USP5 Is Dispensable for Monoubiquitin Maintenance in Drosophila*

Received for publication, November 11, 2015, and in revised form, February 17, 2016  Published, JBC Papers in Press, February 25, 2016, DOI 10.1074/jbc.M115.703504

Gorica Ristic1, Wei-Ling Tsou1, Ermal Guzi1, Adam J. Kanack2, Kenneth Matthew Scaglione2, and Sokol V. Todi1,*1

From the Departments of 1Pharmacology and 2Neurology, Wayne State University School of Medicine, Detroit, Michigan 48201 and the 3Department of Biochemistry and the Neuroscience Research Center, Medical College of Wisconsin, Milwaukee Wisconsin 53226

Ubiquitination is a post-translational modification that regulates most cellular pathways and processes, including degradation of proteins by the proteasome. Substrate ubiquitination is controlled at various stages, including through its reversal by deubiquitinases (DUBs). A critical outcome of this process is the recycling of monoubiquitin. One DUB whose function has been proposed to include monoubiquitin recycling is USP5. Here, we investigated whether Drosophila USP5 is important for maintaining monoubiquitin in vivo. We found that the fruit fly orthologue of USP5 has catalytic preferences similar to its human counterpart and that this DUB is necessary during fly development. Our biochemical and genetic experiments indicate that reduction of USP5 does not lead to monoubiquitin depletion in developing flies. Also, introduction of exogenous ubiquitin does not suppress developmental lethality caused by loss of endogenous USP5. Our work indicates that a primary physiological role of USP5 is not to recycle monoubiquitin for reutilization, but that it may involve disassembly of conjugated ubiquitin to maintain proteasome function.

Ubiquitination is an important post-translational modification of numerous proteins in the cell, where it is involved in the regulation of various processes ranging from DNA transcription to protein degradation. Three different classes of enzymes are responsible for carrying out this modification: E1 (ubiquitin-activating enzymes), E2 (ubiquitin-conjugating enzymes), and E3 (ubiquitin ligases) (1). Through the coordinated action of these proteins, a ubiquitin molecule is conjugated most commonly to a lysine residue of a substrate protein through an isopeptide bond. Because ubiquitin itself has seven lysine residues, which are available for isopeptide bond formation, and because ubiquitin moieties can also be connected “head to tail,” ubiquitin chains of different conformations are generated (2, 3). Different chains impart specific outcomes on the fate of the protein to which they are conjugated. For example, Lys48-linked ubiquitin targets proteins for proteasomal degradation, whereas Lys63-linked species have been associated with autophagy and other non-proteasomally dependent events (4–7).

Ubiquitination and ubiquitin recycling are tightly controlled to fine-tune the process, to bring a cellular event to an end, to regulate protein fate, and to recycle ubiquitin for reuse. The reversal of ubiquitination is carried out by a class of proteases known as deubiquitinating enzymes (DUBs). DUBs are divided into five families, based on similarity in their catalytic domains: ubiquitin-specific proteases, ubiquitin C-terminal hydrolases, Machado-Joseph disease proteins, otubain proteases, and the JAB1/MPN/Mov34 metalloenzymes. Nearly 100 genes encoding DUBs have been identified in humans, but the functions of many of them remain to be discovered (3, 7–10).

Ubiquitin-specific protease 5 (USP5, also known as isopeptidase T) is one DUB whose structural properties are well understood. USP5 is reportedly an exopeptidase that hydrolyzes isopeptide bonds in polyubiquitin from the free C-terminal end to produce monoubiquitin, which can then be reconjugated to substrate proteins (11–13). Depletion of USP5 orthologues in yeast and in mammalian cells leads to accumulation of unanchored ubiquitin chains and causes proteasomal inhibition (14). These and other findings place USP5 at the proteasome: before a protein is degraded by the proteasome, the ubiquitin chain signaling its degradation is removed en bloc by another DUB, RPN11/POH1, leaving unanchored polyubiquitin. USP5 processes this unanchored chain to yield monoubiquitin (3, 10). Thus, this DUB is thought of as a ubiquitin recycler, helping to maintain a monoubiquitin pool for reutilization (7, 15, 16). Most ubiquitin is found in conjugated forms in various tissues tested, leaving only a small portion available in the unconjugated, monoubiquitin pool (16–18). Because there is persistent demand for protein modification through ubiquitination, there is a constant need to generate monoubiquitin through recycling or through new synthesis via ubiquitin-encoding genes.

It is not entirely clear whether a primary role for USP5 in vivo is monoubiquitin maintenance. Here, we tested this possibility in the fruit fly Drosophila melanogaster, whose USP5 is necessary during development (16, 19, 20). Our biochemical and genetic experiments indicate that Drosophila USP5 is not necessary for maintaining a ready pool of monoubiquitin in vivo.

* This work was supported by a Thomas C. Rumble Fellowship (to G. R.) from Wayne State University; by a grant from the Research and Education Program, a component of the Advancing a Healthier Wisconsin endowment at the Medical College of Wisconsin (to K. M. S.); and by NINDS, National Institutes of Health Grants R00NS073936 (to K. M. S. and R01NS086778 (to S. V. T.). The authors declare that they have no conflicts of interest with the contents of this article.

1 To whom correspondence should be addressed: 540 E. Canfield, Scott Hall, Rm. 3108, Detroit, MI 48201. Tel.: 313-577-1173; E-mail: stodi@med.wayne.edu.

