Notch-dependent expression of the archipelago ubiquitin ligase subunit in the Drosophila eye

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SUMMARY
archipelago (ago)/FBw7 encodes a conserved protein that functions as the substrate-receptor component of a polyubiquitin ligase that suppresses tissue growth in flies and tumorigenesis in vertebrates. Ago/Fbw7 targets multiple proteins for degradation, including the G1-S regulator Cyclin E and the oncoprotein dMyc/c-Myc. Despite prominent roles in growth control, little is known about the signals that regulate Ago/Fbw7 abundance in developing tissues. Here we use the Drosophila 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 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. A fragment of the ago promoter that contains consensus binding sites for the N pathway transcription factor Su(H) is bound by Su(H) and confers N-inducibility in cultured cells. 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 is rescued by re-expression of ago. These data suggest 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 the developing eye.

KEY WORDS: Archipelago, Notch, Cyclin E, Drosophila

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 (also known as FBXW7), which is mutated in a wide array of human cancers (reviewed by Welcker and Clurman, 2008). ago encodes an F-box/WD (tryptophan/aspartic acid) protein that 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 polyubiquitylation and subsequent degradation, including the G1/S cell cycle regulator Cyclin E (CycE) and the growth regulator dMyc (Diminutive – FlyBase) in mitotically active imaginal disc cells (Moberg et al., 2001; Moberg et al., 2004), the basic helix-loop-helix (bHLH)-PAS domain transcription factor Tracheless (Trh) in postmitotic tracheal cells (Mortimer and Moberg, 2007), and the glial cell factor Glial cells missing (also known as Glide) (Ho et al., 2009). Loss of ago in imaginal discs causes an accumulation of Cyclin E and dMyc, which drive cell proliferation by balanced increases in the rates of cell division and cell growth (Moberg et al., 2004). Fbw7 acts in a similar manner to promote ubiquitylation and degradation of Cyclin E and c-Myc (Koepf et al., 2001; Welcker et al., 2003; Welcker et al., 2004a; Welcker et al., 2004b; Ye et al., 2004). Additional targets of vertebrate Fbw7 include the Notch1 and Notch4 intracellular domains, c-Jun, sterol regulatory element binding protein (SREBP) and mTor kinase (reviewed by Welcker and Clurman, 2008; Mao et al., 2008). The Fbw7 gene is biallelically lost in a variety of primary tumor types (reviewed by 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 (Trp53 – Mouse Genome Informatics) 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 (MF) (Moberg et al., 2001), where cells arrest in G1 (Wolf and Ready, 1991). ago protects developing eye cells in the anterior region of the MF from apoptotic cell death driven via the Rbf/dE2f1 (E2f – FlyBase) pathway (Nicholson et al., 2009), and blocking this cell death 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 contains a consensus binding site for the Drosophila p53...
ortholog (dp53), which acts downstream of metabolic stress induced by loss of mitochondrial *Cytchrome 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 disc specifically induces expression of the ago-RA mRNA transcript, which initiates transcription from a unique first exon not shared by the other annotated ago transcripts ago-RR and ago-RC (FlyBase, http://flybase.org). DNA sequences upstream of 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 can be bound by Su(H) protein in vitro and 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 summary, these data suggest that ago is an 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.

**MATERIALS AND METHODS**

**Genetics**

Flies were cultured at 25°C except where otherwise noted. N^03^ larvae were cultured at 18°C and shifted to 31°C for 2, 6, or 6 hours. Hsp70> N^Hspa^ larvae were shifted to 37°C for 1 hour, then back to 25°C for 1 hour before dissection. Hsp70>Gal4 driver larvae were shifted to 37°C for 1 hour and back to 25°C for 2 hours before dissection. Egfr mutant clones were generated by shifting eyFLP; FRT42D, Egfrtsla/FRT42D, ubiGFP flies to 30°C for 24 hours immediately prior to analysis. Genotypes used: eyFLP; DF[2]loda^29^-FRT40A, ubiGFP, FRT40A; eyFLP; smo^3^, FRT40A, ubiGFP, FRT40A; eyFLP; Psn^ts^277,FRT80B, ubiGFP, FRT80B; eyFLP; FRT82B, Df[1-2]Fvj^2279^, FRT82B, ubiGFP; eyFLP; FRT42D, Egfrtsla/FRT42D, ubiGFP; eyFLP; smo^3^, FRT40A, armlacZ, FRT40A; ago>Gal4, UAS-R-DHFRr-EGFP; eyFLP; UAS-R-DHFRr-EGFP, ago>Gal4, FRT82B, Df[1-2]Fvj^2279^, Ser^ex23^-FRT82B, armlacZ, eyFLP, Su(H)^Hspa^, FRT40A, armlacZ, FRT40A; ago>Gal4, UAS-R-DHFRr-EGFP, husFLP, FRT40A, ubiGFP, FRT40A; Hsp70>Gal4; and husFLP, FRT40A, ubiGFP, FRT40A; Hsp70>Gal4; and husFLP, FRT40A, ubiGFP, FRT40A; ago>YFP, FRT82B, Df[1-2]Fvj^2279^, Ser^ex23^, FRT82B, tubGal80.

