The Drosophila ubiquitin-specific protease Puffyeye regulates dMyc-mediated growth

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ABSTRACT
The essential and highly conserved role of Myc in organismal growth and development is dependent on the control of Myc protein abundance. It is now well established that Myc levels are in part regulated by ubiquitin-dependent posttranslational degradation. Using a genetic screen for modifiers of Drosophila Myc (dMyc)-induced growth, we identified and characterized a ubiquitin-specific protease (USP), Puffyeye (Puf), as a novel regulator of dMyc levels and function in vivo. We show that puf genetically and physically interacts with dMyc and the ubiquitin ligase archipelago (ago) to modulate a dMyc-dependent cell growth phenotype, and that varying Puf levels in both the eye and wing phenocopies the effects of altered dMyc abundance. Puf containing point mutations within its USP enzymatic domain failed to alter dMyc levels and displayed no detectable phenotype, indicating the importance of deubiquitylating activity for Puf function. We find that dMyc induces Ago, indicating that dMyc triggers a negative-feedback pathway that is modulated by Puf. In addition to its effects on dMyc, Puf regulates both Ago and its cell cycle substrate Cyclin E. Therefore, Puf influences cell growth by controlling the stability of key regulatory proteins.

KEY WORDS: Drosophila, Myc, Deubiquitinase, Growth, Protein degradation

INTRODUCTION
The mammalian Myc gene family, comprising Myc, Mycn and Mycl, is known to be crucial for growth and development (Grandori et al., 2000). Myc proteins control multiple cellular processes, including cell growth, proliferation, metabolism and apoptosis, and deregulation of Myc plays an important role in oncogenesis (Dang, 2012). Non-mammalian Myc has been most intensively studied in Drosophila melanogaster where the absolute requirement for Drosophila Myc (dMyc) (dm – FlyBase) function during development has been demonstrated by the fact that dMyc-null mutants die at an early larval stage (Gallant, 2006; Pierce, 2004). dMyc is necessary and sufficient to regulate organismal growth through cell-autonomous control of cell size (Bellota and Gallant, 2010; Gallant, 2009; Gallant et al., 1996; Johnston et al., 1999; Maines et al., 2004; Prober and Edgar, 2001).

Myc transcript and protein abundance are subject to regulation at multiple levels ranging from transcriptional control by numerous mitogenic signaling pathways to extensive post-translational modifications (Hann, 2006; Liu and Levens, 2006; Thomas and Tansey, 2011; Vervoorts et al., 2006). Of particular interest, given the relatively short half-life of Myc proteins, is the post-translational modification of Myc by the ubiquitin system (Müller and Eilers, 2009; Thomas and Tansey, 2011). Protein ubiquitylation is a fundamental and versatile post-translational modification that controls multiple cellular events by marking proteins as substrates for either degradation or non-degradative processing (Bhat and Greer, 2011). In mammals, distinct ubiquitin E3 ligase complexes, including Skp2 and Fbw7, have been reported to influence Myc protein stability and activity (Müller and Eilers, 2009; Thomas and Tansey, 2011).

The Drosophila ortholog of Fbw7, Archipelago (Ago) is the only ligase identified thus far as involved in proteasome-mediated ubiquitin-dependent turnover of dMyc proteins (Moberg et al., 2004). Ago mutant alleles were first identified in a genetic screen for regulators of tissue growth in the eye, where it was initially shown to bind and regulate Cyclin E (CycE) levels (Moberg et al., 2001). Later work demonstrated that Ago also physically interacts with dMyc, and controls dMyc stability and biological function (Moberg et al., 2004). Unlike c-Myc, which was shown to have a single Myc Box1 phosphodegragon associated with Fbw7 binding, several domains containing putative Ago-interacting motifs were shown in dMyc to mediate Casein kinase 1 (CK1)α-, CK1ε- and GSK3β-dependent protein degradation. Although their link to Ago function has not been precisely established, it is clear that GSK3β plays a key role in Ago-mediated dMyc ubiquitylation and degradation (Galletti et al., 2009; Moberg et al., 2004; Parisi et al., 2011).

Protein ubiquitylation is a reversible process in which removal of ubiquitin chains is mediated by deubiquitylating enzymes (DUBs), and the role of DUBs in controlling various cellular processes has attracted considerable interest (Clague et al., 2012; Reyes-Turcu et al., 2009). DUBs are classified into five subfamilies based on their deubiquitylating domain. Ubiquitin-specific proteases (USPs), which constitute the largest DUB subfamily, share a structurally conserved USP domain of ~350 to 450 amino acids. The USP domain is the catalytic core that mediates the cleavage of ubiquitin conjugates, whereas domains required for protein-protein interaction and substrate specificity are located within N and/or C termini of the USP protein (Komander et al., 2009; Ventii and Wilkinson, 2008). Although several ubiquitin E3 ligases have been implicated in modulating c-Myc stability, only one deubiquitylating enzyme, USP28, has been demonstrated to catalyze the deubiquitylation of Myc in mammals (Popov et al., 2007a). Thus far, no deubiquitylating enzyme has been identified that modulates dMyc function or antagonizes Ago-mediated dMyc degradation. Of the 41 predicted Drosophila DUBs, 21 are predicted to have a mammalian USP ortholog (Tsou et al., 2012). Interestingly, Drosophila does not encode an USP28 ortholog, suggesting that a distinct USP may be responsible for reversing dMyc ubiquitylation in Drosophila. Here, we report the identification and characterization of Puffyeye (Puf), a Drosophila USP that antagonizes Ago function and interacts...
genetically and physically with dMyc. We present evidence that Puf regulates dMyc activity at the level of cell and organ growth.