2 The abbreviations used are: DUB, deubiquitinating enzyme; USP5, ubiquitin-specific protease 5; DmUSP5, D. melanogaster ubiquitin-specific protease 5; CHIP, C terminus of HSC70-interacting protein; qRT, quantitative real time.
TABLE 1

**Drosophila lines**

| Stocks | Source | ID   | Description |
|--------|--------|------|-------------|
| w[1118]; P[G2D7641]v17567 | Vienna Drosophila RNAi Center | 17567 | DmUSP5 UAS-RNAi-1 |
| w[1118]; P[G2D7641]v17568 | Vienna Drosophila RNAi Center | 17568 | DmUSP5 UAS-RNAi-2 |
| y[1] v[1]; P[Trip2F02163]attP2 | Bloomington Drosophila Stock Center | 31866 | DmUSP5 UAS-RNAi-3 |
| y[1] v[1]; P[Trp+t7.7 = CaryP]attP2 | Bloomington Drosophila Stock Center | 36303 | Host strain for RNAi-3 |
| w[1118] | Vienna Drosophila RNAi Center | 60000 | Host strain for RNAi-1, -2 |
| w[1]; UAS-HA-Ubiquitin | Bloomington Drosophila Stock Center | 32055 | Expresses HA-tagged ubiquitin under UAS control |
| M{UAS-CG5384. ORF.3xHA}ZH-86Fb | FlyORF | F001032 | Expresses HA-tagged USP14 under UAS control |
| w[1]; tubP-Gal80\* | Bloomington Drosophila Stock Center | 7018 | Temperature-sensitive Gal80 that is expressed under the control of the tubS48B promoter |

**Experimental Procedures**

**Drosophila Lines and Related Procedures—**RNAi-1 and RNAi-2 targeting USP5 were from the Vienna Drosophila RNAi Center (21). These two fly lines contain the same targeting sequence inserted at different chromosomal sites. RNAi-3, the UAS-monoubiquitin overexpression line, and the Gal80\* line were from the Bloomington Drosophila Stock Center. The UAS-USP14 line was from FlyORF. The UAS-CL1-GFP line was a generous gift from Dr. Udai Pandey (University of Pittsburgh), the actin-Gal4 and the sqh-Gal4 lines were a generous gift from Dr. Daniel Eberl (University of Iowa), and the da-Gal4 line was generously donated by Dr. R. J. Wessells (Wayne State University). Flies were maintained at 25 °C and ~40–60% humidity in regulated diurnal environments. Where noted in figures and legends, the flies were maintained at 18 and 30 °C, ~40–60% humidity under diurnal cycle for Gal80 experiments. Fly lines are listed in Table 1.

**SDS-PAGE, Western Blotting, and Quantification—**Larvae, pupae, or flies, as indicated in figures and legends, were homogenized in boiling SDS lysis buffer (50 mM Tris, pH 6.8, 2% SDS, 10% glycerol, and 100 mM DTT), sonicated, boiled for 10 min, centrifuged for 10 min at 13,000 × g at room temperature, loaded onto SDS-PAGE gels, electrophoresed at 160–170 V, and transferred onto PVDF membranes (Bio-Rad) for Western blotting, as previously described (19, 22–25). 10 larvae, 5 pupae, and 20 adults were collected per group in 15 l of lysis buffer per adult. Protein expression was induced by 0.5 mM of isopropyl-1-D-galactopyranoside (A. G. Scientific) for 2 h at 30 °C. DmUSP5 was purified by GST pull-down. Bacterial cells were pelleted by centrifugation and resus-

**Cloning and Protein Purification—**DmUSP5 was cloned in two fragments from a Drosophila w[1118] cDNA library generated in our laboratory. Fragment A was PCR-amplified by using forward primer (5'-GGT TAT TAT GAG AAA AGA GCT TGT GGG) and reverse primer (5'-CAA TAC TGC TGT ACT TCC CGG ACT-3'). Fragment B was PCR-amplified by using forward primer (5'-GCG AAC TTC TGG TAC AAT AGC-3') and reverse primer (5'-CGA ATA ATA TTA GCT TGT GGG ACT G-3'). The fragments were ligated and inserted into pCR Blunt II TOPO vector (ThermoFisher). Full-length DmUSP5 was then subcloned into pGE65p1 (GE Healthcare). The human USP5 construct was purchased from Addgene, a generous gift of the Arrowsmith Lab (plasmid 25299).

DmUSP5 in pGE65p1 and human USP5 in pET28 were transformed into BL21 Escherichia coli. Individual colonies were grown at 37 °C overnight in LB with ampicillin or kanamycin, as needed. 10 ml was used to inoculate 500 ml of LB and was grown for an additional 3 h at 37 °C. Protein expression was induced by 0.5 mM of isopropyl-1-β-D-galactopyranoside (A. G. Scientific) for 2 h at 30 °C. DmUSP5 was purified by GST pull-down. Bacterial cells were pelleted by centrifugation and resus-

