Drosophila USP5 Controls the Activation of Apoptosis and the Jun N-Terminal Kinase Pathway during Eye Development

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Abstract
The Jun N-terminal kinase pathway plays an important role in inducing programmed cell death (apoptosis) and is activated in a variety of contexts. The deubiquitinating enzymes (DUBs) are proteases regulating the protein stability by ubiquitin-proteasome system. Here, for the first time, we report the phenotypes observed during eye development that are induced by deleting Drosophila USP5 gene, which encodes one of the USP subfamily of DUBs. usp5 mutants displayed defects in photoreceptor differentiation. Using genetic epistasis analysis and molecular markers, we show that most of these phenotypes are caused by the activation of apoptosis and JNK pathway. These data may provide a mechanistic model for understanding the mammalian usp5 gene.

Introduction
The establishment and maintenance of animal homeostasis depends on the balance between cell death and cell proliferation. Programmed cell death, or apoptosis, is a key physiological process that is conserved between nematodes, insects, and vertebrates, which is used to eliminate cells[1]. Caspases are a family of cysteine proteases (cysteinyl aspartate-specific proteases) that play a crucial role in apoptosis[1,2,3]; they destroy vital cellular proteins and thus cause cell death.

Caspases are present in all animal cells; however, their activity is suppressed by the inhibitor of apoptosis proteins (IAPs)[4,5,6]. In Drosophila, the proapoptotic genes reaper (rpr), grim, and head involution defective (hid) have been identified as activators of apoptotic machinery within cells[7]. Their products bind to the Drosophila IAP-1 (DIAP-1), which leads to the release of caspases. One target of DIAP1 is the caspase-9 ortholog DRONC (Drosophila NEDD2-like caspase)[8]. There are two tandem repeats known as the Baculovirus IAP Repeats (BIR) and the RING domain at the carboxyl terminus of DIAP1 [9]. The BIR domains are necessary for binding with caspases [10,11,12]; however, binding of DIAP1 with DRONC is not sufficient for inhibition of DRONC as ubiquitination of DRONC is required to regulate its apoptotic activity[13]. The RING domain of DIAP1 provides the E3-ubiquitin ligase activity that is required for ubiquitination of the target proteins [6,14]. DIAP1-mediated ubiquitination of DRONC does not lead to its degradation by proteasomes; rather, ubiquitination directs the activation of DRONC[13].

The Jun N-terminal kinase (JNK) pathway has been identified as one of the major proapoptotic factors in Drosophila and vertebrates. JNK activates hid and rpr genes [15,16], which in turn initiate the cell death process. JNK not only functions upstream of the activation of proapoptotic genes, but also downstream of DRONC to initiate secondary activation of additional proapoptotic genes[17].

Ubiquitination is an enzymatic process in which proteins are modified by the 76-amino acid protein ubiquitin. Ubiquitination is important not only for protein degradation, but also for the regulation of protein function, such as protein trafficking and protein interactions[18,19,20]. The ubiquitination process is reversible. DUBs are proteases that process ubiquitin or ubiquitin-like gene products, remodel polyubiquitin-chains on target proteins, and reverse the modification of proteins by a single ubiquitin protein[21]. It has recently been shown that knock-down of the deubiquitinating enzyme USP5 (isopeptidaseT) causes p53 activation[22]. USP5 is involved in the disassembly of free polyubiquitin by removing ubiquitin from the proximal end of unanchored polyubiquitin chains[23,24]. When USP5 is knocked-down, unanchored polyubiquitin accumulates. Inhibition of proteasomal degradation of p53, without a defect in p53 ubiquitination, is consistent with a mechanism involving the competition of free polyubiquitin with ubiquitinated p53 for proteasomal recognition.

