The E1 ubiquitin-activating enzyme Uba1 in *Drosophila* controls apoptosis autonomously and tissue growth non-autonomously

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Ubiquitination is an essential process regulating turnover of proteins for basic cellular processes such as the cell cycle and cell death (apoptosis). Ubiquitination is initiated by ubiquitin-activating enzymes (E1), which activate and transfer ubiquitin to ubiquitin-conjugating enzymes (E2). Conjugation of target proteins with ubiquitin is then mediated by ubiquitin ligases (E3). Ubiquitination has been well characterized using mammalian cell lines and yeast genetics. However, the consequences of partial or complete loss of ubiquitin conjugation in a multi-cellular organism are not well understood. Here, we report the characterization of *Uba1*, the only E1 in *Drosophila*. We found that weak and strong *Uba1* alleles behave genetically differently with sometimes opposing phenotypes. Whereas weak *Uba1* alleles protect cells from cell death, clones of strong *Uba1* alleles are highly apoptotic. Strong *Uba1* alleles cause cell cycle arrest which correlates with failure to reduce cyclin levels. Surprisingly, clones of strong *Uba1* mutants stimulate neighboring wild-type tissue to undergo cell division in a non-autonomous manner giving rise to overgrowth phenotypes of the mosaic fly. We demonstrate that the non-autonomous overgrowth is caused by failure to downregulate Notch signaling in *Uba1* mutant clones. In summary, the phenotypic analysis of *Uba1* demonstrates that impaired ubiquitin conjugation has significant consequences for the organism, and may implicate *Uba1* as a tumor suppressor gene.

KEY WORDS: *Uba1*, E1, Ubiquitin-activating enzyme, Apoptosis, Proliferation, *Drosophila*, Autonomous control, Non autonomous control

INTRODUCTION

Ubiquitination refers to the covalent attachment of the small protein ubiquitin to target proteins. This modification usually targets ubiquitinated proteins for proteolytic degradation by the proteasome (reviewed by Glickman and Ciechanover, 2002; Pickart, 2004; Welchman et al., 2005). In this capacity, ubiquitination is essential for turnover of proteins involved in many cellular processes including the cell cycle, cell death, signal transduction, etc. (Petroski and Deshaies, 2005). For example, ubiquitin-mediated degradation of cyclins is essential for progression through the cell cycle (reviewed by Pines, 2006). Inhibitor of apoptosis proteins (IAPs) need to be ubiquitinated and degraded in cells undergoing apoptosis (Herman-Bachinsky et al., 2007; Holley et al., 2002; Ryoo et al., 2002; Yang et al., 2000; Yoo et al., 2002) (reviewed by Cashio et al., 2005). However, ubiquitin-mediated degradation of caspases, the principal executioners of apoptosis, has been reported to protect cells from apoptosis (Cashio et al., 2005; Chai et al., 2003; Huang et al., 2000; Suzuki et al., 2001; Wilson et al., 2002). In addition, non-traditional functions of ubiquitination, which do not target proteins for proteolysis, have been reported (Chen, 2005; Mukhopadhyay and Riezman, 2007; Welchman et al., 2005). In this regard, it is noteworthy that activated cell surface signaling receptors are ubiquitinated, usually

mono-ubiquitinated, for endocytosis and protein sorting at the early endosome (Gruenberg and Stenmark, 2004; Haglund and Dikic, 2005).

Alterations in the ubiquitination machinery are often associated with human diseases such as cancer, neurodegenerative disorders and inflammation (Devoy et al., 2005; Mukhopadhyay and Riezman, 2007; Reinstein and Ciechanover, 2006). On the other hand, targeting the ubiquitination system for therapeutic purposes holds promise in the clinic (Nalepa et al., 2006). Thus, a detailed understanding of the role of ubiquitination for proper homeostasis and physiology of multi-cellular organisms is critical.

E1 ubiquitin-activating enzymes catalyze the first step in the ubiquitination cycle, the ATP-dependent formation of a thioester bond between the C-terminal glycine residue of ubiquitin and the active site cysteine of the E1 (Haas and Siepmann, 1997; Pickart, 2001). This is followed by the transfer of ubiquitin from the E1 to a ubiquitin-conjugating enzyme (E2). The final step is the conjugation of ubiquitin to target proteins mediated by ubiquitin ligases (E3). The specificity of the ubiquitination process is conferred to by E3 ubiquitin ligases. The genomes of eukaryotic organisms contain hundreds of different E3-encoding genes required for the regulated protein turnover in many cellular processes (Hicke et al., 2005; Petroski and Deshaies, 2005). By contrast, there are considerably fewer E1 and E2 enzymes. For example, the *Drosophila* genome encodes only one E1 enzyme, termed *Uba1* (Watts et al., 2003). This low complexity suggests that the primary function of the E1 enzyme is to provide activated ubiquitin for all ubiquitin-dependent reactions. This has indeed been observed in yeast. Genetic inactivation of the yeast gene *Uba1* blocks most, if not all ubiquitin conjugation (Ghaboosi and Deshaies, 2007; McGrath et al., 1991; Swanson and Hochstrasser, 2000). There are mammalian cell lines containing temperature-

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sensitive alleles of E1. These cell lines have been of great importance for understanding the role of ubiquitin-mediated degradation of cyclins for progression through the cell cycle, and have further suggested an essential function of E1 enzymes to provide activated ubiquitin for conjugation of target proteins (Ciechanover et al., 1984; Ciechanover et al., 1985; Finley et al., 1984; Kulka et al., 1988; Salvat et al., 2000).

However, despite these valuable analyses of ubiquitin conjugation in single cell organisms and cell lines, a systematic analysis of partial or complete loss of ubiquitin conjugation in multi-cellular organisms has not been reported. This can be accomplished by reducing the activity of the only E1 enzyme in *Drosophila*, *Uba1* (Watts et al., 2003). To date, a role of *Drosophila Uba1* (from now on referred to as *Uba1*) has only been reported for axon pruning in the nervous system, and the precise mechanistic function of *Uba1* in this process is unknown (Kuo et al., 2006; Watts et al., 2003). Here, we report the isolation and characterization of weak and strong alleles of *Uba1* in *Drosophila*. We show that, depending on the strength of the *Uba1* allele, different and sometimes opposing phenotypes are observed. For example, weak *Uba1* alleles protect cells from apoptosis, whereas mutant clones of strong alleles are highly apoptotic. Strong *Uba1* alleles which we show affect significantly ubiquitin conjugation, cause cell cycle arrest that correlates with increased cyclin levels. Unexpectedly, clones of strong *Uba1* alleles induce cell proliferation in neighboring tissue, triggering non-autonomous overgrowth. These *Uba1* clones fail to downregulate Notch activity which stimulates Jak/STAT signaling, and thus growth, in neighboring wild-type tissue. In summary, this analysis demonstrates

that the lack of ubiquitin conjugation has significant consequences for the organism, and may implicate *Uba1* as a tumor suppressor gene.

MATERIALS AND METHODS
Isolation of *Uba1* alleles
The *Uba1* alleles were obtained in the *GMR-hid ey-FLP* (*GheF*) suppressor screen for chromosome arm 2R, which is described elsewhere (Srivastava et al., 2007). Mapping was done relative to mapped P element insertions according to published procedures (Zhai et al., 2003). To identify the mutation in the *Uba1* alleles, genomic DNA was amplified by PCR and prepared for DNA sequencing. *Uba1^{s3484}* and *Uba1^{03405}* were obtained from the Bloomington Stock Center.

Fly stocks
Uba1^{D6}, *Uba1^{H33}* and *Uba1^{H42}* (this study); *Uba1^{s3484}* (Watts et al., 2003); *Uba1^{03405}*, *Notch^{264-39}* (obtained from Bloomington Stock Center); *GMR-hid ey-FLP* (*GheF*) (Xu et al., 2005); *GMR-hid[w^-]* (Herz et al., 2006); *UASp-p35* (Werz et al., 2005); *ey-FLP*; *FRT42D P[ubi-GFP]* and *E(spl)m8 2.61-lacZ* (obtained from G. Halder, MD Anderson Cancer Center, Houston, TX); *stat92^{85c9}* (obtained from E. Bach, New York University School of Medicine, NY).