**Antibodies—**Anti-ubiquitin (DAKO rabbit polyclonal, 1:500, catalog no. Z0458), P4D1 (mouse monoclonal, 1:500, Santa Cruz Biotechnology, catalog no. SC2017, used only in Fig. 3C), anti-tubulin (mouse monoclonal, 1:5,000, Sigma-Aldrich, catalog no. T5168), anti-actin (JLA20 mouse monoclonal, 1:500, Developmental Studies Hybridoma Bank), anti-HA (Y11, rabbit polyclonal, 1:1,000, Santa Cruz Biotechnology, catalog no. SC805), anti-GFP (mouse monoclonal, 1:1,000, Roche, catalog no. 11814460001), anti-CHIP (rabbit monoclonal, 1:1,000, Cell Signaling Technology, catalog no. 20805), anti-HSP70 (mouse monoclonal, 1:1,000, Rockland, catalog no. 200–301-A27), anti-cyclin A (A12 mouse monoclonal, 1:100, Developmental Studies Hybridoma Bank), anti-Sin3 (rabbit polyclonal, 1:2,000), anti-VCP (valosin-containing protein; rabbit monoclonal, 1:1,000; Cell Signaling Technology catalog no. 2648S) peroxidase-conjugated secondary antibodies (goat anti-rabbit and goat anti-mouse, 1:5,000; Jackson Immunoresearch). Sin3 antibody was a generous gift from Dr. Lori Pile (Wayne State University) (28). The JLA20 and A12 antibodies were procured from the Developmental Studies Hybridoma Bank, created by the NICHD, National Institutes of Health and maintained at the Department of Biology of the University of Iowa (Iowa City, IA). JLA20 was deposited to the Developmental Studies Hybridoma Bank by J. J.-C Lin. A12 was deposited to the Developmental Studies Hybridoma Bank by C. F. Lehner.
Results

Drosophila and Human USP5 Disassemble Unanchored Ubiquitin Chains Similarly in Vitro—CG12082 is the Drosophila gene whose product most closely aligns with mammalian USP5 (19). Like its human counterpart, CG12082 contains the ubiquitin-specific protease (USP) domain, the ubiquitin-associated domains that bind to ubiquitin moieties in polyubiquitin chains, and a zinc finger-like region (11–13, 29–31) (Fig. 1A).

Recombiant, human USP5 has been reported to hydrolyze polyubiquitin chains of different linkages in vitro (11–13, 29–31). We began our studies of Drosophila USP5 (DmUSP5) by comparing ubiquitin chain cleavage preferences between it and the human counterpart in vitro. We carried out deubiquitination reactions at 37 °C, optimal for human USP5, and 25 °C, optimal for Drosophila USP5, although the same results were also obtained at 37 °C (Fig. 1 and data not shown). We observed that DmUSP5 and human USP5 both hydrolyze unanchored polyubiquitin chains of different linkages (Fig. 1B). The recombinant proteases have different efficiencies: Lys11, Lys48, Lys53, and linear (head to tail) species were cleaved more rapidly than Lys6- and Lys44-linked diubiquitin. Both DUBs were also able to cleave rapidly di- and tetraubiquitin chains (Fig. 1B). We noticed that as Lys6 and Lys53 diubiquitin disappear, monoubiquitin does not always seem to mirror the reduction of the substrate species (Fig. 1B). This is not because of indiscriminate cleaving of ubiquitin, e.g. from the presence of non-ubiquitin proteases, because the chains are stable over time in the absence of DmUSP5 (Fig. 1C); instead, it might result from reduced antibody affinity toward ubiquitin with only specific lysines present, which could affect epitope exposure/recognition in different monoubiquitin. Based on these data, we conclude that the Drosophila and human USP5 enzymes have similar catalytic affinity toward most ubiquitin linkages. As shown in Fig. 1D, the catalytic cysteine at position 341 of the Drosophila USP5 is necessary for its protease activity.

There is evidence that in mammalian USP5 can disassemble not only unanchored chains, but that it can also deubiquitinate specific substrates (26, 32). We thus examined whether DmUSP5 and human USP5 could remove ubiquitin from ubiquitinated, recombinant proteins in an in vitro setting. We used a previously published protocol to ubiquitinate CHIP (an E3 ubiquitin ligase) and HSP70 (the molecular chaperone (26)). As shown in Fig. 1E, both human and Drosophila USP5 deubiquitinate monoubiquitinated and polyubiquitinated CHIP, but do not affect ubiquitinated HSP70. These findings support the notion that USP5 can act on specific substrates.

DmUSP5, Important during Fly Development, Is Dispensable in Adults.—To progress toward our major goal, to determine whether DmUSP5 is required for monoubiquitin maintenance in vivo in Drosophila, we next tested the importance of this DUB during development and in adults. Our group and others previously reported that RNAi-dependent knockdown of DmUSP5 and mutations in its gene lead to developmental lethality in the fruit fly (16, 19, 20). To examine whether this DUB is also important in adults, we employed the power of the Gal4-UAS system (33).

