRNA was also measured in *ago¹/M(3),eyGal4,UAS-p35* discs in order to prevent the loss of *hid* mRNA in dying cells. (C-D) *ago¹* clones, marked by the absence of β-galactosidase (red), in *eyGal4,UAS-p35* discs stained with (C-C") α-Hid or (D-D") α-Rpr (both blue in C and D panels). Opposing arrowheads mark the MF (posterior to the left). Arrows mark accumulation of Hid or Rpr in *ago¹* clones.

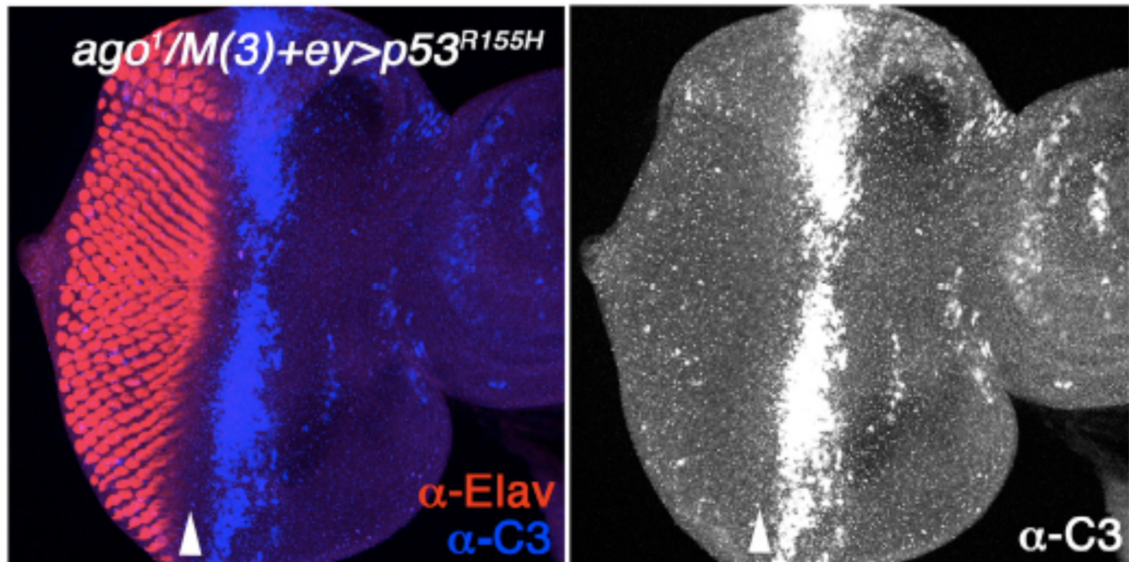
Figure II.8. Death of *ago* mutant eye cells is sensitive to extracellular signaling pathways



(A-A') Merged confocal sections of *ago*^{1}*/M(3)* discs generated in a background overexpressing the hyperactive *EGFR*^{Elp} allele (*eyGal4,UAS-Elp*) transgene stained for Elav (red) or C3 (blue) to mark dying cells (arrowhead marks MF) . (B) Light microscopic images of *eyGal4,UAS-Elp* and *ago*^{1}*/M(3),eyGal4,UAS-Elp* discs (the

ago^{1}*/M(3),eyGal4,UAS-Elp* genotype is pupal lethal and could not be included in the analysis in Figure 6C). Merged confocal sections of (C) *ago*^{1}*,sty*^{D5}*/M(3)*, (D) *ago*^{1}*,aos*^{D7}*/M(3)*, (E) *ago*^{1}*,Gap1*$^{F2.4\text{-}A3}$*/M(3)*, or (F) *wg*$^{l\text{-}17}$*/+;ago*^{1}*/M(3)* larval eye discs stained with α-C3. Arrows in panel C mark persistence of the α-C3 signal at the lateral margins of this disc. Bracket in panel F marks enhanced C3 expression in cells posterior to the MF. Arrowheads mark position of the MF.

Figure II.S5. Death of *ago^1* mutant eye cells is insensitive to dominant-negative p53



Merged confocal sections of an *ago^1/M(3)* larval eye disc expressing *UAS-p53^{R155H}* under control of the *eyGal4* driver. C3 (blue) and Elav (red) are indicated. Arrowhead marks position of the MF.

II.D. Discussion

Many mammalian tumor suppressors simultaneously restrict cell proliferation and apoptosis. As a result, the oncogenic effect of inactivating these genes is balanced by increased apoptosis. Data presented here show this central tenet of vertebrate cancer biology also applies to the *Drosophila* growth suppressor gene *ago*, a homolog of the mammalian tumor suppressor *Fbw7*. Eye overgrowth resulting from loss of *ago* is retarded by apoptosis that occurs as *ago* mutant cells encounter the MF. Blocking this death leads to eye hypertrophy, demonstrating that *ago* mutations can synergize with anti-apoptotic mutations to drive organ overgrowth. The pattern of apoptosis in *ago* mutant eye discs resembles that caused by loss of the retinoblastoma homolog *rbf1* (MOON *et al.* 2006), and we find that *ago* acts as required inhibitor of the *de2f1/rbf1* pathway in this organ. Removing *de2f1 trans*-activation function from *ago* mutant cells blocks apoptosis but does not retard their clonal overgrowth, indicating that *ago* acts through *de2f1* to activate transcription of genes required for apoptosis but not tissue growth. Together these data link two tumor suppressor gene homologs, *ago* and *rbf1*, in a common developmental pathway and show that the role of *ago* as a required regulator of the *rbf1/de2f1* pathway activity can have a significant impact on the phenotypic outcome of *ago* loss.

The tissue-specificity of the *ago* apoptotic phenotype indicates that a signal associated with the MF provides a pro-apoptotic cue to *ago* cells. As this signal, or

combination of signals, is not known, it is difficult to predict where and when *ago* cells in other organs will die. Part of the pro-death stimulus may come from an inability of *ago* cells to respond to cell cycle arrest pathways that begin to be activated as cells approach the MF. However, since cells in other organs (e.g. antenna) are also required to exit the cell cycle as part of their own differentiation programs, cell cycle exit per se does not kill *ago* mutant cells. Thus signals that are either eye- or MF-specific, or that are associated with sharply timed synchronous arrest (as in the ZNC in the larval wing), may also play important roles in the death of *ago* cells.

rbf1 and *ago* mutant eye disc cells appear to share a common apoptotic mechanism that requires intact *de2f1* function and involves elevated expression of pro-death genes like *hid*. However, the variable ability of EGFR pathway alleles to rescue death of *ago* and *rbf1* mutant cells argues that *ago* loss may elicit a more complex and perhaps stronger pro-apoptotic stimulus than loss of *rbf1*. As studies of *rbf1*-regulated apoptosis were done with both hypomorphic ($rbf1^{120a}$) and null ($rbf1^{14}$) alleles of *rbf1* (Moon *et al.* 2006), differences between *ago* and *rbf1* mutant phenotypes are not easily attributable to differences in allele strength. We favor a model in which additional factors that act downstream of *ago* synergize with *de2f1* to provide a pro-death stimulus that is stronger than that in *rbf1* cells. *de2f1* hyperactivation is still required for death in both situations, but because of these additional pro-death inputs, the threshold of EGFR/RTK signaling required to rescue *ago* cells is only met by the *ey*>*Elp* or $sty^{\Delta 5}$ alleles. The reciprocal finding that an *EGFR* allele shrinks *ago/M(3)* eyes supports a model in which *EGFR* signaling is

limiting for the survival of *ago* cells, and suggests that the fate of individual *ago* cells just anterior to the MF may depend on local variations in EGFR signaling strength in a manner similar to that shown for *rbf1* cells (MOON *et al.* 2006).

Based on studies that show that *Fbw7* degrades the Notch intracellular domain (WELCKER and CLURMAN 2008) and that Notch-1 promotes the death of *Fbw7* mutant MEFs (ISHIKAWA *et al.* 2008), Notch is a candidate pro-apoptotic factor in *ago* cells. Indeed, since Notch is antagonistic to EGFR signaling (DOROQUEZ and REBAY), elevated Notch signaling is one potential explanation for the differential sensitivity of *ago* and *rbf1* apoptosis to EGFR pathway alleles. Studies that place *Drosophila* Notch upstream of *de2f* in the larval eye disc (FERRES-MARCO *et al.* 2006) and within the MF (BAONZA and FREEMAN 2005; FIRTH and BAKER 2005) suggest it could synergize with CycE and dMyc to elevate *de2f1* activity in *ago* cells. However since CycE, dMyc and Notch are all proposed to have *de2f1*-independent roles in cells, the differences between *ago* and *rbf1* apoptotic phenotypes could involve roles for *ago* in pathways beyond the *rbf1/de2f1* pathway.

In light of the high rates of apoptosis among *ago*^{1} eye cells, it now appears that *ago* mutations were identified in mosaic screening only because the proliferative advantage conferred by *ago* loss could not be completely counterbalanced by increased death. A similar mechanism has been postulated to explain the relationship between *ago* loss and excess numbers of interommatidial cells in the pupal eye disc (MOBERG *et al.* 2001). If this phenomenon can be generalized to other *Drosophila* anti-growth genes, then traditional mosaic screens carried out in the background of intact apoptotic signaling may be limited in their ability to identify

factors that simultaneously restrict growth and apoptosis. Such genes may be more easily identified in screens designed to recover mutations that synergize with a block in apoptosis to drive hyperplasia.

The synergistic effect of *ago* and *H99/hid* loss on growth is quite similar to cooperativity between alleles of mammalian tumor suppressors like *Rb* and *p53* (e.g. WILLIAMS *et al.* 1994). Whether *Fbw7* mutations require a collaborating block in death in order to promote vertebrate tumorigenesis has not been carefully examined. If so, then *Drosophila* may provide an excellent system in which to identify second-site modifiers of the *ago* small-eye phenotype whose vertebrate homologs are candidates to modulate the rate of growth of *Fbw7* mutant tumors. While some of these modifiers are expected to block apoptosis and reverse the *ago* small-eye phenotype (e.g. *hid*), others could increase apoptosis of *ago*^{1} cells and shrink or ablate *ago* mutant organs (e.g. *wg* and *EGFR*). Thus the fly eye may be an ideal system in which to identify genetic or pharmacological conditions that mimic conditions anterior to the MF and lead to widespread apoptosis of *ago* mutant cells. Such manipulations may be useful tools to induce the apoptotic death of human cancer cells lacking *Fwb7* function.

II.E. Experimental Procedures

II.E1. Genetics

Crosses were performed at 25°C. The following genotypes were used for 'cell lethal' experiments: *eyFLP;P[m-w*$^{+}$*]RpL14*^{1}*,FRT80B*, *ago*^{1}*FRT80B/TM6B*, *ago*^{x2}*,Df(3L)H99,FRT80B/TM6B*, *ago*^{1}*,hid*^{05014}*,FRT80B/TM6B*, *ago*^{1}*,sty*^{D5}*,FRT80B/TM6B*, *ago*^{1}*,aos*^{D7}*,FRT80B/TM6B*, *ago*^{1}*,Gap1*$^{F2.4A3}$*,FRT80B/TM6B*, *EGFR*^{t1}*/CyO;ago*^{1}*FRT80B/TM6B*, *wg*^{xx}*/CyO;ago*^{1}*FRT80B/TM6B,* *cycE*^{AR95}*/CyO;ago*^{1}*FRT80B/TM6B*, and *ago*^{1}*,FRT80B,de2f1*^{rM729}*/TM6B*. The following genotypes were used for '*ago* mosaic' experiments: *w;P[m-w+;PCNA-EmGFP];ago*^{1}*,FRT80B/TM6B,* *eyFLP;P[m-w*$^{+}$*;arm-LacZ],FRT80B/TM6B*, *ago*^{1}*,FRT80B/TM6B*, *eyFLP;P[m-w*$^{+}$*;ubi>GFP],FRT80B*, *eyFLP;;ubi>GFP,FRT80B,de2f1*^{rM729}, and *ago*^{1}*,FRT80B,de2f1*^{i2}*/TM6B*. The following genotypes were used for transgenic expression: *UAS-rbf1/CyO:twi-GFP;ago*^{1}*,FRT80B/TM6B,* *UAS-dap/CyO:twi-GFP;ago*^{1}*,FRT80B/TM6B,UAS-Elp/CyO:twi-GFP;ago*^{1}*,FRT80B/TM6B,* *eyFLP;act>y*$^{+}$*>Gal4;P[m-w*$^{+}$*]RpL14*^{1}*,FRT80B/TM6B*, *w;UAS-p35;ago*^{1}*,FRT80B/TM6B,* *eyFLP;act>y*$^{+}$*>Gal4;P[m-w*$^{+}$*,arm-LacZ],FRT80B/TM6B, UAS-cycE, UAS-dMyc* and *UAS-dp53*^{R155H}*. PCNA-EmGFP* gift of R. Duronio. *UAS-Elp* and *EGFR*^{t1} gifts A. Mortimer. *de2f1*^{rM729}, *de2f1*^{i2}, and *wg*$^{l-17}$ obtained from the BDSC. *sty*^{D5} and *aos*^{D7} gift of N. Moon and I. Rebay.

II.E2. Eye measurements

Eye and wings were photographed with a Leica DFC500 CCD digital camera and sizes quantitated with Adobe Photoshop. Minimum of 10 eyes & wings were counted per genotype.

II.E3. Immunohistochemistry & Microscopy

Immunostaining and confocal microscopy was performed as described previously (MOBERG *et al.* 2004). Antibodies used: rabbit α-cleaved Caspase-3 (Cell Signaling) 1:100; rat α-Elav 1:200 (DSHB); guinea pig α-dE2f1 1:1000 (T. Orr-Weaver); rabbit α-GFP (Molecular Probes) 1:1000; mouse α-βgal 1:1000 (Promega); rabbit α-Hid 1:2500 (H. Steller); rabbit α-Reaper 1:1000 (S. Kornbluth). SEM was performed by the Apkarian Integrated Electron Microscopy Core (Emory) using a Topcon DS-130F Field Emission SEM.

II.E4. Real Time RT-PCR

Total RNA isolated from 30 eye discs (TRIzol/Invitrogen) was reverse transcribed (SuperScript II RT/Invitrogen) and analyzed by qPCR (SYBR Green 1 Master/Roche) Primers: *hid* 5'-GTGGAGCGAGAACGACAAA-3', 5'-TTGGCCAAGTGAAGCTCTGT-3'; *rpr* 5'-TCGATTTCTACTGCAGTCAAGG-3', 5'GAGTAAACTAAAATTGGGTGGGTGT-3'; *b-tub* 5'-CGCACAGAGTCCATGGTG-3', 5'-AAATCGTTCACATCCAAGCTG-3'.

II.E5. Cell culture

S2 cells were cultured under standard conditions. Double-stranded RNA interference was carried out as described previously (STEVAUX *et al.* 2005). Reporter experiments were done in triplicates in two separate experiments. Briefly, cells were pretreated with dsRNA for 4 days, transfected with *PCNA-luciferase* and *pIE4-lacZ* plasmids (CellFectin; Invitrogen), and analyzed 48hrs later. Error bars represent the standard deviation of the mean.