Hsp70> x^Hspa^ and UAS-Di were gifts of K. Moses (Janelia Farm/HHMI). P[GawB] [ago] [NP0850] was obtained from the Drosophila Genetic Resource Center, Kyoto Institute of Technology, Japan. UAS-R-DHFRr-EGFP was a gift of K. Broadie (Vanderbilt University). A^Hspa^ and smo^3^ T(2;3)TSSTL, Cyo^TM6>, Tb^6> were gifts of A. Mortimer (Emory University). The 3R MARCM stock was a gift of J. Treisman (New York University). Su(H)^Hspa^, FRT40A was a gift of W. Du (University of Chicago). FRT42D, Egfrtsla was a gift of D. Marenda (Drexel University). UAS-2xEGFP, A1H2, FRT82B, Df[1-2]Fvj^2279^, Ser^ex23^, Psn^ts^277 and DF[2]loda^29^-FRT40A were obtained from the Bloomington Drosophila Stock Center.

**Immunohistochemistry and microscopy**

Confocal microscopy was performed as described previously (Moberg et al., 2004). Antibodies used: rabbit anti-GFP (Molecular Probes, 1:1000); guinea pig anti-Ago (1:2500-1:5000); mouse anti-β-gal (Promega, 1:1000); mouse anti-dMyic (gift of B. Clurman, Fred Hutchinson Cancer Research Center; used undiluted); rat anti-Cyclin E (gift of H. McNell, Mount Sinai Hospital, Toronto; 1:400); rat anti-Elav (Developmental Studies Hybridoma Bank, 1:200); guinea pig anti-Ato (1:1000); and rabbit anti-Ato (1:625) (Melichar et al., 2008).

**Real-time reverse transcription PCR**

Total RNA isolated from 20 eye discs using TRizol (Invitrogen) was reverse transcribed (SuperScript II reverse transcriptase, Invitrogen) and analyzed by quantitative PCR (SYBR Green 1 Master Mix, Roche). Primers used: ago-RA 5’-GGAGATTTGCAGATCTCTGG-3’ and 5’-AAGTTGGAATGGTGTCCTCAA-3’; ago-RC 5’-TGGTCACGGATTCATGCTCA-3’ and 5’-CAAGGAGACCAAGCTCTAAA-3’; and β-tub 5’-CGCACAGAGTCCATGGT-3’ and 5’-AAATGTTACATCCAA-GCTG-3’.

**S2R+ cell manipulations**

S2R+ cells plated to confluency in 24-well plates were co-transfected with 100 ng of both ago2kb-luc/ago2kb6smut-luc and a coxa-Remilla-luciferase reporter. Wells were also treated with either empty vector, 300 ng of a plasmid encoding the Notch intracellular domain (NICD), 50 ng of a plasmid encoding the first 545 amino acids of Daughterless (DaN), or 300 ng of NICD and 50 ng of DaN. Transfections were performed in triplicate using FUGENE HD (Roche). Forty-eight hours post-transfection, cells were lysed and reporter assays were performed using the Dual-Luciferase reporter system (Promega). Data shown are representative of three independent transfections.