We began by testing the efficacy of various RNAi lines targeting DmUSP5. DmUSP5 is expressed throughout the fly (20); therefore, we targeted this protease in the whole organism by using the ubiquitous drivers sqh-Gal4, actin-Gal4, and da-Gal4 with similar results. We used three different RNAi lines specific for DmUSP5 that target different sites of its mRNA. RNAi-1 and -2 were generated through P-element-mediated insertions of a UAS-based transgene (21), whereas RNAi-3 was generated.
USP5 Is Not Needed to Maintain Monoubiquitin

**TABLE 2**

| Primers used                | Forward primer 5′-3′                   | Reverse primer 5′-3′                   |
|-----------------------------|----------------------------------------|----------------------------------------|
| rp49                        | AGATCGTGGAGACACCCACCACAG              | CACGAGAAGACCTTGGTAGTCC                 |
| hsp1/1/calypso              | CCCAAGGTCGATCCGACAAAGAC              | GACGATGACTTGTACTGACC                   |
| rpn11                       | CTGACTCCAGTACACGCGTCCAG             | CTGAGTTTGACTGACGAG                     |
| uch                         | GCTACGCGGAGCTAGACCGAAG               | CCGAGTTTGGAGCTGACGAG                   |
| uch-15/uchl3                | ACGCTGGCTGAGAAATCCTGACCG             | CTGACGTTTGGAGCTGACGAG                   |
| usp1                        | GCCACTCATGACGAGCGTCG                | CTGACGTTTGGAGCTGACGAG                   |
| usp5                        | GCTACGAGCTGAGACCGAAG               | CCGAGTTTGGAGCTGACGAG                   |
| usp8                        | GCTTGGACGAGATCTGACGAG               | CACGACGTTTGGAGCTGACGAG                 |
| usp14                       | ACGCTGGCTGAGAAATCCTGACCG             | CTGACGTTTGGAGCTGACGAG                   |
| usp32                       | CTGACGAGCTGAGACCGAAG               | CCGAGTTTGGAGCTGACGAG                   |
| usp20-33                    | CTTGGGAGATCTGACGAGAC              | CACGACGTTTGGAGCTGACGAG                 |
| usp47                       | ACCTGACTTGGGAGTTGACGAG              | CCGAGTTTGGAGCTGACGAG                   |
| CG2960                      | CTGACGCGGAGACACCCACCACAG              | CACGAGAAGACCTTGGTAGTCC                 |
| CG5271                      | CCCACGACGACGACGACGAC              | CACGAGAAGACCTTGGTAGTCC                 |
| CG32744                     | GACGACGACGACGACGAC              | CACGAGAAGACCTTGGTAGTCC                 |
| CG11624                     | CTTGGGAGATCTGACGAGAC              | CACGACGTTTGGAGCTGACGAG                 |
| CL1-GFP                     | ACGTACGAGCTGAGACCGAAG            | CCGAGTTTGGAGCTGACGAG                   |

**Alternative DmUSP5 primers used, with similar results**

| Primers used | Forward primer 5′-3′ | Reverse primer 5′-3′ |
|--------------|----------------------|----------------------|
| usp5         | GCCGCGGCAAAAAGCTGAAAT  | CACGAGAAGACCTTGGTAGTCC |
| usp5         | CTTGGGAGATCTGACGAGAC  | CACGACGTTTGGAGCTGACGAG |
| usp5         | CTCAGTGGACTTGGACTCCCAC  | CACGAGAAGACCTTGGTAGTCC |

Intrigued by a lack of depletion of monoubiquitin when DmUSP5 is knocked down in the fruit fly, we observed increased levels of monoubiquitin alongside higher levels of conjugated ubiquitin (brackets in Fig. 3A; quantified in Fig. 3B). Interestingly, we did not observe depletion of monoubiquitin alongside higher levels of conjugated ubiquitin (Fig. 3, A and B). In fact, we observed increased levels of monoubiquitin when DmUSP5 is knocked down. The important point, however, is that DmUSP5 knockdown in the fruit fly does not lead to decreased levels of monoubiquitin. We used two different anti-ubiquitin antibodies to assay conjugated and monoubiquitin in fly lysates, with similar results (Fig. 3, A and C, and other supportive data not shown). Based on these outcomes, knockdown of DmUSP5 does not lead to a reduction of monoubiquitin in developing flies.
ubiquitin-encoding genes in Drosophila. We assessed by qRT-PCR the levels of different ubiquitin-encoding genes in the fly: the monoubiquitin encoding genes CG2960 and CG5271, which encode ribosomal fusion proteins RpL40 and RpS27A, respectively, and two polyubiquitin encoding genes, CG32744 and CG11624. Another gene, CG11700, which closely aligns with CG32744 (36), was assayed through CG32744 primer sets. We identified these genes by using the FlyBase resource (a database of Drosophila genes and genomes), through our own alignments with mammalian ubiquitin-encoding genes through BLASTp, and based on previously published work (36). Based on qRT-PCR results, there is no overall increase in ubiquitin gene expression when DmUSP5 is knocked down everywhere in the fly (Fig. 4A). In fact, in some instances we observe a statistically significant decrease in ubiquitin gene expression. These data suggest that lack of depletion of monoubiquitin when DmUSP5 is knocked down is not due to increased ubiquitin production at the transcript level.

Next, we examined the expression levels of other DUBs that have been linked to monoubiquitin maintenance and that func-
tion, at least in part, by associating with the proteasome, where DmUSP5 is also proposed to act: USP14, RPN11, and UCH-L5 (19). Based on qRT-PCR, reduced DmUSP5 mRNA levels coincide with mildly, but statistically significantly, increased levels of USP14, RPN11, and UCH-L5 (Fig. 4B). The levels of expression of these DUBs during fly development and in adults vary from moderate to very high, similar to the expression of DmUSP5 (transcriptional data on FlyBase). This could suggest

**FIGURE 2.** DmUSP5 is necessary during fly development but not in adults. A, summary of phenotypes when DmUSP5 is knocked down throughout the fly. The flies were heterozygous for all transgenes. The results and percentages are from independent crosses conducted at least 10 times each, with similar outcomes. No larvae (RNAi-3) or pupae (RNAi-1 and -2) varied from the indicated stage of death when DmUSP5 was knocked down. 8, qRT-PCR results from flies expressing DmUSP5-RNAi driven by sqh-Gal4. The flies were heterozygous for UAS-RNAi and sqh-Gal4. Control, sqh-Gal4 on the background host line for each RNAi. *Black font* p values compare RNAi-1, -2 and -3 to the control line. *Red font* p values compare RNAi-2 and RNAi-3 to RNAi-1. p values are from analysis of variance with Tukey’s post hoc correction. Error bars indicate S.D., n = 6 independently conducted experiments. D, diagram summarizing the mechanism of Gal80. Please see text for additional details for this method. *Error bars* indicate S.D., n = 11005 independently conducted experiments.