RESULTS
Identification of puffyeye (puf, CG5794) as a novel regulator of dMyc function
Overexpression of \textit{dmyc} in the developing eye using three copies of \textit{UAS-dmyc} under the control of GMR-Gal4 (denoted GMM) results in a rough eye phenotype, i.e. the adult eyes display disorganized ommatidia and are larger than wild-type eyes (Fig. 1C-D'). (Secombe et al., 2007). Previously, we described a screen to identify genes that modify the GMM-dependent eye phenotype, which led to the discovery of the histone demethylase \textit{Little imaginal discs} (\textit{Lid}) (Secombe et al., 2007). This screen also revealed other deficiency strains that suppress the GMM phenotype, one of which is \textit{Df(3R)ED622}. Further analysis mapped the region to cytological band 96A13, which deletes about eight genes. Among them is \textit{absent small or homeotic discs 2} (\textit{ash2}), a trithorax-group gene that...

Fig. 1. \textit{puffyeye} (CG5794) is a novel regulator of the dMyc-dependent rough eye phenotype. (A) The \textit{puf} locus. Two of the five predicted transcript isoforms (\textit{puf-RD} and -\textit{RE}) are indicated (FlyBase R5.48). Coding exons are indicated as black boxes. The \textit{puf}\texttt{RD} insertion is situated downstream of \textit{puf-RE}. The insertion sites of \textit{EP(3)3472}, \textit{EY03971} and two RNAi targeting regions are indicated. (B) Schematic of PufWT, PufWT-S, PufCA/HA and USP34 proteins. Dark-grey box indicates the USP catalytic domain. Overall percentage of amino acid sequence identity and the USP domain between Puf and human USP34 are shown. Light-grey bars indicate regions used for antisera production. Black dots indicate the wild-type conserved cysteine and histidine boxes, and the white dots indicate the mutated catalytic sites. (C-N') Scanning electron micrographs (SEMs) of the lateral view of adult compound eyes. (C-J') SEM images show that Puf modifies GMM eye phenotype. (K-N') SEM images show that Puf and Ago genetically interact with each other. \textit{ago}\texttt{1} and \textit{ago}\texttt{4} represent different mutant alleles. Original SEM magnification: 160× for C-N; 750× for C'-N'.
had previously been linked to dmyc function (Secombe et al., 2007). As ash2 mutants suppressed the GMM phenotype, we examined whether increased ash2 expression could enhance the phenotype. We therefore induced the P-element insertion strains EP(3)3472 and EY03971 (Bellen et al., 2004; Roth et al., 1998), both of which contain insertions within the ash2 locus (Fig. 1A) and have the potential to induce expression of neighboring genes, including ash2. Interestingly, we observed enhancement of the GMM rough eye phenotype by both EP(3)3472 and EY03971 strains (Fig. 1F-G). The enhanced GMM phenotype was similar to the phenotype caused by increased dMyc levels when another copy of UAS-dmyc was added (Fig. 1E,E'). To ascertain whether this effect was due to ash2 expression, we generated a UAS-ash2 transgene. However, overexpression of UAS-ash2 had no impact on the GMM phenotype (data not shown). EP(3)3472 and EY03971 therefore enhance the GMM phenotype by inducing the expression of a gene other than ash2.

To identify gene(s) induced by EP(3)3472 or EY03971, we performed in situ hybridization using antisense RNA probes against seven genes flanking these two P-element insertions. When EP(3)3472 or EY03971 were crossed to apterous-Gal4 (apGal4), which drives expression in the dorsal compartment of the wing disc, we found that only CG5794 expression was induced (supplementary material Fig. S1A-F; data not shown). As a positive control we showed that ash2 was appropriately expressed when apGal4 was crossed to UAS-ash2 (supplementary material Fig. S1G,H). We conclude that activating EP(3)3472 and EY03971 induced expression of CG5794, but not of ash2. CG5794 is an uncharacterized gene on the 3rd chromosome adjacent to ash2, but is transcribed in the opposite direction (Fig. 1A). CG5794 was deleted in the deficiency strains that mapped to the cytological band 96A13, suggesting that reducing this gene contributes to the suppression of the GMM phenotype originally observed in our screen. We renamed CG5794 puffyeye (puf) to reflect its role in the dMyc-induced rough eye phenotype. Sequence comparison indicates that puf encodes a protein containing an ubiquitin C-terminal hydrolase 2 (UCH) domain (Fig. 1B). Several isoforms of puf are predicted by FlyBase, including a long (3912 amino acids, PufWT) and a short (2832 amino acids, PufWT-S) protein isoform (see Fig. 1A). Based on the sequence of the de-ubiquitylating domain, Puf belongs to the ubiquitin-specific protease (USP) subfamily, and is orthologous to human USP34 (Komander et al., 2009).