Induction of *Uba1* clones and immunohistochemistry
Uba1 clones were induced by the *FLP/FRT*-mitotic recombination system using either *ey-* or *hs-FLP* (Newsome et al., 2000; Xu and Rubin, 1993). If *hs-FLP* was used, heat-shock was induced in first instar larvae at 37°C for 1 hour in a water bath. Dissection and immunohistochemistry of larval and pupal discs was done using standard protocols (Fan et al., 2005). Antibodies against the following proteins were used: mono and poly-ubiquitylated conjugates
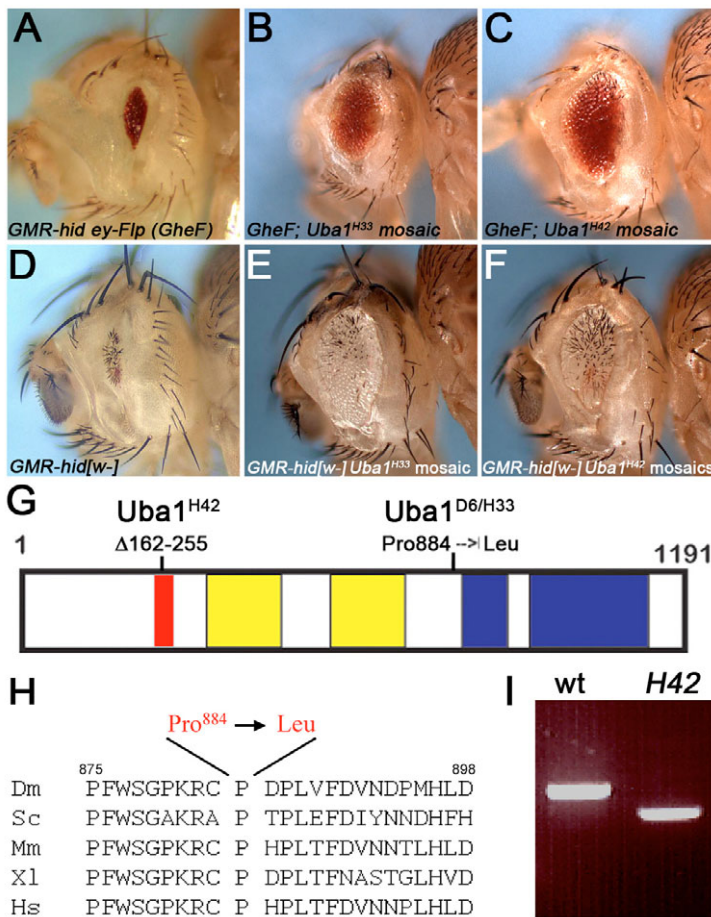

Fig. 1. *Uba1* mutants are autonomous suppressors of *GMR-hid*-induced apoptosis. (**A**) The unmodified small eye phenotype caused by *GMR-hid ey-FLP* (*GheF*). (**B,C**) The *GheF* phenotype is moderately strongly suppressed by *Uba1* alleles at 25°C. Genotype: *GheF*; *FRT42D Uba1^{H33}* and *Uba1^{H42}/FRT42 P[w^+]*, respectively. (**D**) The unmodified small eye phenotype caused by a *GMR-hid* transgene that lacks the *white^+* marker gene (*GMR-hid[w^-]*). Note, the *GMR-hid* transgene in (A) carries the *white^+* minigene. Genotype: *ey-FLP*; *GMR-hid[w^-]*. (**E,F**) The suppression of *GMR-hid[w^-]* is mediated by *Uba1* mutant tissue which is phenotypically *white^-*. Flies were incubated at 25°C. Genotype: *ey-FLP*; *FRT42D Uba1^{H33}* and *Uba1^{H42}/FRT42D P[w^+]*; *GMR-hid[w^-]*, respectively. (**G**) Outline of the Uba1 protein and locations of the mutations of the *Uba1* alleles isolated in this study. The red box indicates the internal deletion of Uba1^{H42}. The yellow boxes mark the Thif domains, and the blue boxes highlight the location of the catalytic Ube domains. (**H**) A highly conserved stretch of residues, incorporating Pro884 is present in a range of species from yeast to humans. Dm, *Drosophila melanogaster*; Sc, *Saccharomyces cerevisiae*; Mm, *Mus musculus*; Xl, *Xenopus laevis*; Hs, *Homo sapiens*. (**I**) RT-PCR of mRNA isolated from wild-type and *Uba1^{H42}* mutant larvae revealing an aberrantly spliced product. This results in an in frame deletion from amino acid residue 162 to 255 of Uba1^{H42} (see G). Primers were directed against the N-terminal third of *Uba1*.

(clone FK2; 1:100; Biomol); ubiquitin (Sigma, 1:10; or Chemicon, 1:500); cleaved caspase 3 (Cas3*; 1:100; Cell Signaling Technology); pSTAT (1:100; Cell Signaling Technology); Diap1 [1:500 (Ryoo et al., 2002)]; Dronc [1:500 (Wilson et al., 2002)]; BrdU (1:50; Becton Dickinson); β-gal (1:250; Promega); Discs large (Dlg) (1:250), cyclin A (1:20), Cyclin B (1:20), Elav (1:60), Notch (clone C17.9C6; 1:20) (all DHSB).

RESULTS

Uba1 alleles were identified as autonomous suppressors of *GMR-hid*

Expression of the pro-apoptotic gene *hid* [also known as *Wrinkled* (*W*) – FlyBase] under control of the eye-specific enhancer *GMR* (*GMR-hid*) causes a small eye phenotype due to excessive apoptosis (Fig. 1A) (Grether et al., 1995). Taking advantage of the *GMR-hid* small eye phenotype we have performed genetic mutagenesis screens aimed at identifying components of the cell death pathway in *Drosophila* (Srivastava et al., 2007; Xu et al., 2005; Xu et al., 2006). In these screens, we induced homozygous mutant clones of mutagenized chromosome arms using *ey-FLP/FRT*-induced mitotic recombination (Newsome et al., 2000; Xu and Rubin, 1993), and screened for suppressors of the small eye phenotype of *GMR-hid* (referred to as *GheF* screens for *GMR-hid ey-FLP*). In the *GheF* screen for chromosome arm 2R, we recovered a lethal complementation group consisting of three alleles (*D6*, *H33*, *H42*) as moderately strong recessive suppressors of *GMR-hid* (Fig. 1B,C).

To molecularly identify the gene, we meiotically mapped the mutant phenotype to 46A1 on the polytene chromosome. One of the genes mapping to this region is *Uba1*, encoding an E1 ubiquitin-activating enzyme. Two existing *Uba1* alleles, *Uba1^{s3484}* and *Uba1^{03405}*, failed to complement the lethality of *D6*, *H33* and *H42*. *Uba1^{s3484}* and *Uba1^{03405}* are caused by P element insertions (www.flybase.org). Precise excision of *Uba1^{s3484}*, which carries the P element insertion in the first intron (Watts et al., 2003), restores viability of *Uba1^{s3484}* itself, and viability in trans to *D6*, *H33* and *H42*. This analysis demonstrates that the lethality of *Uba1^{s3484}* is indeed caused by the P element insertion in *Uba1*, and that the new mutants are allelic to *Uba1*. Sequencing of the *Uba1* gene in these mutants revealed that Pro884 is changed to Leu in *D6* and *H33*. Pro884 is a highly conserved residue in all E1 ubiquitin-activating enzymes ranging from yeast to humans (Fig. 1H). As determined by RT-PCR (Fig. 1I), the third allele, *H42*, contains a new splicing donor site, which results in inappropriate splicing causing an internal in-frame deletion of residues 162-255 (Fig. 1G). Finally, for clonal analysis, we recombined the P allele *Uba1^{s3484}* onto the *FRT42D* chromosome and obtained similar results to the analysis of the new alleles (see below; data not shown). Taken together, these results indicate that the suppressors of *GMR-hid*, *D6*, *H33* and *H42* encode new alleles of *Uba1*. We refer to these mutants as *Uba1^{D6}*, *Uba1^{H33}* and *Uba1^{H42}*.