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Chapter 3: *Notch*-dependent expression of the *archipelago* ubiquitin ligase subunit in the *Drosophila* eye

Notch-dependent expression of the _archipelago_ ubiquitin ligase subunit in the _Drosophila_ eye

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III.A. Abstract

The *Drosophila archipelago* gene (*ago*) encodes a conserved protein (Ago) that functions as the substrate-receptor component of a ubiquitin-ligase that suppresses tissue growth in flies and tumorigenesis in vertebrates. Ago and its vertebrate homolog Fbw7 target multiple proteins for degradation, including the G1-S regulator Cyclin E and the oncoprotein c-Myc/dMyc. Given the significant effect that loss of *ago* or *Fbw7* have on growth control, it is notable that very little is known about signals that act upstream of Ago/Fbw7 to regulate their abundance in developing tissues. Here we use the fly eye as a model to identify developmental signals that regulate *ago* expression. We find that expression of *ago* mRNA and protein is induced by passage of the morphogenetic furrow (MF) and identify the *Hedgehog (Hh)* and *Notch* (*N*) pathways as required elements of this inductive mechanism. Cells mutant for *N* pathway components, or *Hh* defective cells that express reduced levels of the Notch ligand Delta, fail to upregulate *ago* transcription in the region of the MF; reciprocally, ectopic *N* activation in eye discs induces expression of *ago* mRNA. The *ago* promoter contains consensus binding sites for the *N* pathway transcription factor Su(H) and a fragment of the *ago* promoter containing these sites confers *N*-inducibility in cultured cells. Finally, we find that the failure to upregulate *ago* in *N* pathway mutant cells correlates with accumulation of the SCF-Ago target Cyclin E in the area of the MF, and this can be rescued by re-expression of *ago*. These data are consistent with a model in which *N* acts through *ago* to restrict levels of the pro-mitotic factor Cyclin E. This

N/Ago/Cyclin E link represents a significant new cell-cycle regulatory mechanism in cells of the developing eye.

III.B. Introduction

The *archipelago* (*ago*) gene was first identified in a screen for growth suppressor genes in the *Drosophila melanogaster* eye (MOBERG *et al.* 2001) and was subsequently shown to have a human ortholog, *Fbw7*, which is mutated in a wide array of human cancers <reviewed in (WELCKER and CLURMAN 2008). *ago* encodes an F-box/WD (tryptophan/aspartic acid) protein (Archipelago or Ago) which acts as the substrate specificity component of a Skp/Cullin/F-box (SCF) E3 ubiquitin ligase (SCF-Ago). SCF-Ago acts in various developmental contexts to target proteins for polyubiquitination and subsequent degradation, including the G1/S cell-cycle regulator Cyclin E (Cyclin E) and the growth regulator dMyc in mitotically active imaginal disc cells (MOBERG *et al.* 2001; MOBERG *et al.* 2004), the bHLH-PAS domain transcription factor Trachealess (Trh) in post-mitotic tracheal cells (MORTIMER and MOBERG 2007), and the glial cell factor Glial Cells Missing/Glide in neuronal cells (HO *et al.* 2009). Loss of *ago* in imaginal disc cells impairs degradation of Cyclin E and dMyc proteins, which then accumulate and drive excess cell proliferation by balanced increases in the rates of cell division and cell growth (MOBERG *et al.* 2004). The mammalian Ago ortholog, Fbw7, acts in a similar manner to promote ubiquitination and degradation of Cyclin E and c-Myc (KOEPP *et al.* 2001; WELCKER *et al.* 2004a; WELCKER *et al.* 2004b; WELCKER *et al.* 2003; YE *et al.* 2004). Additional candidate targets of vertebrate Fbw7 include the Notch1 and Notch4 intracellular domains, c-Jun, sterol regulatory element binding protein (SREBP), and mTor kinase ((MAO *et al.* 2008) and reviewed in (WELCKER and CLURMAN 2008)). The *Fbw7* gene is biallelically lost in a variety of primary tumor types (reviewed in WELCKER and

CLURMAN 2008), including colorectal (RAJAGOPALAN *et al.* 2004) endometrial (SPRUCK *et al.* 2002) and pancreatic (CALHOUN *et al.* 2003) cancers, indicating that the Fbw7 protein is growth-inhibitory *in vivo*. In addition, *Fbw7* mutations occur frequently in T-cell acute lymphoblastic leukemia (T-ALL) (MALYUKOVA *et al.* 2007; MASER *et al.* 2007; O'NEIL *et al.* 2007; THOMPSON *et al.* 2007) and loss of a single copy of *Fbw7* can synergize with *p53* loss to accelerate tumorigenesis and broaden the spectrum of epithelial tumors in mice (MAO *et al.* 2004).

Given the significant effect of *ago/Fbw7* loss on cell proliferation and tumor progression, it is notable that very little is known about signals that act upstream of either fly Ago or vertebrate Fbw7 to regulate their abundance in developing tissues. *Drosophila ago* is expressed ubiquitously in the eye disc but exhibits a strong pulse of mRNA expression in the area of the morphogenetic furrow (MOBERG *et al.* 2001), where cells arrest in G1 (WOLFF and READY 1991). *ago* protects developing eye cells in the anterior region of the morphogenetic furrow (MF) from apoptotic cell death driven via the Rbf/dE2f1 pathway (NICHOLSON *et al.* 2009), and blocking dE2f1-dependent apoptosis of *ago* cells at the MF reverts a small-eye phenotype caused by *ago* loss and leads to dramatically enlarged organs (NICHOLSON *et al.* 2009). Thus, Ago activity in the MF plays a significant role in determining the phenotypic outcome of *ago* loss on organ size by ensuring inactivity of pro-apoptotic dE2f1. Although a variety of juxtacrine and paracrine pathways are active in the eye disc, their contributions to the MF-associated pulse of *ago* expression in the developing eye disc are undefined. The *ago* promoter does contain a consensus binding site for the *Drosophila* p53 ortholog dp53, and dp53 acts downstream of metabolic stress

produced by loss of the mitochondrial *cytochrome c oxidase subunit Va* (*CoVa*) to upregulate *ago* transcription and induce G1 arrest (MANDAL *et al.* 2010). However, other than its induction as part of this metabolic checkpoint, nothing is known about the developmental signals that pattern *ago* expression.

Here we show that the pulse of *ago* expression occurs within and immediately behind the MF and requires the activity of two pathways with established roles in the MF, the *Notch* (*N*) pathway and the *Hedgehog* (*Hh*) pathway, and that the requirement for *Hh* activity arises due to an established role for *Hh* signaling in proper expression of the N ligand Delta (Dl) (VRAILAS and MOSES 2006). Cells mutant for *N* pathway components fail to upregulate *ago* transcription in the MF, and reciprocally, ectopic *N* activation in eye discs specifically induces expression of the *ago-RA* mRNA transcript, which initiates transcription from a unique 1st exon not shared by the other annotated *ago* transcripts *ago-RB* and *ago-RC* (Flybase.org). DNA sequences upstream of the *ago-RA* exon-1 contain consensus binding sites for the *N* pathway transcription factor Suppressor of Hairless (Su(H)) and a fragment of the *ago* promoter containing these sites confers *N*-inducibility in a cultured cell system. Finally, we find that the failure to upregulate *ago* in *N* pathway mutant MF cells correlates with accumulation of the SCF-Ago target Cyclin E in cells immediately posterior to the MF, and this phenotype can be rescued by expression of *ago* from an exogenous transgene. In sum, these data suggest that *ago* is a *N* target gene in the developing eye and indicate that N can act through *ago* to restrict the levels of the pro-mitotic factor Cyclin E in cells immediately behind the MF.

III.C. Results

III.C1. *ago* protein levels and promoter activity are elevated within the MF

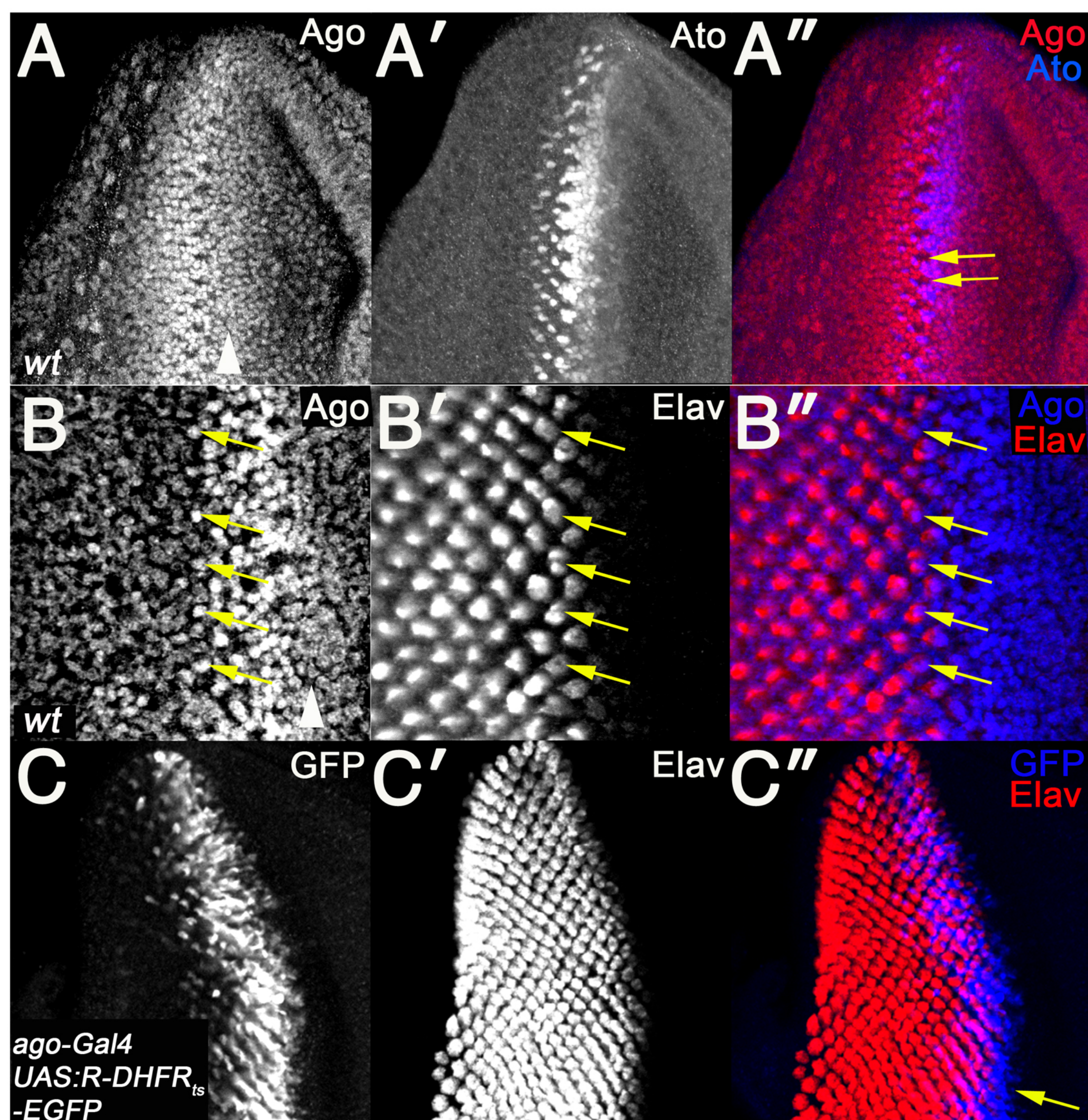
Immunostaining of 3rd instar eye imaginal discs with a polyclonal anti-Ago antiserum (MORTIMER and MOBERG 2007) reveals a strong pulse of nuclear Ago protein expression in the area of the MF (Fig 1A) that coincides with the previously described location of highest *ago* mRNA expression (MOBERG *et al.* 2001). Ago levels begin to rise above baseline among cells in the region of the MF (see Fig 1A and Fig 1B for different optical planes and magnifications; posterior is to the left in this and all subsequent images), and are subsequently refined into a pattern in the first few rows of cells posterior to the MF; 'holes' in this pattern appear to overlap with gaps in expression of the Atonal (Ato) neuronal transcription factor between emerging R8 precursor equivalence groups (DOKUCU *et al.* 1996) (see arrows Fig, 1A"). Higher magnification analysis of discs co-stained for Ago and the neuronal marker Elav (ROBINOW and WHITE 1991) shows the rise in Ago levels in the nuclei of cells anterior to Elav-positive neuronal nuclei (Fig 1B); in apical sections at the level of the Elav-positive photoreceptor nuclei, Ago appears to become restricted to a single cell nucleus that occupies an anterior location within the nascent Elav-positive cluster (see yellow arrows in Fig 1B-B'). Baseline levels of Ago are also detected in the Elav-negative cells surrounding the developing preclusters (Fig 1B"). This pattern of expression defines two phases of Ago expression at the MF: (1) an early inductive phase in the area of the MF, and (2) a later refinement of this to produce patterned Ago expression among cells in its wake. In this study we have focused on identifying

the Ago-inductive pathways involved in the initial phase of elevated Ago expression in the area of the MF.

The correlation between elevated Ago protein (this study) and mRNA (MOBERG *et al.* 2001) in the region of the MF suggests that transcriptional mechanisms may contribute to the induction of Ago in this area of the eye disc. To assay *ago* promoter activity in intact discs, the *GawB*-type transposable element *P{GawB}ago[NP0850]*, which contains a minimal promoter upstream of the *Gal4* open reading frame (BRAND and PERRIMON 1993) and is inserted 53 basepairs (bp) upstream of the transcriptional start site of the *ago-RA* transcript (the locus encodes three transcripts *ago-RA, RB* and *RC* that each initiate from a unique non-coding first exon; Flybase.org), was combined with a *UAS:R-DHFR*$_{ts}$*-EGFP* transgene (SPEESE *et al.* 2003), which expresses a temperature-sensitive DHFR-GFP fusion protein that is rapidly degraded in response to heat exposure. As *P{GawB}* elements function as enhancer-trap reporters of the loci into which they insert (BRAND and PERRIMON 1993), we surmised that expression of *P{GawB}ago[NP0850]* (hereafter referred to as *ago>Gal4*) would depend on *ago* regulatory sequences and that the heat-labile EGFP would allow visualization of ongoing transcription, as opposed to the cumulative promoter activity reported by more stable forms of EGFP. Consistent with this hypothesis, *UAS:R-DHFR*$_{ts}$*-EGFP*;*ago>Gal4* animals shifted to the restrictive temperature 37°C and allowed to recover for 1 hour at the permissive temperature of 18°C show a stripe of EGFP accumulation associated with the posterior region of the MF (Fig 1C); in the absence of a 37°C shift, EGFP protein is expressed throughout the posterior region of *UAS:R-DHFR*$_{ts}$*-EGFP*;*ago>Gal4* eye discs (data not shown),

presumably due to perdurance of EGFP protein. The GFP signal in *UAS:R-DHFR*$_{ts}$-*EGFP*;*ago>Gal4* discs exposed to the 37°C-to-18° shift can be detected among Elav-positive cells in the rows immediately posterior to the MF and in cells lying just anterior to the region of Elav expression (medial region of the disc in Fig 1C"; see yellow arrow). Given the predicted temporal delay in EGFP expression produced by the binary *Gal4,UAS* system, these data indicate that the *ago>Gal4* element likely reports *ago* promoter activity in cells a few rows anterior to the location of EGFP appearance, which is the area within and immediately behind the MF. Notably, EGFP does not accumulate elsewhere in *UAS:R-DHFR*$_{ts}$-*EGFP;ago>Gal4* eye discs following the 37°C-to-18° shift, indicating that the *ago>Gal4* element does not detect the baseline promoter activity associated with the uniform expression of *ago* throughout the eye/antennal disc.