**puffyeye is an essential gene that modifies the dMyc dependent rough eye phenotype**

Using an existing puf mutant allele, puf4659, which is generated by a piggyBac transposon insertion (Bellen et al., 2004), we found puf to be essential, with homozygous mutants dying throughout larval and pupal development. A few homozygous mutants survive to adulthood; however, they are short lived and exhibit male sterility. To verify that the lethality and adult phenotypes are caused by disruption of puf expression, we excised the piggyBac transposon. Two independent precise excision revertant alleles were recovered that were both viable and fertile with normal life spans, confirming that disruption of puf by the piggyBac transposon is responsible for the lethality and mutant phenotypes. Transcripts of both isoforms were detected by qRT-PCR in puf4659 animals, indicating that the piggyBac transposon affects translation but not transcription of puf (supplementary material Fig. S1I). However, puf4659 is unlikely to be a null mutation because the piggyBac is inserted in a C-terminal exon contained in the long (PufWT) isoforms and therefore will affect expression only in the long but not the short (PufWT-S) isoform (Fig. 1A). In addition, we obtained two RNA interference strains (RNAi, puf-RNAiGD and puf-RNAiPK) that targeted all isoforms of puf, with no predicted off-target effects (Fig. 1A; Dickson et al., 2007). Ubiquitous knockdown with puf-RNAiPK using actin-Gal4 resulted in pupal lethality with no escapers when compared with the puf4659 mutant allele. Together, these data indicate that the puf4659 mutant allele is likely to be a strong hypomorph and the RNAi strains are effective in knocking down puf expression.

To test whether reduced puf levels influence the GMM phenotype in a similar manner to our original deficiency line, we crossed the GMM strain to the puf4659 mutant allele or the puf-RNAi strains, and found that all of these strains suppressed the GMM rough eye phenotype. The extent of GMM suppression was similar to that obtained with dmyc-RNAi (Fig. 1H-J'). To determine whether the genetic interaction between puf and dMyc is specific to Puf, rather than to a general decrease in USP function, we examined the effect on the GMM phenotype of RNAi strains against four other genes predicted to encode USP proteins (out of a total of 21 predicted USP genes): CG4165 (USP45), CG8494 (USP20), CG12082 (USP5) and CG30421 (USP43). None of these affect the GMM phenotype (data not shown), indicating that the genetic interaction between puf and dmyc is specific. To determine whether the puf mutation affected growth phenotypes independent of dmyc, we examined the effect of puf on the rough eye phenotype caused by GMR-Gal4 induced overexpression of UAS-cyclinD and UAS-Cdk4 (designated as GDK) (Datar et al., 2000). Varying puf levels did not modify the GDK rough eye phenotype (supplementary material Fig. S2A-E).

Taken together, our data indicate that puf specifically modifies dMyc dependent growth in the eye.

**Antagonism between puf and ago**

Because puf is predicted to encode a deubiquitylating enzyme, and was identified as a regulator of dMyc-mediated cell growth, we asked whether puf antagonizes the dMyc ubiquitin ligase archipelago (ago). Consistent with its role in regulating dMyc levels, flies with null mutations of ago have elevated levels of dMyc due to stabilization of the dMyc protein (Moberg et al., 2004). Although ago heterozygotes alone did not display any eye phenotype, they strongly enhanced the GMM rough eye phenotype (Fig. 1K,K'). Enhancement of the GMM phenotype caused by reducing ago gene dose was further augmented when Puf expression was increased using the P-element insertion strains EP(3)3472 or EY03971 (Fig. 1L,L'). Conversely, this phenotype was mildly suppressed by reduced Puf levels (Fig. 1M,M'). These findings demonstrate that puf and ago act antagonistically to regulate dMyc function in the eye.

**Puf promotes growth in the eye**

Based on its predicted ability to regulate dMyc stability, we examined in greater detail the ability of Puf to promote cell growth. Overexpressing Puf in the eye using EP(3)3472 or EY03971 caused a mild rough eye phenotype with increased ommatidial size when compared with the control, an effect similar to that caused by dMyc overexpression (Fig. 2A-C'). This rough eye phenotype was suppressed when puf levels were reduced by loss of one puf allele or RNAi knockdown (Fig. 2D-E'), consistent with the ability of EP(3)3472 or EY03971 to induce puf expression. Importantly, the Puf eye phenotype was modified by altering dMyc levels (Fig. 2F-G'), suggesting that misregulation of dMyc is an essential contributor to the Puf-induced phenotype. We also noted that Puf overexpression (resulting from GMR driven expression of EY03971) in the context of the ago heterozygous mutant produced a very
strong rough eye phenotype even in the absence of dMyc overexpression (Fig. 1N), consistent with the notion that Ago antagonizes Puf function. This may be due to an effect of Puf on endogenous dMyc, as well as on other proteins important for eye development, such as CycE (Secombe et al., 1998) (see Discussion). To analyze the role of Puf in greater detail, we generated UAS-puf transgenic flies and antisera against either the N-terminal (anti-PufN) or the C-terminal region (anti-PufC) of Puf. To date, five mRNA isoforms of puf have been described, all of which are predicted to contain identical N-terminal regions and the conserved core deubiquitylating domain of ~400 amino acids, but differ in their C-terminal regions (Fig. 1A,B) (Tweedie et al., 2009). Protein domains outside of the core deubiquitylating domain may be important for substrate recognition and protein-protein interaction, suggesting the possibility that the long and short isoforms of Puf might possess different activities. We therefore generated UAS-puf transgenic flies for both isoforms. Transgenic flies containing the wild-type Puf-RD long-isoform cDNA encoding a protein of 3912 amino acids are designated as UAS-pufWT, whereas flies containing the Puf-RE short-isoform cDNA encoding a 2832 amino acid protein are designated as UAS-pufWT-S (Fig. 1B). To determine the functional relevance of its enzymatic activity, we carried out site directed mutagenesis to mutate conserved critical residues within the UCH catalytic domain (Komander et al., 2009).
transgenic flies containing either (1) a single mutation in the conserved cysteine box by replacing the catalytic cysteine (Cys2024) with alanine, designated UAS-pufC2024A, or (2) triple mutations in which the cysteine box mutation was combined with alanine substitutions of histidine (His2974) and asparagine (Asp2302) within the histidine box, designated UAS-pufC2024A,H2974A,N2302A (Fig. 1B). The transgenic flies containing cDNA of triple mutations for the short isoform are designated UAS-pufC2024A,H2974A,N2302A.