It is unknown if USP5 is involved in ocular development or in the regulation of other signaling pathways. To explore USP5 function, we generated an usp5 null allele and characterized the usp5 mutant phenotype during eye development. The usp5 mutant contained a reduced number of photoreceptors (R8, R3/R4) and cone cells compared to wild type. Genetic interaction analysis indicated that usp5 regulates apoptosis and the JNK pathway during eye development, consistent with its proposed role as a negative regulator of apoptosis and the JNK pathway.
Materials and Methods

Drosophila genetics

All stocks were maintained and crossed at 25 °C according to standard procedures. The en-Gal4, ap-Gal4/BCG, ey-Gal4, yw, hs-dlpβCd8-GFPFRT2A/Tm6B, yw;flipf;M3LhsGFPFRT2A/Tm6B,yw;D2-3/Tm6B,yw;hs-flp;Act>y+;Gal4-UAS-CD8-GFP/Cyo;Gal80FRT2A/Tm6B provided by Xinhua Liu lab, yw;P[EPgy2]CG12082EY23 569,yw;P[EPgy2]CG12082EY20760/TM3,mirr-Gal4;puc-lacZ/Tm6B UAS-p35 got out from bloomington. Usp5-RNAi (VDRC#17567, 17568) we obtained from VDRC.

Usp5 knockout flies

The usp5 knockout flies were generated using “P-element Hopping” technology. The details described as this paper [25]. The sequence of the primers used to PCR the usp5 coding sequence was: 5’-GACAGGTCGCTTAATAGTGTGACC-3’; 3’-CATCCCGACACTTTCCAGCAGC-5’. The deletion DNA sequence was 1.673 kb include the UBP and part of the UCH domain.

Generation of clones

Mutant clones in the eye discs were generated by the FLP-FRT method [26,27]. The fly was heat-shocked at 37°C for 2 h in first and second instars larvae to induce clones. The disc was dissected at third instar stage. The usp5 mutant clones were marked by the absence of GFP. Mosaics Analysis with a Repressible Cell Marker (MARCM) clones were induced in first and second instar larvae by heat-shock for 2 h at 37°C. The disc was dissected at third instar stage. Expression of UAS-p35 in usp5 mosaic discs was accomplished by act-Gal4.

Polyclonal Antibodies

A fragment of the usp5 cDNA encoding the amino acids 226–515 was amplified by PCR using primers that inserted a 5’in-frame EcoRI site and a 3’NotI site. This fragment was cloned into the EcoRI and NotI 1 sites of the pGEX-4T-1 vector and used to produce the GST fusion protein to immunize mice.

Immunohistochemistry

Eye imaginal discs from the indicated larval and pupal stage were dissected at third instar stage. Fixation and antibody staining of eye imaginal discs were performed as described [28]. Primary antibodies were used as the following dilutions: Rat anti-Elav 1:50 (Developmental Studies Hybridoma Bank, DSHB); Mouse anti-ubiquitin, Lys48 specific 1:500 (Millipore); Rabbit anti-Spalt 1:200 (made in our laboratory); Mouse anti-Cut 1:500 (DSHB); Guinea pig anti-Sns 1:200 (made in our laboratory); Mouse anti-USP5 1:300; Rabbit anti HID 1:10 (Santa Cruz); Mouse anti-DIAP1 1:5was generously provided by Dr. Bruce A. Hay (California Institute of Technology); Rabbit anti-cleaved Caspase-3 1:100 (Cell Signaling Technology, Danvers, MA, USA); Mouse anti-pJNK1:150 (Cell Signaling Technology, Danvers, MA, USA); Mouse anti-β-gal 1:1000 (Abcam); The primary antibodies were detected by fluorescent-conjugated secondary antibodies (Invitrogen). We used the Zeiss 710 laser confocal microscopy to observe the samples and take images.

Scanning electron microscopy

Adult flies were anesthetized, mounted on stages and observed under a scanning electron microscope, the VE-7000 (keyence, Tokyo, Japan) in the low-vacuum mode and high-vacuum mode.