**Molecular cloning**

To create ago2kb-luc, the minimal promoter from *Heat shock protein 70 (Hsp70)* was cloned into the *HindIII* and *BglII* sites of the pGL2-basic luciferase vector (Promega). A 2 kb fragment (−302 to +1697 bp relative to the transcriptional start site of ago-RC (FlyBase)) from the ago locus was PCR amplified from genomic DNA isolated from Canton S flies and then subcloned (in the sense orientation) into the SacI and Nhel sites upstream of the *Hsp70* minimal promoter in pGL2-basic. ago2kb6smut-luc was generated from ago2kb-luc using QuikChange II Site-Directed Mutagenesis (Agilent). Primer sequences are listed in Table S1 in the supplementary material.

**Gel shift assays**

Gel shift assays were performed as described (Bailey and Posakony, 1995) using GST-Su(H) protein preparations made as described previously (Yan et al., 2004). Briefly, 200 ng of purified GST-Su(H) protein were incubated for 10 minutes at 4°C in 10 mM Tris-HCl pH 7.5, 50 mM NaCl, 1 mM DTT, 1 mM EDTA pH 8.0 and 1 μg sonicated salmon sperm DNA, in the presence of competitor DNA; 1 μl of [γ^32P]ATP end-labeled ago probe (2×10^6 cpm) was then added, reactions were incubated for a further 10 minutes at 25°C, then electrophoresed on 4% polyacrylamide 0.5X TBE non-denaturing gels.

**RESULTS**

Ago protein levels and promoter activity are elevated within the MF

Immunostaining of third instar eye imaginal discs with a polyclonal anti-Ago antiserum (Mortimer and Moberg, 2007) revealed 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 began to rise above baseline among cells in the region of the MF (see Fig. 1A,B for different optical planes and magnifications), and were subsequently refined into a pattern in the first few rows of cells posterior to the MF. ‘Holes’ in this pattern appeared to overlap
with gaps in expression of the Atonal (Ato) neuronal transcription factor among emerging R8 precursor equivalence groups (Dokucu et al., 1996) (Fig. 1A, arrows). Higher magnification analysis of discs co-stained for Ago and the neuronal marker Elav (Robinow and White, 1991) showed 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 appeared to become restricted to a single cell nucleus that occupied an anterior location within the nascent Elav-positive cluster (Fig. 1B,B’, arrows). Baseline levels of Ago were 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 that elevates Ago levels above a uniform background level; 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 might contribute to the induction of Ago in this area. To assay ago promoter activity in vivo, the P[GawB]ago[NP0850] insertion, which contains a minimal promoter upstream of the Gal4 open reading frame (Brand and Perrimon, 1993) and is inserted 53 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), was combined with a UAS:R-DHFRts-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] ago elements function as enhancer-trap reporters of the loci into which they are inserted (Brand and Perrimon, 1993), we surmised that expression of P[GawB] ago (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 GFP. Consistent with this hypothesis, UAS:R-DHFRts-EGFP; ago>Gal4 animals shifted to the restrictive temperature of 37°C and allowed to recover for 1 hour at the permissive temperature of 18°C showed 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 was expressed throughout the posterior region of UAS:R-DHFRts-EGFP; ago>Gal4 eye discs (data not shown), presumably owing to perdurence of EGFP protein. The EGFP signal in UAS:R-DHFRts-EGFP; ago>Gal4 discs exposed to the 37°C to 18°C shift was 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, arrow). Given the predicted temporal delay in EGFP expression produced by the binary Gal4,UAS system, these data indicate that the ago>Gal4 element is likely to report 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 did not accumulate elsewhere in UAS:R-DHFRts-EGFP; ago>Gal4 eye discs following the 37°C to 18°C shift, indicating that the ago>Gal4 element does not report baseline ago promoter activity associated with the uniform expression of ago mRNA throughout the eye/antennal disc.