We have recently shown that mutants obtained in our screens can either suppress *GMR-hid* in an autonomous or non-autonomous manner (Herz et al., 2006; Srivastava et al., 2007). Thus, we tested whether the new *Uba1* alleles suppress *GMR-hid* in an autonomous or non-autonomous manner. A *GMR-hid* transgene that does not carry the *white$^+$* minigene (*GMR-hid*[*w$^-$*]; Fig. 1D) allows determination of the genetic identity of the rescued eye tissue based on red/white pigment selection in a clonal analysis. This analysis showed that the rescued eye tissue in *GMR-hid*[*w$^-$*]; *Uba1* mosaics is genetically *Uba1$^-$*, which is marked by the absence of red eye pigment, i.e. is phenotypically white$^-$ (Fig. 1E,F). Thus, the moderately suppressed *GMR-hid* eye phenotype
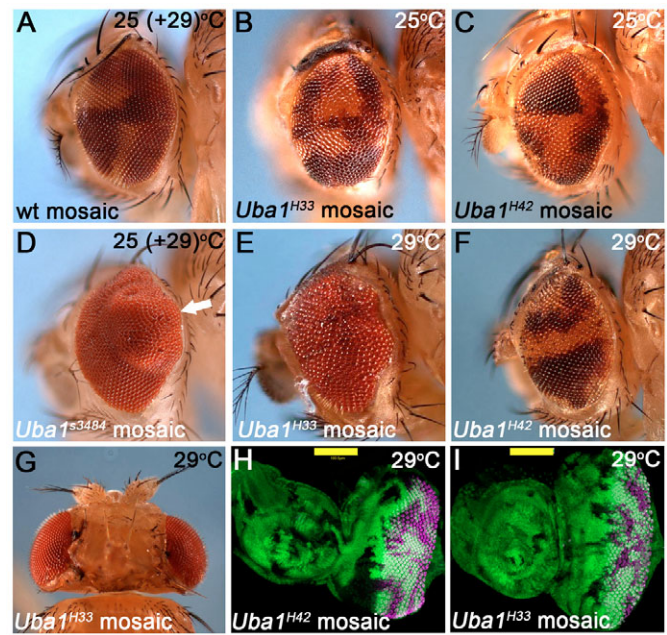

Fig. 2. Strong *Uba1* alleles cause non-autonomous overgrowth. (**A**) Control mosaics: *ey-FLP*-induced clones in wild-type background. This phenotype is identical at 25°C and 29°C (shown is 25°C). Genotype: *ey-FLP*; *FRT42D+/FRT42D* P[*w$^+$*]. (**B,C**) Clones of weak *Uba1* mutants (phenotypically *white$^-$*) develop normally. Genotype: *ey-FLP*; *FRT42D Uba1^{H33}* and *Uba1^{H42}/FRT42D* P[*w$^+$*], respectively. (**D**) Eye mosaics of the strong *Uba1^{s3484}* allele are overgrown with bulges (arrow). Genotype: *ey-FLP*;*FRT42D Uba1^{s3484}/FRT42D* P[*w$^+$*]. This phenotype was obtained at 25°C, and is similar at 29°C. (**E**) Eye mosaic of the ts allele *Uba1^{H33}* at 29°C. *Uba1^{H33}* clones, which would be phenotypically *white$^-$*, are not detectable. The eyes appear rough, irregular and are often enlarged compared to wild-type eyes. Identical results were obtained for *Uba1^{D6}*. Genotype as in B. (**F**) Eye mosaic of the weak *Uba1^{H42}* allele at 29°C. This allele does not behave as a ts allele. Phenotype and genotype are identical to those at 25°C (C). (**G**) Mosaic head of an *Uba1^{D6}* animal raised at 29°C. The left half of the *Uba1^{H33}* mosaic head is larger than the right half, suggesting that the overgrowth phenotype depends on the number and location of the *Uba1* clones. Genotype as in B. (**H,I**) Eye-antennal imaginal discs of wild-type (H) and *Uba1^{D6}* (I) mosaic animals raised at 29°C. These discs were labeled with Elav antibody which labels photoreceptor neurons to identify discs of comparable age. Photoreceptor differentiation does occur in strong *Uba1* clones, but is weakly delayed (see also Fig. S1 in the supplementary material). Genotype as in A and B, respectively. Size bar: 100 μm.

is largely due to *Uba1* mutant tissue, indicating that the suppression of *GMR-hid*-induced apoptosis occurs in an autonomous manner.

Strong *Uba1* alleles cause non-autonomous overgrowth

To further characterize the *Uba1* mutant phenotype, we performed a clonal analysis using *ey-FLP* without *GMR-hid*. When the flies were incubated at 25°C, *ey-FLP*-induced *Uba1* mutant clones survived well and occasionally occupied an even larger area than the wild-type twin-spot (Fig. 2A-C). However, during the course of this work, we noticed that *Uba1^{H33}* and *Uba1^{D6}* are temperature-sensitive (ts) alleles, whereas the third allele, *Uba1^{H42}*, is not. At 29°C, *ey-FLP*-induced clones of *Uba1^{H33}* (Fig. 2E) and *Uba1^{D6}* (not shown) could not be recovered in adult eyes whereas *ey-FLP*-

induced clones of the non-ts allele $Uba1^{H42}$ survived well (Fig. 2F). This suggests that $Uba1^{D6}$ and $Uba1^{H33}$ mutant clones are cell lethal at 29°C. As will be shown below, $Uba1^{D6}$ and $Uba1^{H33}$ clones undergo apoptosis at 29°C. Surprisingly, however, despite the absence of $Uba1^{H33}$ and $Uba1^{D6}$ clones, the eyes were often larger than normal and contained folds, indicative of overgrowth (Fig. 2D,E). The overgrowth phenotype also affected the size of the heads of $Uba1^{D6}$ and $Uba1^{H33}$ mosaics. Strikingly, we recovered several $Uba1^{H33}$ and $Uba1^{D6}$ mosaic animals (Fig. 2G) in which one half of the head did not contain $Uba1$ clones (as judged by the absence of twin-spots) and this half was normal in size, thus serving as an internal control (see right half of the head in Fig. 2G), whereas the other side of the head contained twin-spots and was overgrown (left half in Fig. 2G). Third instar larval eye-antennal imaginal discs of $Uba1^{D6}$ and $Uba1^{H33}$ mosaics reared at 29°C were overgrown compared to similarly staged wild-type controls (Fig. 2H,I). Interestingly, in imaginal discs clones with strong $Uba1$ alleles are present. However, although the entire disc was overgrown, $Uba1^{H33}$ clones are relatively small compared with controls (Fig. 2H,I) and thus do not contribute to the overgrowth. To verify that the overgrowth phenotype is caused by mutations in $Uba1$ itself, and not by an unrelated ts mutation, we tested the independently obtained $Uba1^{s3484}$ allele. In mosaic animals, this allele gives rise to a similar overgrowth phenotype at both 25°C and 29°C, i.e. in a temperature-independent manner (Fig. 2D). $Uba1^{s3484}$ mosaic eye discs are also overgrown in a temperature-independent manner (data not shown). Precise excision of the P element in $Uba1^{s3484}$ reverts the overgrowth phenotype (data not shown), suggesting that this phenotype is caused by genetic inactivation of the $Uba1$ locus.

