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The deubiquitinase Leon/USP5 regulates ubiquitin homeostasis during *Drosophila* development

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ABSTRACT

Ubiquitination and the reverse process deubiquitination regulate protein stability and function during animal development. The *Drosophila* USP5 homolog Leon functions as other family members of unconventional deubiquitinases, disassembling free, substrate-unconjugated polyubiquitin chains to replenish the pool of mono-ubiquitin, and maintaining cellular ubiquitin homeostasis. However, the significance of Leon/USP5 in animal development is still unexplored. In this study, we generated *leon* mutants to show that Leon is essential for animal viability and tissue integrity during development. Both free and substrate-conjugated polyubiquitin chains accumulate in *leon* mutants, suggesting that abnormal ubiquitin homeostasis caused tissue disorder and lethality in *leon* mutants. Further analysis of protein expression profiles in *leon* mutants shows that the levels of all proteasomal subunits were elevated. Also, proteasomal enzymatic activities were elevated in *leon* mutants. However, proteasomal degradation of ubiquitinated substrates was impaired. Thus, aberrant ubiquitin homeostasis in *leon* mutants disrupts normal proteasomal degradation, which is compensated by elevating the levels of proteasomal subunits and activities. Ultimately, the failure to fully compensate the dysfunctional proteasome in *leon* mutants leads to animal lethality and tissue disorder.

1. Introduction

Ubiquitination is an important cellular process to respond to signal stimulation, leading to tissue patterning and differentiation during animal development. Protein substrates are ubiquitinated repeatedly by the series of E1 activating, E2 conjugating and E3 ligating enzymes to build up polyubiquitinated chains [1]. Polyubiquitinated substrates are then recognized, deubiquitinated, and degraded by the proteasome. Deubiquitinases (DUBs), the large family of enzymes that deconjugate polyubiquitin chains from protein substrates, are often considered as rivalry to ubiquitination in many cellular functions [2,3].

DUBs cleave ubiquitin from mono- or poly-ubiquitin-conjugated protein substrates, linear ubiquitin precursors and unanchored polyubiquitin chains [4]. The universal enzymatic action of DUBs is to hydrolyze the ester or amide bond at carboxyl-terminus of ubiquitin [4]. However, the large DUB family of more than one hundred members in the human genome, for example, can be classified into five classes according to their protein sequences [3–5]. In four of the five classes, the DUBs are cysteine proteases, including ubiquitin specific proteases (USPs), ubiquitin C-terminal hydrolases (UCHs), otubian proteases (OTUs) and Machado–Joseph disease proteases (MJDs). The DUBs of the non-cysteine protease contain JAB1/MPN domain-associated metalloisopeptidase (JAMM domain) activity [3–5]. Mutations in many DUBs have been shown to associate with cancers and neurodegenerative diseases [6,7]. There are 41 DUBs in the *Drosophila* genome with only a handful of them being revealed for their developmental roles [8]. Nonstop is required for photoreceptor axon growth regulated by glial cells [9], Fat facets regulates the ubiquitination status of Liquid facets in retina patterning [10], and DUBAI stabilizes DIAP1 to suppress apoptosis [11]. However, the functions for most of *Drosophila* DUBs are still unknown.

In contrast to other DUBs that deconjugate mono-ubiquitin or polyubiquitin chains from protein substrates, human USP5 recognizes and deconjugates free, unanchored polyubiquitin chains to mono-ubiquitin, thus recycling ubiquitin for further usage [12]. This cleavage process requires conserved domains of USP5: the N-terminal Zn^{2+}-stabilized ZnF UBP domain that forms a pocket to recognize the di-glycine motif in the C-terminal ubiquitin of

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the polyubiquitin chain, two UBA domains that bind ubiquitin of the polyubiquitin chain, and conserved cysteine and histidine boxes that constitute the catalytic site [13]. The homologues in yeast and *Arabidopsis thaliana* also have the same structure and cleavage capability [14–16]. The USP5 family of DUBs assumes a key regulatory role for ubiquitin homeostasis in cells. In yeast, free polyubiquitin chains serve as the ubiquitin reservoir that maintains ubiquitin at adequate levels in normal conditions and rapidly provides ubiquitin for substrate conjugation under stress conditions [17]. In addition to the disassembly of polyubiquitin chains, USP5 is also important to the stability of protein substrates. Accumulation of polyubiquitin chains in USP5 knockdown cells promotes p53 accumulation [15].

Drosophila is an excellent model system to study gene functions in developmental processes. We study the function of the USP5 homologue Leon first by isolating three mutant alleles. In *leon* mutants, brain lobes and imaginal disks are dramatically reduced. Leon is expressed ubiquitously throughout developmental stages. Consistent with human USP5, Leon is capable to cleave unanchored lysine48-linked polyubiquitin chains. The animal lethality, abnormal tissues and accumulation of polyubiquitin chains are completely restored to normality by ubiquitous expression of Leon. However, expression of the enzyme-dead version of Leon fails to rescue these defects. Furthermore, proteasomal degradation of protein substrates is impaired in *leon* mutants. Surprisingly, proteasomal enzymatic activities and proteasomal subunit levels are elevated in *leon* mutants, suggesting a compensatory mechanism when ubiquitin homeostasis is interfered. Thus, *leon* is an essential gene for *Drosophila* development and required for ubiquitin homeostasis to avoid dysfunction of proteasomal degradation.