**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 to cell communication mediated by morphogens and membrane-bound ligands (Rouignant and Teisson, 2009). The pattern of induction of the ago promoter suggests that one or more of these pathways might drive ago expression in this region. To test this, clones of cells mutant for either the Drosophila Epidermal growth factor receptor (Egfr), the Dpp receptor thickveins (tkv), the Hh receptor smothing (smo), the N activator Presenilin (Pan), or both of the N ligands Dl and Serrate (Ser), were examined for their effect on Ago protein levels. Whereas neither Egfr nor tkv loss had a profound effect on Ago expression (see Fig. S1 in the supplementary material; data not shown), cells mutant for the smo2 null allele (Chen and Struhl, 1998) failed to upregulate Ago protein among MF cells (Fig. 2A-A’); these clones also lacked 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 was 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 could be rescued in smo3 cells if they lay adjacent to wild-type cells at a clonal boundary (Fig. 2B-B', arrows). Thus, smo is not absolutely required for Ago induction but promotes a second juxtacrine pathway that is required for Ago expression. As smo promotes expression of the N ligand Dl in eye cells (Vrailas and Moses, 2006), we hypothesized that the effect of the smo3 allele on Ago induction could result from reduced N signaling. Consistent with this, loss of multiple N pathway components blocked the upregulation of Ago protein in the area of the MF. Cells homozygous for a null allele of Psn (Psnzzzz), which encodes a component of the γ-secretase complex that cleaves and activates N in response to ligand binding (Struhl and Greenwald, 2001), failed to upregulate Ago protein at the MF (Fig. 2C-C'). Similarly, clones of cells carrying mutations in both N ligands, Dl (DlRevF10) and Ser (SerRxx), also failed to upregulate Ago protein at the MF (Fig. 2D-D'). Furthermore, flies homozygous for a temperature-sensitive N allele (Nts3) showed reduced Ago levels in the area of the MF in larval eye discs following a 2-hour shift to the restrictive temperature (Fig. 3). Ago expression posterior to the MF also became irregular in Nts3 discs upon longer exposure to the restrictive temperature, appearing as disorganized clumps of Ago-expressing nuclei (insets in Fig. 3A' show Nts3 discs exposed to 0 and 6 hours of heat shock). Thus, N appears to have a second role in patterning Ago expression among cells posterior to the MF. As ago protects eye disc cells in the anterior region of the MF from apoptotic cell death driven via the Rbf/de2f1 pathway (Nicholson et al., 2009), DlRevF10,SerRxx clones were also stained for the pro-apoptotic marker cleaved Caspase 3 (C3; Decay – FlyBase) (see Fig. S2 in the supplementary material). C3 levels were not elevated in DlRevF10,SerRxx clones, indicating that the N-dependent pulse of ago expression at the MF is not required to protect cells from apoptosis.

In light of the requirement for N signaling 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 smo3 clones. Re-expression of Dl restored Ago levels in smo3 clones lying within or just behind the MF (Fig. 4, arrows); this Ago protein appeared to be shifted to the 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.

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-DHFRttt-EGFP,ago-Gal4 system was reduced to background levels in smo3 mutant clones at the MF (Fig. 5A-A', arrows). As observed with Ago protein, smo3 mutant cells that lay adjacent to wild-type cells showed normal levels of ago-Gal4 activity (Fig. 5B-B', arrowheads). Discs mosaic for mutations in Dl and Ser also showed a failure to express GFP from the UAS:R-DHFRttt-EGFP,ago-Gal4 transgene at the MF (Fig. 5C-D'), and this also appeared to be rescued by the presence of adjoining wild-type cells. This juxtacrine rescue of ago-Gal4 activity is likely to be due to the ability of Dl and Ser proteins in wild-type cells to signal into adjacent Dl,Ser mutant cells. Eye disc cells mutant for Su(H),
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and in the case of the NICD target gene E(Spl)m8, co-expression of the NICD and the N-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 2 kb fragment of the ago promoter (ago2kb-luc; Fig. 6B) containing all six 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. The NICD and DaN expression plasmids individually produced ~1.5-fold increases in luciferase activity, whereas their co-expression led to a ~13-fold synergistic induction of ago2kb-luc (Fig. 6A). Parallel analysis in intact discs showed that clones of da mutant cells fail to upregulate Ago protein at the MF (Fig. 6D-D*), indicating that da is required for proper Ago expression in this region. To test whether optimal activation of the ago2kb-luc construct requires the six Su(H) binding sites, two point mutations were introduced into each of the six putative sites to change them to RAGRCAR, which reduces Su(H) binding in vitro (Morel and Schweisguth, 2000). Synergistic activation of this mutant construct (ago2kb6mut-luc) by NICD and DaN was considerably reduced relative to ago2kb-luc (Fig. 6A); it was not, however, abolished completely. Gel shift analysis confirmed that Su(H) can bind a radiolabeled double-stranded DNA probe corresponding to a portion of the ago2kb sequence that contains two of the six candidate Su(H) binding sites; this in vitro interaction was efficiently competed by addition of excess unlabeled wild-type probe, but not by a version of this probe in which both of the Su(H) sites had been changed to the RAGRCAR sequence (Fig. 6E).

