Development 138, 251-260 (2011) doi:10.1242/dev.054429

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

Sarah C. Nicholson[1], Brandon N. Nicolay[2], Maxim V. Frolov[2] and Kenneth H. Moberg[1,*]

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 Trachealess (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 (Koepp 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 (Wolff 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

[1]Department of Cell Biology, Emory University School of Medicine, Atlanta, GA 30322, USA. [2]Department of Biochemistry and Molecular Genetics, University of Illinois at Chicago, Chicago, IL 60607, USA.

*Author for correspondence (kmoberg@cellbio.emory.edu)

Accepted 15 November 2010

ortholog (dp53), which acts downstream of metabolic stress induced by loss of 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 first exon not shared by the other annotated *ago* transcripts *ago-RB* 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^{ts3} larvae were cultured at 18°C and shifted to 31°C for 0, 2 or 6 hours. *Hsp70>*N^{intra} 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,*$Egfr^{tsla}$*/FRT42D,ubiGFP* flies to 30°C for 24 hours immediately prior to analysis. Genotypes used:
eyFLP;DF(2L)da^{10}*FRT40A/ubiGFP,FRT40A*;
eyFLP;smo^{3}*,FRT40A/ubiGFP,FRT40A*;
eyFLP;;Psn^{227}*,FRT80B/ubiGFP,FRT80B*;
eyFLP;;FRT82B,Dl^{RevF10}*,*Ser^{RX82}*/FRT82B,ubiGFP*;
eyFLP;FRT42D,$Egfr^{tsla}$*/FRT42D,ubiGFP*;
eyFLP;smo^{3}*,FRT40A/armlacZ,FRT40A;ago>Gal4,UAS:R-DHFR*$_{ts}$*-EGFP*;
eyFLP;UAS:R-DHFR$_{ts}$*-EGFP;ago>Gal4,FRT82B,*Dl^{RevF10}*,*Ser^{RX82}*/FRT82B,armlacZ*;
eyFLP;Su(H)$^{\Delta 47}$*,FRT40A/armlacZ,FRT40A;ago>Gal4,UAS:R-DHFR*$_{ts}$*-EGFP*;
UAS>Dl/eyFLP;smo^{3}*,FRT40A/ubiGFP,FRT40A;Hsp70>Gal4*; and
hsFLP,UAS:GFP;tubGal4/UAS-ago;YFP;FRT82B,Dl^{RevF10}*,*Ser^{RX82}*/FRT82B,tubGal80*.

Hsp70>N^{intra} and *UAS>Dl* were gifts of K. Moses (Janelia Farms/HHMI). *P{GawB}ago[NP0850]* was obtained from the Drosophila Genetic Resource Center, Kyoto Institute of Technology, Japan. *UAS:R-DHFR*$_{ts}$*-EGFP* was a gift of K. Broadie (Vanderbilt University). N^{ts3} and *smo*^{3}*/T(2;3)TSTL,CyO:TM6B,Tb*^{1} were gifts of A. Mortimer (Emory University). The 3R MARCM stock was a gift of J. Treisman (New York University). *Su(H)*$^{\Delta 47}$*,FRT40A* was a gift of W. Du (University of Chicago). *FRT42D,*$Egfr^{tsla}$ was a gift of D. Marenda (Drexel University). *{UAS-2xEGFP}AH2*, *FRT82B,*Dl^{RevF10}*,*Ser^{RX82}*, Psn*^{227} and *DF(2L)da*^{10}*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-dMyc (gift of B. Clurman, Fred Hutchinson Cancer Research Center; used undiluted); rat anti-Cyclin E (gift of H. McNeill, 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) (Melicharek 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′-GGAATTTCGGATCATCTTGG-3′ and 5′-AAGTGGGTAATGCGTTCTCAA-3′; *ago-RC* 5′-TGGTCACGGATTCACTGCTA-3′ and 5′-CGAGGAGCAACGTCCTTAAA-3′; and β-*tub* 5′-CGCACAGAGTCCATGGTG-3′ and 5′-AAATCGTTCACATCCAAGCTG-3′.

S2R+ cell manipulations

S2R+ cells plated to confluency in 24-well plates were co-transfected with 100 ng of both *ago2kb-luc/ago2kb6xmut-luc* and a *copia-Renilla*-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 first cloned into the *Hin*dIII and *Bgl*II 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 *Sac*I and *Nhe*I sites upstream of the *Hsp70* minimal promoter in *pGL2-basic*. *ago2kb6xmut-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^{32}P]ATP end-labeled *ago* probe (2×10^4 cpm) was then added, reactions were incubated for a further 10 minutes at 25°C, then electrophoresed on 4% polyacrylamide 0.5×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