**FIGURE 3.** Knockdown of DmUSP5 does not deplete monoubiquitin. A, Western blots from larval or pupal lysates when DmUSP5-RNAi was driven by sqh-Gal4. 4–20% gradient gels were used. All flies were heterozygous for UAS-RNAi (1, 2, or 3) and sqh-Gal4. *Brackets* indicate conjugated ubiquitin species that were quantified for B. Control, Gal4 driver on the background host line of RNAi. B, quantifications of blots in A and other similar, independent experiments. p values are from Student’s t tests. Error bars indicate S.D., n = 4 independently conducted experiments. C, Western blots of whole larval or pupal lysates where sqh-Gal4 was driving the expression of UAS-RNAi-3 (larvae) or UAS-RNAi-2 (pupae). 4–20% gradient gels were used. Anti-ubiquitin antibody used was different from the one in the other panels. This antibody (monoclonal P4D1) also shows increased levels of conjugated ubiquitin whereas monoubiquitin is also not depleted, similar to what we observe with the other antibody, shown in A and in the rest of the figures of this manuscript.
that some DmUSP5 functions might be complemented by increased transcription of these other DUBs, even though ultimately they fail to suppress lethality caused by the absence of DmUSP5 during development. Increased transcription of endogenous USP14, a DUB demonstrated to maintain monoubiquitin in mice (37–40), may account for monoubiquitin not being depleted when DmUSP5 is knocked down in developing flies. When we tested whether exogenous USP14 alleviates lethality caused by knockdown of DmUSP5, we found that this was not the case (Fig. 4C): USP14 overexpression alongside ubiquitous DmUSP5 knockdown results in developmental lethality at the same stage as DmUSP5 knockdown without exogenous USP14. Overexpression of USP14 by itself is not detrimental to the fly (data not shown).

Lastly, we used a UAS-based, HA-tagged monoubiquitin transgenic fly line to overexpress wild type ubiquitin (41). Expression of ubiquitin through this line leads to an overall increase in levels of conjugated ubiquitin in intact flies (Fig. 5A). Importantly, as shown in the right portion of Fig. 5A, the HA-tagged monoubiquitin that we overexpress is conjugated in flies (also see Fig. 5C). Monoubiquitin expression simultaneously with DmUSP5 knockdown, however, does not affect the stage at which developing flies die (Fig. 5B). Thus, lethality caused by DmUSP5 knockdown in Drosophila is unlikely to be due to a lack of monoubiquitin available for conjugation to various proteins. As shown in Fig. 5C, exogenous ubiquitin does not lead to an appreciable difference in ubiquitin species observed by Western blotting when DmUSP5 levels are reduced. Collectively, the data in Figs. 3–5 led us to conclude that DmUSP5 is not necessary for monoubiquitin maintenance in Drosophila.

Accumulation of Conjugated Ubiquitin Species during DmUSP5 Knockdown Is an Early Event—The results shown in the previous figures were from samples collected preceding larval (RNAi-3) or pupal (RNAi-1 and -2) death when DmUSP5 is knocked down. To examine more temporally the changes in ubiquitin species when DmUSP5 levels are reduced, we analyzed developing flies of different stages when DmUSP5 is knocked down through RNA-1 or -2, which cause pupal death. As summarized in Fig. 6, when DmUSP5 is knocked down, accumulation of conjugated ubiquitin species begins at least as early as third instar larvae and continues through early and mid pupal stages, when they die. This increase in conjugated species is accompanied by an increase in monoubiquitin that appears more robust over time (Fig. 6).

DmUSP5 Knockdown Leads to Impaired Proteasome Activity—Excess ubiquitin chains can compete for proteasomal binding with substrates destined for degradation, thereby impairing ubiquitin-dependent proteasome activity (4, 14, 42). As shown in earlier figures, there is an accumulation of conjugated ubiquitin species that results from knocking down DmUSP5 throughout the fly. Could these species coincide with impaired proteasome function? To examine this possibility, we used a reporter of ubiquitin-dependent proteasome function, CL1-GFP. This construct consists of a GFP moiety fused to the CL1 degron, a signal that targets GFP for proteasomal degradation through ubiquitination (43, 44). We used the ubiquitous driver, da-Gal4, to express the reporter throughout the fly while simultaneously knocking down USP5 with RNAi-1. da-Gal4 also leads to death at mid pupal stages with RNAi-1 and -2, similar to what we observe with the other driver, sqh-Gal4. As the blots in Fig. 7A show, there is an increase in
CL1-GFP protein when DmUSP5 is knocked down compared with its respective, no knockdown control. Increased levels of CL1-GFP mRNA (Fig. 7B), indicative of impaired ubiquitin-dependent proteasomal activity.

