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

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Summary

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.

Introduction

Genetic screens in the fruit fly Drosophila melanogaster have identified many genes that restrict growth of developing tissues [reviewed in 1, 2]. 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 [3]. ago encodes an F-box/WD...
(tryptophan/aspartic acid) protein (Ago) that acts as the substrate-adaptor for an Skp/Cullin/E-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 [3, 4]. 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 [4]. In addition to this mitotic role, ago also regulates hypoxia-sensitivity and post-mitotic morphogenesis of the tracheal system via degradation of the Tracheless transcription factor [5,6].

Fbw7, the human ago ortholog, is frequently mutated in a wide array of human tumor types, including those of endometrial, colorectal and hematopoetic origin [reviewed in 7]. Moreover, deletion of murine Fbw7 increases cancer incidence and collaborates with p53 mutations to promote epithelial carcinogenesis [8]. 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 [7], [9]). 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 [reviewed in 1, 2]. Notably, ectopic expression of pro-growth factors such as dMyc in larval discs also increases adult organ size [10]. It is therefore somewhat surprising that adult eyes composed mainly of ago mutant cells expressing very high levels of dMyc are not obviously enlarged [3,4]. One explanation of this may be that excess dMyc causes ago cells to behave ‘super competitors’ that kill adjacent normal cells [11,12], 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.

Results

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 ago1 allele and the 3L Minute (M) allele RpL141 [13] as a recessive ‘cell-lethal’ mutation to kill M/M cells, allowing ago1/ago1 cells to populate the disc and adult structures derived from it. ago1 encodes a prematurely truncated protein that cannot bind CycE and dMyc and increases levels of these proteins in vivo [3,4]. Thus the ‘ago1/M(3)’ genotype provides a model of organ development in the absence of normal ago activity. Adult ago1/M(3) eyes are smaller than control eyes (Figs. 1A, B). This phenotype is specific to the eye: other organs in ago1/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 [14]. 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, ago3 [3], produces a similar pattern of apoptosis (Fig. S2). Co-staining for C3 and Atonal protein, which marks cells within the MF [15-17], 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/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 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/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.

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/M(3) eyes. To test whether blocking this could reverse the ago small-eye phenotype, the ago3 allele was combined with the Df(3)H99 genomic deletion, which removes the pro-apoptotic genes rpr, grim and hid [18]. This completely blocks the C3 ‘stripe’ (Figs. 2A′ vs. 2B′) and produces adult heads that are much larger than ago/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 ago1 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 ago1 and the hid05014 allele [19] 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 ago1 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.

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 [20], 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 [reviewed in 21]. 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 [22] and the pro-apoptotic genes hid [23] and rpr [24], which leads to MF-associated death of rbf1 mutant cells [20].

To test whether ago controls de2f activity in the eye, the de2f-reporter transgene PCNA-GFP [25] was placed into the background of ago1 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 ago1 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 ago\(^1\) 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 [3] and acts upstream of de2f1 [reviewed in 21], CycE-associated kinase activity may contribute to ago\(^1\) MF-associated apoptosis. To test this hypothesis, the CycE/cdk2 inhibitor dacapo was expressed ubiquitously in ago\(^1/M\)(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.

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\(^{12/M729}\) viable allele combination was used to reduce de2f1 activity in the background of ago mosaic eye discs (Fig. 4). de2f1\(^{1/M729}\) is an amorph [26], while de2f1\(^{12}\) is a hypomorph that lacks the activation domain and Rbf1-binding motif [27]. The de2f1\(^{12/M729}\) allele combination has no residual dE2f1 trans-activation function but supports normal patterns of division and MF progression in the eye disc [20]. 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\(^{12/M729}\) 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\(^{12/M729}\) animals are proportionately smaller than de2f1\(^+\) animals, ago\(^1\) mosaic eyes generated in the de2f1\(^{12/M729}\) 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\(^{630/H}/M\)(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 ago1/M(3) eye size (Figs 6A-C). A similar effect is observed with a null allele of cyclin E (cyclER95; [28]). 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 ago1/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 ago1 eye cells, these data are consistent with a model in which reducing the dose of de2f1 and cyclE 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 [29].