Figure III.1. Ago accumulates at the MF in a subset of Ato-positive and Elav-positive cells. Ago promoter activity is detected several rows of cells anterior to the wave of Elav-positive clusters.



(A-A'') Merged confocal sections of a wild type larval eye disc stained for Ago (red) and Ato (blue). Yellow arrows mark overlapping 'holes' in Ato and Ago expression immediately posterior to the MF (A''). White arrowheads indicate the position of the morphogenetic furrow (MF). In these and all following images, posterior is to the left.

(B-B'') Merged confocal sections of a wild type larval eye disc stained for Ago (blue) and Elav (red). Yellow arrows in B-B'' mark Ago-positive cells that reside anteriorly within the Elav-positive clusters.

(C-C'') Merged confocal sections of *UAS:R-DHFR$_{ts}$-EGFP; ago>Gal4* larval eye discs stained for GFP (blue) and Elav (red). Yellow arrow denotes GFP expression extending into the MF anterior to the first row of Elav-positive nuclei.

III.C2. Hedgehog and Notch pathway mutations block Ago induction at the MF

The coordinate control of cell division and neuronal cell specification in the area of the MF is the product of a highly coordinated series of events that rely on cell:cell communication mediated by morphogens and membrane-bound ligands (ROIGNANT and TREISMAN 2009). The pattern of induction of the *ago* promoter suggests one or more of these pathways drives *ago* expression in this region. To test this, clones of cells mutant for either the Dpp receptor *thickveins* (*tkv*), the Hedgehog receptor *smoothened* (*smo*), the Notch (N) activator *Presenilin* (*Psn*), or both of the N ligands *Delta* (*Dl*) and *Serrate* (*Ser*), were examined for their effect on Ago protein levels. While *tkv* loss had no effect on Ago expression (data not shown), cells mutant for the smo^3 null allele (CHEN and STRUHL 1998) fail to upregulate Ago protein among MF cells (Fig 2A-A''); these clones also lack the elevated Ago normally found in cells posterior to the MF, suggesting that Ago must be induced within the MF in order to perdure in the rows of cells immediately behind it. Notably, the uniform baseline expression of Ago elsewhere in the eye disc is unaffected by *smo* loss. These observations indicate that *smo* is required for the pulse of Ago expression specifically in the area of the MF, but that distinct mechanisms contribute to the uniform expression of Ago throughout the eye disc.

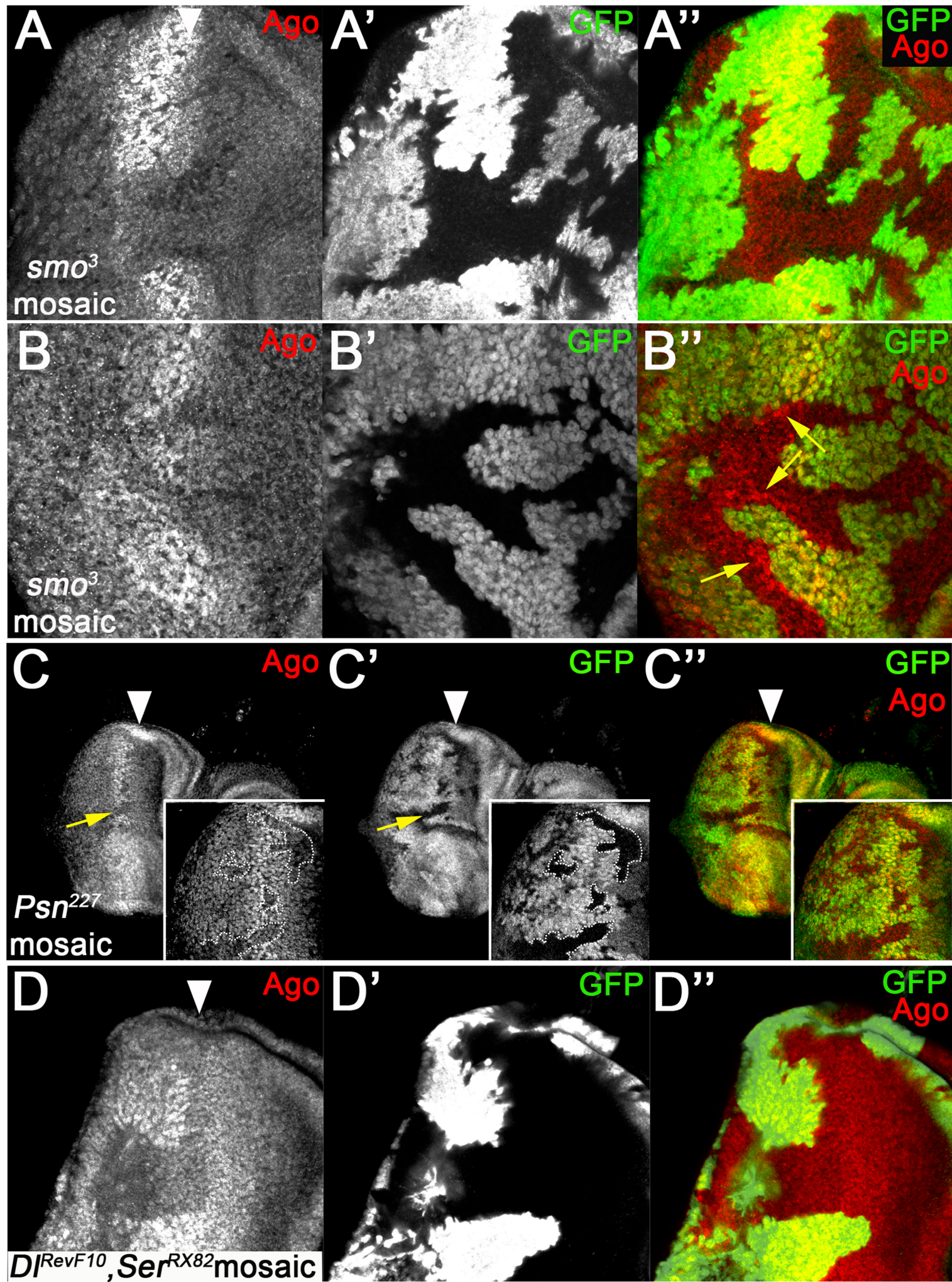
Interestingly, the pulse of Ago expression in MF cells can be rescued in smo^3 cells if they lie adjacent to wild type cells at a clonal boundary (Fig 2B-B''; yellow arrows). This indicates that *smo* itself is not absolutely required for Ago induction,

but that *smo* is required for the activity of a second juxtacrine pathway that is required for Ago expression. As *smo* is required for expression of the N ligand Dl protein in eye cells (VRAILAS and MOSES 2006), we hypothesized that the effect of the *smo*^{3} allele on Ago induction could result from reduced N signaling. Consistent with this, loss of multiple N pathway components blocks the upregulation of Ago protein in the area of the MF. Cells homozygous for a null allele of the *Presenilin* gene (*Psn*^{227}), which encodes a component of the γ-secretase complex that cleaves and activates N in response to ligand binding (STRUHL and GREENWALD 2001), fail to upregulate Ago protein at the MF (Fig 2C-C''). Similarly, clones of cells with null mutations in both N ligands, *Delta* (*Dl*^{RevF10}) and *Serrate* (*Ser*^{RX82}), also fail to upregulate Ago protein at the MF (Fig 2D-D''; and see also Supplemental Fig. 1). Furthermore, flies homozygous for a *N* temperature sensitive allele (*N*^{ts3}) show reduced Ago levels in the area of the MF in larval eye discs following a 2hr shift to the restrictive temperature (Fig 3A-A'). Ago expression posterior to the MF also becomes irregular in *N*^{ts3} discs upon longer exposure to the restrictive temperature, appearing as disorganized clumps of Ago-expressing nuclei (insets in Fig 3A' show *N*^{ts3} discs exposed to 0hr and 6 hrs of hs). Thus, *N* appears to have a second role in patterning Ago expression among cells posterior to the MF.

In light of the requirement for N signals in Ago expression in the MF, we tested whether generating a pulse of Dl protein using a *UAS>Dl* transgene in combination with the *hsp70-Gal4* driver could rescue Ago protein levels in *smo*^{3} clones. Re-expression of *Dl* restored Ago levels in *smo*^{3} clones lying within or just behind the MF (Fig 4A-A'' and B-B'', yellow arrows); this Ago protein appears to be

shifted posterior relative to the location of Ago expression in adjacent wild type cells, indicating that MF progression is delayed in *smo* clones as reported previously (STRUTT and MLODZIK 1997). Cumulatively, these data suggest that the *Hh* and *N* pathways are required for the pulse of Ago protein expression associated with passage of the MF, and that *smo* appears to act upstream of the N ligand Dl in this pathway.

Figure III.2. Hh and N pathway mutants cause a decrease in Ago levels at the MF.

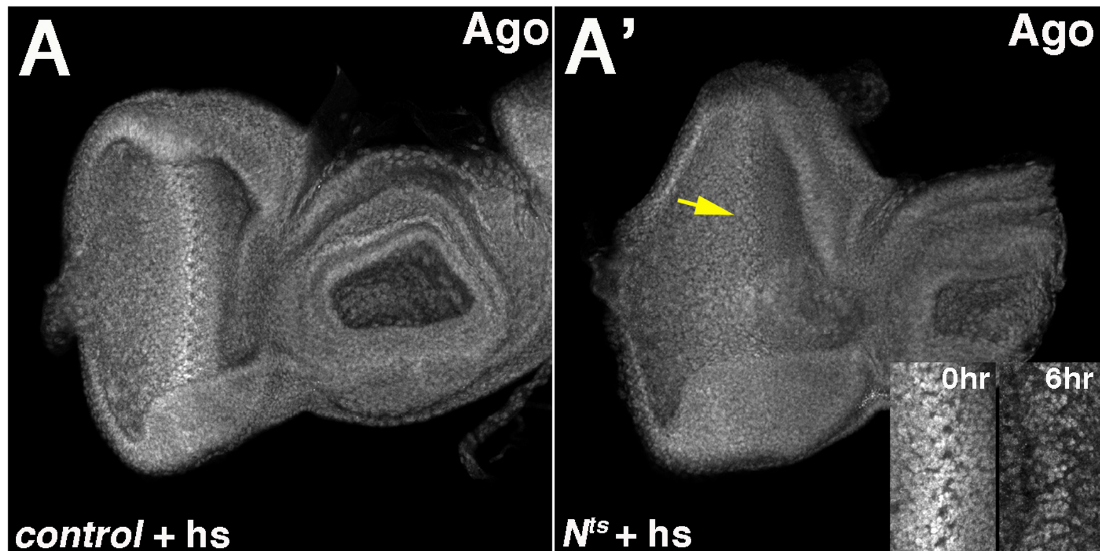


(A-A'' and B-B'') Ago (red) fails to accumulate at the MF in *smo*^{3} clones marked by the absence of GFP. Higher magnification (B-B'') shows rescue of Ago accumulation at clonal boundaries that cross the MF (yellow arrows in B'').

(C-C'') Ago (red) fails to accumulate at the MF in *Psn*^{227} clones (yellow arrows in C-C') marked by the absence of GFP (yellow arrow in C and C'). Insets show higher magnification view of clones (outlined by dotted lines) in C-C''.

(D-D'') Ago (red) fails to accumulate at the MF in *Dl*^{RevF10},*Ser*^{RX82} clones marked by the absence of GFP. White arrowheads indicate the position of the MF.

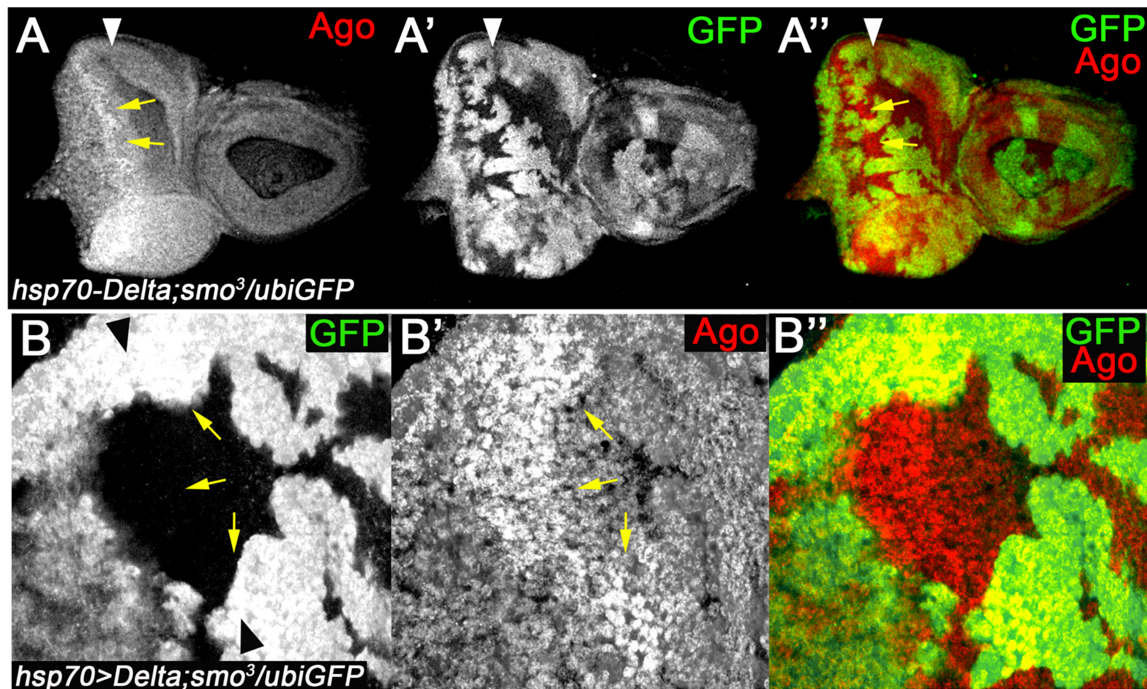
Figure III.3. N is necessary for elevated Ago expression and patterning at the MF.



(A) Confocal section of a heat-shocked control *FRT80B* eye disc stained for Ago.

(B) Confocal section of a N^{ts3} eye disc stained for Ago after a 2hr temperature shift to 31°C. Yellow arrow indicates residual Ago expression at the MF. Inset shows high magnification views of a N^{ts3} eye disc at t=0hr (left) and t=6hr (right) of heat-shock. Note the overall drop in Ago levels, and the appearance of disordered groups of Ago positive nuclei posterior to the MF.

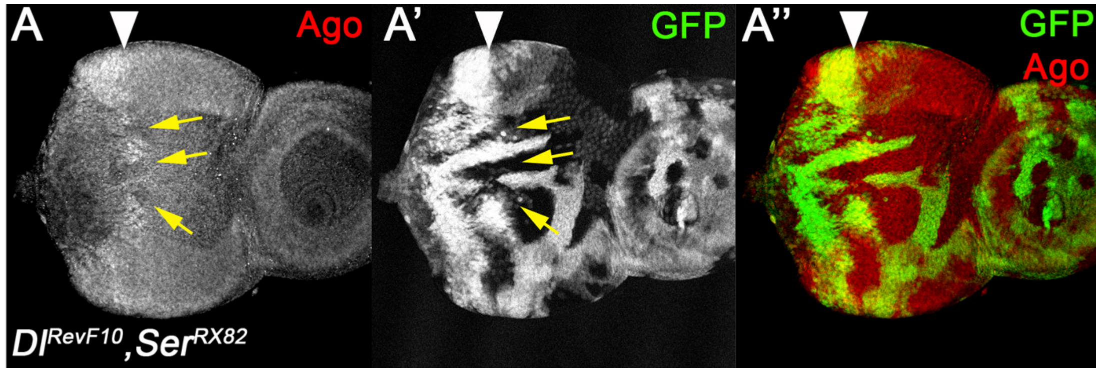
Figure III.4. Elevated *Dl* restores Ago in *smo* mutant cells.