To confirm that ago transcript levels are also N-responsive in the in vivo context of the larval eye disc, the levels of ago-R4 and ago-RC transcripts (the ago-RB transcript is undetectable in larval discs; N.T.M. and K.H.M., unpublished) were measured in eye discs of third instar larvae 1 hour after a 1-hour heat shock induction of an Hsp70-Nextra transgene (Fig. 6C), which expresses an active form of the Notch receptor (Struhl et al., 1993). This produced a ~3.5-fold increase in the abundance of the ago-R4 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 in vitro and in vivo data presented above support a model in which ago transcription responds to both elevated and reduced N activity, and that this mechanism is required for the pulse of ago mRNA expression associated with passage of the MF.

**Fig. 4. Elevated Dl restores Ago in smo mutant cells.**

(A-A*) Confocal sections of smo clones marked by the absence of GFP (green) generated in the presence of Hsp70-Dl and stained for Ago (red). Arrows denote a delayed rescue of Ago levels in mutant clones. (B-B*) High-magnification view of a confocal section of smo clones marked by the absence of GFP (green) generated in the presence of Hsp70-Dl and stained for Ago (red). Arrows denote the delayed Ago expression in the mutant clone. Arrowheads denote the location of the MF.

which encodes a protein that complexes with the Notch intracellular domain (NICD) and mediates transcriptional responses to N pathway activity (Bailey and Posakony, 1995), also failed to activate the ago>Gal4 transgene (Fig. 5E-E*), indicating that this nuclear component of the N pathway is required for ago promoter activity. Notably, ago>Gal4 activity could be elevated in wild-type MF cells that lay immediately adjacent to Su(H) mutant cells (e.g. see clone of wild-type cells indicated by red arrow in Fig. 5E). As Su(H) is required to repress Dl expression and prevent hyperaccumulation of DI protein within and behind the MF (Tsuda et al., 2002), this ‘boundary’ effect on ago>Gal4 is likely to be due to increased N activity in wild-type cells that lie adjacent to DI-overexpressing Su(H) mutant cells. In summary, these data confirm that the components of 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 that ago might be a transcriptional target of the N pathway. The ago promoter was examined for DNA sequences resembling those bound by the Su(H) transcription factor (Nellesen et al., 1999), which complexes with NICD and mediates transcriptional responses to N pathway activity (Bailey and Posakony, 1995). Thirteen putative Su(H) binding sites [RTGRGAR (Bailey and Posakony, 1995; Nellesen et al., 1999; Morel and Schweisguth, 2000)] were identified within a 5.5 kb region of the ago locus (spanning base 4,250,442 to base 4,244,682 on chromosome 3L; numbering according to FlyBase). Notably, six of these putative Su(H) binding sites lie upstream of the first exon of the ago-R4 transcript in the immediate area of the N-dependent ago>Gal4 element (see Fig. 6B). Of these six candidate Su(H) binding sites, five are in the forward orientation with respect to ago gene transcription and one lies on the opposite strand in the reverse orientation, a configuration that has been reported in other N-responsive promoters (Bailey and Posakony, 1995). Two putative ‘A’ sites for 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