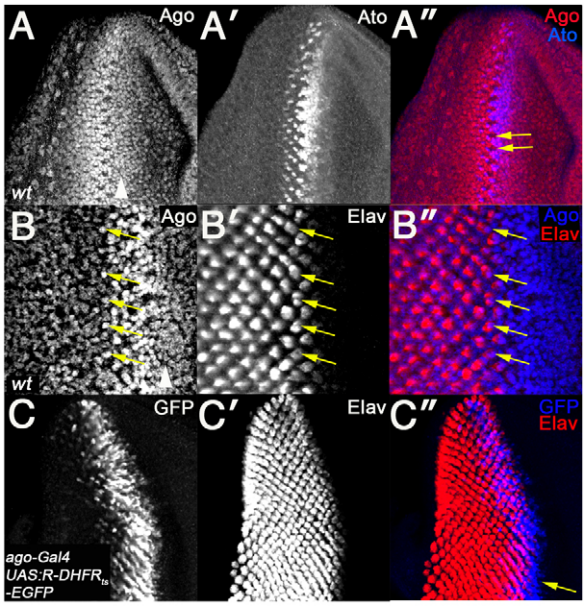

Fig. 1. Ago accumulates at the MF in a subset of Ato-positive and Elav-positive cells. *Drosophila ago* promoter activity is detected several rows of cells anterior to the wave of Elav-positive clusters. (**A-A″**) Merged confocal sections of a wild-type (wt) *Drosophila* larval eye disc stained for Ago (red) and Ato (blue). Arrows mark overlapping 'holes' in Ato and Ago expression immediately posterior to the MF. Arrowhead indicates 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). Arrows in 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). Arrow indicates GFP expression extending into the MF anterior to the first row of Elav-positive nuclei.

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-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 are inserted (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 GFP. Consistent with this hypothesis, *UAS:R-DHFR$_{ts}$-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-DHFR$_{ts}$-EGFP;ago>Gal4* eye discs (data not shown), presumably owing to perdurance of EGFP protein. The EGFP signal in *UAS:R-DHFR$_{ts}$-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-DHFR$_{ts}$-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 (Roignant and Treisman, 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 *smoothened* (*smo*), the N activator *Presenilin* (*Psn*), 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 *smo^{3}* 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.

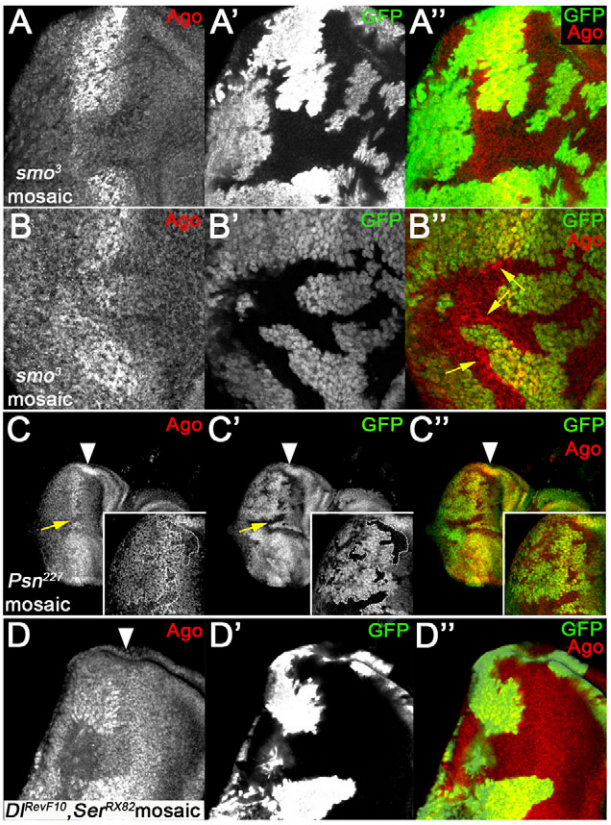

Fig. 2. *hh* and *N* pathway mutants cause a decrease in Ago levels at the MF. (**A-B″**) Ago (red) fails to accumulate at the MF in *smo*^{3} clones marked by the absence of GFP (A-A″). Higher magnification (B-B″) shows rescue of Ago accumulation at clonal boundaries that cross the MF (arrows). (**C-C″**) Ago (red) fails to accumulate at the MF in *Psn*^{227} clones (arrow in C) marked by the absence of GFP (arrow in C′). Insets show higher magnification views of clones (outlined by dotted lines). (**D-D″**) Ago (red) fails to accumulate at the MF in *Dl*^{RevF10},*Ser*^{RX82} clones marked by the absence of GFP. Arrowheads indicate the position of the MF.

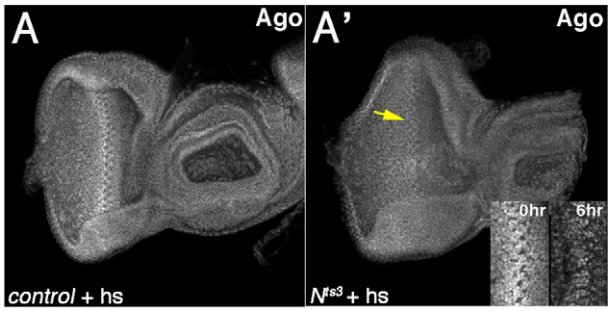

Fig. 3. *N* is necessary for elevated Ago expression and patterning at the MF. (**A**) Confocal section of a heat-shocked control *FRT80B* eye disc stained for Ago. (**A′**) Confocal section of an *N*^{ts3} eye disc stained for Ago after a 2-hour temperature shift to 31°C. Arrow indicates residual Ago expression at the MF. Insets show higher magnification views of an *N*^{ts3} eye disc at *t*=0 hours (left) and *t*=6 hours (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.