We then examined the levels of endogenous proteins that are proteasome substrates: cyclin A (45) and Sin3 (whose orthologue in mammals is degraded by the proteasome, (50)). None of these proteins has been linked to DmUSP5 so far. We also tested the protein levels of VCP/p97, whose expression is induced when proteasome function is reduced (46). As indicated by data in Fig. 7 (C–E), knockdown of DmUSP5 leads to statistically significantly higher levels of each of these endogenous proteins in developing flies. Based on data in Fig. 7, we conclude that ubiquitin-dependent proteasome activity is inhibited by reduced levels of USP5 in Drosophila.

Discussion

Ubiquitination is a critical regulator of most cellular pathways. Therefore, the process of protein ubiquitination and ubiquitin availability both are carefully monitored (1–3, 47). Important regulators of protein ubiquitination are the DUBs, one of which, USP5, has been proposed to function in part by maintaining monoubiquitin (3, 7, 15, 16). Here, our primary question was whether the fly orthologue of this protease is important for monoubiquitin recycling in vivo. We found that this is not the case in Drosophila.

Prior work conducted on DmUSP5 delivered some information on its role on monoubiquitin levels. One study (20) found either increased levels or nondepleted monoubiquitin in DmUSP5 mutants in Drosophila. However, whether this meant that fly USP5 is dispensable for monoubiquitin recycling was not clarified. Another study observed increased levels of conjugated ubiquitin, whereas the levels of monoubiquitin did not appear to be consistently depleted when the gene was mutated in the fruit fly (16). Both of these studies arrived at a similar conclusion: USP5 is important for ubiquitin homeostasis in Drosophila. The issue raised in our present work, whether USP5 is needed for monoubiquitin recycling in Drosophila, requires further investigation.
We here presented evidence that *Drosophila* USP5, which functions similarly to its human counterpart in vitro, is required during fly development but seems dispensable in adults. These findings correspond well with previously published reports by others and us, which showed that this DUB is required in developing flies: ubiquitous knockdown and knockdown in select tissues (e.g., neurons and glial cells) during development has negative effects (16, 19, 20).

In reconstituted systems, both human and fly USP5 efficiently cleave ubiquitin chains linked head to tail and through Lys11, Lys48, and Lys63. Lys11- and Lys48-linked ubiquitin have been connected to the degradation of proteins by the proteasome, whereas Lys63-linked chains have been associated with other pathways, including DNA repair, endosomal recycling, and autophagy (6, 48). Our in vitro data also hint at the possibility that USP5 may have specific substrates. This DUB was able to rapidly and efficiently deubiquitinate at least one recombinant, ubiquitinated protein in a reconstituted system, but not a second. There have been other reports of mammalian USP5 acting on specific substrates in vitro and in vivo (26, 32).

Although we found a clear importance for the presence of this DUB during larval and pupal stages, USP5 did not seem important when it was knocked down only during adult stages, even though it is well expressed in adults, based on transcriptional data aggregated on Flybase. If DmUSP5 is acting on specific substrates, perhaps they are not as critical in adults as in earlier stages. Alternatively, expression of other DUBs may at this point complement some of the USP5 functions that they could not in larval and pupal stages. We hope to identify specific DmUSP5 substrates in the near future through quantitative mass spectrometry.

Our collective genetic and biochemical experiments indicate that the function of DmUSP5 in flies is not to maintain monoubiquitin. Perhaps it is useful to compare our results with data from work conducted with another DUB, USP14, which is important for maintaining monoubiquitin in the nervous sys-

**FIGURE 6.** **Conjugated ubiquitin accumulates early when DmUSP5 is knocked down.** A, Western blots of larval and pupal lysates (17% gel) when DmUSP5-RNAi-1 or -2 were driven by sqh-Gal4. Samples were collected during three stages of development, as noted. The flies were heterozygous for RNAi and sqh-Gal4. Control, sqh-Gal4 on the host background of RNAi. Direct blue staining shows total protein loaded. B, quantifications of Western blots from A and other similar, independent experiments. p values are from Student’s t tests. Error bars indicate S.D., n = 4 independently conducted experiments.
Mutations in USP14 lead to reduced monoubiquitin protein levels and increased transcription of ubiquitin-encoding genes (39). Importantly, introduction of wild type monoubiquitin alleviates some of the phenotypes caused by loss of USP14 (39). In our case, depletion of DmUSP5 does not lead to lower levels of monoubiquitin (in fact, we see a trend of increased monoubiquitin at the protein level), transcription of fly ubiquitin-encoding genes is not increased, and introduction of exogenous ubiquitin does not suppress the lethality effect of DmUSP5 knockdown. Thus, our work argues that DmUSP5 is not critically needed to maintain the monoubiquitin pool in vivo in Drosophila.

qRT-PCR results hint that maintenance of monoubiquitin when DmUSP5 is knocked down may result from increased transcription of other DUBs that can yield monoubiquitin, such as USP14. Other investigators also described increased levels of various DUBs when DmUSP5 was reduced or absent in the fly (16, 20). Up-regulation of these other DUBs, however, appears unable to suppress lethality caused by DmUSP5 knockdown or to normalize the levels of conjugated ubiquitin species that we observe in Western blots. These findings suggest a highly regulated and flexible system for DUB expression in vivo, while also underscoring the nonredundant roles that these proteases play in intact organisms, as we also described before (19).