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). Identification of N as a transcriptional regulator of ago suggests that N might execute some of these anti-mitotic roles in part by indirectly promoting the turnover of SCF-Ago targets. Consistent with previous studies of the effect of N pathway mutations on Cyclin E (Sukhanova and Du, 2008), levels of Cyclin E protein were elevated only in DI,Ser double-mutant clones located within or posterior to the MF (Fig. 7A,B). This effect did not occur in DI,Ser clones in the anterior region, and
only became apparent as cells traversed the area of the MF (compare areas of the GFP-marked clone indicated by arrows in Fig. 7B). This phenotype is distinct from that observed in ago null clones, which accumulate Cyclin E regardless of their spatial location in the disc (Moberg et al., 2001), but does correlate 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 an UAS-ago, YFP transgene. Although expression of ago, YFP was not sufficient to reduce Cyclin E levels in other areas of the eye disc (e.g. the large clone in the posterior region of the disc in Fig. 7C'), it was sufficient to substantially rescue the elevated Cyclin E phenotype in Dl, Ser mutant clones lying within or just posterior to the MF (Fig. 7C, D). These clones still contained slightly more Cyclin E protein than surrounding control cells, indicating either that Dl, Ser loss may have a second Ago-independent effect on Cyclin E expression or that the Ago:YFP protein does not function optimally. However, the substantial rescue of Cyclin E levels in Dl, Ser clones argues that the N pathway acts through ago to control Cyclin E turnover in a subset of cells in the developing eye. Interestingly, levels of dMyc (Moberg et al., 2004) were not elevated in smo mutant or Dl, Ser mutant clones located in the area of the MF (see Fig. S3 in the supplementary material). Since complete loss of ago elevates dMyc protein levels in cells regardless of their location in the disc (Moberg et al., 2004), this observation suggests that baseline levels of Ago that persist in these mutant backgrounds are sufficient to support the turnover of dMyc but are insufficient to regulate Cyclin E.

**DISCUSSION**

The ago gene and its vertebrate ortholog Fbw7 have well established roles in controlling cell division, cell growth and apoptosis in developing tissues, yet only a few studies have investigated pathways that regulate ago activity in cells. It is known that dimerization of Fbw7 enhances its ability to degrade Cyclin E (Zhang and Koepp, 2006), and that ago and Fbw7 are both 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). 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. DaN can bind to Su(H) and drive elevated expression of the N-target E(spl)m8 (Cave et al., 2005), and the requirement for da in Ago expression at the MF suggests that a similar mechanism might occur here. Interestingly, mutating the six putative Su(H) sites in the ago2kb-luc reporter does not completely abolish transactivation by NICD and DaN, suggesting that they have a secondary effect on ago transcription that is independent of the Su(H) sites. Finally, we find that the defect in the MF-associated pulse of ago expression in N pathway-defective cells results in hyperaccumulation 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-S phase transition in eye disc cells.

Although 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 pattern of Ago expression. The regular pattern of Ago expression posterior to the MF appears to overlap with gaps in Ato expression between the R8 cells and for restriction of Ato expression in cells immediately posterior to the MF to refine the pattern of Ago expression. The regular pattern of Ago expression posterior to the MF might play a similar dual activator/inhibitor role upstream of the Ago-RA start site (see B). GST-Su(H) binds the wild-type probe (lane 2), and is efficiently competed by excess unlabeled wt DNA (lanes 3-5 contain 50, 100 and 500 ng DNA, respectively), but not by equivalent amounts of an unlabeled DNA containing mutations (altered bases indicated by dots) in the two consensus Su(H) sites (lanes 6-8). Free probe and shifted Su(H):DNA complexes are indicated. 

Fig. 6. The ago promoter is responsive to changes in levels of N signaling. (A) Relative luciferase activity in Drosophila S2 cells transfected with ago2kb-luc or ago2kb6mut-luc. Cells were transfected with either 200 ng of NICD plasmid, 50 ng of DaN plasmid, or both, as indicated. Error bars indicate mean ± s.d. (B) The ago locus. Three transcript variants, RA, RB and RC, have alternate first exons but share the same coding sequence (red bars, exons). Splicing patterns are indicated. Location of the P[GawB]ago[NP0850] element 53 bp upstream of the ago-RA start site is indicated. The 2 kb region cloned into the luciferase vector is indicated (black bar) as are the locations of putative transcription factor binding sites: six Su(H) binding sites (purple; arrows indicate orientation), two A sites (green), and the p53 binding sequence (blue). (C) Gel shift analysis of an interaction between recombinant Su(H) protein [GST-Su(H)] and a radiolabeled fragment of the wild-type (wt) ago promoter containing two putative Su(H) sites closest to the ago-RA start site (see B). GST-Su(H) binds the wild-type probe (lane 2), and is efficiently competed by excess unlabeled wt DNA (lanes 3-5 contain 50, 100 and 500 ng DNA, respectively), but not by equivalent amounts of an unlabeled DNA containing mutations (altered bases indicated by dots) in the two consensus Su(H) sites (lanes 6-8). Free probe and shifted Su(H):DNA complexes are indicated.
Expression of ago-Gal4 is rescued in DI,Ser mutant cells by adjacent wild-type cells, and Su(H) mutant cells appear to be able to upregulate ago expression in adjacent wild-type cells, probably via increased DI expression (Tsuda et al., 2002). Alleles of other N pathway components might thus be expected to exhibit effects on ago expression at clonal boundaries. However, expression of Ago in Psm mutant clones that span the MF is not obviously rescued by adjacent wild-type cells, and, reciprocally, these cells do not induce higher levels of Ago in adjacent wild-type cells (see Fig. S5 in the supplementary material), indicating that the cross-border induction of ago transcription might be restricted to alleles of genes required for repression of DI.