Interestingly, the pulse of Ago expression in MF cells could be rescued in *smo*^{3} 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 *smo*^{3} 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* (*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), failed to upregulate Ago protein at the MF (Fig. 2C-C″). Similarly, clones of cells carrying mutations in both N ligands, *Dl* (*Dl*^{RevF10}) and *Ser* (*Ser*^{RX82}), also failed to upregulate Ago protein at the MF (Fig. 2D-D″). Furthermore, flies homozygous for a temperature-sensitive *N* allele (*N*^{ts3}) 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 *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 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), *Dl*^{RevF10},*Ser*^{RX82} 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 *Dl*^{RevF10},*Ser*^{RX82} 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 *smo*^{3} clones. Re-expression of *Dl* restored Ago levels in *smo*^{3} 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-DHFR*$_{ts}$*-EGFP;ago>Gal4* system was reduced to background levels in *smo*^{3} mutant clones at the MF (Fig. 5A-A″, arrows). As observed with Ago protein, *smo*^{3} 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-DHFR*$_{ts}$*-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)*,

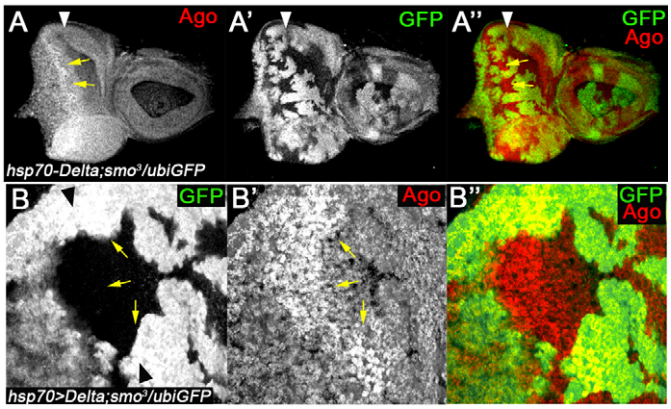

Fig. 4. Elevated *Dl* restores Ago in *smo* mutant cells.
(**A-A″**) Confocal sections of *smo*^{3} clones marked by the absence of GFP (green) generated in the presence of *Hsp70>Dl* and stained for Ago (red). Arrows denote a delayed rescue of Ago levels in mutant clones. (**B-B″**) High-magnification view of a 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). 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 Dl 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 Dl-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-RA* 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 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 (*ago2kb6xmut-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-RA* 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-N*^{intra} 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-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 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.

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 *Dl,Ser* double-mutant clones located within or posterior to the MF (Fig. 7A,B). This effect did not occur in *Dl,Ser* clones in the anterior region, and