As expected, GMR-Gal4 > UAS-pufC2024A,H2974A,N2302A retained the ability to mimic the GMM eye phenotype (Fig. 2P,Q; supplementary material Fig. S2H). Expression of Puf-containing catalytic domain mutations did not drastically alter the rough eye phenotype caused by wild-type Puf (Fig. 2M,N), suggesting that Puf affects targets in addition to dMyc. A similar phenotype was observed when the Puf isoform UAS-pufWT was overexpressed (supplementary material Fig. S2I). Adult eyes overexpressing Puf using UAS-pufWT displayed marked disorganization of the ommatidia compared with control (Fig. 2L). This phenotype was strongly suppressed following RNAi knockdown of Puf, further confirming that the puf encoding transgene is responsible for the rough eye effect (Fig. 2M,N).

In contrast to our findings with wild-type Puf, overexpression of enzymatic mutant pufCA, pufC2024A,H2974A,N2302A or the short isoform pufC2024A,H2974A,N2302A failed to elicit any detectable eye phenotype (Fig. 2K,L; supplementary material Fig. S2G), despite being expressed at similar levels (supplementary material Fig. S4A), indicating that the rough eye phenotype caused by wild-type Puf depends on its deubiquitylating activity. Our data also show that a single mutation in the cysteine box of the catalytic domain is as efficient in inactivating Puf activity as triple mutations in both the cysteine and histidine boxes. Furthermore, we demonstrate that expression of pufC2024A,H2974A,N2302A strongly enhances the GMM eye phenotype by causing massive cell death, as indicated by the extreme disorganization of ommatidia and reduction in overall size of the eye (Fig. 2O; supplementary material Fig. S2H). As expected, expression of Puf-containing catalytic domain mutations did not modify the GMM eye phenotype (Fig. 2P,Q; supplementary material Fig. S2I).

**Puf is necessary for wing growth**

Because different tissues have been reported to exhibit differences in growth regulation (Herranz and Milán, 2008), we next examined wing development to determine whether the strong interaction between puf and dmyc can be detected in organs other than the eye. We used the BxAK-Gal4 driver to overexpress UAS-dmyc in the dorsal part of the larval wing disc that gives rise to the upper layer of adult wing bilayer. As shown in Fig. 3A,B, this caused the adult wing to curve downwards when compared with the straight wing of the control. Such a downward curving wing is indicative of increased cell size in the dorsal layer relative to the ventral layer, as previously observed for other genes known to promote cell growth (Montagne et al., 1999). Similarly, BxAK-Gal4 driven puf overexpression through either EP(3)3472 or EY03971 displayed the same downward curved wing phenotype, suggesting that puf overexpression also increased cell size (Fig. 3C,D). Furthermore increased Puf expression enhanced the downward wing curvature caused by Dmyc overexpression (Fig. 3E,F), consistent with the genetic interaction we observed between puf and dmyc in the eye (Fig. 2). Conversely, when levels of either Dmyc or Puf were reduced by RNAi knockdown, the wing exhibited upward curvature, indicating that cells on the dorsal side are now smaller relative to their ventral counterparts (Fig. 3G,H).

The expression of high levels of either wild-type Puf isoform caused a much stronger wing phenotype, whereas no wing phenotype was detected following expression of the catalytic PufC2024A,H2974A,N2302A and PufCA/HA-S (supplementary material Fig. S3A-F). The severe wing phenotype observed with the wild-type isoforms resulted from extensive cell death, as evidenced by increased cleaved caspase 3 staining (supplementary material Fig. S3G). The increased cell death phenotype was partially suppressed when UAS-pufWT was co-expressed with UAS-Diap, an inhibitor of apoptosis (data not shown) (Orme and Meier, 2009; Steller, 2008), suggesting that Puf also plays a role in the apoptotic pathway. As reported in previous studies, we also observed that DMyC induced apoptosis in a dose-dependent manner in the wing discs. However Puf overexpression has a stronger effect in inducing apoptosis than dMyc. For example, when Puf was overexpressed using BxAK-Gal4 and engrailed-Gal4, it caused cell death, whereas when DMyC was induced with the same Gal4 drivers, no apoptosis was observed. This suggests that apoptosis due to Puf is largely independent of DMyC, a notion that is consistent with previous studies indicating that Myc-induced apoptosis is highly context dependent (Montero et al., 2008).

To further explore interactions between Dmyc, Puf and Ago, we used engrailed-Gal4 (enGal4) to drive their expression in the posterior compartment of the wing disc. Dmyc overexpression, or reduction of ago activity, using an ago RNAi knockdown transgene, significantly increased the area of the posterior compartment, as well as the ratio between the posterior and anterior areas, owing to enlarged cell size (Fig. 3I-K,Q; supplementary material Fig. S3K-M) (Flockhart et al., 2006; Johnston et al., 1999; Moberg et al., 2004). The effect of Dmyc overexpression was mitigated by increasing Ago levels (Fig. 3L,Q). Conversely, reducing Dmyc or Puf levels via RNAi knockdown caused reduction of the posterior region (Fig. 3M-O,Q). This was due to decreased cell size and not to apoptosis, as indicated by the increased density of bristles and an absence of cleaved caspase 3 staining (supplementary material Fig. S3N-O; data not shown). puf RNAi knockdown partially suppressed the size increase caused by Dmyc overexpression in the posterior compartment (Fig. 3P,Q; supplementary material Fig. S3P). Interestingly, this combination caused a mild wing vein phenotype, which was also observed after puf and ago RNAi knockdown (Fig. 3K,N,P), suggesting the existence of other Puf and Ago substrates in the wing because no vein phenotype was observed in dMyc overexpression or knockdown (Fig. 3J,O).