In summary, because the mutant clones of the ts alleles $Uba1^{D6}$ and $Uba1^{H33}$ do not survive to adulthood at 29°C, yet $Uba1^{D6}$ and $Uba1^{H33}$ mosaics display overgrowth phenotypes, we conclude that these $Uba1$ mutants cause tissue overgrowth in a non-autonomous manner. A similar conclusion can be drawn for $Uba1^{s3484}$. Because $Uba1^{s3484}$ and $Uba1^{H42}$ are non-ts alleles, the temperature-sensitive nature of $Uba1^{D6}$ and $Uba1^{H33}$ is not an intrinsic feature of the process controlled by the $Uba1$ locus. Instead, it is likely that the ts nature of $Uba1^{D6}$ and $Uba1^{H33}$ reflects a transition from weak to strong loss-of-function alleles, since it is common for ts alleles to lose activity at the non-permissive temperature. This will also be confirmed in the next section. Therefore, at low temperature (<25°C) these alleles and the temperature-insensitive allele $Uba1^{H42}$ behave as weak loss-of-function alleles, whereas $Uba1^{s3484}$ and the ts alleles $Uba1^{D6}$ and $Uba1^{H33}$ at >29°C are strong loss-of-function alleles. It is interesting to note that the ts alleles $Uba1^{D6}$ and $Uba1^{H33}$ carry the same point mutation, causing a mutation of Pro884 to Leu (Fig. 1G,H), although both alleles were obtained in independent mutagenesis events. A $Uba1$ allele with exactly the same mutation and the same genetic behavior has recently been described by others (Pfleger et al., 2007). Because $Uba1^{D6}$ and $Uba1^{H33}$ behave genetically identically, we refer to them from now on as $Uba1^{D6/H33}$.

Strong *Uba1* alleles impair general ubiquitin conjugation

E1 ubiquitin-activating enzymes catalyze the first step in the ubiquitination cycle, the ATP-dependent formation of a thioester bond between the C-terminal glycine residue of ubiquitin and the active site cysteine of E1 (Haas and Siepmann, 1997; Pickart, 2001). Thus, E1 enzymes are required to provide most, if not all, activated ubiquitin for conjugation of target proteins. $Uba1$ encodes the only

E1 ubiquitin-activating enzyme in *Drosophila* (Watts et al., 2003). Therefore, genetic inactivation of $Uba1$ should result in reduction of general ubiquitination.

To test this prediction, we analyzed the $Uba1$ mutants for loss of ubiquitin conjugation using the FK2 antibody which specifically recognizes mono- and poly-ubiquitinated proteins, but not unconjugated ubiquitin (Fujimuro et al., 1994; Fujimuro et al., 1997). We tested this prediction first in larval eye imaginal discs of mosaics of the strong P allele $Uba1^{s3484}$. A significant decrease in FK2 immunolabeling was detected in $Uba1^{s3484}$ mutant clones (Fig. 3A). However, due to the apoptotic phenotype of strong $Uba1$ alleles (see below), clones were small and difficult to recover. The ts alleles $Uba1^{D6/H33}$ provide a convenient alternative to obtain large clones for analysis. After induction of $Uba1^{D6/H33}$ clones, these mosaics were incubated at 25°C. The animals were shifted to 29°C 12 hours before dissection and fixation of the discs. When $Uba1^{D6/H33}$
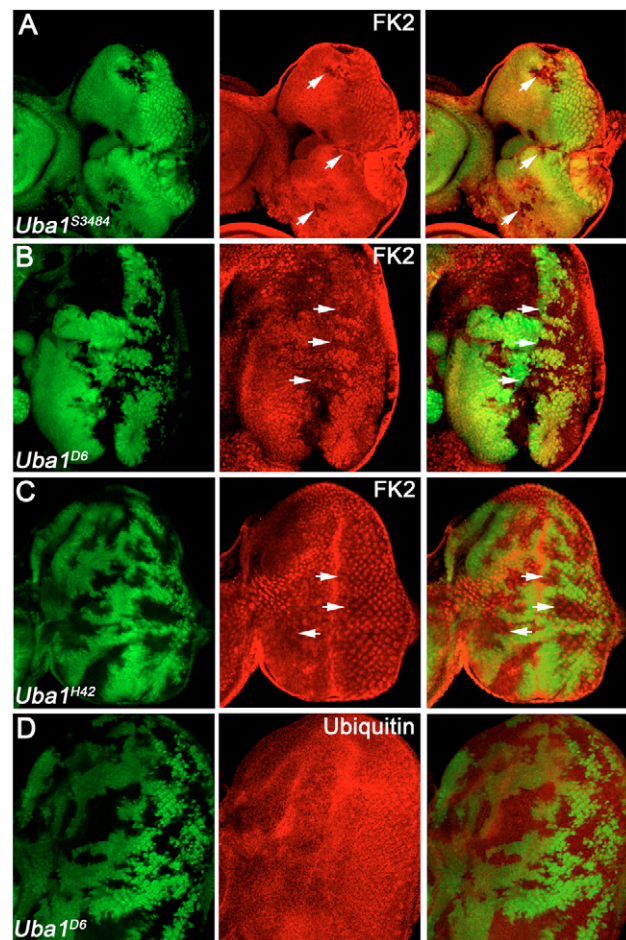

Fig. 3. *Uba1* **mutant clones affect ubiquitin conjugation.** (**A-D**) The left panels indicate the positions of the *Uba1* clones by absence of GFP, the middle panels show the experiment and the right panels are the merged images of left and middle panels. Genotype in all panels: *ey-FLP*; *FRT42D Uba1/FRT42D* P[*ubi-GFP*]. The allele is indicated in the panels. (A-C) *Uba1* mutant discs were labeled with the FK2 antibody which specifically recognizes mono- and poly-ubiquitinated conjugates, but not unconjugated ubiquitin (Fujimuro et al., 1994). The strong alleles $Uba1^{s3484}$ (A) and $Uba1^{D6}$ (B, incubated at 29°C) significantly reduce FK2 immunoreactivity (see arrows). Clones of the weak allele $Uba1^{H42}$ (C) mildly reduce FK2 immunoreactivity (arrows). (D) $Uba1^{D6}$ mosaics incubated at 29°C. The overall protein levels of ubiquitin are unchanged in *Uba1* mutant clones.

mosaics were treated in this way, larger clones were obtained. FK2 labeling was significantly reduced in $Uba1^{D6/H33}$ clones incubated at 29°C (Fig. 3B), suggesting that mono- and poly-ubiquitination was affected in strong $Uba1$ allele clones. Weak alleles also reduced FK2 immunoreactivity; however, this reduction was much more subtle compared to the strong alleles (Fig. 3C).

We considered that the absence of FK2 labeling in strong $Uba1$ clones may be due to an overall reduction of the protein levels of ubiquitin. Thus, as a control we tested the total ubiquitin protein levels. An antibody recognizing both conjugated and unconjugated ubiquitin did not reveal significant alterations of ubiquitin protein in $Uba1^{D6/H33}$ clones compared with wild-type tissue at both 25°C and 29°C (Fig. 3D) suggesting that the loss of $Uba1$ has no effect on total protein levels of ubiquitin. Thus, the lack of FK2 immunolabeling in $Uba1$ clones is due to reduction in ubiquitin conjugation, and not to reduced levels of ubiquitin.

Taken together, these data illustrate that $Uba1$ is required to provide activated ubiquitin for protein conjugation, which is essential for ubiquitin-mediated protein degradation and other ubiquitin-dependent processes. With this knowledge in mind, we analyzed the observed apoptotic and growth phenotypes in more detail.

Weak *Uba1* alleles protect from cell death, whereas clones of strong *Uba1* alleles are apoptotic

We have identified weak $Uba1$ alleles as suppressors of GMR-hid-induced apoptosis (Fig. 1). Therefore, we analyzed the cause of the suppression of GMR-hid by $Uba1$. GMR-hid eye discs are characterized by two zones of apoptotic cell death, as shown by cleaved caspase 3 (Cas3*) labeling (Fig. 4A) (Srivastava et al., 2007). Consistent with the expectation, Cas3* activity induced by GMR-hid is significantly reduced in $Uba1^{D6/H33}$ mutant clones in GMR-hid eye imaginal discs at 25°C (white arrows in Fig. 4B,B′). However, we also noticed that suppression of GMR-hid-induced cell death does not occur in all $Uba1$ clones. In some areas (red arrow in Fig. 4B,B′), Cas3*-positive cell death still occurs in $Uba1$ mutant clones. This region-specific behavior was consistently observed, even at lower temperature (22°C). It appears to be located at the midline, although we have not been able to pinpoint what distinguishes this region from others. Interestingly, this region-specific behavior suggests that there may be a form of hid-induced apoptosis that is independent of ubiquitin conjugation. This selective behavior may also explain why the suppression of GMR-hid by $Uba1$ is only moderately strong, and not as complete as observed for mutants of other components of the cell death pathway (Srivastava et al., 2007; Xu et al., 2005). Nevertheless, this analysis demonstrates that $Uba1$ directly or indirectly controls caspase activation.