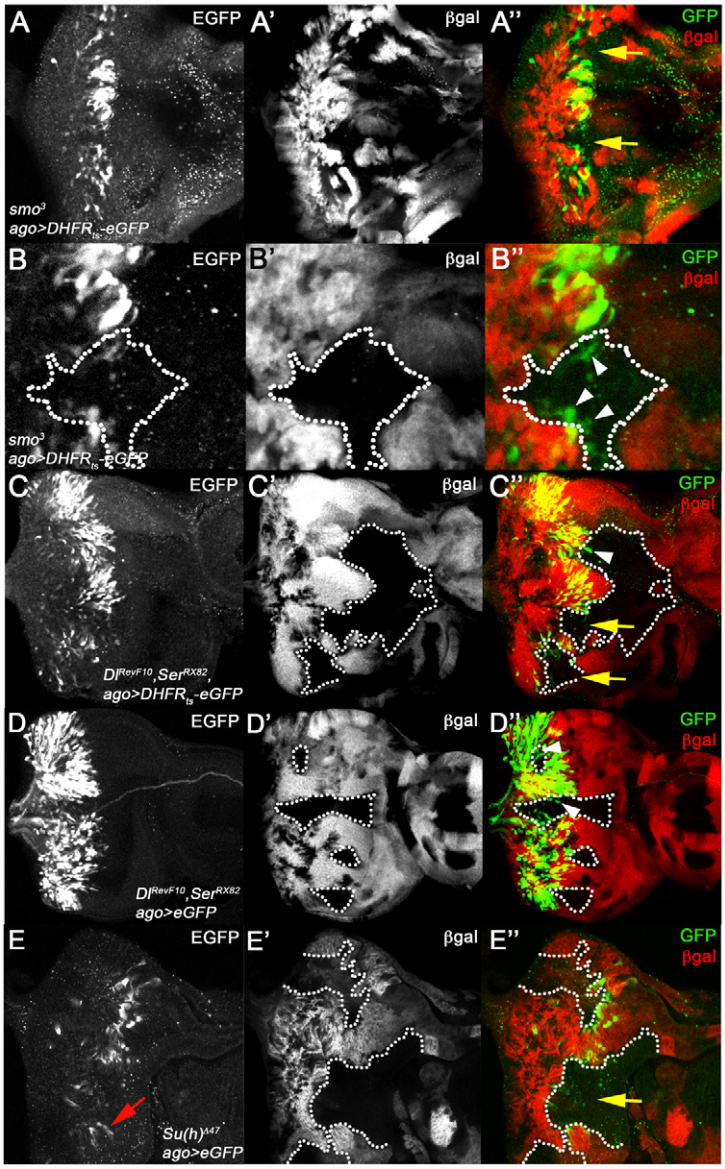

Fig. 5. Hh and N pathway mutants cause a decrease in *ago* promoter activity at the MF. (**A-A″**) Confocal sections of *smo*^{3} clones marked by the absence of β-gal expression (red). *ago* promoter activity detected by the *ago>Gal4,UAS:R-DHFR*$_{ts}$*-EGFP* transgene (green) is much lower in the mutant clones (arrows). (**B-B″**) A higher magnification view of the disc in A-A″ shows rescued *ago* promoter activity in MF cells along clonal borders (arrowheads). (**C-C″**) *Dl*^{RevF10}*,Ser*^{RX82} clones marked by the absence of β-gal (red) generated in *ago>Gal4,UAS:R-DHFR*$_{ts}$*-EGFP* (green) discs. *ago* promoter activity at the MF drops in the mutant clones (arrows) but is rescued in cells along clonal borders (arrowhead). (**D-D″**) *Dl*^{RevF10}*,Ser*^{RX82} clones marked by the absence of β-gal (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. *ago* promoter activity is rescued in cells along clonal borders (arrowheads). (**E-E″**) Confocal sections of *Su(H)*$^{\Delta 47}$ clones marked by the absence of β-gal (red). *ago* promoter activity detected by the *ago>Gal4,UAS:R-DHFR*$_{ts}$*-EGFP* transgene (green) is reduced in the mutant clones (yellow arrow). The red arrow (E) indicates an example of elevation of *ago>Gal4* activity in wild-type MF cells immediately adjacent to *Su(H)* mutant cells.

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

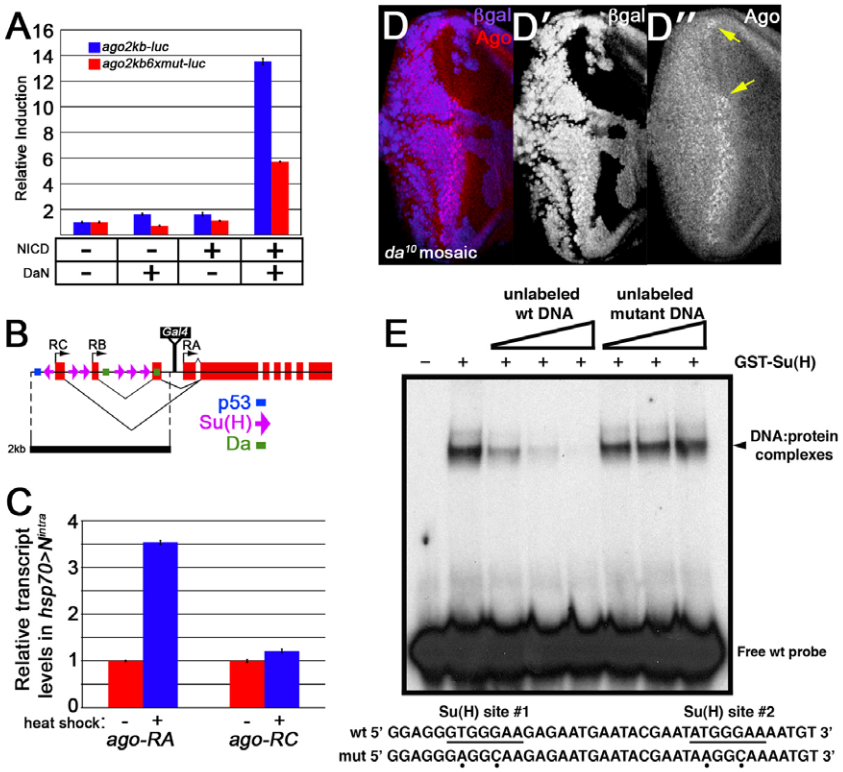

Fig. 6. The *ago* promoter is responsive to changes in levels of *N* signaling. (**A**) Relative luciferase activity in *Drosophila* S2 cells transfected with *ago2kb-luc* or *ago2kb6xmut-luc*. Cells were co-transfected with either 300 ng of *NICD* plasmid, 50 ng of *DaN* plasmid, or both, as indicated. 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**) Quantitative real-time PCR analysis of the expression of *RA* and *RC* mRNA levels in *Hsp70>N^{intra}* with (+) or without (–) a 1-hour heat shock as indicated. Error bars indicate mean ± s.d. (**D-D″**) Ago protein (red) fails to accumulate at the MF in *da^{10}* mutant clones marked by the absence of *lacZ* expression (magenta). Arrows indicate Ago protein in wild-type cells that flank a large *da^{10}* clone. (**E**) 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.