(A-A'') Confocal sections of *smo*^{3} clones marked by the absence of GFP (green) generated in the presence of *hsp70>Dl* and stained for Ago (red). Yellow arrows in (A) denote a delayed rescue of Ago levels in mutant clones.

(B-B'') High magnification confocal section of *smo*^{3} clones marked by the absence of GFP (green) generated in the presence of *hsp70>Dl* and stained for Ago (red). Yellow arrows denote the delayed Ago expression in the mutant clone.

Figure III.S1. *Dl/Ser* mutations block the pulse of Ago expression at the MF.



(A-A'') Confocal sections of a whole disc bearing *Dl^{RevF10}*, *Ser^{RX82}* double mutant clones marked by the absence of GFP (green) and stained for Ago (red). Yellow arrows denote the lack of MF-associated Ago expression in *Dl^{RevF10}*, *Ser^{RX82}* clones.

III.C3. The *ago* promoter is *N*-responsive

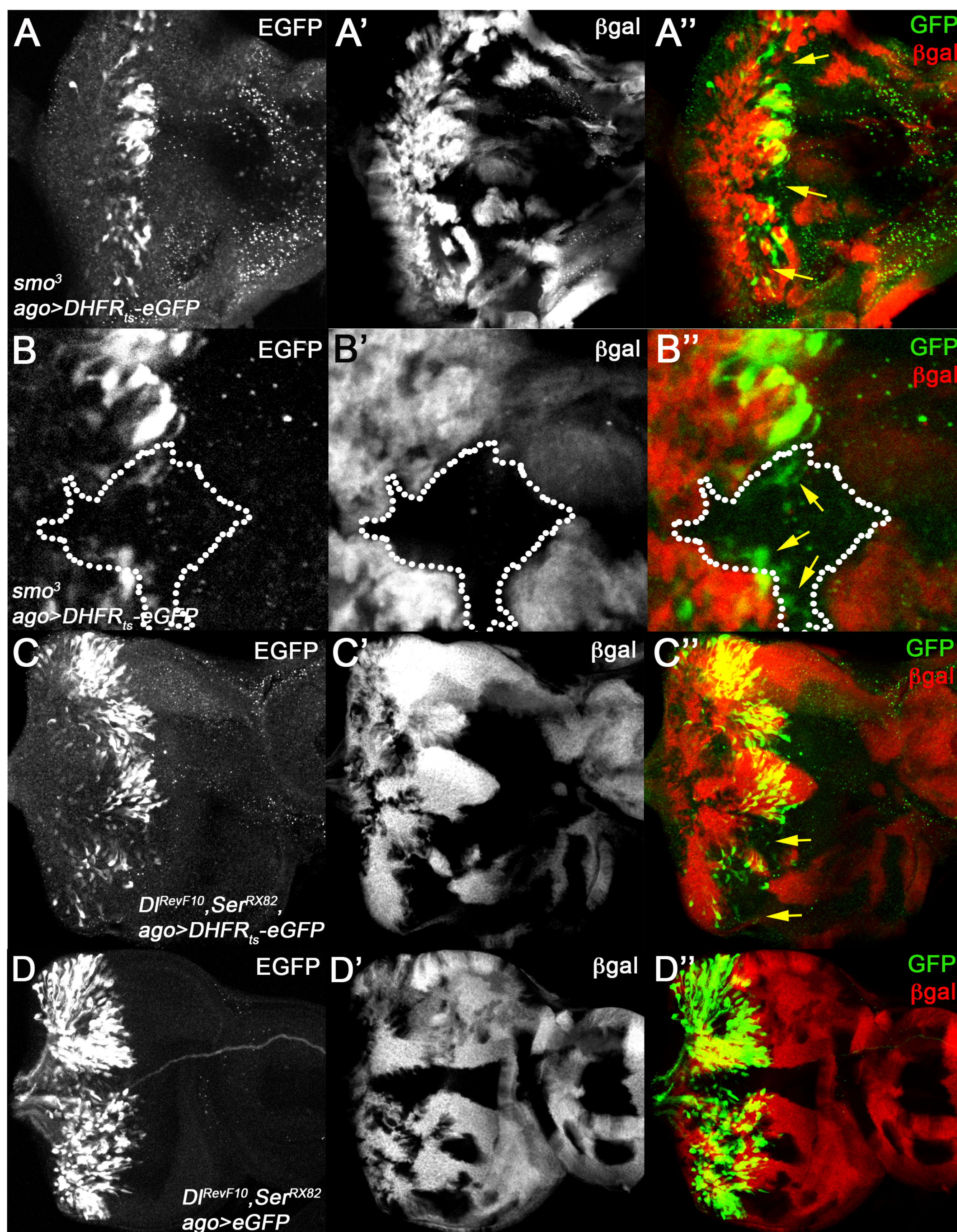
To determine whether the *Hh* and *N* pathways have an effect on *ago* transcription in the area of the MF, the activity of the *ago>Gal4* transcriptional reporter was tested in *Hh* and *N* pathway mutants. *ago* promoter activity measured by the *UAS:R-DHFR*$_{ts}$*-EGFP;ago>Gal4* system is reduced to background levels in *smo*^{3} mutant clones at the MF (Fig 5A-A'', yellow arrows). As observed with Ago protein, *smo*^{3} mutant cells that lie adjacent to wild type cells show normal levels of *ago-Gal4* activity (Fig 5B-B'', yellow arrows). Discs mosaic for mutations in *Dl* and *Ser* also show a failure to express GFP from the *UAS:R-DHFR*$_{ts}$*-EGFP;ago>Gal4* at the MF (Fig 5C-C''), that also appears to be rescued by the presence of adjoining wild type cells. This juxtacrine rescue of *ago>Gal4* activity is likely due to the ability of Dl and Ser proteins in wild type cells to signal into adjacent *Del/Ser* mutant cells. In sum, these data confirm that the *Hh* and *N* pathways are necessary for *ago* promoter activity in MF cells and are consistent with an indirect requirement for *smo* in this mechanism.

The effect of *N* pathway alleles on the *ago>Gal4* reporter suggests *ago* may be a transcriptional target of N. The *ago* promoter was examined for DNA sequences resembling those bound by the Su(H) transcription factor (NELLESEN *et al.* 1999), which complexes with the Notch intracellular domain (NICD) and mediates transcriptional responses to *N* pathway activity (BAILEY and POSAKONY 1995). Eleven putative Su(H) sites (RTGRGAR; (NELLESEN *et al.* 1999)) were identified within a 5.5kb region of the *ago* locus (spanning from base 4,250,442 to base 4,244,682 on chromosome 3L; numbering according to Flybase.org); two of these eleven sites contain a single base deviation from the RTGRGAR consensus that are still able to be

bound by Su(H) *in vitro* (MOREL and SCHWEISGUTH 2000). Four of these lie upstream of the first exon of the *ago-RA* transcript in the immediate area of the *N*-dependent *ago>Gal4* element (see Fig 6B; one of these four contains a single-base divergence from the RTGRGAR sequence). Two putative “A” sites for basic helix-loop-helix (bHLH) transcription factors (CAGSTG; (CAVE *et al.* 2005)) were also identified within the putative *ago* promoter. NICD target promoters commonly have binding sites for bHLH proteins and in the case of the NICD target gene *E(Spl)-m8*, co-expression of the NICD and the amino terminus of the bHLH protein Daughterless (DaN) synergistically activates gene transcription (CAVE *et al.* 2005). To more directly test the effect of N on *ago* promoter activity, a fragment of the *ago* promoter corresponding to a 2kb region (Fig 6B) (*ago2kb-luc*) containing all four putative Su(H) binding sites, the two putative A sites, and another site previously shown to be bound by the p53 transcription factor (MANDAL *et al.* 2010), was placed in front of the *luciferase* gene. S2 cells were then transfected with this *ago2kb-luc* reporter, NICD and DaN expression constructs, and a *Renilla* luciferase expression plasmid as an internal control for transfection efficiency. Addition of the *NICD* expression plasmid produced an approximately 3-fold increase in *ago2kb-luc* activity relative to its baseline expression, and this effect was potentiated by co-expression of DaN to an approximately 10-fold induction of promoter activity (Fig 6A). To confirm that *ago* transcript levels are also *N*-responsive in the *in vivo* context of the larval eye disc, the levels of *ago-RA* and *ago-RC* transcripts (the *ago-RB* transcript is undetectable in larval discs; NTM and KHM, unpublished) were measured in the eye discs of 3rd instar larvae 1-hour following a 1-hour heat-shock induction of a *hsp70-*

N^{intra} transgene (STRUHL *et al.* 1993), which expresses an intracellular, active form of the Notch receptor (Fig 6C). This resulted in a 3.5-fold increase in the abundance of the *ago-RA* transcript but not the *ago-RC* transcript (Fig 6C), suggesting that the effect of *N* on the *ago* promoter is transcript-specific. Considered together, the data from the *ago>Gal4* element, the *ago2kb-luc* reporter, and the *hsp70-N^{intra}* transgene, support a model in which *ago* gene transcription responds to both elevated and reduced *N* activity, and that this mechanism is required for the pulse of *ago* mRNA associated with passage of the MF.

Figure III.5. Hedgehog and Notch pathway mutants cause a decrease in *ago* promoter activity at the MF.



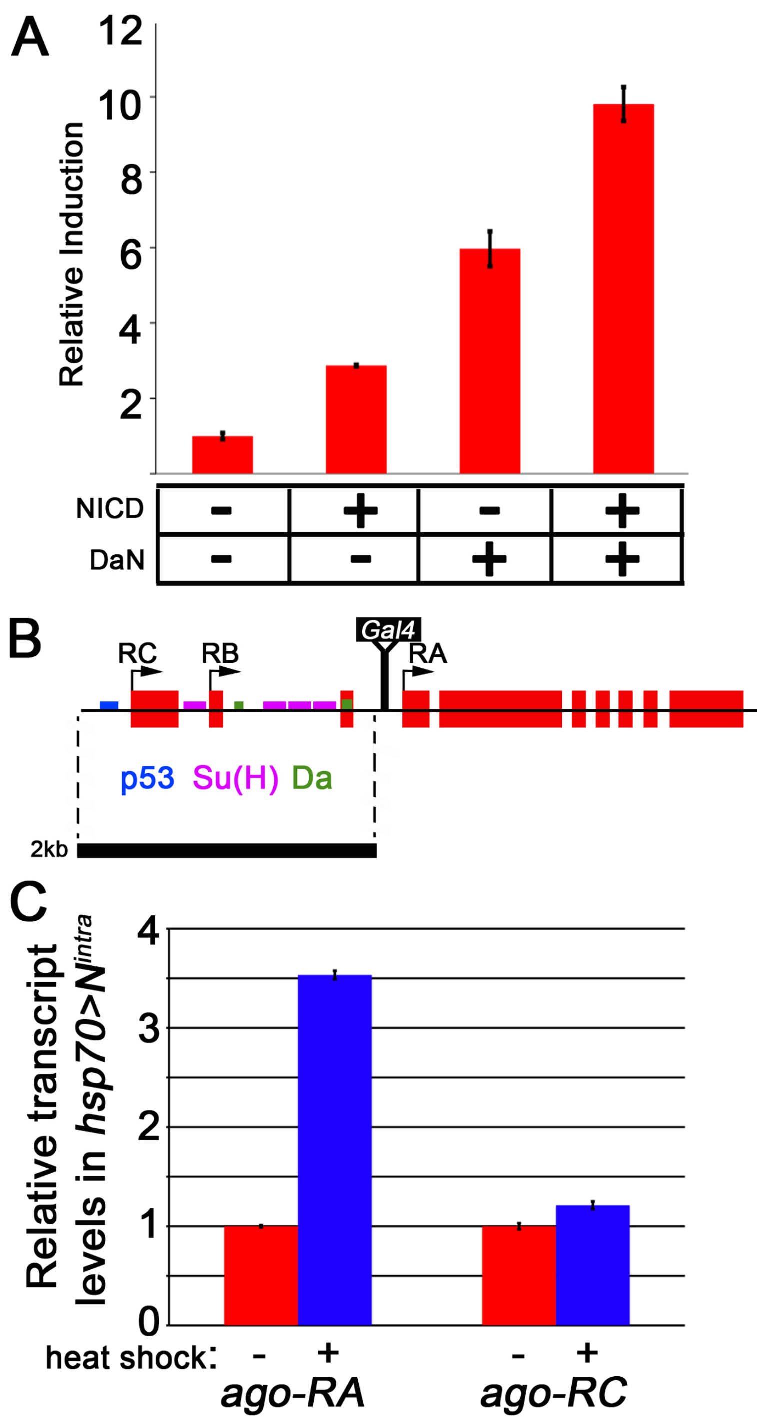
(A-A'') Confocal sections of *smo*^{3} clones marked by absence of βgal expression (red). *ago* promoter activity detected by the *ago>Gal4,UAS:R-DHFR*$_{ts}$*-EGFP* transgenes (green) is much lower in the mutant clones (yellow arrows in A-A').

(B-B'') A higher magnification view of the disc in A-A" shows rescued *ago* promoter activity in MF cells along clonal borders in the MF (yellow arrows in B'').

(C-C'') *Dl*^{RevF10}*,Ser*^{RX82} clones marked by the absence of βgal (red) generated in *ago>Gal4,UAS:R-DHFR*$_{ts}$*-EGFP* (green) discs. *ago* promoter activity drops in the mutants clones.

(D-D'') *Dl*^{RevF10}*,Ser*^{RX82} clones marked by the absence of βgal (red) generated in *ago>Gal4,UAS>eGFP* (green) discs. *ago>Gal4* driven expression of stable eGFP results in perdurance of GFP posterior to the MF only in wild type cells.

Figure III.6. The *ago* promoter is responsive to changes in levels of *N* signaling.



(A) Relative luciferase activity in S2 cells transfected with a plasmid containing a 2kb fragment of the *ago* promoter cloned in front of the luciferase coding sequence (*ago2kb-luc*). Cells were co-transfected with either 300ng of plasmid coding for the NICD, 50 ng of plasmid coding for DaN, or both, as indicated.

(B) Diagram of the *ago* gene locus. Three transcript variants, *RA*, *RB*, and *RC*, have alternate first exons but share the same coding sequence. Location of the *P{GawB}ago[NP0850]* element is indicated (53bp upstream of the annotated *ago-RA* transcriptional start-site; Flybase.org). The 2kb region cloned into the luciferase vector is indicated (black bar) as are the locations of putative transcription factor binding sites: four Su(H) binding sites (purple), two A sites (green), and the p53 binging sequence (blue).