We also analyzed whether $Uba1$ function is needed for normal physiologically occurring cell death. During normal eye development between 26 and 30 hours after puparium formation (APF), cell death is required to remove all cells that have not adopted a cell fate (Cagan and Ready, 1989; Cordero et al., 2004; Wolff and Ready, 1991). $Uba1^{D6/H33}$ mutant clones raised at 25°C contain significantly less Cas3*-positive cells compared with wild-type tissue (Fig. 4C-C″), consistent with a genetic requirement of $Uba1$ for normal cell death. This lack of cell death results in increase in the number of interommatidial cells (IOCs). Wild-type ommatidia contain exactly nine IOCs. However, $Uba1$ clones contain up to 16 IOCs (Fig. 4D-D″). Thus, consistent with the $Uba1$ mutants being suppressors of GMR-hid, $Uba1$ is generally required for the control of normal cell death during *Drosophila* development.
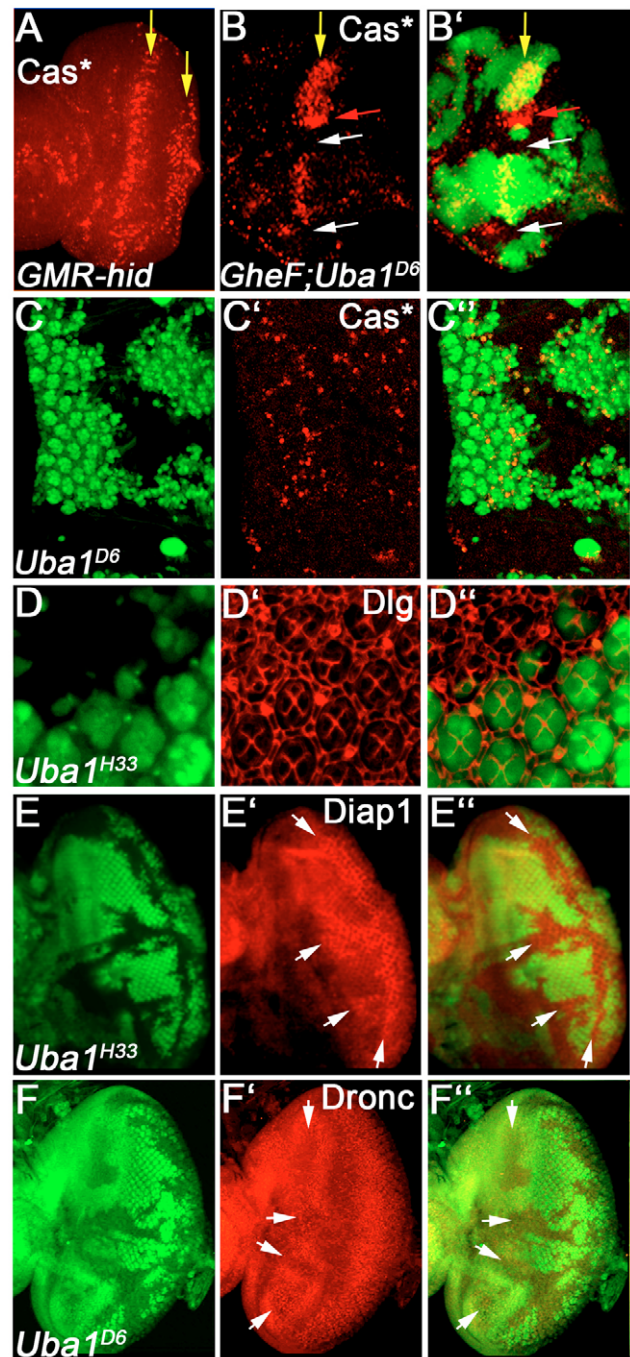

Fig. 4. Weak *Uba1* alleles suppress apoptosis. (**A**) *GMR-hid* eye disc labeled with cleaved Caspase-3 (Cas3*) antibodies. Yellow arrows indicate two zones of dying cells in *GMR-hid*. (**B,B′**) Suppression of Cas3*-positive cell death in *GMR-hid* discs. The yellow arrow indicates the position of the first zone of Cas3*-positive cell death in *GMR-hid*. *Uba1^{D6}* clones suppress Cas3*-positive cell death (white arrows). However, this suppression is not uniform and occurs in a region-specific manner. Clones in an area of the disc marked by the red arrow are still apoptotic. Genotype: *GMR-hid ey-FLP; FRT42D Uba1^{D6}/FRT42D P[ubi-GFP]*; (**C-C″**) *Uba1^{D6}* clones suppress most normal developmental cell death in 28 hours APF pupal discs. Genotype as in B. (**D-D″**) *Uba1^{H33}* clones contain additional IOCs in 42 hours APF pupal discs. Genotype: *ey-FLP; FRT42D Uba1^{H33}/FRT42D P[ubi-GFP]*. (**E-E″**) Diap1 protein levels are increased in *Uba1^{H33}* mutant clones (arrows). Genotype as in D. (**F-F″**) Dronc protein levels are decreased in *Uba1^{D6}* mutant clones (arrows). Genotype as in B.

Because activation of ubiquitin is the only known function of Uba1, these observations suggest that *Uba1* is required for a ubiquitination-dependent event during cell death. Two components of the cell death pathway, the caspase inhibitor Diap1 [also known as Thread (Th) – FlyBase] and the initiator caspase Dronc [also known as Nedd2-like caspase (Nc) – FlyBase], are subject to ubiquitin-mediated degradation (Chai et al., 2003; Herman-Bachinsky et al., 2007; Holley et al., 2002; Ryoo et al., 2002; Wilson et al., 2002; Yoo et al., 2002). Whereas Dronc degradation occurs in surviving cells, Diap1 degradation is triggered in response to apoptotic stimuli. The degradation of both proteins depends on the E3-ligase activity of Diap1 (Herman-Bachinsky et al., 2007; Holley et al., 2002; Ryoo et al., 2002; Wilson et al., 2002; Yoo et al., 2002). Thus, we analyzed the protein levels of Diap1 and Dronc in *Uba1* clones. Interestingly, whereas Diap1 protein levels are increased in *Uba1* mutant clones (Fig. 4E-E″), Dronc protein levels are reduced (Fig. 4F-F″), consistent with previous reports showing that Diap1 controls the protein levels of Dronc (Chai et al., 2003; Ryoo et al.,

2004; Wilson et al., 2002). Because increased levels of Diap1 do protect cells more efficiently from cell death (Hay et al., 1995), this observation suggests that the anti-apoptotic phenotype of *Uba1* clones may be mediated by increased levels of Diap1. The fact that Dronc levels are reduced implies that ubiquitin conjugation still occurs in *Uba1^{D6/H33}* mutant clones at 25°C, providing further evidence that the *Uba1* alleles isolated in our study are weak loss-of-function alleles.