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 equivalence groups (Baker and Yu, 1998) (see Fig. 1A), arguing that these expression patterns might share some regulatory inputs. Interestingly, *N* is required both for induction of Ato within MF cells and for restriction of Ato expression in cells immediately posterior to the MF via a lateral inhibition mechanism (Jarman et al., 1994; Jarman et al., 1995; Baker et al., 1996; Dokucu et al., 1996). Thus, although Ago does not display precisely the same pattern as that of Ato restriction posterior to the MF (e.g. expression only in the presumptive R8), it seems possible that *N* might 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 (Oberg et al., 2001; Fryer et al., 2004; Gupta-Rossi et al., 2004; Tsunematsu et al., 2004), the pattern of Ago expression posterior to the MF might also reflect a requirement for SCF-Ago to inhibit NICD activity in

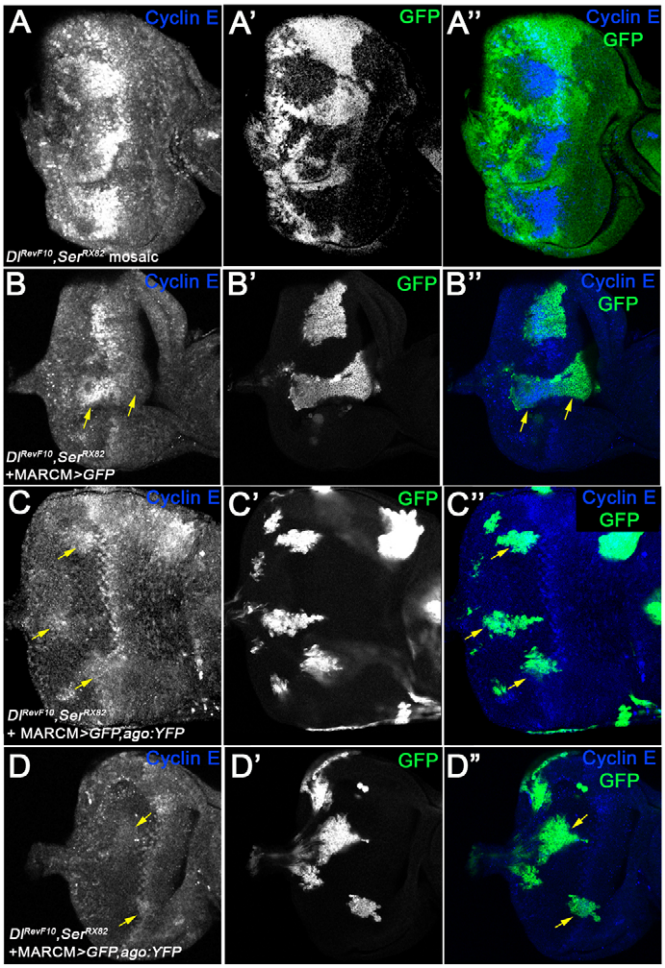

Fig. 7. The decrease in Ago levels in *N* pathway mutant clones results in a build-up of the SCF-Ago substrate Cyclin E. (**A-A″**) Confocal sections of an eye disc containing Dl^{RevF10},Ser^{RX82} double-mutant clones marked by the absence of GFP (green) stained for Cyclin E (blue). Note the elevated 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). Arrows highlight the fact that the effect of Dl^{RevF10},Ser^{RX82} on Cyclin E only occurs in cells within and behind the MF. (**C-D″**) Merged confocal sections of Dl^{RevF10},Ser^{RX82} MARCM clones (GFP positive; green) expressing an Ago:YFP fusion protein. Arrows indicate *Dl,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).

differentiating neurons. In support of this type of feedback model, expression of the *N* pathway reporter *E(spl)mβ-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. Expression of *ago>Gal4* is rescued in *Dl,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 Dl 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 *Psn* 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 *Dl*.

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* fulfils 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 *N* pathway upstream of *ago*, we propose that this phenotype is 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; 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

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 *dp53* 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 *dp53* 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.

Acknowledgements
We thank K. Moses, K. Broadie, J. Treisman, B. Clurman, H. McNeill, A. Mortimer, J. Cave, A. Veraksa, W. Du and D. Marenda for gifts of stocks and antibodies; S. J. Yan for providing recombinant GST-Su(H); and members of the K.H.M. and M.V.F. laboratories for helpful discussions and comments. B.N.N. was supported by National Research Service Award predoctoral fellowship 1F31AG032169, M.V.F. is a Leukemia and Lymphoma Society Scholar and was also supported by National Institutes of Health (NIH) grant GM079774. S.C.N. was supported by NIH grant 5T32GM008490 and by NIH grant GM079242 to K.H.M. Deposited in PMC for release after 12 months.

Competing interests statement
The authors declare no competing financial interests.