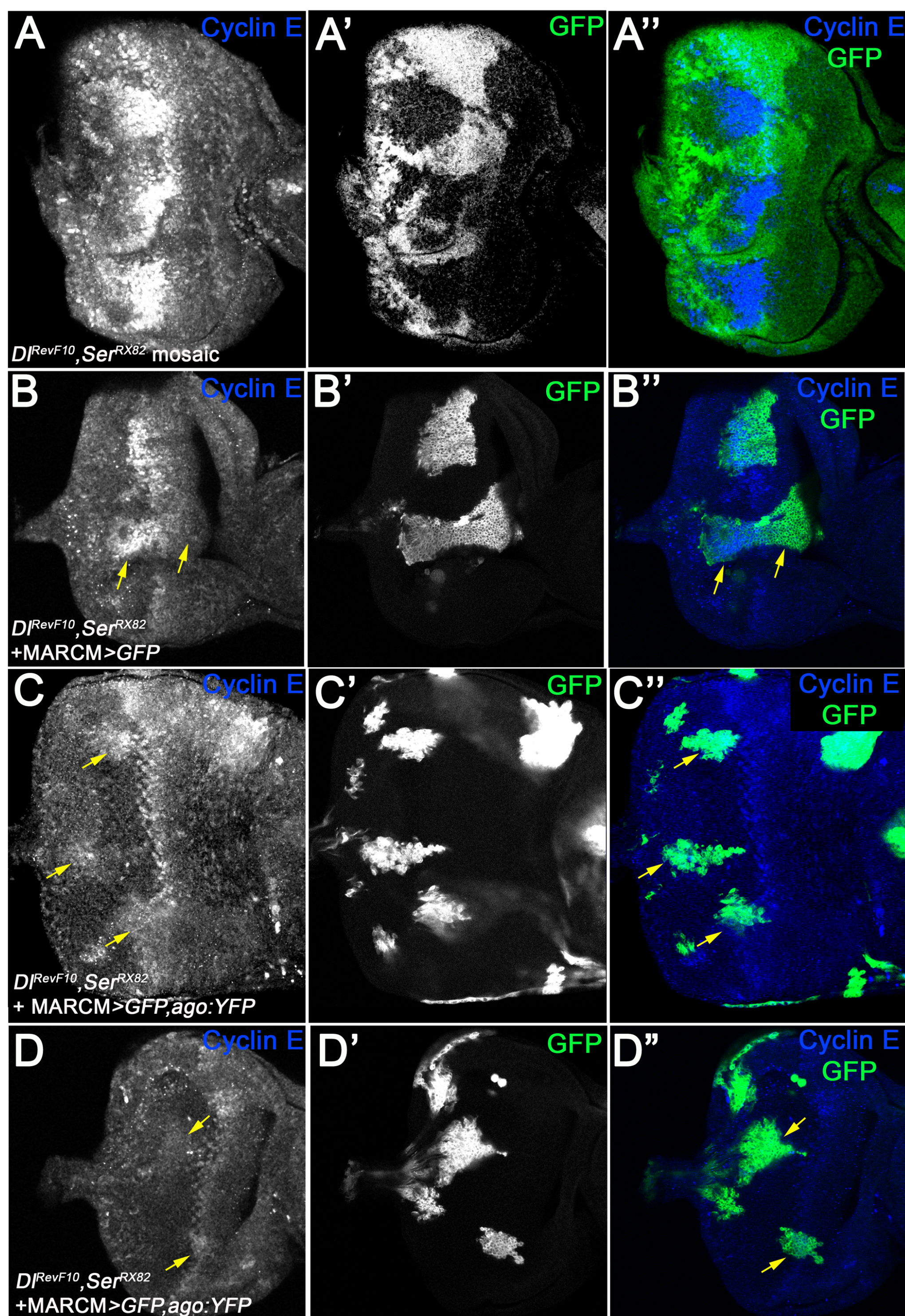
(C) Quantitative real-time PCR analysis of the expression of *RA* and *RC* mRNA levels in *hsp70>N^{intra}* with (+) or without (-) a 1hr heat shock as indicated.

III.C4. The induction of *ago* by the *N* pathway is required to limited levels of the SCF-Ago target Cyclin E

N has well-documented anti-mitotic roles in cells of the bristle lineage (SIMON *et al.* 2009), the imaginal wing disc (HERRANZ *et al.* 2008), and the ovarian follicle (DENG *et al.* 2001; LOPEZ-SCHIER and ST JOHNSTON 2001), and vertebrate *N* genes are proposed to be context-specific tumor suppressors (MAILLARD and PEAR 2003). The positioning of *Drosophila N* upstream of *ago* suggests that *N* may execute some of these anti-mitotic roles in part by indirectly promoting the turnover of SCF-Ago target proteins. Consistent with its positive role upstream of *ago*, levels of the SCF-Ago target Cyclin E are elevated in *Dl/Ser* double mutant clones located within or posterior to the MF (Fig. 7A,B and as described in (SUKHANOVA and DU 2008)). This effect does not occur in *Dl/Ser* clones in the anterior region of the disc, and only becomes apparent as cells traverse the area of the MF (compare areas of GFP-marked clone denoted by arrows in Fig. 7B). This phenotype is distinct from *ago* null clones, which accumulate Cyclin E regardless of their spatial location in the disc (MOBERG *et al.* 2001), and correlates spatially with the requirement for *Dl* and *Ser* in the MF-associated pulse of *ago* expression. To test whether the link between *Dl/Ser* loss and elevated Cyclin E might be caused by reduced *ago* expression, the MARCM technique (LEE and LUO 2001) was used to re-express *ago* in *Dl/Ser* clones using a *UAS-ago;YFP* transgene. Although this excess Ago is not sufficient to reduce Cyclin E levels in other areas of the eye disc (e.g. large clone in posterior region of disc in Fig 7C"), it is sufficient to substantially rescue the elevated Cyclin E otherwise seen in *Dl/Ser* mutant clones lying within or just posterior to the MF (Fig. 7C,D). Ago-

expressing *Dl/Ser* clones still contain slightly more Cyclin E protein than surrounding control cells, indicating either that *Dl/Ser* loss has a second Ago-independent effect on Cyclin E expression, or that the Ago:YFP protein does not function optimally in cells. In sum, these data argue that the *N* pathway acts through *ago* to control Cyclin E turnover in a specific subset of cells in the developing eye. Interestingly the levels of the other major SCF-Ago target, dMyc (MOBERG *et al.* 2004), are not elevated in *Dl/Ser* mutant clones located in the area of the MF (Supplemental Fig. 2). Since complete loss of *ago* elevates dMyc protein levels in cells regardless of their location in the disc (MOBERG *et al.* 2004), this effect suggests that the baseline level of Ago protein that persists in *Dl/Ser* clones are sufficient to support turnover of dMyc but are insufficient to regulate Cyclin E.

Figure III.7. The decrease in Ago levels in *N* pathway mutant clones results in a build-up of the SCF-Ago substrate Cyclin E.

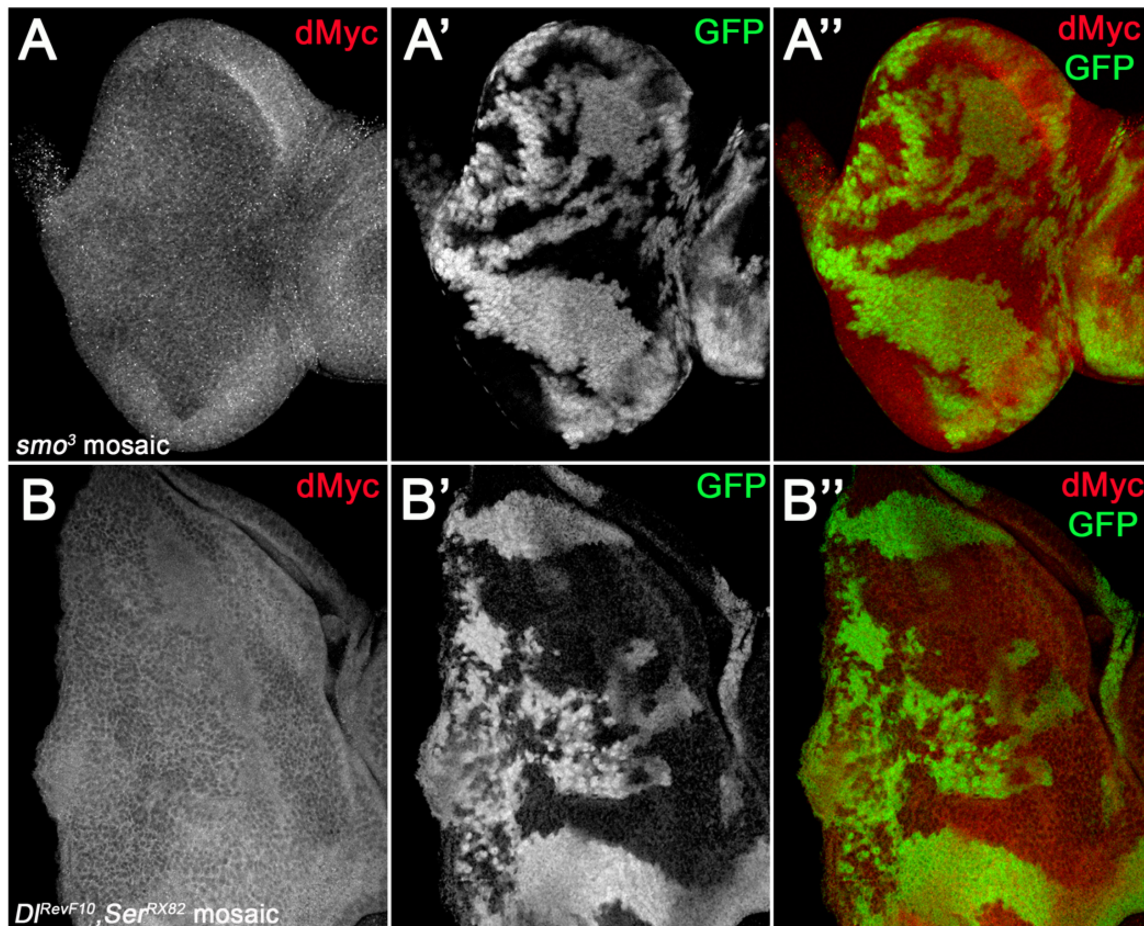


(A-A") Confocal sections of an eye disc containing *Dl*^{RevF10}, *Ser*^{RX82} double mutant clones marked by the absence of GFP (green) stained for Cyclin E (blue). Note the elevated Cyclin E in *Dl*^{RevF10}, *Ser*^{RX82} cells behind the MF.

(B-B") Merged confocal sections of *Dl*^{RevF10}, *Ser*^{RX82} MARCM clones positively marked by GFP (green) and co-stained for Cyclin E (blue). Yellow arrows (B and B") highlight the effect of *Dl*^{RevF10}, *Ser*^{RX82} on Cyclin E only occurs in cells within and behind the MF.

(C-C" and D-D") Merged confocal sections of *Dl*^{RevF10}, *Ser*^{RX82} MARCM clones (GFP-positive; green) expressing an Ago:YFP fusion protein. Yellow arrows (C,C" and D,D") denote *Dl/Ser* mutant clones in which the Cyclin E levels are partially rescued by expression of Ago:YFP (compare intensity of Cyclin E levels in C and D with those in A and B).

Figure III.S2. The effect of *N* loss on dMyc levels at the MF.



(A-A'') Confocal sections of *smo*^{3} clones marked by the absence of GFP (green) and stained for dMyc (red).

(B-B'') Confocal sections of *Dl*^{RevF10}, *Ser*^{RX82} double mutant clones marked by the absence of GFP (green) and stained for dMyc (red).

III.D. Discussion

The *ago* gene has been studied for its role in controlling cell division, growth and death in developing tissues, but only a few studies have provided insight into pathways that might regulate *ago* activity in cells. Dimerization of the Ago ortholog Fbw7 enhances its ability to degrade Cyclin E (ZHANG and KOEPP 2006), although it is not clear whether this mechanism is conserved across species. *ago* and its vertebrate homolog *Fbw7* each appear to be transcriptionally induced by *p53/dp53* via a checkpoint pathway that responds to either energy starvation (MANDAL *et al.* 2010) or oncogenic mutations (KIMURA *et al.* 2003; MAO *et al.* 2004; MATSUMOTO *et al.* 2006). Patterned transcription of *ago* in the *Drosophila* eye disc indicates that developmental pathways control Ago levels and activity, and thus play important roles in Ago-dependent processes in cells. However, the extent to which MF-associated patterning pathways regulate Ago expression has not been examined. Here we show that the *N* and *Hh* pathways are necessary for the proper regulation of Ago levels in the developing *Drosophila* eye, specifically by increasing *ago* transcription in the region within and immediately behind the MF. This effect correlates with the presence of Su(H) binding sites in the *ago* promoter, and can be enhanced by co-expression of the N-terminal activation domain of Da. The DaN protein fragment is able to bind to Su(H) and drive high expression of the N-target *E(spl)m8* (CAVE *et al.* 2005), and the presence of predicted Da binding sites in the *ago* promoter region suggests a similar mechanism may occur here. Moreover, the defect in the MF-associated pulse of *ago* expression in *N*-mutant cells results in

hyper-accumulation of the SCF-Ago target protein Cyclin E, indicating that this novel transcriptional link between *N* and *ago* is an important mechanism through which *N* regulates the G1-to-S phase transition in eye disc cells.

While these data shed light on the initial inductive phase of Ago expression at the MF, additional mechanisms must operate immediately posterior to the MF to refine the Ago expression into a pattern. The regular gaps in the Ago pattern posterior to the MF appear to overlap with gaps in Ato expression between the R8 equivalence groups (BAKER and YU 1998) (see Fig 1A), arguing that a common mechanism may underlie both expression patterns. Interestingly, *N* is required for induction of Ato within the MF and for the restriction of Ato expression posterior to the MF via a lateral inhibition mechanism (BAKER *et al.* 1996; DOKUCU *et al.* 1996; JARMAN *et al.* 1994; JARMAN *et al.* 1995). Thus, although Ago does not display precisely the same pattern of Ato restriction posterior to the MF (e.g. expression only in the presumptive R8), it seems possible that *N* may play a similar dual activator/inhibitor role upstream of *ago*. The effect of the N^{ts} allele on Ago patterning behind the MF (see Fig 3A', insets) supports such a model. Moreover, since Fbw7 can target mammalian NICDs for proteasomal degradation (FRYER *et al.* 2004; GUPTA-ROSSI *et al.* 2004; OBERG *et al.* 2001; TSUNEMATSU *et al.* 2004), the pattern of Ago expression posterior to the MF may also reflect a requirement for SCF-Ago to inhibit NICD activity in differentiating neurons. In this regard *N* and *ago* may be capable of acting in a feedback loop in which the NICD stimulates its own turnover by elevating *ago* expression and activity.