Depending on the developmental context, N can be either pro- or anti-mitotic (reviewed by 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, provide 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) hyperaccumulate 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 the N pathway in promoting ago expression. The latter pro-mitotic effect of N has been attributed to a requirement for N for relief of Rbf-mediated repression of dE2f1 activity (Baonza and Freeman, 2005), but the former role of the N pathway in antagonizing Cyclin E levels is not well understood. Based on data presented here that place the ago pathway in promoting ago expression. The identity of the N target that promotes SMW entry has remained controversial (Baonza and Freeman, 2005; Firth and Baker, 2005; Escudero and Freeman, 2007). 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 might 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 discernible effect on the levels of dMyc. One logical explanation for this is 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 selectively regulates some SCF-Ago targets but not others; alternatively, N might have a second role in this pathway by protecting dMyc from SCF-Ago activity, although there is no clear evidence of such a role.

The role for N upstream of Ago and Cyclin E could theoretically be significant in mediating N effects in tissues outside of 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 suggest that the N-ago link might be fairly context specific and not generalized to N signaling in all cell types. First, the pattern of Ago protein in developing larval discs (including the eye) does not generally mirror that of N pathway activity in each organ. 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 a clear candidate to fulfill this role: Da expression peaks in the MF (Brown et al., 1996) and is required for

differentiating neurons. In support of this type of feedback model, expression of the N pathway reporter E(spl)m6-Cd2 (de Celis et al., 1998) is elevated in ago mutant cells posterior to the MF, indicating that Ago might also regulate N activity. As overall N protein levels are not obviously altered in ago mutant eye cells (see Fig. S4 in the supplementary material), as assessed by both indirect immunofluorescence and immunoblotting with the C17.9C6 antibody that recognizes the cytoplasmic tail of the N receptor (Fehon et al., 1990), it remains to be established whether or not this potential feedback loop involves changes in N protein turnover.

In addition to the potential for a cell-autonomous feedback mechanism between Ago and N, ago expression is subject to N pathway-mediated, non-cell-autonomous effects at the MF.

Fig. 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 DfRevF10,SerP052 double-mutant clones marked by the absence of GFP (green) stained for Cyclin E (blue). Note the elevated Cyclin E in DfRevF10,SerP052 cells behind the MF. (B-B') Merged confocal sections of DfRevF10,SerP052 MARCM clones positively marked by GFP (green) and co-stained for Cyclin E (blue). Arrows highlight the fact that the effect of DfRevF10,SerP052 on Cyclin E only occurs in cells within and behind the MF. (C-D') Merged confocal sections of DfRevF10,SerP052 MARCM clones expressing an Ago:YFP fusion protein. Arrows indicate DI,Ser mutant clones in which the Cyclin E levels are partially rescued by expression of Ago:YFP (compare the intensity of Cyclin E in C and D with that in A and B).
the pulse of Ago protein expression at the MF. Thus, it seems likely that N signals synergize with Da, and perhaps with additional unidentified factors, to pattern ago expression. Given the role that dp35 plays in ago induction and in the control of Cyclin E protein under conditions of metabolic stress (Mandal et al., 2010), it will be interesting to determine how pathways involving N, da and dp35 interact on the ago promoter under various stresses and developmental conditions. Furthermore, it will important to determine whether additional signaling and checkpoint mechanisms, especially those that interact functionally with the N pathway, act through the ago promoter to pattern Cyclin E levels in developing tissues.

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References