Results

Isolation and characterization of the USP5 mutants

To perform a deubiquitase RNA interference (RNAi) screen, we used en-Gal4 and ey-Gal4 to induce the gene knockdown in the wing and eye in Drosophila. We examined phenotypes of adult wings and eyes to determine whether deubiquitinases were involved in wing and eye development in Drosophila. We characterized a related, novel Drosophila gene, CG12082, which encodes a USP family protein and shares 58.38% homology with human USP5 (Figure 1 D). The two independent usp5 RNAi alleles confirmed that the small eye phenotype is caused by usp5 knock-down. To further analyze usp5 gene function, we generated one mutant allele usp5p by P-element hopping; the usp5p mutant allele is homozygous lethal. DNA sequencing revealed a deletion mutation in usp5 that affected the UBP and part of the UCH domain (Figure 1 D). A USP5 antibody confirmed the specificity by overexpression of exogenous USP5 in wing discs. En-Gal4 was crossed with UAS-usp5 to induce usp5 overexpression in the posterior compartment of the wing disc. The antibody we developed for this study was sensitive enough to detect the overexpression of USP5 (Figure 1 A’). To test whether this antibody recognized endogenous USP5 protein, we used ap-Gal4 to induce usp5 RNAi in the dorsal compartment of the wing disc. USP5 staining was reduced following usp5 knock-down by RNAi (Figure 1 B’). These results show that the USP5 antibody specifically recognizes the USP5 protein. Thus, we next used this antibody to test USP5 protein levels in the usp5 mutant clone (Figure 1 C’), and found that the protein level was greatly reduced. These data suggest that usp5p is a null allele for the gene usp5. There is another P-element insertion line of usp5 named CG12082D120765b, in which the P-element is inserted into the second annotated intron of usp5, and the associated lethality is reversed by precise excision of the P-element. USP5 and CG12082D120765b did not complement each other, and exhibited similar phenotypes (data not shown). We therefore used usp5p to carry out all of the following experiments.

USP5 is involved in Drosophila eye development

USP5 is known to recycle unanchored polyubiquitin from proteasomes back to free ubiquitin [29]. An in vitro study showed that the preferred substrate of USP5 was free/unanchored polyubiquitin chains. The free/unanchored polyubiquitin linked by isopeptide bonds was not conjugated to the substrate protein [24,30,31]. The mutation of usp14 and the usp5 homologues in Saccharomyces cerevisiae, Dictyostelium, and Arabidopsis causes accumulation of free polyubiquitin [32,33,34]. In the usp5 mutant mosaic clones (Figure 2 D), which were marked by no GFP expression, we used the Lys48 specific antibody to label free polyubiquitin chains. The chains accumulated in the usp5 mutant clone in the eye disc of Drosophila (Figure 2 A’), which suggests that the function of usp5 is conserved.

Accumulation of free polyubiquitin is detrimental since this protein acts as a competitive inhibitor of substrates that bind to the proteasome and other ubiquitin receptors [35]. We are interested in whether the accumulation of free polyubiquitin chains affects Drosophila eye development; therefore, we employed the allele for eye-specific expression of hid under the GMR promoter to generate an usp5 homozygous mutant clone in the eye by ey-FLP/FRT. 


mediated mitotic recombination. Compared to control fly eyes carrying FRT-2A alone (Figure 2 B, B”), the eye of the usp5 homozygous mutant clone manifested as rough, small, and disordered bristles (Figure 2 C, C”). Furthermore, some of the ommatidias failed to develop in the usp5 mutant clone eye (Figure 2 C”).

Numerous factors are involved in Drosophila eye development, including cell signaling, cell death, apoptosis, and protein degradation. To focus on the mechanism behind usp5 mutant eye defects, we first examined Elav, a photoreceptor cell marker, which is required for correct differentiation and maintenance of the nervous system. We labeled eye discs in the third larval stage with an Elav-specific antibody. In the usp5 mutant clone, Elav protein level was reduced (Figure 2 D”). This result indicates that the usp5 gene is involved in Drosophila eye development.

USP5 regulates differentiation of photoreceptors during Drosophila eye development

To identify whether usp5 mutant clones display eye differentiation defects, we examined important photoreceptor markers during development of the ommatidium. During ommatidium development, the R8 cell forms in the center, around which all photoreceptors are subsequently incorporated into the ommatidia. The key regulator in the specification of R8 is the proneural gene atonal (ato), a central determinant in peripheral nervous system development [36,37]. We tested Ato protein levels in the usp5 mutant using the minute clone technique; we found that the Ato was slightly reduced and the morphogenetic furrow (MF) was delayed in the mutant clone compared to wild-type cells. Another factor required for precise R8 specification is Senseless (Sens), which is activated by Ato. Sens is not only required for early R8 selection, but also plays a role in controlling terminal differentiation of the R8 photoreceptor. Sens was used as a photoreceptor R8 marker, so we tested the Sens in usp5 mutant clones; the amount of R8 photoreceptors was reduced, but R8 photoreceptor survivors showed outgrowth (Figure 3 B”). This illustrates that USP5 is involved in R8 specification and differentiation.