**Post-transcriptional regulation of Dmyc abundance by Puf**

Because Puf is predicted to be a deubiquitylating enzyme, we determined whether Puf influences DMyC protein abundance by altering DMyC stability. We examined Dmyc levels in wing imaginal discs of wild-type control (w1118) or the pufA459 hypomorphic mutant. Western blots of protein lysates from wing disc cells show that endogenous Dmyc protein levels are reduced in pufA459 mutant cells relative to wild-type cells (w1118), whereas Dmyc transcript levels were not significantly changed (Fig. 4A; supplementary material Fig. S11). Moreover, Dmyc levels increased when UAS-pufWT was overexpressed, whereas Dmyc was unaffected by UAS-pufCA/HA-S overexpression (Fig. 4B). Immunostaining of wild-type wing disc cells show that endogenous DMyC is widely expressed, with highest levels in the wing pouch and the notum, as previously reported (Galletti et al., 2009; Wu and Johnston, 2010). Endogenous Puf, by contrast, is ubiquitously expressed at low levels without any evident pattern (Fig. 4C,C'). Widespread overexpression of UAS-DMyC in the wing pouch using the rntGal4 driver does not alter Puf levels or expression pattern, indicating that DMyC does not regulate Puf protein levels (Fig. 4D,D').
However, when UAS-puf\(^{WT}\) is overexpressed using \(rnGal4\), dMyc levels are markedly upregulated in domains overexpressing Puf (Fig. 4E,E'). Similarly, UAS-puf\(^{WT}\) expression driven by dppGal4 resulted in elevated dMyc levels in dpp-expressing domains. Importantly, expression of catalytic mutant puf\(^{CA/HA}\) had no effect on dMyc (Fig. 4F-H'). In western blots, the elevation of dMyc levels by puf\(^{WT}\) is even more apparent when dMyc and Puf WT are co-overexpressed, whereas this effect is not observed when either PufCA or PufCA/HA are co-expressed with dMyc (Fig. 4I,J). Overexpression of wild-type and mutant forms of Puf\(^{WT-S}\), the short isoform of Puf, gave the same results as with full-length Puf (supplementary material Fig. S4A,B). Therefore, the ability of both isoforms of Puf to regulate dMyc levels requires a wild-type catalytic deubiquitylation domain.

The effect of Puf on dMyc levels is not limited to mitotic tissue as Puf also regulates dMyc levels in fatbody, a larval tissue.

To determine whether Puf regulates dMyc directly, we asked whether these proteins are localized to the same subcellular location. In control wing discs, endogenous dMyc protein levels dropped to ~50% of untreated control levels by 30 minutes after cycloheximide addition, and was barely detectable after a 2-hour chase (Fig. 5A,B). This rate of dMyc degradation is unchanged by overexpression of catalytic domain mutant Puf\(^{CA/HA}\) (Fig. 5A,B). By contrast, when Puf WT is overexpressed, dMyc abundance remained at 60% of initial levels following 2 hours of treatment with cycloheximide (Fig. 5A,B). We also performed qRT-PCR to determine whether Puf controls dMyc abundance at the transcriptional level. No change in \(dMyc\) transcript levels was observed in wing discs overexpressing Puf WT (Fig. 5D). Together, these results are consistent with the notion that Puf deubiquitylating activity antagonizes Ago to regulate dMyc protein stability.

**Interactions between dMyc, Puf and Ago proteins**

To determine whether Puf regulates dMyc directly, we asked whether these proteins are localized to the same subcellular location.
compartment in vivo and associate in a protein complex. dMyc functions as a transcription factor and is predominantly localized in the nucleus; however, Puf lacks a conventional nuclear localization signal. We examined Puf localization using anti-PufC in immunostaining experiments. Employing antisera against the nuclear envelope structural protein lamin C (Riemer et al., 1995) to demarcate nuclei, we found that endogenous Puf localizes within the lamin C-marked nuclear regions (Fig. 6A). In addition, we find that both Puf isoforms are nuclear localized, as is dMyc (Fig. 6B; data not shown).

To examine whether Puf and dMyc proteins interact, we performed co-immunoprecipitation experiments using protein lysates from wing discs co-expressing dMyc and PufWT or PufCA/HA. We first used anti-Puf to immunoprecipitate Puf and then carried out immunoblotting of the precipitate with anti-dMyc. The results show that dMyc is present in anti-PufWT immunoprecipitates.
Precipitates with pre-immune serum showed only a low intensity background band. Importantly, the ability of Puf to form a protein complex with dMyc is independent of its deubiquitylating function as PufCA/HA also co-immunoprecipitated with dMyc (Fig. 6C). Although expressed at much lower levels, endogenous dMyc was detected with anti-PufC, but not with pre-immune sera in wing discs expressing either PufWT or PufCA/HA (Fig. 6D). To further verify the interaction between Puf and dMyc, we performed immunoprecipitation using anti-sera against dMyc and immunoblotted with anti-Puf. Both PufWT and PufCA/HA co-immunoprecipitated with dMyc, albeit with somewhat lower efficiency than in anti-Puf immunoprecipitates (supplementary material Fig. S5A). These data suggest that mutations in the Puf catalytic domain do not impair its ability to interact with dMyc.