Excess conjugated ubiquitin may hinder ubiquitin-dependent proteasomal degradation of proteins by competing for binding to the 19S proteasome. Based on our work with the reporter CL1-GFP and the endogenous proteins cyclin A, Sin3, and VCP/p97, proteasome function is indeed reduced when DmUSP5 is knocked down throughout the fly.

Our work leads us to propose that a primary function of DmUSP5 in Drosophila is the disassembly of conjugated ubiquitin, which helps, at least in part, to maintain proteasome activity. When this protease is unavailable, increased transcription of other DUBs could assist with the processing of some conjugated ubiquitin to recycle monoubiquitin. However, these proteases appear unable to fully complement DmUSP5 activities. We suggest that as the proteasome is inhibited by species that would have been disassembled by DmUSP5, conjugated and monoubiquitin levels increase as ubiquitinated substrates and ubiquitin (itself a proteasome substrate (47)) are degraded less rapidly. Ultimately death ensues, probably because of proteasomal inhibition.

In summary, we presented evidence that DmUSP5 is not required for monoubiquitin maintenance in vivo. Based on our additional work that DmUSP5 has wide, although not universal, ubiquitin chain linkage preferences, it will be of interest to con-

FIGURE 7. Knockdown of DmUSP5 causes impairment of proteasome activity. A and C–E, left panels, Western blot of pupal lysates where the ubiquitous drivers da-Gal4 (A) or sqh-Gal4 (C–E) express DmUSP5 UAS-RNAi-1 throughout the fly. RNAi Ctrl, ubiquitous driver for each panel on the isogenic background of RNAi-1. A and B, UAS-CL1-GFP is also expressed in these pupae. C–E, no UAS-CL1-GFP is present. Right panels, quantification of data from each Western blot shown and other, independently conducted experiments. p values are from Student’s t tests. Error bars indicate S.D., n = 3 independently conducted experiments for each panel. Arrows in D, Sin3 has different isoforms, both of which are detected by this antibody. 4–20% gradient gels were used. B, qRT-PCR results from pupae with the same genotype and age as in A. n = 3 independent experiments. Error bar indicates S.D. p value is from Student’s t test.
to examine the various pathways and processes in which this developmentally required DUB is involved.

Author Contributions—G. R., W.-L. T., K. M. S., and S. V. T. designed the study. G. R., W.-L. T., E. G., A. J. K., K. M. S., and S. V. T. prepared the reagents and conducted the experiments. G. R., W.-L. T., E. G., K. M. S., and S. V. T. analyzed the data and prepared the figures. G. R., K. M. S., and S. V. T. wrote the manuscript.

Acknowledgments—We thank Drs. Udai Pandey (University of Pittsburgh), Daniel Eberl (University of Iowa), and R. J. Westells (Wayne State University) for fly stocks, as well as Dr. Lori Pile (Wayne State University) for transgenic Drosophila Stock Center (funded by National Institutes of Health Grant R01GM084947), the Vienna Drosophila RNAi Center, the FlyORF, and the TRIP at Harvard Medical School (funded by National Institutes of Health Grant R01GM084947) for transgenic stocks.

References
1. Pickart, C. M. (2000) Ubiquitin in chains. Trends Biochem. Sci. 25, 544–548
2. Pickart, C. M., and Fushman, D. (2004) Polyubiquitin chains: polymeric protein signals. Curr. Opin. Chem. Biol. 8, 610–616
3. Ristic, G., Tsou, W. L., and Todi, S. V. (2014) An optimal ubiquitin-proteasome pathway in the nervous system: the role of deubiquitinating enzymes. Front Mol. Neurosci. 7, 72
4. Thrower, J. S., Hoffman, L., Rechsteiner, M., and Pickart, C. M. (2000) Recognition of the polyubiquitin proteolytic signal. EMBO J. 19, 94–102
5. Hao, R., Nanduri, P., Rao, Y., Panichelli, R. S., Ito, A., Yoshida, M., and Yao, T. P. (2013) Proteasomes activate aggresome disassembly and clearance by producing unanchored ubiquitin chains. Mol. Cell. 51, 619–828
6. Nathan, J. A., Kim, H. T., Ting, L., Gygi, S. P., and Goldberg, A. L. (2013) Why do cellular proteins linked to K63-polyubiquitin chains not associate with proteasomes? EMBO J. 32, 552–565
7. Komander, D., Cлагe, M. J., and Urbé, S. (2009) Breaking the chains: structure and function of the deubiquitinases. Nat. Rev. Mol. Cell Biol. 10, 550–563
8. Todi, S. V., and Paulson, H. L. (2011) Balancing act: deubiquitinating enzymes in the nervous system. Trends Neurosci. 34, 370–382
9. Cлагe, M. J., Couolon, J. M., and Urbé, S. (2012) Cellular functions of the DUBs. J. Cell Sci. 125, 277–286
10. Cлагe, M. J., Barsukov, I., Couolon, J. M., Liu, H., Rigden, D. J., and Urbé, S. (2013) Deubiquitylases from genes to organism. Physiol. Rev. 93, 1289–1315
11. Wilkinson, K. D., Tashayev, V. L., O’Connor, L. B., Larsen, C. N., Kasperek, E., and Pickart, C. M. (1995) Metabolism of the polyubiquitin degradation signal: structure, mechanism, and role of isopeptidase T. Biochemistry 34, 14535–14546
12. Reyes-Turcu, F. E., Horsot, J. R., Mullally, J. E., Heroux, A., Cheng, X., and Wilkinson, K. D. (2006) The ubiquitin binding domain Znf UBPI recognizes the C-terminal diglycine motif of unanchored ubiquitin. Cell 124, 1197–1208
13. Reyes-Turcu, F. E., Shanks, J. R., Komander, D., and Wilkinson, K. D. (2008) Recognition of polyubiquitin isoforms by the multiple ubiquitin binding modules of isopeptidase T. J. Biol. Chem. 283, 19581–19592
14. Amerik, A., Swaminathan, S., Krantz, B. A., Wilkinson, K. D., and Hochstrasser, M. (1997) In vivo disassembly of free polyubiquitin chains by yeast Ubpl4 modulates rates of protein degradation by the proteasome. EMBO J. 16, 4826–4838
15. Grou, C. P., Pinto, M. P., Mendes, A. V., Domingues, P., and Azevedo, J. E. (2015) The de novo synthesis of ubiquitin: identification of ubiquitinases acting on ubiquitin precursors. Sci. Rep. 5, 12836
16. Kovacs, L., Nagy, O., Pal, M., Udvardy, A., Popescu, O., and Deak, P. (2015) Role of the deubiquitylating enzyme DmuSp5 in coupling ubiquitin equi-
USP5 Is Not Needed to Maintain Monoubiquitin