**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 [30]). Expression of dominant-negative form of Drosophila p53 (UAS-dp53R155H; [31]) does not block C3 accumulation in ago1 cells (Fig. S5). e2f1-driven death of ago1 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 [24], are both overexpressed in ago1/M(3) eye discs (Figs. 7A,B), and Hid and Rpr proteins accumulate in ago1 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 ‘ago1+p35’ cells in the area anterior to the MF accumulate Hid and Rpr, indicating either that these proteins accumulate transiently in ago1 cells even the presence of p35, or that additional factors are limiting for their expression. Together these molecular data indicate that ago1 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 [32,33] and this function is proposed to underlie a pro-survival role for EGFR in rbf1 mutant MF cells [20]. Given that ago acts upstream of de2f1 pathway and hid, we sought to determine whether the death of ago1 cells also proceeds by an EGFR-sensitive mechanism. eyGal4-driven expression of the EGFREllipse gain-of-function allele (Elp) [34] blocks MF-associated C3 in ago1/M(3) discs (Figs. 8A-A’) and synergizes with ago1/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 ago1/M(3)+ey>Elp discs is also observed in ey>Elp discs (data not shown), indicating it occurs independent of ago1. In the reciprocal experiment, EGFR activity was reduced in ago1/M(3) discs with the EGFR1 hypomorphic allele [35]. This dramatically shrank ago1/M(3) adult eyes (Fig. 6C). A similar effect was observed with a second allele EGFR24 (data not shown). These data indicate that ago1 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 (Gap1F2.4-A3; K. Moberg and I.K. Hariharan, unpub.) or argos (argoA7; [36]), or the RTK inhibitor sprouty (styA5; [37]). Whereas each of these alleles is able to rescue rbf1 cell death in the MF [20], they are not equally capable of rescuing the death of ago1 MF cells (Figs. 8C-E): styA5 largely blocks C3 accumulation, with two areas of C3 remaining laterally (Fig. 8C), whereas Gap1F2.4-A3 or argoA7 do not detectably alter the pattern of C3 accumulation (Figs. 8D,E). As agoA5 rescues the death of rbf1 mutant MF cells [20] these data argue that although MF-associated apoptosis of ago and rbf1 cells is similar in that they both require de2f1, ago1 cells require stronger EGFR/RTK signals (i.e. styA5 and ey>Elp) to survive than do rbf1 mutant cells. In support of this, styA5/M(3) eyes are larger than
Gap1^{F2.4-A3}/M(3) and aos^{Δ7}/M(3) (Fig. 6C) indicating that the sty^Δ5 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^I and the sty^Δ5 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^I/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 [38,39]. To test whether this lateral C3 might require wg, one copy of the wg^I-17 loss-of-function allele [40] was placed in the ago^I/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^I 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^I 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^I 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.

**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 [20], 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 (rbf1120a) and null (rbf114d) alleles of rbf1 [20], 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 styΔ5 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 [20].

Based on studies that show that Fbw7 degrades the Notch intracellular domain [7] and that Notch-1 promotes the death of Fbw7 mutant MEFs [41], Notch is a candidate pro-apoptotic factor in ago cells. Indeed, since Notch is antagonistic to EGFR signaling [reviewed in 42], 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 de2f1 in the larval eye disc [43] and within the MF [44,45] 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 ago1 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 [3]. 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. 46]. 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 ago1 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.
Experimental Procedures