Previous studies have demonstrated that some deubiquitylating enzymes associate with multiple proteins in addition to their substrates, including their antagonistic E3 ligases (Brooks et al., 2007; Hu et al., 2006; Sheng et al., 2006; Sowa et al., 2009; Ventii and Wilkinson, 2008). We therefore asked whether Puf co-immunoprecipitates with Ago. Because endogenous levels of Ago are extremely low, we used wing discs co-overexpressing Ago with PufCA/HA co-immunoprecipitated with dMyc, albeit with somewhat lower efficiency than in anti-Puf immunoprecipitates (supplementary material Fig. S5A). These data suggest that mutations in the Puf catalytic domain do not impair its ability to interact with dMyc.

### Puf regulates Ago and Cyclin E

Several ubiquitin ligases have been shown to be regulated post-translationally by ubiquitylation and deubiquitylation (de Bie and Ciechanover, 2011). Recently, Fbw7 was reported to be auto-ubiquitylated in a phosphorylation- and substrate-dependent manner (Min et al., 2012). To examine whether Puf affects Ago levels, we carried out immunostaining experiments demonstrating that endogenous Ago is expressed throughout the larval wing disc (Fig. 7A). Overexpressing either isoform of wild-type Puf (PufWT or PufWT-S) in either the dorsal or the anterior compartment of the wing disc using a temperature-inducible ap-Gal4,tub-Gal80ts or ci-Gal4,tub-Gal80ts driver results in higher levels of Ago (Fig. 7B; supplementary material Fig. S6B; Fig. S7B), whereas overexpressing the catalytically inactive Puf (PufCA/HA or PufCA/HA-S) does not affect Ago (Fig. 7C; supplementary material Fig. S6C; Fig. S7C). This result was confirmed by an immunoblot from late 3rd instar larval wing discs overexpressing Ago, showing overexpressed PufWT, but are both reduced when Ago is knocked down by ago RNAi, and increased by PufWT overexpression (Fig. 6F).

#### Fig. 5. Post-translational regulation of dMyc abundance by Puf.

(A) Endogenous dMyc levels at increasing times following treatment with cycloheximide (CHX chase) to determine the stability of dMyc in control wing disc cells (lanes 1-4) or in cells overexpressing PufWT (lanes 5-8) or PufCA/HA (lanes 9-11). Protein lysates were isolated from 12 wing discs at the indicated time points after CHX treatment. (B) The quantification of dMyc levels after the addition of CHX. The amount of dMyc remaining was obtained by normalizing signals generated by mouse monoclonal dMyc antibody to that of γ-tubulin using ImageJ software. Time zero was set at 100%. The data are plotted as percentages of dMyc remaining over time zero for each point. (C) Stability of overexpressed dMyc measured in CHX-chase experiments. Protein lysates from wing discs overexpressing dMyc (lanes 1-4), dMyc+ PufWT (lanes 5-8) or dMyc+PufCA/HA (lanes 9-11). (D) qRT-PCR determination of relative levels of dMyc mRNA following overexpression of Puf (tub-Gal4, UAS-pufWT). Relative expression levels (ΔΔCT) were calculated using RpS16 as internal control. Data represent mean of three biological samples analyzed in duplicate. Error bars indicate s.e.m. Transgenes were induced for 20 hours using temperature-inducible Gal4 drivers.
not the catalytic mutant PufCA, increased Ago levels (Fig. 6F). Furthermore, downregulation of Puf using an RNAi transgene mildly reduces endogenous Ago levels (Fig. 7D; supplementary material Fig. S7D). The physical interaction between Puf and Ago, and the requirement for Puf and its functional deubiquitylase domain in the regulation of Ago expression suggests that Ago is likely to be a substrate of Puf. Conversely, although RNAi knockdown of Ago in the dorsal wing disc compartment resulted in elevated levels of the known Ago substrate dMyc, it did not elevate endogenous Puf levels, suggesting Puf itself is not an Ago substrate (supplementary material Fig. S6F,K,L; Fig. S7K,L).

Because Ago in Drosophila and Fbw7 in mammalian cells have been shown to promote protein turnover of CycE (Koepp et al., 2001; Moberg et al., 2001), we asked whether Puf is also involved in CycE regulation. Western blots of protein lysates from wing discs overexpressing wild-type Puf showed markedly increased levels of overexpressed CycE, whereas overexpression of the deubiquitylation domain mutant PufCA had no effect on CycE abundance (Fig. 7H). Furthermore, cycloheximide chase experiments using wing discs from late 3rd instar larvae show that high levels of CycE are maintained in wing discs overexpressing CycE and PufWT after 6 hours of cycloheximide treatment. By contrast, CycE levels were strongly reduced in wing discs overexpressing CycE alone or co-overexpressed with mutated PufCA (Fig. 7I), indicating that Puf regulates CycE levels by increasing its stability dependent on a wild-type deubiquitylation domain. qRT-PCR confirms that Puf has no significant effect on the transcript levels of ago or cycE (supplementary material Fig. S6M).

Interestingly, Ago levels are also elevated when dMyc is overexpressed, and reduced when dMyc was knocked down (Fig. 7E,F; supplementary material Fig. S7E,H). Importantly, the upregulation of Ago by overexpressed Puf is not solely a consequence of dMyc upregulation as PufWT overexpression induces Ago when dMyc is simultaneously knocked down (Fig. 7G; supplementary material Fig. S6D,I,J; Fig. S7G-J). Because we failed to observe a significant increase in ago RNA expression upon dMyc overexpression (supplementary material Fig. S6N), we surmise that the induction of Ago by dMyc is indirect, possibly as a result of the effect of dMyc on cell growth and mitochondrial function (Mandal et al., 2010). Taken together, our data suggest that negative autoregulation of dMyc can be mediated through dMyc induction of Ago, and Puf plays an important role in modulating dMyc, CycE and Ago functions by regulating their protein levels (summarized in Fig. 7J).