We have shown above that higher temperature converts the alleles *Uba1^{D6}* and *Uba1^{H33}* to stronger loss-of-function alleles, which results in loss of *Uba1* mutant clones and in non-autonomous phenotypes (see Fig. 2). *Uba1^{D6/H33}* clones in eye imaginal discs incubated at 29°C labeled with the Cas3* antibody are highly apoptotic (Fig. 5A) providing an explanation for the loss of *Uba1* mutant clones in adult eyes. In control discs carrying clones of the non-ts *Uba1^{H42}* allele, Cas3* activity is low at 29°C (Fig. 5B). However, despite the fact that *Uba1^{D6/H33}* clones are highly apoptotic, Diap1 protein levels are still increased (Fig. 5C), yet they do not appear to be able to protect *Uba1^{D6}* mutant clones from apoptosis at 29°C. Interestingly, in contrast to incubation at 25°C, we found that the protein levels of Dronc are increased in *Uba1^{D6/H33}* clones at 29°C (Fig. 5D). Clones of the non-ts allele *Uba1^{H42}* do not contain increased levels of Dronc at 29°C (data not shown) suggesting that the increase of Dronc protein levels is caused
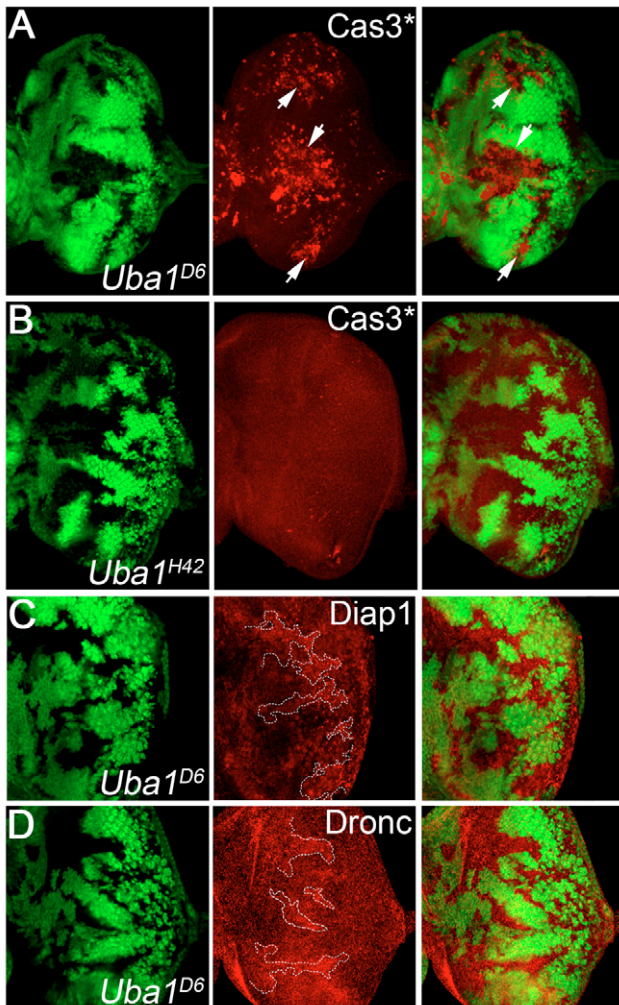

Fig. 5. The apoptotic phenotype of strong *Uba1* alleles. All discs were incubated at 29°C. (**A,C,D**) *ey-FLP*; *FRT Uba1^{D6}/FRT* P[*ubi-GFP*]; (**B**) *ey-FLP*; *FRT Uba1^{H42}/FRT* P[*ubi-GFP*]. (A) *Uba1^{D6}* clones are highly Cas3*-apoptotic (see arrows). (B) *Uba1^{H42}* clones lack apoptotic labeling. (C) *Uba1^{D6}* clones (outlined by dashed line) contain increased levels of Diap1 protein. (D) *Uba1^{D6}* clones (outlined by dashed line) contain increased levels of Dronc protein.
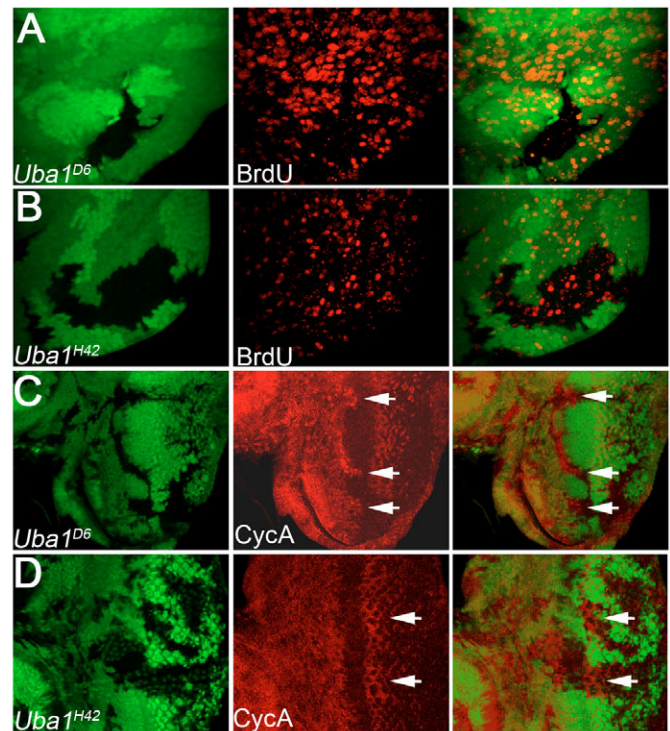

Fig. 6. Cell cycle arrest and non-autonomous proliferation in *Uba1* mosaics. (**A,B**) Third instar mosaic *Uba1^{D6}* (A) and *Uba1^{H42}* (B) wing discs incubated at 29°C. Note lack of BrdU labeling in *Uba1^{D6}* mutant clones, and increased BrdU labeling in wild-type tissue surrounding *Uba1^{D6}* clones. Proliferation in *Uba1^{H42}* mosaics is homogenous within and outside the clones (B). (**C,D**) Cyclin A labeling of eye discs from third instar larval eye discs containing *Uba1^{D6}* (C) and *Uba1^{H42}* (D) mutant clones. Cyclin A levels are increased in *Uba1^{D6}* clones spanning the morphogenetic furrow (arrows in C). Clones of the weak allele *Uba1^{H42}* contain slightly increased Cyclin A levels (D, arrows). However, this increase does not affect cell proliferation (B).

by the transition of $Uba1^{D6/H33}$ to stronger alleles at 29°C, and thus by loss of ubiquitin conjugation. Therefore, owing to the lack of activated ubiquitin, Diap1 can no longer efficiently act as an E3 ubiquitin-ligase for Diap1 and Dronc degradation. In conclusion, our study demonstrates that different proteins require different ubiquitination activity for efficient degradation. Diap1 requires much higher ubiquitination activity for degradation than Dronc (see also Discussion).

Strong *Uba1* alleles cause autonomous cell cycle arrest and non-autonomous cell proliferation

Ubiquitin-induced degradation of cyclins is essential for propagation through the cell cycle (reviewed by Pines, 2006). Thus, we analyzed the effect of loss of ubiquitin conjugation on cell proliferation in strong *Uba1* mutant clones. In $Uba1^{D6/H33}$ clones at 29°C, cell proliferation, as determined by BrdU labeling, is strongly reduced (Fig. 6A). This cell cycle arrest phenotype correlates with increased Cyclin A and Cyclin B levels in $Uba1^{D6/H33}$ mutant clones. This is particularly striking in the morphogenetic furrow (MF) in the eye disc, which does not contain cyclin proteins in wild-type tissue. However, $Uba1^{D6/H33}$ clones spanning the MF fail to downregulate Cyclin A (Fig. 6C) and Cyclin B (not shown) at 29°C. The weak allele $Uba1^{H42}$ and the ts alleles at 25°C do not affect cyclin levels in the MF, but do contain slightly increased cyclin levels in areas of the disc where cyclins are normally detected (Fig. 6D, arrow). However, these increased cyclin levels do not appear to significantly affect the cell cycle, as no obvious alterations in cell proliferation in $Uba1^{H42}$ clones are detected (Fig. 6B).