Depending on the developmental context, *N* can be either pro- or anti-mitotic (reviewed in MAILLARD and PEAR 2003; RADTKE and RAJ 2003). Exactly how *N* fulfills these roles is not fully understood. The finding that *ago* is regulated by *N*, and that *N* appears to act through *ago* to control Cyclin E levels in a subset of eye disc cells, provides a novel link between *N* and the core cell cycle machinery. This link could explain certain cell cycle phenotypes described in *N* mutant disc cells. For example *N* deficient cells in the second mitotic wave (SMW) hyper-accumulate Cyclin E but also simultaneously fail to enter the SMW properly (BAONZA and FREEMAN 2005; SUKHANOVA and DU 2008). *Su(H)* mutant cells also accumulate Cyclin E (FIRTH and BAKER 2005), which is consistent with a role for *N* in promoting *ago* expression. The latter pro-mitotic effect of *N* has been attributed to a requirement for *N* for relief of Rbf1-mediated repression of dE2f activity (BAONZA and FREEMAN 2005), but the former role of the N pathway in antagonizing Cyclin E levels is not well understood. Based on the role of the *N* pathway upstream of *ago*, we propose that this phenotype may be due to defective turnover of Cyclin E resulting from insufficient *ago* expression. The identity of the *N* target that promotes SMW entry has remained controversial (BAONZA and FREEMAN 2005; ESCUDERO and FREEMAN 2007; FIRTH and BAKER 2005). Interestingly, high Cyclin E activity can downregulate levels of the pro-S-phase transcription factor dE2F1 in the developing wing (REIS and EDGAR 2004) and eye (NICOLAY and FROLOV 2008). Thus, *N* induced *ago* transcription may promote Cyclin E turnover following S-phase, but may also ensure that cells are only able to enter the SMW with an appropriate amount of Cyclin E present.

Interestingly, the reduced Ago expression in *N* pathway mutants has no

discernable effect on levels of dMyc, indicating that the threshold of SCF-Ago activity required to degrade dMyc is lower than that required for Cyclin E. Such a mechanism would imply that the *N*/*ago* link is specific for some SCF-Ago targets (e.g. Cyclin E) but not others (e.g. dMyc).

The role for *N* upstream of Ago and Cyclin E could theoretically play a significant role in mediating *N* effects in tissues outside the eye, and it will thus be important to examine whether *N* is required for optimal *ago* expression in other developing tissues. However, available data suggests that the *N/ago* link may be fairly context specific and not generalized to *N* signaling in all cell types. The pattern of Ago protein in developing larval discs (including the eye) does not generally mirror the pattern of *N* pathway activity as detected with reporters such as *E(spl)mb-CD2* (DE CELIS *et al.* 1998). Other signals, present at the MF but not elsewhere, must thus cooperate with *N* to induce *ago* within the MF. The proneural transcription factor Da is one likely candidate for this role. Da expression peaks in the MF and, like *N*, *da* is required for expression of Ato in the MF (BROWN *et al.* 1996). It seems likely that N and Da may synergize with each other, and perhaps with additional unidentified factors, to regulate *ago* expression in eye cells.

In sum these data identify *N* as a required regulator of *ago* expression in the MF and reveal a previously unappreciated cell cycle regulatory role of the *N* pathway involving *ago*-dependent control of Cyclin E levels. Given the role *dp53* plays in *ago* induction under conditions of metabolic stress (MANDAL *et al.* 2010), it will be interesting to determine how the *N* and *dp53* pathways interact to regulate *ago*

transcription, and whether other signaling and checkpoint pathways act through *ago* to pattern division in developing tissues, especially those pathways that interact functionally with the *N* pathway.

III.E. Experimental Procedures

III.E1. Genetics

Crosses were performed at 25°C except for those involving the N^{ts3} allele, which were performed at 18°C and shifted to 31°C for 0, 2, or 6hrs before dissecting. Experiments using the *hsp70>*N^{intra} construct were done by shifting larvae to 37°C for 1hr and back to 25°C for 1hr before dissecting. Experiments using the *hsp70>Gal4* driver were done by shifting larvae to 37°C for 1hr and back to 25°C for 2hrs before dissecting. Genotypes used for mosaic experiments were *eyFLP;ubiGFP,FRT40A*, *eyFLP;;ubiGFP,FRT80B*, *eyFLP;;FRT82B,ubiGFP, eyFLP;armLacZ,FRT40A*, *eyFLP;;FRT82B,armLacZ,* smo^3*, FRT40A/T(2;3)TSTL,CyO:TM6B,*Tb^1, Psn^{227}*,FRT80B/TM6B*, *FRT82B,*Dl^{RevF10}*,* Ser^{RX82}*/TM6B,* smo^3*,FRT40A;ago>Gal4,UAS:R-*$DHFR_{ts}$*-EGFP/T(2;3)TSTL,CyO:TM6B,* Tb^1, *UAS:R-*$DHFR_{ts}$*-EGFP/CyO:twi-GFP;ago>Gal4,FRT82B,*Dl^{RevF10}*,*Ser^{RX82}*/TM6B*, *UAS:eGFP/CyO:twi-GFP;ago>Gal4,FRT82B,*Dl^{RevF10}*,*Ser^{RX82}*/TM6B*, *UAS>Dl;*smo^3*, FRT40A/T(2;3)TSTL,CyO:TM6B,*Tb^1, *ey>FLP;ubiGFP,FRT40A;hsp70>Gal4*. MARCM experiments used *hsFLP,UAS:GFP;tubGal4;FRT82B,tubGal80/TM6B* and *UAS-ago;YFP/CyO;FRT82B,*Dl^{RevF10}*,*Ser^{RX82}*/TM6B*. *Hsp70>*N^{intra} and *UAS>Dl* were gifts of K. Moses. *P{GawB}ago[NP0850]* was obtained from the *Drosophila* Genetic Resource Center, Kyoto Institute of Technology. *UAS:R-*$DHFR_{ts}$*-EGFP* was a gift of K. Broadie. N^{ts3} and smo^3*/T(2;3)TSTL,CyO:TM6B,*Tb^1 were gifts of A. Mortimer. The 3R MARCM stock was a gift of J. Treisman. *P{UAS-2xEGFP}AH2*, *FRT82B,*Dl^{RevF10}*,*Ser^{RX82}*/TM6B,* Psn^{227}*/TM6B* were obtained from the Bloomington *Drosophila* Stock Center.

III.E2. Immunohistochemistry and Microscopy

Immunostaining and confocal microscopy were performed as described previously (MOBERG *et al.* 2004). Antibodies used: rabbit anti-GFP (Molecular Probes, 1:1000); guinea pig anti-Ago (1:2500-5000); mouse anti-βgal (Promega, 1:1000); mouse anti-dMyc (gift of B. Clurman, used undilute); rat anti-Cyclin E (gift of H. McNeill, 1:400); rat anti-Elav (Developmental Studies Hybridoma Bank, 1:200); guinea pig anti-Ato (1:1000) and rabbit anti-Ato (1:625) (MELICHAREK *et al.* 2008).

III.E3. Real-Time Reverse Transcriptase-PCR

Total RNA isolated from 20 eye discs (TRIzol, Invitrogen) was reverse transcribed (SuperScript II RT, Invitrogen) and analyzed by quantitative PCR (SYBR Green 1 Master Mix, Roche). Primers used were *ago-RA*, 5'-GGAATTTCGGATCATCTTGG-3' and 5'-AAGTGGGTAATGCGTTCTCAA-3'; *ago-RC* 5'-TGGTCACGGATTCACTGCTA-3' and 5'-CGAGGAGCAACGTCCTTAAA-3' ; and *ß-tub*, 5'-CGCACAGAGTCCATGGTG-3' and 5'-AAATCGTTCACATCCAAGCTG-3'.

III.E4. S2R+ cell manipulations

S2R+ cells plated to confluency in 24 well plates were co-transfected with 100ng of both the *ago2kb*-luc and a *copia-renilla*-luciferase reporter (used as a transfection efficiency control). Wells were also treated with either 1) empty vector 2) 300ng of plasmid coding for the Notch Intracellular Domain (NICD) 3) 50ng of a plasmid coding for the first 545 amino acids of the Daughterless protein (DaN) or 4) 300ng of NICD and 50ng of DaN. Transfections were done in triplicate using FUGENE HD (Roche). Error bars (Figure 6A) are representative of the standard deviation from the mean. Figure 6A is representative of 3 independent transfections. 48hrs after transfection, cells were lysed and the reporter assay was carried out using the Dual-Luciferase reporter system (Promega).

III.E5. Molecular Cloning

A minimal promoter taken from the *heat shock protein-70* gene was cloned in between the HindIII and BglII sites of the pGL2-basic luciferase vector (Promega). A 2kb (-302bp to +1697bp with respect to the transcriptional start site of *ago-RC* [Flybase version 2010-04]) fragment from the *archipelago* locus was PCR amplified from genomic DNA isolated from *Canton S*. flies then subcloned (in the sense orientation) into the SacI and NheI sites of the pGL2-basic vector upstream of the *hsp70* minimal promoter. Following sequencing analysis, the plasmid was designated *ago2kb-luc* and used as described above. Primer sequences used in the cloning are available upon request.

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Chapter 4: Future Experiments and Concluding Remarks

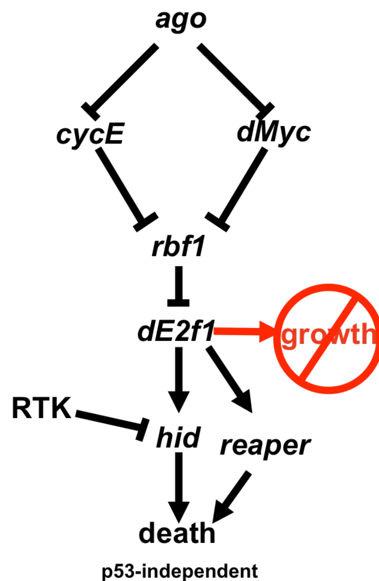
IV.A. Summary

Based on the experiments described here, we can conclude that loss of *ago* function results in the death of a subset of cells in the *Drosophila* eye as a result of the sensitization of these cells to proapoptotic signals through the Rpr/Hid pathway. This death occurs through the inactivation of Rbf1, which happens at least in part through Rbf1 phosphorylation by the ectopic accumulation of Cyclin E (Figure IV.1). Thus, *ago* mutant cells might succumb to apoptosis through a similar mechanism as *rbf1* mutant cells. Interestingly, the pattern of cell death in *ago* mutant eye discs mimics the pattern of death in *rbf1* mutant eye discs. In fact, *rbf1* mutant cells are sensitized to apoptosis when signaling through the epidermal growth factor receptor (EGFR) pathway is low (MOON *et al.* 2006), which suggests that removal or inhibition of EGFR in cells mutant for *ago* may similarly stimulate their removal by programmed cell death. Future experiments which determine if inhibiting the EGFR pathway specifically kills *ago* mutant cells could lead to important advances in treating *ago*-deficient human tumors. Furthermore, a genetic screen for other mutations that modify the *ago*-mutant 'small eye' phenotype could lead to the identification of other therapeutic targets for the treatment of these tumors.

Ago functions as a tumor suppressor because it has the ability to limit the activity of multiple oncogenes. While the N receptor, one of many mammalian Ago targets, has not been validated as a substrate for Ago-dependent degradation in flies, the work described here places *N* in the *Drosophila ago* pathway, only upstream of Ago rather than downstream of it. The fact that N acts as an Ago substrate in vertebrates begs

the question of whether *N* and *ago* act in a feedback loop, where N activates *ago* transcription, and Ago negatively feeds back on the pathway by degrading N (Figure IV.4). However, it remains to be seen if N regulates *ago* by simple activation in a specific subset of cells, or if N has dual roles in *ago* regulation, broadly activating the gene and subsequently repressing it to refine *ago* transcript into a defined pattern, adding another layer of complexity to the N/Ago relationship. In this way, N would be regulating *ago* in a similar way as some of its other targets. For example, the proneural bHLH transcription factor *atonal* (*ato*) is activated and then subsequently repressed by N as the MF sweeps across the eye disc (Figure IV.5). Thorough analysis of *ago* enhancer elements and determination of which elements are needed for each level of regulation may give more clues concerning *ago* regulation by N and other transcription factors. Because mammalian Notch1 is already an established substrate of Fbw7, the studies of N-dependent *ago* regulation should also be extended to mammalian cells to determine whether a N/Ago feedback loop exists in mammalian cells. Clearly, while this work answers a few questions, it raises many more which need to be addressed in future studies.

Figure IV.1. *ago* is required to restrict the apoptotic activity of the Rb/E2f pathway adjacent to the eye-specific MF



ago normally acts to restrict the levels of dMyc and Cyclin E, which have both been shown to negatively regulate the activity of the Rbf1 ((DUMAN-SCHEEL *et al.* 2004), reviewed in (VAN DEN HEUVEL and DYSON 2008)). Rbf1 binds to the dE2f1 transcription factor and inhibits the expression of dE2f1 target genes (reviewed in (VAN DEN HEUVEL and DYSON 2008)). In *ago* mutant fly eyes, the dE2f1 transactivation domain is required for the apoptosis of mutant cells in front of the MF but is not required for the growth advantage of *ago* mutant clones. *rpr* and *hid*, which are inducible by dE2f1 (ASANO *et al.* 1996; MOON *et al.* 2005), are ectopically expressed in *ago* mutant cells, leading to the removal of these cells via p53-independent apoptosis. Finally, *ago* mutant cells are sensitive to levels of RTK signaling, which has been shown to repress the activity of *hid* (BERGMANN *et al.* 2002; KURADA and WHITE 1998).

IV.B. Screen for modifiers of *ago* 'small eye'

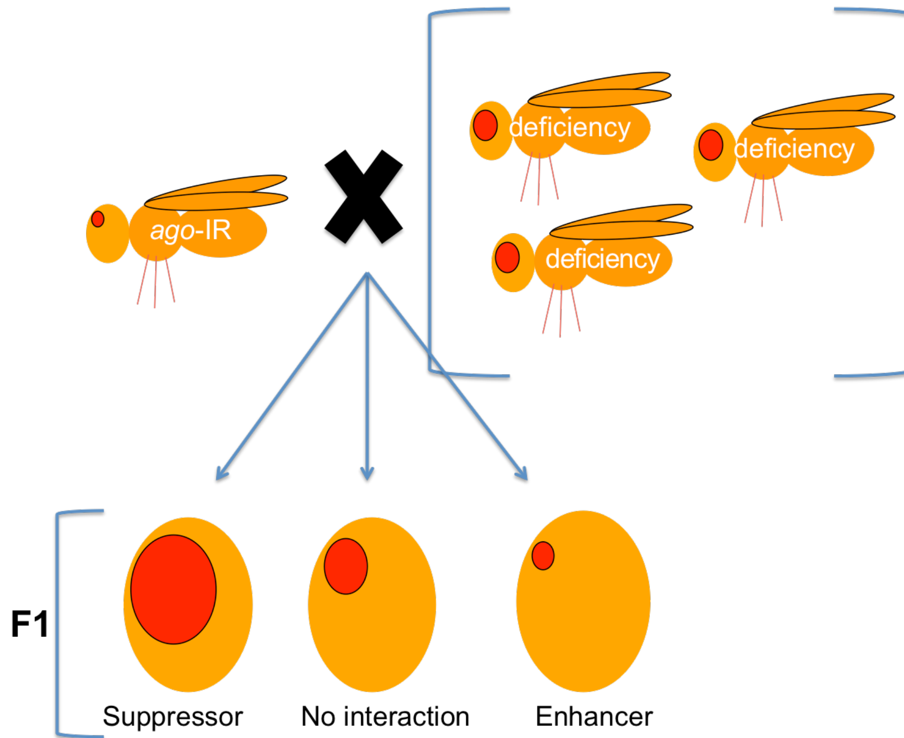
The ability to measure a *Drosophila* eye 'en face' as a quantitative measure of organ size was used here to identify *epidermal growth factor receptor (EGFR)* and *wingless (wg)* as dominant enhancers of the 'small eye' phenotype that is characteristic of eyes lacking wild-type *ago*. This assay was also used to validate the genetic interactions of *ago* with other genes, such as the three pro-death genes, *rpr, grim,* and *hid*, along with genes encoding the cell cycle regulators dE2F1 and Cyclin E. Presumably, an unbiased screening of the whole or part of the *Drosophila* genome would uncover many other *ago* interactions.