**DISCUSSION**

Ubiquitin ligases act as key components of the ubiquitin machinery by specifically ubiquitylating protein substrates to mark them for proteasome-mediated destruction (Varshavsky, 2012). Importantly, protein ubiquitylation is reversible through the activity of deubiquitylating enzymes (DUBs), which act to maintain a dynamic balance of ubiquitylated substrates and to control ubiquitin-dependent signaling pathways (Clague et al., 2012; Ventii and Wilkinson, 2008). Systematic RNAi knockdowns in Drosophila demonstrated that most DUBs have non-redundant functions (Tsou et al., 2012). In this report, we describe a novel DUB that regulates Myc and CycE protein stability in Drosophila.
Although a great deal has been learned recently concerning ubiquitin ligases that interact with Myc proteins (Müller and Eilers, 2009), to date only one DUB has been reported that targets Myc (Popov et al., 2007b). Here, we have employed a genetic screen based on the rough eye phenotype induced by dMyc overexpression in the eye (GMM) in Drosophila. This screen led to the identification of a USP-type DUB, which we have named Puffyeye (Puf; CG9754), as a novel regulator of dMyc function in vivo. We found that reduced puf expression suppresses, whereas puf overexpression augments, the GMM phenotype. This phenotype is largely an effect of cell overgrowth (Secombe et al., 2007), yet overgrowth in the eye due to cyclin D/Cdk4 was not influenced by altered Puf abundance. Moreover, knockdown of four other USPs had no effect on the GMM phenotype. This suggests that puf possesses specificity for dMyc-induced growth in the eye. Indeed, puf itself induced a dose-dependent rough eye phenotype, displaying augmented ommatidial size that can be modulated by altering dMyc levels. In the wing disc, dMyc and Puf also were found to collaborate in cell growth. We also found that Puf is essential for normal development, consistent with a crucial role for Puf in cell growth.

dMyc levels markedly increase in cells in which puf is overexpressed, whereas dMyc levels are decreased in Puf hypomorphic mutants. We show that these changes in dMyc are predominantly post-translational. This is consistent with our finding that Puf overexpression results in a dramatic increase in dMyc protein stability. Importantly, all of the biological effects of Puf, as well as its effects on dMyc abundance and turnover, are abolished by point mutations in the highly conserved Puf USP catalytic domain. We surmise that Puf stabilizes Myc through its function as a deubiquitylating enzyme that antagonizes the activity of the Ago ubiquitin ligase, previously shown to target Myc for ubiquitylation and degradation (Moberg et al., 2004). Importantly, increased Puf exacerbates, and decreased Puf suppresses, the effect of Ago heterozygotes in enhancing the GMM phenotype. The notion that Puf and Ago act as antagonists receives further support from our findings that Puf protein physically associates with both dMyc and Ago in vivo. Interactions between DUBs and their antagonistic E3 ligases, as well as their substrates have been reported previously (Popov et al., 2007b; Sowa et al., 2009). The ability of both the Puf short and long isoforms (Fig. 1A) to modify the dMyc-mediated eye phenotype, and stabilize dMyc and Ago proteins in an ubiquitylation
domain-dependent manner suggests that domain(s) required for Puf to interact with dMyc or Ago are located in a region N-terminal to the core catalytic domain.

We have also found that Puf stabilizes CycE, another known Ago substrate, suggesting that Puf antagonizes Ago function in regulating other targets that are crucial for cell cycle control (Nakayama and Nakayama, 2006). Indeed, flies homozygous for puf and ago double mutations do not survive, raising the possibility that, in addition to regulating common substrates, they each possess unique targets, as shown for other ubiquitin ligases and DUBs (Komander et al., 2009). Notch would be another potential candidate for Puf activity (Moberg et al., 2004); however, we have failed to find a significant effect of Puf on Notch levels in wing discs (data not shown).

In mammalian cells, the ubiquitin-specific protease USP28 was demonstrated to regulate the turnover of c-Myc by binding and antagonizing the activity of Fbw7α, the vertebrate ortholog of Ago (Popov et al., 2007b). However, Puf and USP28 are not homologs: they appear to be two very distinct USPs in terms of their overall size and amino acid sequence similarity in both their core enzymatic domains and the protein sequence as a whole. The closest mammalian homolog of Puf is USP34 (3546aa) (Quesada et al., 2004). Puf and USP34 are highly homologous in their core catalytic domains (67% identity; 80% similarity) with the catalytic triad conserved, whereas the overall similarity between the two proteins is ~52% (~37% identity).

Previous studies have shown that multiple signaling pathways regulate Ago and Fbw7 expression and activity (Nicholson et al., 2011). Here, we find that Ago levels are increased upon dMyc, as well as upon Puf overexpression. Although the mechanisms by which dMyc and Puf regulate Ago expression are unclear, dMyc-dependent Ago expression may provide a mechanism for dMyc autoregulation (Goodcliffe et al., 2005), whereas Puf may stabilize Ago by deubiquitylating it. Indeed, Fbw7 has been shown to be regulated through ubiquitylation (Min et al., 2012). A similar type of dynamic relationship has been reported for the ubiquitin ligase Mdm2 and deubiquitylase HAUSP/USP7 in regulating the stability and function of the tumor suppressor p53 (Brooks and Gu, 2011).