Interestingly, cell proliferation in $Uba1^{D6/H33}$ mosaics incubated at 29°C as demonstrated by BrdU incorporation is significantly increased in tissue adjacent to the mutant clones (Fig. 6A). This was also observed for the P allele $Uba1^{s3484}$ (data not shown). Control discs (mosaic for the weak non-ts allele $Uba1^{H42}$) show a homogenous distribution of proliferating cells both within and outside the clones (Fig. 6B). Thus, strong *Uba1* clones appear to be the origin of increased proliferation in adjacent tissue, and the overgrowth phenotype in $Uba1^{D6/H33}$ mosaics (Fig. 2) can most likely be explained by emission of signaling molecules from the mutant cells initiating non-autonomous proliferation in the adjacent wild-type tissue (see below).

In summary, consistent with previous reports, lack of ubiquitin conjugation causes cell cycle arrest, presumably through failure to proteolytically degrade cyclin proteins (Pines, 2006). Surprisingly, however, this study also reveals an unanticipated function of *Uba1*, the negative control of the cell cycle in neighboring cells.

Notch activity causes non-autonomous overgrowth in mosaics of strong *Uba1* alleles

The non-autonomous overgrowth of mosaics of strong *Uba1* alleles came as a surprise. However, we and others have recently reported a similar non-autonomous overgrowth phenotype caused by mutations in the ESCRT (endosomal sorting complex required for transport) components *vps23* [also known as *erupted* (*ept*) and *tsg101* – FlyBase] and *vps25* (Herz et al., 2006; Moberg et al., 2005; Thompson et al., 2005; Vaccari and Bilder, 2005). ESCRT components are required at the early endosome for the sorting and targeting of activated cell surface receptors for lysosomal degradation (Babst, 2005; Gruenberg and Stenmark, 2004; Katzmann et al., 2002). Similar to *Uba1* clones, *vps23* and *vps25* clones are highly apoptotic, but stimulate cell proliferation in neighboring tissue and non-autonomous overgrowth (Herz et al., 2006; Moberg et al., 2005; Thompson et al., 2005; Vaccari and
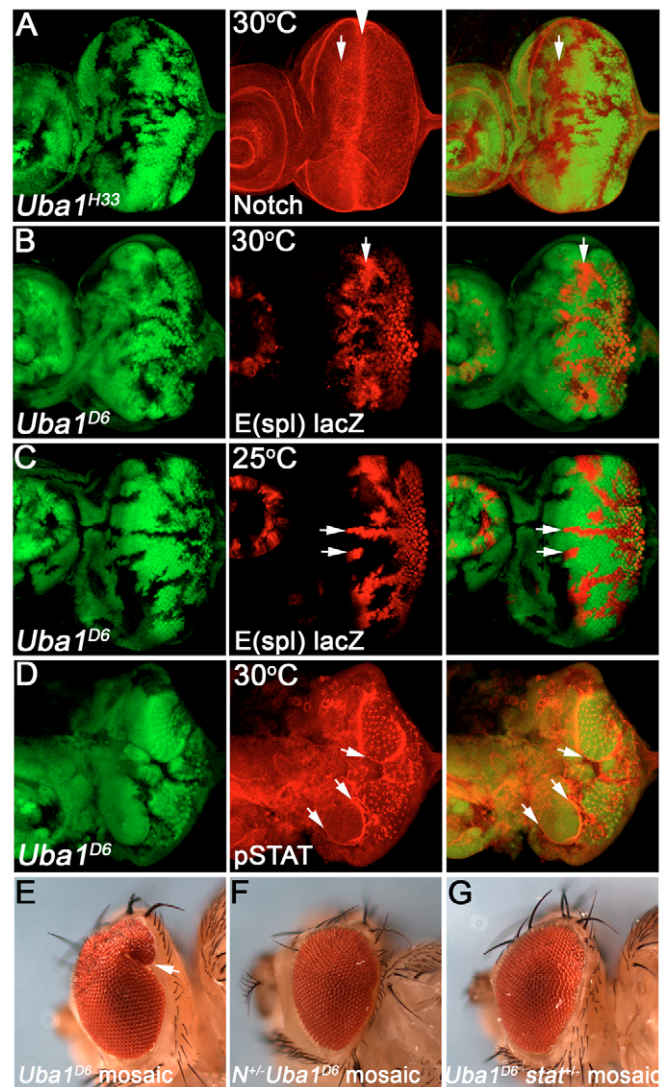

Fig. 7. Notch signaling induces non-autonomous overgrowth in *Uba1* mosaics. All discs were incubated at 30°C, except C which was incubated at 25°C. (**A**) $Uba1^{H33}$ mosaic eye discs labeled with Notch antibody C17.9C6 which was raised against the intracellular domain of Notch. Arrows indicate increase of Notch protein in *Uba1* clones. This is most prominent immediately anterior to the morphogenetic furrow (arrowhead). Genotype: *ey-FLP*; *FRT Uba1^{D6}/FRT* P[*ubi-GFP*]. (**B,C**) Notch activity as visualized by the Notch reporter *E(spl)m8 2.61-lacZ* is increased in $Uba1^{D6}$ clones at 30°C (B) and 25°C (C). Genotype: *ey-FLP*; *E(spl)m8 2.61-lacZ FRT uba^{D6}/FRT* P[*ubi-GFP*]. (**D**) pSTAT labeling is increased in a non-autonomous manner in wild-type tissue immediately adjacent to *Uba1* clones (arrow). Genotype as in A. (**E**) Eyes of $Uba1^{D6}$ mosaic animals are overgrown with bulges (arrow). Genotype as in A. (**F,G**) Heterozygosity of *Notch* (F) and *stat92* (G) dominantly suppresses the $Uba1^{D6}$ overgrowth phenotype. Genotype: *Notch$^{264-39}$/ey-FLP*; *FRT42 Uba1^{D6}/FRT42* P[*ubi-GFP*] (F) and *ey-FLP*; *FRT42 Uba1^{D6}/FRT42* P[*ubi-GFP*]; *stat92^{85c9}* (G).

Bilder, 2005). Interestingly, the signal for endocytosis and endosomal protein sorting is provided by ubiquitination of activated cell surface receptors (Haglund and Dikic, 2005); Gruenberg and Stenmark, 2004). Thus, in *Uba1* mutants, endocytosis and endosomal protein sorting may be affected, too. In the case of *vps23* and *vps25*, signaling by the Notch receptor is inappropriately

increased, leading to secretion of the cytokine Unpaired which stimulates proliferation of neighboring cells by activation of the Jak/STAT pathway (Herz et al., 2006; Moberg et al., 2005; Thompson et al., 2005; Vaccari and Bilder, 2005). Therefore, we tested whether a similar mechanism causes non-autonomous proliferation and overgrowth in *Uba1* mosaics.

In accord, we observe increased protein levels of Notch in *Uba1^{D6/H33}* mutant clones raised at 29°C (Fig. 7A). Using the Notch reporter *E(spl)m8 2.61-lacZ*, increased Notch activity was found in *Uba1* mutant clones (Fig. 7B). Moreover, increased STAT activity, as judged by labeling with an antibody recognizing phosphorylated, i.e. activated STAT protein (pSTAT), is increased in a band of cells immediately adjacent to *Uba1* clones, i.e. in a non-autonomous manner (Fig. 7D). Thus, similar to *vps23* and *vps25*, *Uba1* clones contain increased Notch activity resulting in activation of the Jak/STAT pathway in neighboring cells. Surprisingly, we also detected Notch activity in *Uba1^{D6}* clones raised at 25°C (Fig. 7C), although at this temperature non-autonomous activation of Jak/STAT signaling and overgrowth are not observed (data not shown; see Discussion).

To test for a genetic requirement of Notch and Jak/STAT for the non-autonomous overgrowth phenotype, we analyzed *Uba1* mosaics in a heterozygous *Notch* or *stat92* mutant background. As a marker in this assay, we used the bulging phenotype of *Uba1* mosaic eyes (Fig. 2D, Fig. 7E). Heterozygosity for *Notch* or *stat92* dominantly suppressed the overgrowth phenotype of strong *Uba1* mosaics (*Uba1^{D6/H33}* at 29°C; Fig. 7F,G). Together, these data strongly suggest that the overgrowth phenotype of strong *Uba1* mosaics is caused by inappropriate Notch signaling, which triggers activation of the Jak/STAT signaling pathway in neighboring cells (see Discussion).