(2003) Spatiotemporal rescue of memory dysfunction in Drosophila. Science 302, 1765–1768
36. Lu, C., Kim, J., and Fuller, M. T. (2013) The polyubiquitin gene Ubi-p63E is essential for male meiotic cell cycle progression and germ cell differentiation in Drosophila. Development 140, 3522–3531
37. Anderson, C., Crimmins, S., Wilson, J. A., Korbel, G. A., Ploegh, H. L., and Wilson, S. M. (2005) Loss of Usp14 results in reduced levels of ubiquitin in ataxia mice. J. Neurochem. 95, 724–731
38. Chen, P. C., Qin, L. N., Li, X. M., Walters, B. J., Wilson, J. A., Mei, L., and Wilson, S. M. (2009) The proteasome-associated deubiquitinating enzyme Usp14 is essential for the maintenance of synaptic ubiquitin levels and the development of neuromuscular junctions. J. Neurosci. 29, 10909–10919
39. Chen, P. C., Bhattacharyya, B. J., Hanna, J., Minkel, H., Wilson, J. A., Finley, D., Miller, R. J., and Wilson, S. M. (2011) Ubiquitin homeostasis is critical for synaptic development and function. J. Neurosci. 31, 17505–17513
40. Hallengren, J., Chen, P. C., and Wilson, S. M. (2013) Neuronal ubiquitin homeostasis. Cell Biochem. Biophys. 67, 67–73
41. Lee, F. K., Wong, A. K., Lee, Y. W., Wan, O. W., Chan, H. Y., and Chung, K. K. (2009) The role of ubiquitin linkages on α-synuclein induced-toxicity in a Drosophila model of Parkinson’s disease. J. Neurochem. 110, 208–219
42. Beal, R., Deveraux, Q., Xia, G., Rechsteiner, M., and Pickart, C. (1996) Surface hydrophobic residues of mult ubiquitin chains essential for proteolytic targeting. Proc. Natl. Acad. Sci. U.S.A. 93, 861–866
43. Bence, N. F., Sampat, R. M., and Kopito, R. R. (2001) Impairment of the ubiquitin-proteasome system by protein aggregation. Science 292, 1552–1555
44. Bennett, E. J., Bence, N. F., Jayakumar, R., and Kopito, R. R. (2005) Global impairment of the ubiquitin-proteasome system by nuclear or cytoplasmic protein aggregates precedes inclusion body formation. Mol. Cell 17, 351–365
45. Chen, W., Lee, J., Cho, S. Y., and Fine, H. A. (2004) Proteasome-mediated destruction of the cyclin a/cyclin-dependent kinase 2 complex suppresses tumor cell growth in vitro and in vivo. Cancer Res. 64, 3949–3957
46. Lundgren, J., Masson, P., Mirzai, Z., and Young, P. (2005) Identification and characterization of a Drosophila proteasome regulatory network. Mol. Cell. Biol. 25, 4662–4675
47. Shabek, N., and Ciechanover, A. (2010) Degradation of ubiquitin: the fate of the cellular reaper. Cell Cycle 9, 523–530
48. Ikeda, F., and Dikic, I. (2008) Atypical ubiquitin chains: new molecular signals. “Protein Modifications: Beyond the Usual Suspects” review series. EMBO Rep. 9, 536–542
49. Crimmins, S., Jin, Y., Wheeler, C., Huffman, A. K., Chapman, C., Dobrunz, L. E., Levey, A., Roth, K. A., Wilson, J. A., and Wilson, S. M. (2006) Transgenic rescue of ataxia mice with neuronal-specific expression of ubiquitin-specific protease 14. J. Neurosci. 26, 11423–11431
50. Kong, Q., Zeng, W., Wu, J., Hu, W., Li, C., and Mao, B. (2010) RNF20, an E3 ubiquitin ligase that targets Sin3B for ubiquitination. Biochem. Biophys. Res. Commun. 393, 708–713