Recruitment of photoreceptors into the advancement ommatidia by the founder R8 follows a stereotypical pattern[38].
first photoreceptors to be recruited are R2 and R5, followed by R3 and R4, and subsequently R1 and R6. The R3/R4 photoreceptor specification depends on the spalt gene complex, which is a target gene of the Dpp pathway. Spalt is also required for R8 photoreceptor terminal differentiation[39]. Our results showed that Spalt was reduced in the usp5 mutant clone (Figure 3 D9). The eye disc was fully differentiated 42 hours after puparium formation, but it is not known whether usp5 regulates the differentiation of other cells aside from just photoreceptors. We labeled the eye disc with a Cut antibody and a cone cell marker; this revealed that most cone cells failed to differentiate in the usp5 mutant clone (Figure 3 C9). Combined, all of the above results indicate that the loss of usp5 caused defects in the specification and differentiation of eye photoreceptors.

USP5 regulates the apoptosis pathway in the Drosophila eye

To focus on the mechanisms by which USP5 regulates eye development, we first wanted to know whether apoptosis is induced by the knock-out of usp5, since there is a possibility that interference with the differentiation process leads to apoptosis. Apoptosis has occasionally been observed with a rough eye phenotype[40]. We hypothesized that inappropriate activation of DRONC caused the apoptotic phenotype in the usp5 mutant. To explore this possibility, we tested DRONC in the usp5 mutant cells of the eye imaginal disc using minute technology, and labeled apoptosis using an activated CASPASE-3 (Cas3*) antibody. Consistent with our hypothesis, immunostaining of the eye disc with the anti-active Cas* antibody revealed an increase in apoptotic cells in the usp5 mutant clone (Figure 4 A9), especially in the clone anterior to MF of the eye imaginal disc. We also targeted usp5 with RNAi in the eye disc using the mirr-Gal4 line, which is only expressed in the dorsal part of the eye disc and functions relatively late in development. The Cas3* accumulated at extremely high levels in the dorsal region, anterior to MF, in the eye discs of the third larval instar (Figure 4 B9). This suggests that the apoptosis pathway is activated in usp5 mutant clones.

The caspase DRONC is ubiquitinated by E3 ligase DIAP1. The protein DIAP1 also suppresses the effector caspase drICE and targets drICE for ubiquitination. As Cas3* accumulated in the usp5 mutant clones, we first speculated that the phenotype was caused by changes in DIAP1 protein levels. We tested DIAP1 levels in the usp5 mutant clone using a DIAP1 antibody. As

Figure 3. Photoreceptors fail to differentiate in usp5 mutant eyes. Usp5 mutant clones were identified by the absence of GFP in all panels. A-A” Ato antibody staining shows delayed Ato expression in the usp5 mutant clone. B-B” Within the usp5 mutant clones, the quantity of R8 photoreceptors is reduced. C-C” The cone cells marker Cut is strongly reduced in the usp5 mutant clones. D-D” The Spalt antibody was used to mark R3/R4 photoreceptors; Spalt expression was reduced in the usp5 mutant clone.

doi:10.1371/journal.pone.0092250.g003
expected, DIAP1 protein level was significantly lower in the usp5 mutant clone, especially in the anterior portion of the eye disc (Figure 4C'). The inhibition of drICE mediated by DIAP1 could be countered by RPR, HID, and GRIM[41]. We observed that HID protein levels increased in the usp5 mutant clones (Figure 4D'). Since HID binds to DIAP1, the accumulation of HID could reduce DIAP1 protein levels. This result shows that apoptosis is activated upstream of HID in the usp5 mutant eye discs. To identify the photoreceptor defects were caused by inappropriate activation of apoptosis, we employed p35 to suppress apoptosis; P35 is cleaved and forms a thioester bond with the caspase active site, thereby causing irreversible inhibition of the caspase[42]. Furthermore, p35 effectively inhibits a broad range of active caspases and can block cell death [43]. We used the MARCM system to over-express p35 in the usp5 mutant clones. The clones were marked by GFP expression (Figure 4E). We found that over-expression of p35 can rescue the Elav-reduced phenotype in the usp5 mutant clone of the eye disc (Figure 4E'). Therefore, usp5 suppression of apoptosis is necessary for the differentiation of photoreceptors.