DISCUSSION

Before this study, E1 ubiquitin-activating enzymes have only been characterized in yeast and in mammalian cell lines (Ciechanover et al., 1984; Ciechanover et al., 1985; Finley et al., 1984; Ghaboosi and Deshaies, 2007; Kulka et al., 1988; McGrath et al., 1991; Salvat et al., 2000; Swanson and Hochstrasser, 2000). Here, we analyzed the only E1 gene in *Drosophila*, *Uba1*, and uncovered two unexpected mutant phenotypes. First, while partial loss of ubiquitin conjugation caused by weak *Uba1* alleles inhibits cell death, strong *Uba1* alleles are highly apoptotic. Second, while strong *Uba1* clones are cell cycle arrested, they do stimulate neighboring wild-type cells to undergo cell proliferation and induce non-autonomous overgrowth. We also found that photoreceptor differentiation occurs both in clones of weak and strong *Uba1* alleles. However, the onset of photoreceptor differentiation is slightly delayed in clones of strong alleles (see Fig. S1 in the supplementary material). Similar observations have been made in a different study (Pfleger et al., 2007).

Phenotypes affecting cell death

We identified *Uba1* alleles as suppressors of the apoptotic phenotype caused by *GMR-hid*, and showed that *Uba1* is also required for normal developmental cell death. This requirement is probably mediated through the control of Diap1 protein levels which in turn mediates ubiquitination of the caspase Dronc (Chai et al., 2003; Wilson et al., 2002). However, the *GMR-hid*-suppressing *Uba1* alleles are weak. They affect overall ubiquitin conjugation only mildly (Fig. 3C) suggesting that ubiquitin-mediated degradation can still occur in an almost normal manner. In accord, the increased protein levels of Diap1 are even able to reduce the protein levels of Dronc in clones expressing weak *Uba1* alleles.

It is interesting to note that whereas Diap1 protein levels are increased in clones expressing weak *Uba1* alleles (Fig. 4E), other proteins such as Ci, Arm (not shown) or Dronc (Fig. 4F) are normal in abundance or even reduced, respectively. This suggests that some proteins such as Diap1 respond in a very sensitive manner to partial loss of activated ubiquitin, whereas other proteins do not. Because Diap1 has a fairly short half-life (~30-40 minutes) compared to Dronc (~3 hours) (Wilson et al., 2002; Yoo et al., 2002), the requirement of a fully functional ubiquitination machinery may be much stricter for Diap1, providing an explanation for why Diap1 responds so sensitively to a small reduction of activated ubiquitin for protein conjugation.

Alternatively, it is also possible that the *Uba1* alleles isolated in this study specifically affect the interaction with UbcD1, the E2-conjugating-enzyme which targets Diap1 for ubiquitin-mediated degradation (Ryoo et al., 2002; Yoo, 2005). Thus, the interaction with other E2 enzymes may be normal, so that ubiquitin conjugation and degradation of other proteins may be normal. We have not tested which of these two possibilities applies.

Strong *Uba1* alleles, which significantly reduce ubiquitin conjugation, affect the levels of all proteins we have analyzed. For example, although Diap1 levels are increased with strong *Uba1* alleles, Dronc is no longer efficiently degraded. Instead, Dronc protein accumulates, suggesting that activated ubiquitin required for conjugation and degradation is no longer available. However, it is unclear why cells in *Uba1* clones die. Dronc needs to be cleaved for activation, and Diap1 can directly bind to and inhibit caspases without degradation, at least in vitro (Chai et al., 2003; Meier et al., 2000; Zachariou et al., 2003). Thus, the increased Diap1 levels should still be able to inhibit the accumulated Dronc protein. Mutants in *ark* (also known as *D-Apaf-1*, *hac-1* and *dark*), which encodes an adaptor protein required for Dronc activation (Kanuka et al., 1999; Mendes et al., 2006; Rodriguez et al., 1999; Srivastava et al., 2007; Zhou et al., 1999), block cell death in *Uba1* (data not shown), suggesting that cell death in *Uba1* mutants is indeed mediated via Dronc. Thus, simple binding of Diap1 to Dronc may not be sufficient to completely inhibit Dronc activity. Instead, ubiquitination may be required for full inactivation of Dronc.

Phenotypes affecting cell proliferation

Consistent with the expectation, loss of ubiquitin conjugation in strong *Uba1* alleles causes cell cycle arrest. This correlates with increased protein levels of Cyclins A and B, the ubiquitin-dependent degradation of which is required for cell cycle progression (reviewed by Pines, 2006).

However, the non-autonomous overgrowth phenotype was unexpected. Strong *Uba1* clones appear to be able to secrete a growth factor that promotes cell proliferation and overgrowth in adjacent wild-type tissue. In this capacity, *Uba1* qualifies as a tumor suppressor gene.

It is interesting to note that the phenotypes observed for *Uba1* are very similar to those of *vps23* and *vps25* (Herz et al., 2006; Moberg et al., 2005; Thompson et al., 2005; Vaccari and Bilder, 2005). In both cases, Notch signaling is inappropriately increased. Notch triggers Jak/STAT signaling in neighboring wild-type tissue, presumably through secretion of Unpaired, which encodes an Interleukin-like factor (Harrison et al., 1998) and acts as the ligand of the receptor of the Jak/STAT signaling pathway (Chao et al., 2004; Reynolds-Kenneally and Mlodzik, 2005; Tsai and Sun, 2004). However, the ultimate cause of Notch activation may be different. In the case of *vps23* and *vps25*, Notch is internalized via endocytosis, however, endosomal protein sorting is impaired, thus

turnover of Notch is affected. In the case of *Uba1*, it is not clear whether the lack of ubiquitination affects endocytosis of membrane-localized Notch or ubiquitin-mediated degradation of intracellular Notch in the nucleus. Failure of either may cause inappropriate signaling. The accumulation of Notch in *Uba1* clones is not as striking as in *vps25* clones, making it difficult to identify the subcellular localization of accumulated Notch.

Another interesting observation is the fact that we do observe increased Notch activity in clones of *Uba1^{D6}* at 25°C, at which temperature non-autonomous cell proliferation is not observed. Consistently, we do not detect increased STAT signaling under these conditions. If Notch signaling is increased at 25°C, why does this not induce non-autonomous proliferation? One potential reason may lie in the fact that *Uba1* clones at 25°C are protected from apoptosis, whereas at 29°C they are apoptotic. Thus, an apoptotic environment may be necessary for the induction of non-autonomous proliferation. A similar phenomenon, referred to as apoptosis-induced compensatory proliferation, has recently been reported (Huh et al., 2004; Kondo et al., 2006; Perez-Garijo et al., 2004; Ryoo et al., 2004; Wells et al., 2006). In these studies, apoptotic cells trigger the secretion of Dpp and Wg which promote proliferation in neighboring cells. An involvement of Notch was not reported. However, in the aforementioned studies, apoptosis-induced compensatory proliferation is only detectable if cell death is simultaneously blocked. In the case of *Uba1*, *vps23* and *vps25*, overgrowth occurs without inhibition of apoptosis (Herz et al., 2006; Moberg et al., 2005; Thompson et al., 2005; Vaccari and Bilder, 2005). Therefore, there may be different forms of compensatory proliferation in response to different apoptotic triggers.

In summary, we have largely focused on the effects of loss of ubiquitin conjugation for apoptosis and cell proliferation. Our analysis demonstrates that the loss of ubiquitin conjugation has significant consequences for the organism, and may implicate *Uba1* as a tumor suppressor gene in *Drosophila*. The *Uba1* alleles identified in this study will be of further use to analyze a general requirement of ubiquitination for other cellular processes as well.

Supplementary material
Supplementary material for this article is available at
http://dev.biologists.org/cgi/content/full/135/1/43/DC1

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