Genetics

Crosses were performed at 25°C. The following genotypes were used for ‘cell lethal’ experiments: eyFLP;P[med];RpL14 FRT80B/TM6B, ago1 FRT80B/TM6B, ago1, hid5014, FRT80B/TM6B, ago1, sty5, FRT80B/TM6B, ago1, FRT80B/TM6B, ago1, Gap1F2.4A3, FRT80B/TM6B, EGFRT1/CyO; ago1, FRT80B/TM6B, wg56/CyO; ago1,FRT80B/TM6B, and ago1, FRT80B, de2f1rm729/TM6B. The following genotypes were used for ‘ago mosaic’ experiments: w;P[med];PCNA-EmGFP]; ago1, FRT80B/TM6B, eyFLP;P[med];arm-LacZ], FRT80B/TM6B, ago1, FRT80B/TM6B, eyFLP;P[med];ubi>GFP], FRT80B, eyFLP;ubi>GFP, FRT80B, de2f1rm729, and ago1, FRT80B, de2f1ii/TM6B. The following genotypes were used for transgenic expression: UAS-rbf1/CyO:twi-GFP; ago1, FRT80B/TM6B, UAS-dap/CyO:twi-GFP; ago1, FRT80B/TM6B, UAS-Elp/CyO:twi-GFP; ago1, FRT80B/TM6B, eyFLP;act>y+;Gal4;P[med];RpL14 FRT80B/TM6B, w;UAS-p35; ago1, FRT80B/TM6B, eyFLP;act>y+;Gal4;P[med];arm-LacZ], FRT80B/TM6B, UAS-cycE, UAS-dMyc and UAS-dp53R155H. PCNA-EmGFP gift of R. Duronio. UAS-Elp and EGFRT1 gifts A. Mortimer. de2f1rm729, de2f1ii, and wg56 obtained from the BDSC. sty5 and aos57 gift of N. Moon and I. Rebay.

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.

Immunohistochemistry & Microscopy

Immunostaining and confocal microscopy was performed as described previously [4]. 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.

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′-GTGGAGCAGACACACAAA-3′, 5′-TTGGCAAGTGAAGCTCTGT-3′; rpr 5′-TCGATTCTACTCTGACAGTCAAGG-3′, 5′GAGTAAACTAAAATTGGGTGGTG-3′; β-tub 5′-CGCACAGAGTCTCAGTGTGTG-3′, 5′-AAATCCGTTTCATCAGGCTG-3′.

Cell culture

S2 cells were cultured under standard conditions. Double-stranded RNA interference was carried out as described previously [47]. 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.
Acknowledgments

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References


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

Images of (A) FRT80B/M(3) and (B) *ago*¹/M(3) adult female heads. Black bar denotes depth of FRT80B/M(3) eyes. Arrows indicate increased antennal size in *ago*¹/M(3) heads. (C) Merged confocal sections of FRT80B/M(3) and (D,E) *ago*¹/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 2. Blocking death of ago cells produces enlarged organs
(A) ago\textsuperscript{1}/M(3), (B) ago\textsuperscript{0}, Df(3L)H99/M(3), or (C) ago\textsuperscript{1}, hid\textsuperscript{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\textsuperscript{1}/M(3) eyes; arrows indicate increased antennal size. Arrowheads indicate position of the MF.
Figure 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* 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 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\(^{12/729}\) 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\(^{12/729}\) discs. Paired views of eyes and heads from (C-C\(^{'}\)) ago\(^{1}\) mosaic or (D-D\(^{'}\)) ago\(^{1}\) mosaic+de2f1\(^{12/729}\) adult females in which ago\(^{1}\) mutant tissue is marked by the absence of red pigment; the white:red imbalance persists in the de2f1\(^{12/729}\) background. Reduced organ size in panels D-D\(^{'}\) are due to the effect of the de2f1\(^{12/729}\) genotype on organism size.
Figure 5. Excess *rbf1* suppresses the cell death of *ago* mutant cells in the MF
*ago*1/*M(3)* (A) and *ago*1/*M(3), *ey*Gal4, *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 6. Modification of ago eye size by cell cycle regulators and signaling components
Light microscopic images of ago\(^1//M(3)\) (A) and ago\(^1//M(3),\ de2f1^M729/+\) 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.
Figure 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 8. Death of ago mutant eye cells is sensitive to extracellular signaling pathways

(A–A′) Merged confocal sections of ago\(^{1}/\text{M}(3)\) 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\(^{1}/\text{M}(3)\), eyGal4, UAS-Elp discs (the ago\(^{1}/\text{M}(3)\), 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\(^{55}/\text{M}(3)\), (D) ago\(^{1}\), aos\(^{77}/\text{M}(3)\), (E) ago\(^{1}\), Gap1\(^{F2.4-A3}/\text{M}(3)\), or (F) wg\(^{17+}/+;\ ago^{1}/\text{M}(3)\) 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.