The JNK pathway is activated upon loss of usp5
The JNK pathway appears to be a key component of apoptosis; the JNK and proapoptotic genes establish a positive feedback that amplifies the original apoptotic stimulus [17]. To test whether the JNK pathway is activated by accumulation of DRONC in the usp5 mutant cells, we first used the pJNK antibody, which recognizes the phosphorylated form of JNK, to test this hypothesis. pJNK level is increased in the usp5 mutant clone in the eye disc (Figure 5A'). The activity of JNK was also indicated by the transcriptional activation of puckered (puc), one of the target genes of JNK signaling in Drosophila. Puc encodes a JNK-specific phosphatase that down-regulates the pathway, thus forming a negative feedback loop[44]. We used a mirr-Gal4/puc-lacZ line to induce usp5 RNAi expression in the dorsal part of the eye disc. A USP5 antibody was used to detect the effects of RNAi, and anti-β-GAL used to label the lacZ. Puc transcription levels increased once usp5 was knocked-down compared to wild-type cells in the same disc (Figure 5B'). Taken together, these experiments suggest that the JNK pathway is activated once the usp5 gene is removed.

Discussion
Our phenotypic characterization of usp5 mutants during eye development in Drosophila leads to two important conclusions. First, loss of the usp5 gene causes severe defects in the specification and differentiation of photoreceptors. Second, loss of usp5 activates apoptosis and the JNK pathway.

Loss of usp5 causes severe mid-differentiation defects in photoreceptors
Several important factors are involved in regulating eye development in Drosophila. Furthermore, specification and

Figure 4. Loss of usp5 induces apoptosis in the Drosophila eye disc. A-A'' Caspases accumulated in the usp5 mutant clone, and the accumulation is greatest anterior to the MF (arrow marked). B, Mirr-Gal4 induces usp5 RNAi expression in the dorsal half part of eye discs; GFP labels the mirr gene expression position. B', Caspases are increased when usp5 is knocked-down. C-C'' DIAP1 is reduced in the usp5 mutant clones in eye discs. D-D'' The apoptotic activator HID increased in usp5 mutant clones. E-E'' p35 is over expressed in the usp5 mutant clone as determined using MARCM techniques. E Clones marked with GFP. E' Elav staining was normal in usp5 mutant clones.

doi:10.1371/journal.pone.0092250.g004

Figure 5. The JNK pathway is activated in the usp5 mutant clone. A' Eye imaginal discs from third instar larvae are shown. JNK phosphorylation was used to test the activation of JNK signaling; pJNK is increased in usp5 mutant clones. B' puc transcription level is labeled using the puc-lacZ reporter (β-GAL labeling); puc level is increased in the dorsal eye imaginal disc where USP5 is knocked-down as driven by mirr-Gal4.

doi:10.1371/journal.pone.0092250.g005
differentialiation of the photoreceptors are regulated by various cell signaling pathways, cell proliferation, and cell death factors [45]. Some of these pathways, such as the apoptosis pathway [46] and hedgehog pathway [47], are controlled by the ubiquitination-proteasome system. USP5 is able to cleave at least five types of polyubiquitin linkage, including K6, K29, K48, and K63, as well as linear polyubiquitin chains [48]; we therefore anticipated that it regulates eye development. As expected, our data shows that the number of R8 photoreceptors is reduced (Figure 1 C’). The R8 cell serves as the founder cell around which other photoreceptors are subsequently incorporated into the ommatidium. Accompanied by the defect in the photoreceptor R8, the other photoreceptors and cone cells (Figure 3 C’) also showed defects when the usp5 gene was lost. This is the first time that the in vivo function of USP5 has been demonstrated.