I would propose a deficiency screen using the eye-measuring assay described above to isolate other dominant genetic modifiers of the *ago* 'small eye' phenotype. Because the *eyelessFLP/FRT* system would require every deficiency to first be put in the background of an *ago^1,FRT80B* chromosome, the screen should be done using an eye-specific promoter driving expression of *ago* inverted repeats (*ago-IR*) to eliminate *ago* function in the eyes alone. Once an appropriate *ago-IR*/driver combination has been found and shown to result in a 'small eye' phenotype comparable to that of the *ago* mutants, the driver should be placed on the same chromosome as *ago-IR*, and flies carrying *ago-IR*/driver can be crossed to a library of deficiencies, and F1 flies can be screened by measuring eye size to find those deficiencies that act as suppressors or enhancers of the *ago* 'small eye' phenotype (Figure IV.1). The dominant genetic interactions would then be mapped to single

genes, potentially revealing novel Ago targets in flies or genes that act downstream of *ago*. Genes already shown to interact with *ago* in this assay would serve as controls to ensure the screen was working properly.

The mutations that result in an enhancement of the *ago* 'small eye' phenotype may lead to the identification of new potential therapeutic targets for treating *ago*-deficient tumors, especially those mutations that shrink *ago* mutant eyes but not wild-type eyes. Such modifier genes might potentially encode proteins which could be inhibited by small molecules and thus result in the initiation of apoptosis specifically in cells lacking Ago. The screen would also uncover novel interactions of *ago* with other pathways and as such advance our understanding of the function of *ago* in cells.

Figure IV.2. Deficiency screen for modifiers of the 'small eye' phenotype



Flies with *ago-IR* driven only in the eye will have small eyes. When these flies are crossed individually with various deficiencies removing different parts of the genome, the eye size of F1 flies will be a measure of the interaction of *ago* with genes removed by the deficiency. Offspring of a mating between *ago-IR* flies and flies carrying a deficiency that suppresses the *ago* mutant phenotype will have larger eyes than the *ago-IR* flies, while offspring of a mating between *ago-IR* flies and flies carrying a deficiency that enhances the *ago* mutant phenotype will have even smaller eyes than the *ago-IR* flies.

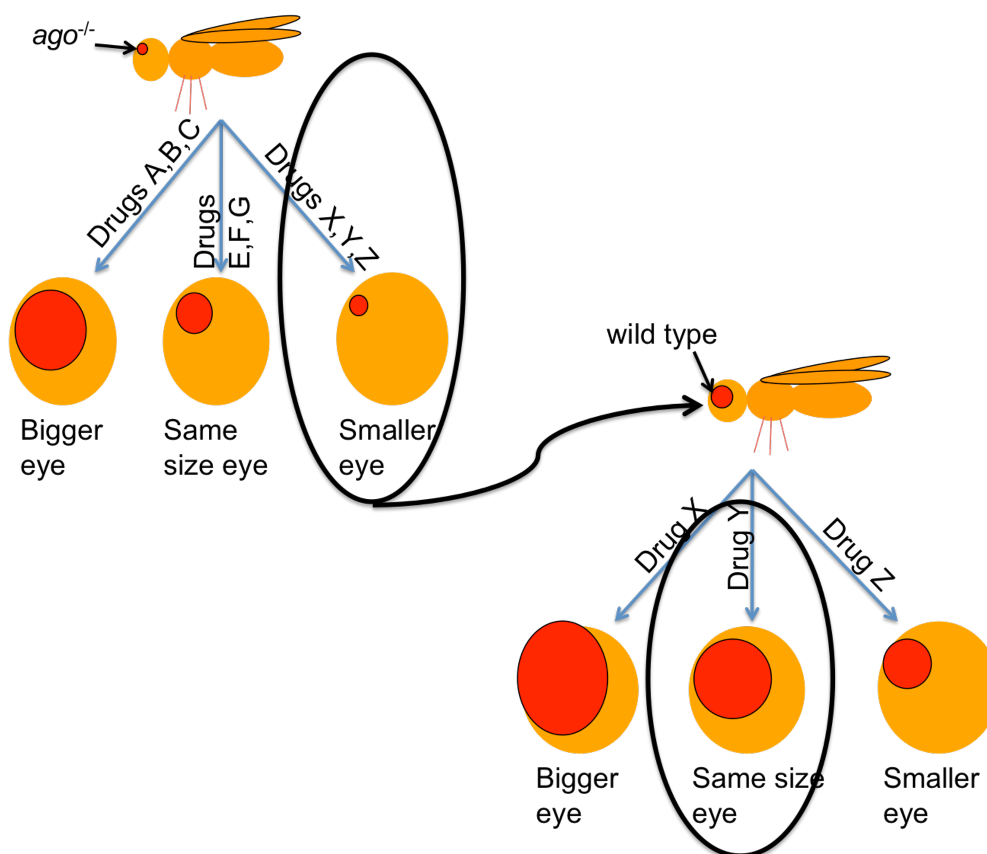
IV.C. Small molecule testing in *Drosophila*

Flies have long been established as useful models for the study of development and growth control, but have recently made their debut in the realm of drug screening. In fact, *Drosophila* have already been used in drug development for the multiple endocrine neoplasia (MEN) types 2A and 2B diseases, which are associated with mutations in the RET proto-oncogene. A *Drosophila* model for MEN2A and MEN2B was developed by targeting oncogenic forms of RET to the developing *Drosophila* eye. The low molecular weight tyrosine kinase inhibitor ZD6474 (aka Vandetanib), which has been found to block enzymatic activity of RET-derived oncoproteins in cultured cell lines, was fed to these flies orally and found to suppress the RET-mediated phenotypes (VIDAL *et al.* 2005), and is currently undergoing clinical trials.

I propose using *Drosophila*, specifically those with *ago* mutant eyes, to test drugs for use in treatment of *Fbw7*-deficient tumors. The deficiency screen described in section IV.B. would uncover potential drug targets, and drugs proposed to act on these targets would be tested in the flies themselves. Also, the toxicity of the drug would be measured so that doses below those leading to observed toxicity or lethality could be tested. For example, *EGFR* dominantly enhances the *ago* 'small eye' phenotype; therefore the EGFR inhibitor gefitinib would be fed to *Drosophila* with *ago* mutant eyes to determine if it makes mutant eyes even smaller. If the drug affects only mutant tissue and not wild-type tissue, then it is highly specific and may be useful in anti-cancer therapies where it would shrink the tumors but not affect

healthy tissue. I carried out a first attempt at the assay described above by feeding gefitinib to flies with *ago* mosaic eyes. The intent was to see specific loss of the white, mutant tissue, with no effect on the red, wild-type tissue. Unfortunately, the drug interferes with pigment formation, distorting eye color and preventing the quantification of the ratio of red-to-white tissue. Instead of determining tissue ratios, the size of the entire eye was measured as a rough estimate of the gain or loss of mutant tissue upon drug treatment. While this experiment did not lead to any definitive conclusions, the results are promising enough to merit a second attempt at the experiment with some modifications (Figure IV.2). Using flies with eyes entirely mutant for *ago* will make the effect of the drug more easily measurable by again quantifying the size of the entire eye. If gefitinib shrinks *ago* mutant eyes but not wild type eyes, it may be considered for use in treating *ago*-deficient tumors. This kind of testing on gefitinib and other drugs in *Drosophila* can provide answers to questions about toxicity, efficacy, and specificity, which would lay important groundwork for future drug development in mammals.

Figure IV.3. Small molecule testing in *ago* mutant eyes



A drug screen would be carried out in which flies with *ago* mutant eyes are fed a small molecule throughout their development, and the adult fly eyes are measured to determine if the small molecule had any effect on the mutant tissue. Any small molecules (e.g. drugs X, Y, and Z) that make the *ago* mutant eye even smaller should then be tested in wild type flies. Any drug (e.g. drug Y) which makes mutant eyes smaller but has no effect on wild type eyes may be considered for use in treating mammalian *ago*-deficient tumors and should next be tested in a mouse model.

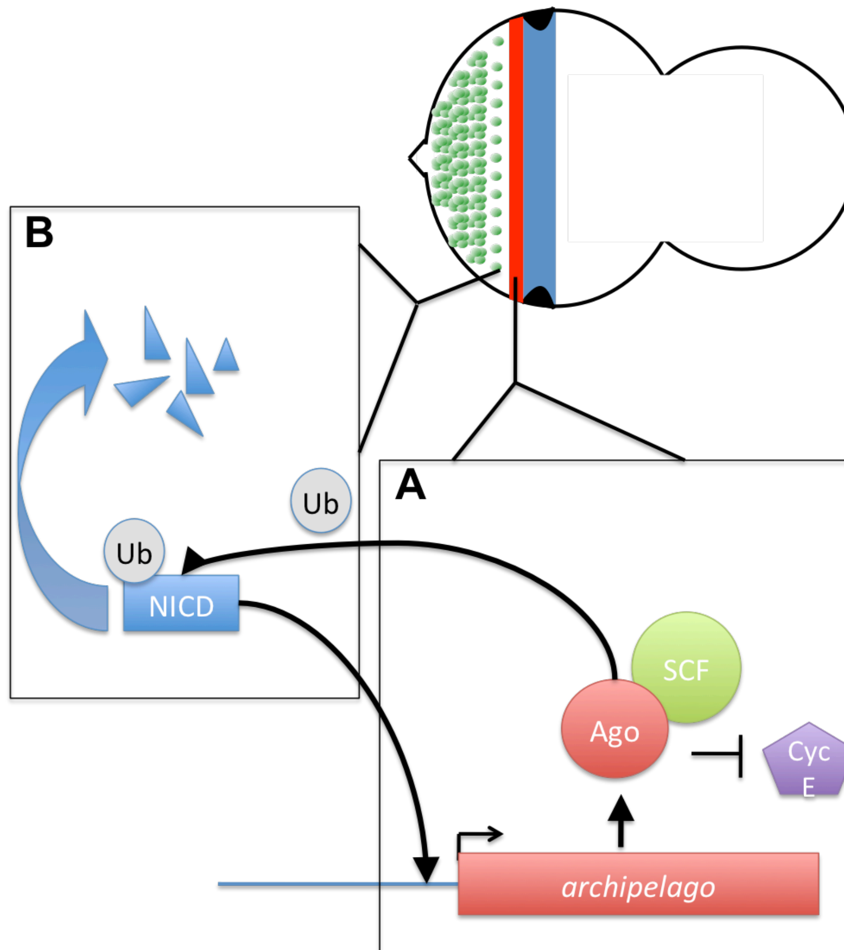
IV.D. Defining a N/Ago feedback loop

There has been extensive work done in vertebrates showing that Notch1 and Notch4 is bound by Fbw7 and targeted for degradation by the proteasome (GUPTA-ROSSI *et al.* 2001; OBERG *et al.* 2001; WU *et al.* 2001; TETZLAFF *et al.* 2004; TSUNEMATSU *et al.* 2004). While ectopic nuclear NICD has not yet been visualized in fly cells mutant for *ago*, a N-inducible transcriptional reporter, *E(spl)mβ-CD2* (DE CELIS *et al.* 1998), shows elevated N activity in *ago* mutant cells in the *Drosophila* eye imaginal disc (K.H. Moberg, unpublished data). If fly Ago indeed binds and degrades NICD, then the data presented in chapter III suggest that these two proteins form a feedback loop which may function to ensure that certain signals through the N pathway only occur transiently. In essence, when N is activated to turn on *ago* transcription, the abundant Ago protein produced by this signal quickly shuts it off by ubiquitinating the NICD and targeting it for degradation (Figure IV.3). This regulation would be especially relevant in the context of the morphogenetic furrow (MF), where N activates *ago*, perhaps in order to degrade Cyclin E so that cells can properly exit the cell cycle after the passing of the second mitotic wave (SMW). Because N plays such diverse roles in eye development, the surge of N activity required to activate *ago* must quickly be turned off to avoid abnormal specification of neuronal cell fates.

One way to test this hypothesis would be to create an *ago* transcriptional reporter transgene, which would consist of the 2kb region shown here to be responsive to activation by the N pathway, a minimal promoter, and the *LacZ* coding sequence

(hereafter referred to as *ago-LacZ).* This *ago-LacZ* transgene should be placed in the background of *ago* mutant clones, where, presumably, NICD signaling activity accumulates due to the lack of *ago*-dependent NICD degradation. As a result, NICD would ectopically activate the *ago* promoter in two places: (1)the endogenous *ago* promoter, and (2)the *ago-LacZ* transgene, which would result in elevate levels of βgal protein only in *ago* mutant tissue. Elevated βgal staining in *ago* mutant clones versus wild type tissue would suggest the presence of a N/Ago feedback loop and would provide more evidence for *Drosophila* Ago binding and ubiquitinating N. These kinds of studies advance our understanding of the highly complex yet precisely regulated events that occur at the MF and the signaling programs that ultimately give rise to a properly formed eye.

Figure IV.4. The N/*ago* feedback loop



Within and behind the furrow, N activity is required to activate transcription of the *ago* gene so that the SCF-Ago complex can then degrade Cyclin E behind the MF (A, in the eye disc schematic the MF is in blue and the SMW is in red). However NICD, which plays a part in many other processes needed to properly specify neuronal fates for the adult eye, cannot be allowed to persist in cells after the need for *ago* activation has passed. NICD is then ubiquitinated and degraded by the Ago protein itself (B, in the eye disc schematic the row of green circles represents the R8s. These photoreceptors are the first to be specified and the spacing of these cells is determined by lateral inhibition through the N pathway).

IV.E. Notch-dependent *ago* expression patterns in the eye

The N pathway has complex interactions with its target genes, activating or repressing the same targets in distinct subsets of cells and at specific times during development. For example, the proneural bHLH gene *atonal* (*ato*) which is required for R8 cell determination, is initially expressed in a band of cells anterior to the MF. This 'prepattern expression' is induced by Hh signaling (HEBERLEIN *et al.* 1995) and is restricted first to regularly spaced intermediate groups within the MF and later to single R8 cells within and behind the MF (JARMAN *et al.* 1994; JARMAN *et al.* 1995, Figure IV.4). Thus developmental timing in the eye disc can be measured in rows of cells, such that the most posterior rows of cells are the farthest in development, and the cells in front of the MF are essentially at t=0. Using the temperature-sensitive mutant allele N^{ts1}, Baker and Yu (1997) performed a time-course to show that loss of *N* function blocked the resolution of intermediate groups as well as reduced *ato* expression in more anterior cells (BAKER and YU 1997). Therefore, while *N* is not required for *ato* expression to be initiated, it is required after this stage for *ato* expression to be enhanced. Furthermore, a few rows of cells later, *ato* expression is refined to single R8 precursor cells by N-induced lateral inhibition of *atonal* expression (BAKER *et al.* 1996). Thus, N has sequentially opposite effects on the same cells, first promoting and then inhibiting functions of proneural genes such as *ato*.