Supplementary material
Supplementary material for this article is available at
http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.054429/-/DC1

References

Bailey, A. M. and Posakony, J. W. (1995). Suppressor of hairless directly activates transcription of enhancer of split complex genes in response to Notch receptor activity. *Genes Dev.* **9**, 2609-2622.
Baker, N. E. and Yu, S. Y. (1998). The R8-photoreceptor equivalence group in Drosophila: fate choice precedes regulated Delta transcription and is independent of Notch gene dose. *Mech. Dev.* **74**, 3-14.
Baker, N. E., Yu, S. and Han, D. (1996). Evolution of proneural atonal expression during distinct regulatory phases in the developing Drosophila eye. *Curr. Biol.* **6**, 1290-1301.
Baonza, A. and Freeman, M. (2005). Control of cell proliferation in the Drosophila eye by Notch signaling. *Dev. Cell* **8**, 529-539.
Brand, A. H. and Perrimon, N. (1993). Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. *Development* **118**, 401-415.
Brown, N. L., Paddock, S. W., Sattler, C. A., Cronmiller, C., Thomas, B. J. and Carroll, S. B. (1996). daughterless is required for Drosophila photoreceptor cell determination, eye morphogenesis, and cell cycle progression. *Dev. Biol.* **179**, 65-78.
Calhoun, E. S., Jones, J. B., Ashfaq, R., Adsay, V., Baker, S. J., Valentine, V., Hempen, P. M., Hilgers, W., Yeo, C. J., Hruban, R. H. et al. (2003). BRAF and FBXW7 (CDC4, FBW7, AGO, SEL10) mutations in distinct subsets of pancreatic cancer: potential therapeutic targets. *Am. J. Pathol.* **163**, 1255-1260.
Cave, J. W., Loh, F., Surpris, J. W., Xia, L. and Caudy, M. A. (2005). A DNA transcription code for cell-specific gene activation by notch signaling. *Curr. Biol.* **15**, 94-104.
Chen, Y. and Struhl, G. (1998). In vivo evidence that Patched and Smoothened constitute distinct binding and transducing components of a Hedgehog receptor complex. *Development* **125**, 4943-4948.
de Celis, J. F., Tyler, D. M., de Celis, J. and Bray, S. J. (1998). Notch signalling mediates segmentation of the Drosophila leg. *Development* **125**, 4617-4626.
Deng, W. M., Althauser, C. and Ruohola-Baker, H. (2001). Notch-Delta signaling induces a transition from mitotic cell cycle to endocycle in Drosophila follicle cells. *Development* **128**, 4737-4746.
Dokucu, M. E., Zipursky, S. L. and Cagan, R. L. (1996). Atonal, rough and the resolution of proneural clusters in the developing Drosophila retina. *Development* **122**, 4139-4147.
Escudero, L. M. and Freeman, M. (2007). Mechanism of G1 arrest in the Drosophila eye imaginal disc. *BMC Dev. Biol.* **7**, 13.
Fehon, R. G., Kooh, P. J., Rebay, I., Regan, C. L., Xu, T., Muskavitch, M. A. and Artavanis-Tsakonas, S. (1990). Molecular interactions between the protein products of the neurogenic loci Notch and Delta, two EGF-homologous genes in Drosophila. *Cell* **61**, 523-534.
Firth, L. C. and Baker, N. E. (2005). Extracellular signals responsible for spatially regulated proliferation in the differentiating Drosophila eye. *Dev. Cell* **8**, 541-551.
Fryer, C. J., White, J. B. and Jones, K. A. (2004). Mastermind recruits CycC:CDK8 to phosphorylate the Notch ICD and coordinate activation with turnover. *Mol. Cell* **16**, 509-520.
Gupta-Rossi, N., Six, E., LeBail, O., Logeat, F., Chastagner, P., Olry, A., Israel, A. and Brou, C. (2004). Monoubiquitination and endocytosis direct gamma-secretase cleavage of activated Notch receptor. *J. Cell Biol.* **166**, 73-83.
Herranz, H., Perez, L., Martin, F. A. and Milan, M. (2008). A Wingless and Notch double-repression mechanism regulates G1-S transition in the Drosophila wing. *EMBO J.* **27**, 1633-1645.
Ho, M. S., Chen, H., Chen, M., Jacques, C., Giangrande, A. and Chien, C. T. (2009). Gcm protein degradation suppresses proliferation of glial progenitors. *Proc. Natl. Acad. Sci. USA* **106**, 6778-6783.
Jarman, A. P., Grell, E. H., Ackerman, L., Jan, L. Y. and Jan, Y. N. (1994). Atonal is the proneural gene for Drosophila photoreceptors. *Nature* **369**, 398-400.