Taken together, our data suggest that Ago and Puf represent a regulatory node that controls degradation of Myc and CycE (as summarized in Fig. 7J), and very likely other growth control factors. Further studies will be required to identify additional substrates of Puf and to understand the physiological importance of Puf-mediated regulation of protein degradation in Drosophila.

**MATERIALS AND METHODS**

**Drosophila stocks**

All the fly stocks were maintained at 22-25°C on standard medium unless otherwise specified. Temperature-inducible experiments were carried out by maintaining crosses at 18°C until they were shifted to 29°C for indicated duration.

The transgenic lines UAS-pufWT, UAS-pufα, UAS-pufαiso, UAS-pufβ iso, UAS-pufCA iso, and UAS-ash2 were generated for this study. Fly stocks used were: w1118 (wild type), UAS-dMyc13; ap-Gal4,UAS-GFP; GDK (GMR-Gal4, UAS-CycD UAS-dMycRNAi); ap-Gal4, UAS-cycE (gifts from Dr B. Edgar, University of Heidelberg, Germany); pufα, pufβ iso (gifts from Dr K. H. Moberg). Alexa Fluor-conjugated secondary antibodies (1:1000) were from Molecular Probes. Samples were analyzed on a Nikon Eclipse Ti or a Zeiss LSM510 confocal microscope. Western blotting was carried out as previously described (Secome et al., 2007). Primary antibodies used were: anti-dMyc 1:500, anti-tubulin 1:5000 (Sigma), anti-Puf 1:10,000 and guinea pig anti-Ago 1:10,000 (Mortimer and Moberg, 2007).

**Cloning and generation of transgenic flies**

For long-isoform puf, PCR primers specific for puf-RD was used to amplify puf cDNA. Site-directed mutagenesis was used to generate pufWT and pufα iso clones. All full-length cDNA clones were subcloned into the EcoRI site of the pUAST vector, and verified by sequencing. The short isoform of puf cDNA, (puf-RE) was generated by replacing C-terminal region of the long-isoform puf cDNA with the Stu-Nor1 fragment from cDNA clone AT30546 (BDGP). UAS-ash2 transgenic flies were generated by cloning the full-length ash2 ORF (clone LD31690 from BDGP). All transgenic flies were generated by Bestgene. Primer sequences used for cloning and quantitative validation of gene expression are listed in supplementary material Tables S1 and S2.

**Puf antibody production**

Rabbit polyclonal antisera were generated using peptides composed of amino acids 13-181 (anti-PufN) or peptides composed of amino acids 3667-3912 (anti-PufC). Specificity of both anti-Puf antisera was confirmed on puf mutant, pufα iso and RNAi knockdown strains.

**Antibodies**

The following primary antibodies were used: rabbit anti-Puf (1:2000), mouse anti-dMyc (1:10), rabbit monoclonal anti-cleaved caspase 3 (1:100, Cell Signaling, #9664), mouse anti-lamC (1:50, DSHB), guinea pig anti-Ago (1:2000, a gift from Dr K. H. Moberg). Alexa Fluor-conjugated secondary antibodies (1:1000) were from Molecular Probes. Samples were analyzed on a Nikon Eclipse Ti or a Zeiss LSM510 confocal microscope. Western blotting was carried out as previously described (Secome et al., 2007). Primary antibodies used were: anti-dMyc 1:500, anti-tubulin 1:5000 (Sigma), anti-Puf 1:10,000 and guinea pig anti-Ago 1:10,000 (Mortimer and Moberg, 2007).

**Immunoprecipitation**

Immunoprecipitations were performed by dissecting discs from wandering 3rd instar larvae (75 discs per sample in overexpression larvae, 150 discs per sample in endogenous larvae) and placing them in PBS with Complete protease inhibitor cocktail (Roche). Discs were lysed in 1 volume of RIPA buffer with inhibitors. Lysate was cleared by centrifugation and diluted to 1 ml with PBS plus inhibitors. Protein was preclared for 1 hour with a combination of protein A/G beads (Invitrogen). Protein was then incubated with antibody diluted to 1:10 for 4 hours, and added to 50 μl protein A/G beads overnight. Immunoprecipitations were washed with PBS plus inhibitors five times, resuspended with Laemmli buffer, and run on a precast gradient gel.

**In situ hybridization**

Antisense RNA probes were generated using cDNA clones (ash2 LD31680, puf AT30546, CG31125 SD02419, CG6695 SD06668, wsc2 LD25626, all GH09383 and syn18 LD37002) (BDGP). cDNAs were linearized with appropriate restriction enzymes and purified with Qiagen PCR Purification Kit. Hybridization was carried out as described previously (Kozopas et al., 1998). Anti-DIG antibody (Roche) was used at a concentration of 1:4000.

**Electron microscopy**

Scanning electron microscopy (SEM) and transmission electron microscopy (TEM) were performed on a JEOL 1230 microscope with Drosophila eyes prepared as described (http://sharedresources.fhcrc.org/sites/default/files/EMProceduresManual.pdf).

**Acknowledgements**

We thank Dr Laura Buttitta and members of the Eisenman Lab for comments and suggestions; Dr Bruce A. Edgar and Dr Kenneth H. Moberg for reagents; Vienna Drosophila RNAi Center, Bloomington Drosophila Stock Center and the TRIP at Harvard Medical School (NIH/NIGMS R01-GM084947) for fly stocks; the Developmental Studies Hybridoma Bank for antibodies; and Berkeley Drosophila Genome Project for cDNA clones.

**Competing interests**

The authors declare no competing financial interests.


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