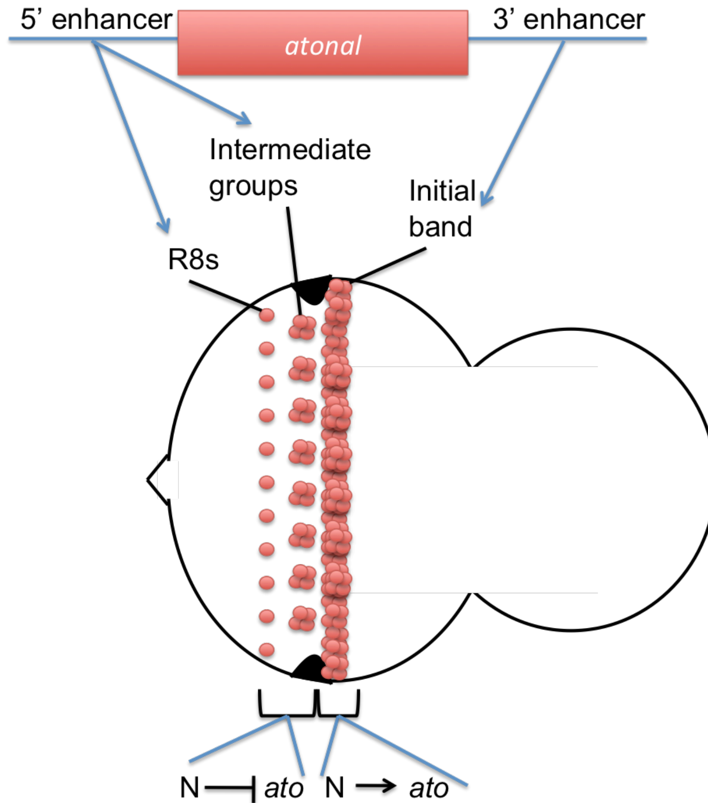
Because *ago* also displays a pattern of expression which starts off in a band of cells and is subsequently refined into a distinct pattern (Figure III.1), we hypothesized that *N* may function on the *ago* promoter similarly as on the *ato* promoter, first activating, then refining. To test this hypothesis, I performed a time course experiment with the N^{ts3} allele to look for progressive changes in *ago* expression. While *ago* expression did become disorganized after 6hrs at the restrictive temperature (Figure III.3A', insets) the quality of the staining in these discs was not such that conclusions could be made concerning the different roles of N in different subsets of cells, either due to limitations of the antibody itself or the low amounts of Ago remaining upon removal of N. Therefore, the *ago>gal4* transcriptional reporter was used in combination with the *UAS:eGFP* transgene to measure promoter activity in the N^{ts3} loss-of-function background. The eGFP protein provides a much stronger signal than that produced by the anti-Ago antibody, and it appears to be more stable than the Ago protein, which causes the GFP to persist in cells posterior to the MF all the way to the most posterior row of developing ommatidial clusters. Interestingly, the GFP signal appears to be stronger in N^{ts3} discs than in wild type discs after the shift to restrictive temperature, suggesting that the *ago>gal4* transcriptional reporter is capable of responding to *N*-dependent transcriptional-repression (Figure IV.5). Perhaps this phenomenon is detectable in conditional mutants and not discs completely deficient for N signaling, e.g. *Dl, Ser* double mutants, because *N* is initially required to induce the inhibition of *ago* expression in response to later *N* signaling, a phenomenon seen in the *N* regulation of *ato* (BAKER and YU 1997). Notably, Ago protein accumulation at the MF decreases in N^{ts3} discs compared to wild-type after

the shift to restrictive temperature (Figure III.3), suggesting that N exerts opposite effects on *ago* RNA and protein, another observation that was also made concerning *N* regulation of *ato* expression (SUN *et al.* 1998).

Regulation of *ago* may mimic *ato* in yet another way. One study shows that the *ato* regulatory element responsible for the initial band of expression anterior to the MF is a 3' enhancer located in a 5.8 kb fragment downstream of the *ato* coding region, while expression in the intermediate groups and R8s is directed by a 5' enhancer located between 7.2 and 9.3 kb upstream of the coding region (SUN *et al.* 1998). Interestingly, the *ago>gal4 in vivo* transcriptional reporter used in the experiments described in chapter III would presumably report the activity of a 5' enhancer upstream of the *ago* coding region, and this enhancer seems to drive expression in cells more posterior than where Ago protein first appears (Figure IV.6, compare panel A with panel B), leaving the possibility that a 3' enhancer exists downstream of the *ago* coding region which is responsible for the initial band of *ago* expression. A thorough investigation into the regulatory elements of the *ago* gene would further our ability to identify *ago* regulators and determine the ability of regulators like *N* to exert different effects in different cells. I propose performing an experiment in which various genomic fragments upstream and downstream of the *ago*-coding region along with a minimal promoter are fused to a *lacZ* reporter gene, and these constructs introduced into flies via germline transformation. The expression pattern of *lacZ* from the various constructs should be determined in the eye disc, antennal disc, wing disc, and embryo using anti-β-galactosidase along with anti-Ago antibodies. If *N* truly plays a dual role in activating and repressing *ago*, I predict that

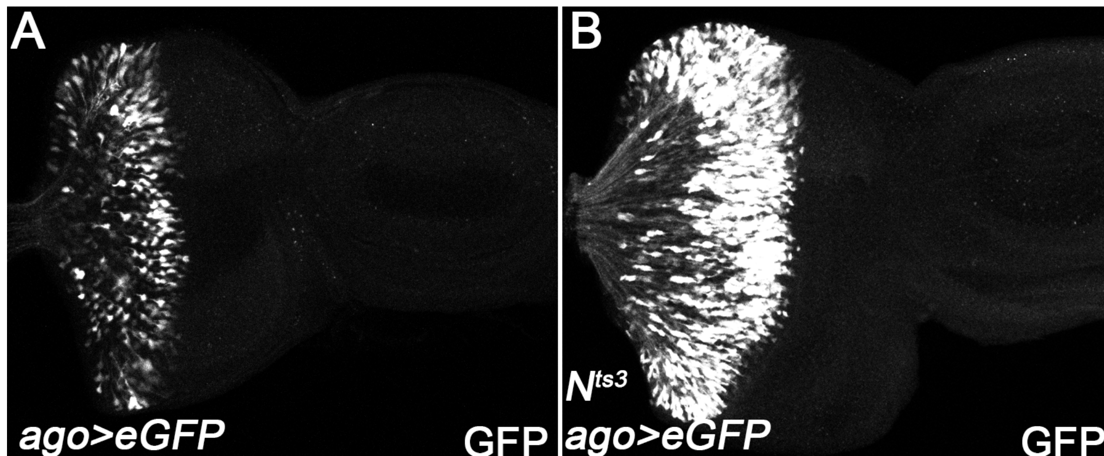
distinct promoter regions will give rise to the broad band of *ago* expression and the refined *ago* pattern. Other enhancers may be uncovered that direct expression in the embryo, wing, and antenna. Once reporter constructs have been characterized to drive expression in either a broad stripe or a distinct pattern in the *Drosophila* eye disc, these constructs can be tested for their ability to respond to changes in N activity, with the expectation that one will be activated by N and the other repressed. An investigation such as this will provide valuable insight into the organization of the *ago* promoter and the constructs will allow the determination of the responsiveness of each enhancer to various transcription factors and in various tissues.

Figure IV.5. Regulation of *ato* expression in the eye disc



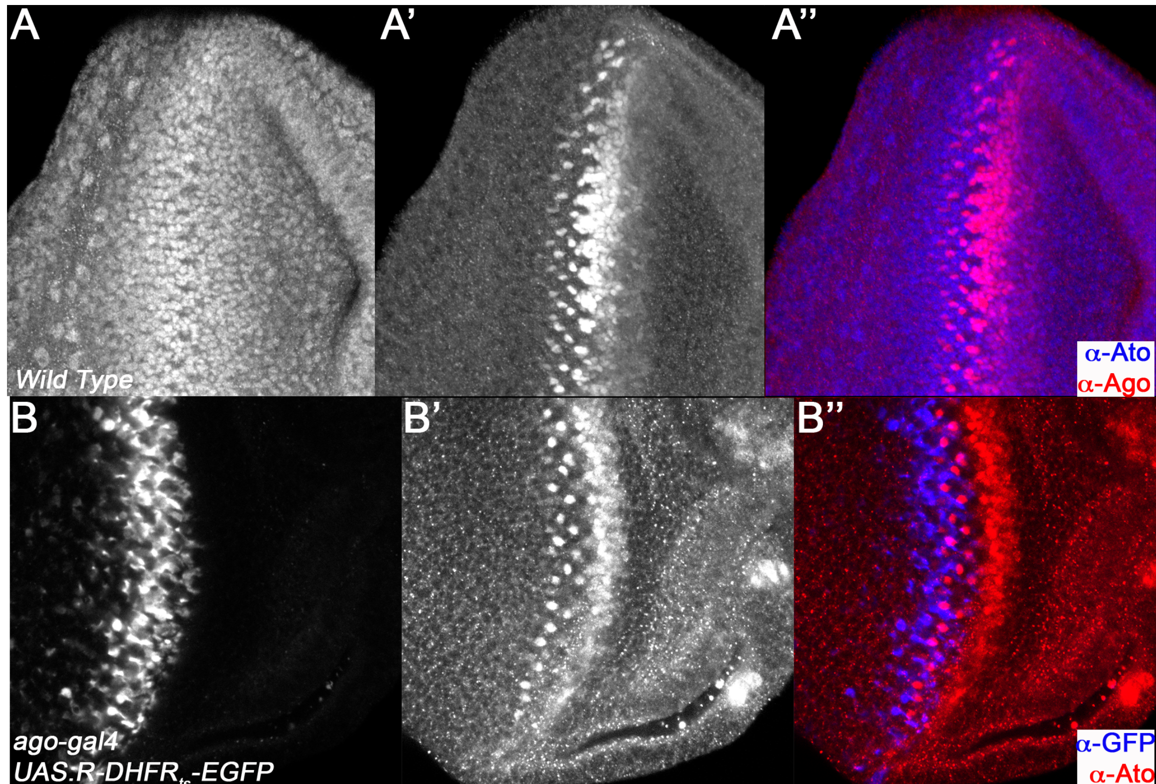
ato is first expressed in an initial band of cells anterior to the MF. This expression is initiated by Hh signaling and is enhanced by N signaling. Subsequently, *ato* expression is refined to intermediate groups of cells and ultimately to single R8 precursor cells. This refinement occurs through N-dependent lateral inhibition. Thus, N changes roles from activating *ato* in front of the furrow to repressing *ato* within and behind the furrow. Interestingly, the enhancer responsible for initiating *ato* expression in the initial band is located downstream from the *ato* coding sequence (3' enhancer), whereas the enhancer responsible for controlling *ato* expression in the intermediate groups and R8 precursor cells is located upstream of the *ato* coding sequence (5' enhancer).

Figure IV.6. Loss of N activity results in an increase in *ago>Gal4* reporter transcription



ago>Gal4 driven expression of stable eGFP results in perdurance of GFP posterior to the MF. After the temperature shift, *ago>Gal4,UAS>eGFP* eye discs express stable eGFP in cells posterior to the MF (A). The levels of GFP expression from *ago>Gal4,UAS>eGFP* after the temperature shift are higher in N^{ts3} discs (B).

Figure IV.7. Ago protein expression and *ago-gal4* enhancer trap activity in the *Drosophila* eye disc



Ago protein expression (A, and blue in A'') begins within the furrow, which is marked by staining for Ato (A', B', and red in A'' and B''). The activity of the *ago-gal4* enhancer trap is measured by GFP staining (B, and blue in B'') which starts a few cell rows later than, or more posterior to, the beginning of Ago protein expression (compare A and B).

IV.F. Ago/N studies in human cells

While the ability of Ago to bind and degrade N has already been firmly established in mammalian systems, it remains to be seen whether N controls *ago/Fbw7* transcription in mammals as it does in flies. Because the *N* gene is abnormally activated in many human malignancies, any new information concerning the function of *N* in these cells may help lead to more effective treatment. In a relatively small number of tumor types, including human hepatocellular carcinoma and small cell lung cancer, N signaling is antiproliferative rather than oncogenic (QI *et al.* 2003; SHOU *et al.* 2001; SRIURANPONG *et al.* 2001). However, most studies have shown an opposite function of N, with up-regulated expression of N receptors and their ligands in cervical, lung, colon, head and neck, renal carcinoma, acute myeloid, Hodgkin and large-cell lymphomas, and pancreatic cancer (ALLENSPACH *et al.* 2002; ASTER *et al.* 2008; BOLOS *et al.* 2007; CHIARAMONTE *et al.* 2005; KOCH and RADTKE 2007; MIYAMOTO *et al.* 2003; SHOU *et al.* 2001; SRIURANPONG *et al.* 2001). Interestingly, Notch-1 is an important prognostic marker in T-cell acute lymphoblastic leukemia (T-ALL), which is the cancer type in which *Fbw7* mutations are the most frequent type of lesion found (O'NEIL *et al.* 2007; THOMPSON *et al.* 2007). Although human N is typically seen as a proto-oncogene, its ability to stimulate the transcription of the *ago* tumor suppressor gene presents a novel anti-mitotic role for N, and perhaps provides a mechanism through which cells with higher levels of N counterbalance the pro-growth message being sent through the N pathway. If this is the case, cancer

cells expressing high levels of the N oncogene would also need to lose both copies of *Fbw7* before transformation could occur. On the other hand, N may only be able to activate *Fbw7* in certain developmental contexts, in which case this regulation may or may not be relevant to tumorigenesis.

To first test the ability of N to activate *Fbw7*, a plasmid carrying the *NICD* coding sequence should be transfected into a mammalian cell line, and *Fbw7* transcript measured by quantitative real-time RT-PCR to determine whether *Fbw7* increases upon stimulation through the N signaling pathway. If the NICD raises transcriptional activity of the *Fbw7* promoter, the next step would be to determine if N-dependent *Fbw7* regulation plays a role in the many types of cancer which show elevated N activity. Cancer cell lines that have increased N expression should be evaluated for mutations at the *Fbw7* locus to determine the correlation between these two cellular events. If loss of *Fbw7* increases the likelihood of tumor formation from cells expressing high levels of N, then a relationship such as the one discussed in chapter III may exist in humans as well.

IV.G. Concluding Remarks

The work described here reveals a novel role for the *ago* tumor suppressor gene in limiting cell death in the developing *Drosophila* retina. This mechanism provides a way for cells that have lost both copies of the anti-growth gene *ago* to antagonize the resulting overgrowth. Thus, loss of *ago* results in both elevated pro-growth signals and also activation of apoptotic signals. Furthermore, we uncovered a novel anti-mitotic role for the N proto-oncogene, in which N activates expression of *ago*, thereby reducing levels of Cyclin E and perhaps initiating exit from the cell-cycle. Although this work focuses on the developmental role of N-dependent *ago* regulation, this interaction may yet play an important role in growth control, providing a mechanism for cells to combat the oncogenic effects of ectopic N through the expression of anti-growth *ago*. Clearly, cells have evolved many powerful ways in which to keep growth, death, and survival in check so that every organism reproducibly grows to the same size and maintains this size throughout its lifetime, with few exceptions. This study provides only the tiniest glimpse into the complexity that is an organism's development, and demonstrates how multiple pathways work together in highly complex yet highly regulated webs of interactions to give rise to the end product. By understanding these processes in a simple organism such as *Drosophila*, we are one step closer to grasping human growth and development, a goal that may appear only more elusive the closer we are to achieving it.

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