Jarman, A. P., Sun, Y., Jan, L. Y. and Jan, Y. N. (1995). Role of the proneural gene, atonal, in formation of Drosophila chordotonal organs and photoreceptors. *Development* **121**, 2019-2030.
Kimura, T., Gotoh, M., Nakamura, Y. and Arakawa, H. (2003). hCDC4b, a regulator of cyclin E, as a direct transcriptional target of p53. *Cancer Sci.* **94**, 431-436.
Koepp, D. M., Schaefer, L. K., Ye, X., Keyomarsi, K., Chu, C., Harper, J. W. and Elledge, S. J. (2001). Phosphorylation-dependent ubiquitination of cyclin E by the SCFFbw7 ubiquitin ligase. *Science* **294**, 173-177.
Lee, T. and Luo, L. (2001). Mosaic analysis with a repressible cell marker (MARCM) for Drosophila neural development. *Trends Neurosci.* **24**, 251-254.
Lopez-Schier, H. and St Johnston, D. (2001). Delta signaling from the germ line controls the proliferation and differentiation of the somatic follicle cells during Drosophila oogenesis. *Genes Dev.* **15**, 1393-1405.
Maillard, I. and Pear, W. S. (2003). Notch and cancer: Best to avoid the ups and downs. *Cancer Cell* **3**, 203-205.
Malyukova, A., Dohda, T., von der Lehr, N., Akhondi, S., Corcoran, M., Heyman, M., Spruck, C., Grander, D., Lendahl, U. and Sangfelt, O. (2007). The tumor suppressor gene hCDC4 is frequently mutated in human T-cell acute lymphoblastic leukemia with functional consequences for Notch signaling. *Cancer Res.* **67**, 5611-5616.
Mandal, S., Freije, W. A., Guptan, P. and Banerjee, U. (2010). Metabolic control of G1-S transition: cyclin E degradation by p53-induced activation of the ubiquitin-proteasome system. *J. Cell Biol.* **188**, 473-479.
Mao, J. H., Perez-Losada, J., Wu, D., Delrosario, R., Tsunematsu, R., Nakayama, K. I., Brown, K., Bryson, S. and Balmain, A. (2004). Fbxw7/Cdc4 is a p53-dependent, haploinsufficient tumour suppressor gene. *Nature* **432**, 775-779.
Mao, J. H., Kim, I. J., Wu, D., Climent, J., Kang, H. C., DelRosario, R. and Balmain, A. (2008). FBXW7 targets mTOR for degradation and cooperates with PTEN in tumor suppression. *Science* **321**, 1499-1502.
Maser, R. S., Choudhury, B., Campbell, P. J., Feng, B., Wong, K. K., Protopopov, A., O'Neil, J., Gutierrez, A., Ivanova, E., Perna, I. et al. (2007). Chromosomally unstable mouse tumours have genomic alterations similar to diverse human cancers. *Nature* **447**, 966-971.
Matsumoto, A., Onoyama, I. and Nakayama, K. I. (2006). Expression of mouse Fbxw7 isoforms is regulated in a cell cycle- or p53-dependent manner. *Biochem. Biophys. Res. Commun.* **350**, 114-119.
Melicharek, D., Shah, A., DiStefano, G., Gangemi, A. J., Orapallo, A., Vrailas-Mortimer, A. D. and Marenda, D. R. (2008). Identification of novel regulators of atonal expression in the developing Drosophila retina. *Genetics* **180**, 2095-2110.
Moberg, K. H., Bell, D. W., Wahrer, D. C., Haber, D. A. and Hariharan, I. K. (2001). Archipelago regulates Cyclin E levels in Drosophila and is mutated in human cancer cell lines. *Nature* **413**, 311-316.
Moberg, K. H., Mukherjee, A., Veraksa, A., Artavanis-Tsakonas, S. and Hariharan, I. K. (2004). The Drosophila F box protein archipelago regulates dMyc protein levels in vivo. *Curr. Biol.* **14**, 965-974.
Morel, V. and Schweisguth, F. (2000). Repression by suppressor of hairless and activation by Notch are required to define a single row of single-minded expressing cells in the Drosophila embryo. *Genes Dev.* **14**, 377-388.
Mortimer, N. T. and Moberg, K. H. (2007). The Drosophila F-box protein Archipelago controls levels of the Trachealess transcription factor in the embryonic tracheal system. *Dev. Biol.* **312**, 560-571.
Nellesen, D. T., Lai, E. C. and Posakony, J. W. (1999). Discrete enhancer elements mediate selective responsiveness of enhancer of split complex genes to common transcriptional activators. *Dev. Biol.* **213**, 33-53.
Nicholson, S. C., Gilbert, M. M., Nicolay, B. N., Frolov, M. V. and Moberg, K. H. (2009). The Archipelago tumor suppressor gene limits Rb/E2F-regulated apoptosis in developing drosophila tissues. *Curr. Biol.* **19**, 1503-1510.
Nicolay, B. N. and Frolov, M. V. (2008). Context-dependent requirement for dE2F during oncogenic proliferation. *PLoS Genet.* **4**, e1000205.