Loss of usp5 activates apoptosis and the JNK pathway

The apoptosis pathway plays an important role in Drosophila eye development, especially at the end of the process, when most cells survive or die based on the state of their internal caspases. This pathway is controlled by the ubiquitination-proteasome system. The IAP is an E3 ligase that negatively regulates caspases. DIAP1-mediated ubiquitination of DRONC is sufficient for controlling DRONC activation. Furthermore, the DIAP1 antagonist Grim [49] and DIAP1 itself are ubiquitinated by DIAP1. DIAP1 levels are increased in uba1, the E1 in Drosophila mutant clones [50,51]. The USP5 serves as a deubiquitinating enzyme, the loss of function of which leads to an accumulation of polyubiquitin chains. Accumulation of free polyubiquitin is probably detrimental to development as it works as a competitive inhibitor of substrate binding to the proteasome [35]. In the usp5 mutant clone, DIAP1 protein levels were reduced (Figure 4 C’). There are two models to explain this phenotype: 1) the ubiquitination of DIAP1 increased and the stability of DIAP1 declined because of degradation by a proteasome; or, 2) the DIAP1 was inhibited by the upstream antagonists HID, RPR, and GRIM. It has previously been shown that over-expression of hid, spr, or grim resulted in a loss of DIAP1 in the wing disc and embryo [52]; HID promotes cell death by stimulating DIAP1 auto-ubiquitination and degradation [9,53]. In the case of the usp5 mutant, we observed an increase in HID protein level (Figure 4 B’). The reduction of the DIAP1 protein is possibly caused by HID accumulation. These results suggest that usp5 regulates the apoptosis pathway upstream of HID. More interestingly, the elevation of HID in the posterior compartment did not cause the same accumulation of caspases as in the anterior compartment. A potential explanation for this is that in the mammalian cell caspase-8 is a key initiator of caspase, and the activation is dependent on the polyubiquitination induced by cullin3 (CUL3) -based E3 ligase [54]. The HIB protein functions as substrate recognition subunits for CUL3-based modular ubiquitin ligases, which are expressed only posterior to the MF [55]. The start of caspase activation may also require polyubiquitination induced by CUL3 in Drosophila. We found that the expression level of hid was weakly reduced in the usp5 mutant clones of eye disc (unpublished data); this may lead to relatively little caspase accumulation in the posterior of the eye disc.

The p53 and JNK pathways are the major proapoptotic factors in Drosophila, playing prominent roles in inducing apoptosis. p53 accumulation is observed when usp5 is knocked down in the ARN8 cell line [22]. However, we did not observe an accumulation of Dp35 in the usp5 mutant clones in Drosophila eyes or wing discs (data not shown). This suggests that usp5 may not involve in Dp35 degradation. The JNK signaling also plays an important role in promoting apoptosis in response to a diverse array of signals, including both the p53-dependent response to DNA damage and developmentally regulated apoptosis [36,57]. JNK-induced up regulation of HID was observed in eye imaginal discs [58]. We observed an increase in phosphorylated JNK in the usp5 mutant clones. Furthermore, transcription levels of the target gene of the JNK pathway, puc, were increased (Figure 5 B’). Puc prevents apoptosis by buffering basal levels of JNK signaling. A pilot study showed that the PUC protein is ubiquitinated by the E3 ligase HIB (also called D-SPOP) in vitro [59]. Also, PUC protein levels were significantly reduced when co-expressed with D-SPOP in S2 cells [60]. Interestingly, we found that hid-lacZ was strongly reduced in the usp5 mutant clones of eye discs (data unpublished). The stability of the HIB protein depends on a high level of hedgehog signaling [55]. The key next step in advancing our knowledge of this system will be to further investigate the precise role of usp5 in regulating puc activity towards JNK and the activation of apoptosis in development. As inappropriate activation of mammalian JNK can lead to various forms of human cancers [61,62], these types of genetic studies in model organisms contribute to the broader understanding of oncogenic processes in mammals.

Acknowledgments

We thank X.H. Lin for reagents; the Iowa Developmental Studies Hybridoma Bank (DSHB) for antibodies; the Bloomington Stock Center and VDRC for Drosophila stocks.

Author Contributions

Conceived and designed the experiments: XF. Performed the experiments: XF XY YL YC. Analyzed the data: XF QH JQ. Contributed reagents/materials/analysis tools: XY YL YC. Wrote the paper: XF QH JQ XL.

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