O'Neil, J., Grim, J., Strack, P., Rao, S., Tibbitts, D., Winter, C., Hardwick, J., Welcker, M., Meijerink, J. P., Pieters, R. et al. (2007). FBW7 mutations in leukemic cells mediate NOTCH pathway activation and resistance to {gamma}-secretase inhibitors. *J. Exp. Med.* **204**, 1813-1824.
Oberg, C., Li, J., Pauley, A., Wolf, E., Gurney, M. and Lendahl, U. (2001). The Notch intracellular domain is ubiquitinated and negatively regulated by the mammalian Sel-10 homolog. *J. Biol. Chem.* **276**, 35847-35853.
Radtke, F. and Raj, K. (2003). The role of Notch in tumorigenesis: oncogene or tumour suppressor? *Nat. Rev. Cancer* **3**, 756-767.
Rajagopalan, H., Jallepalli, P. V., Rago, C., Velculescu, V. E., Kinzler, K. W., Vogelstein, B. and Lengauer, C. (2004). Inactivation of hCDC4 can cause chromosomal instability. *Nature* **428**, 77-81.
Reis, T. and Edgar, B. A. (2004). Negative regulation of dE2F1 by cyclin-dependent kinases controls cell cycle timing. *Cell* **117**, 253-264.
Robinow, S. and White, K. (1991). Characterization and spatial distribution of the ELAV protein during Drosophila melanogaster development. *J. Neurobiol.* **22**, 443-461.
Roignant, J. Y. and Treisman, J. E. (2009). Pattern formation in the Drosophila eye disc. *Int. J. Dev. Biol.* **53**, 795-804.
Simon, F., Fichelson, P., Gho, M. and Audibert, A. (2009). Notch and Prospero repress proliferation following cyclin E overexpression in the Drosophila bristle lineage. *PLoS Genet.* **5**, e1000594.
Speese, S. D., Trotta, N., Rodesch, C. K., Aravamudan, B. and Broadie, K. (2003). The ubiquitin proteasome system acutely regulates presynaptic protein turnover and synaptic efficacy. *Curr. Biol.* **13**, 899-910.
Spruck, C. H., Strohmaier, H., Sangfelt, O., Muller, H. M., Hubalek, M., Muller-Holzner, E., Marth, C., Widschwendter, M. and Reed, S. I. (2002). hCDC4 gene mutations in endometrial cancer. *Cancer Res.* **62**, 4535-4539.
Struhl, G. and Greenwald, I. (2001). Presenilin-mediated transmembrane cleavage is required for Notch signal transduction in Drosophila. *Proc. Natl. Acad. Sci. USA* **98**, 229-234.
Struhl, G., Fitzgerald, K. and Greenwald, I. (1993). Intrinsic activity of the Lin-12 and Notch intracellular domains in vivo. *Cell* **74**, 331-345.
Strutt, D. I. and Mlodzik, M. (1997). Hedgehog is an indirect regulator of morphogenetic furrow progression in the Drosophila eye disc. *Development* **124**, 3233-3240.
Sukhanova, M. J. and Du, W. (2008). Control of cell cycle entry and exiting from the second mitotic wave in the Drosophila developing eye. *BMC Dev. Biol.* **8**, 7.
Thompson, B. J., Buonamici, S., Sulis, M. L., Palomero, T., Vilimas, T., Basso, G., Ferrando, A. and Aifantis, I. (2007). The SCFFBW7 ubiquitin ligase complex as a tumor suppressor in T cell leukemia. *J. Exp. Med.* **204**, 1825-1835.
Tsuda, L., Nagaraj, R., Zipursky, S. L. and Banerjee, U. (2002). An EGFR/Ebi/Sno pathway promotes Delta expression by inactivating Su(H)/SMRTER repression during inductive Notch signaling. *Cell* **110**, 625-637.
Tsunematsu, R., Nakayama, K., Oike, Y., Nishiyama, M., Ishida, N., Hatakeyama, S., Bessho, Y., Kageyama, R., Suda, T. and Nakayama, K. I. (2004). Mouse Fbw7/Sel-10/Cdc4 is required for notch degradation during vascular development. *J. Biol. Chem.* **279**, 9417-9423.
Vrailas, A. D. and Moses, K. (2006). Smoothened, thickveins and the genetic control of cell cycle and cell fate in the developing Drosophila eye. *Mech. Dev.* **123**, 151-165.
Welcker, M. and Clurman, B. E. (2008). FBW7 ubiquitin ligase: a tumour suppressor at the crossroads of cell division, growth and differentiation. *Nat. Rev. Cancer* **8**, 83-93.
Welcker, M., Singer, J., Loeb, K. R., Grim, J., Bloecher, A., Gurien-West, M., Clurman, B. E. and Roberts, J. M. (2003). Multisite phosphorylation by Cdk2 and GSK3 controls cyclin E degradation. *Mol. Cell* **12**, 381-392.
Welcker, M., Orian, A., Grim, J. A., Eisenman, R. N. and Clurman, B. E. (2004a). A nucleolar isoform of the Fbw7 ubiquitin ligase regulates c-Myc and cell size. *Curr. Biol.* **14**, 1852-1857.
Welcker, M., Orian, A., Jin, J., Grim, J. A., Harper, J. W., Eisenman, R. N. and Clurman, B. E. (2004b). The Fbw7 tumor suppressor regulates glycogen synthase kinase 3 phosphorylation-dependent c-Myc protein degradation. *Proc. Natl. Acad. Sci. USA* **101**, 9085-9090.
Wolff, T. and Ready, D. F. (1991). The beginning of pattern formation in the Drosophila compound eye: the morphogenetic furrow and the second mitotic wave. *Development* **113**, 841-850.
Yan, S. J., Gu, Y., Li, W. X. and Fleming, R. J. (2004). Multiple signaling pathways and a selector protein sequentially regulate Drosophila wing development. *Development* **131**, 285-298.
Ye, X., Nalepa, G., Welcker, M., Kessler, B. M., Spooner, E., Qin, J., Elledge, S. J., Clurman, B. E. and Harper, J. W. (2004). Recognition of phosphodegron motifs in human cyclin E by the SCF(Fbw7) ubiquitin ligase. *J. Biol. Chem.* **279**, 50110-50119.
Zhang, W. and Koepp, D. M. (2006). Fbw7 isoform interaction contributes to cyclin E proteolysis. *Mol. Cancer Res.* **4**, 935-943.