2. Materials and methods

2.1. Fly stocks

leon^{1}, *leon*^{2} and *19-2* alleles and *GFP-leon-GR* were described in this study, and wild-type control *w*^{1118} was used to backcross all mutant alleles for at least five generations. All flies were reared at 25 °C.

UAS-3xFlag-leon and *UAS-3xFlag-ED-leon* were constructed by fusing 3 Flag repeats (DYKDHDGDYKDHDIDYKDDDDK) at the N-terminus of *leon* cDNA. *UAS-CL1-GFP* [18] has been described. *tubP-GAL4* and *24B-GAL4* were obtained from Bloomington *Drosophila* Stock Center.

2.2. Immunostaining

Larvae were dissected and fixed as previously described [19]. Primary antibodies: chicken anti-GFP (1:100, Abcam®), goat anti-HRP conjugated FITC or TRITC (1:200 Jackson ImmunoResearch), mouse anti-Leon (1:100) generated against GST-Leon fusion protein (LTK BioLaboratories) and TRITC conjugated phalloidin (1:2000; Sigma Co.) Tissues were mounted in PBS containing 87.5% glycerol and 0.22 M 1,4-diaza-byciclo (2.2.2) octane (Dabco, Sigma Co.). Images were acquired by confocal Z-stack scanning (Zeiss LSM510) using 20× objectives and processed by LSM 5 image examiner and Adobe Photoshop.

2.3. Western blot and deubiquitination assay

Third instar larvae or transfected S2 cells were homogenized in lysis buffer (150 mM NaCl, 5 mM EDTA, 50 mM K_2HPO_4, 1% triton-X-100 and 20 mM Tris–HCl, pH 7.4) supplemented with protease inhibitor cocktails (Roche) and 1 mM PMSF. For deubiquitination assay, purified GST-Leon and GST-ED-Leon were incubated with K48-linked or K63-liked polyubiquitin (1 μg, Enzo Co.) in a reaction mixture (10 ml containing 20 mM Tris–HCl, pH 7.5, and 0.5 mM DTT) for 1 h at 30 °C. For Western blot, antibodies against proteasomal subunits 20S-α, P39A, P42A, and P54 (1:100; Santa Cruz Biotechnology), ubiquitin (P4D1, 1:1000; Santa Cruz Biotechnology), α-Tubulin (1:10,000; Sigma Co.) and Leon (1:1000) were used.

2.4. In vitro enzymatic activity assay

20 μM of each fluorogenic substrates Z-LLE-AMC, Boc-LRR-AMC and Suc-LLVY-AMC [20] (Enzo Co.) were incubated with 20 μg larva lysates of *w*^{1118}, *leon*^{1}/*19-2* and *leon*^{2}/*19-2* in buffer A (Glycerol 10%, 25 mM Tris–HCl, pH 7.4, 10 mM $MgCl_2$, 1 mM ATP and 1 mM DTT) for 1 h at 25 °C. The reactivities were measured with excitation at 380 nm and emission at 460 nm (Enspire Multilabel Plate Reader, Perkin Elmer).

2.5. SILAC method for flies

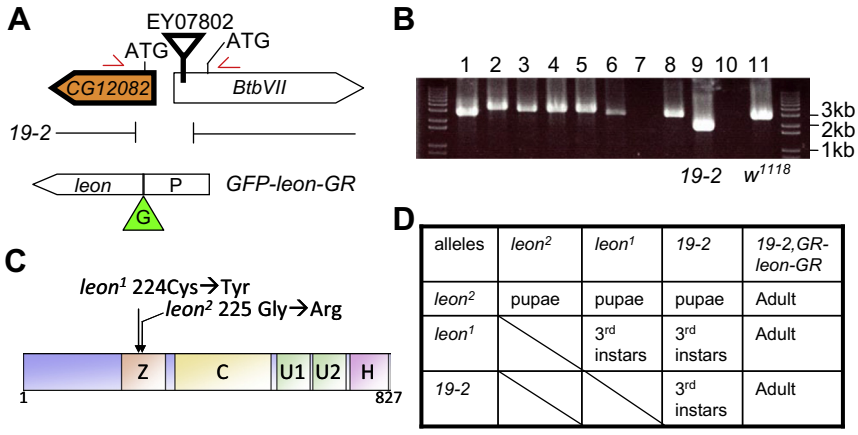
Wild type larvae were reared in normal fly food and *leon*^{1}/*19-2* larvae were reared in food with heavy amino acid (Lys-6). Larvae were homogenized in lysis buffer (150 mM NaCl, 5 mM EDTA, 50 mM K_2HPO_4, 1% triton-X-100 and 20 mM Tris–HCl, pH 7.4) supplemented with protease inhibitor cocktails (Roche) and 1 mM PMSF. Each 0.5 μg of wild type and *leon*^{1}/*19-2* larval lysates were mixed and preformed in 10% SDS–PAGE. Gel with proteins were further digested by trypsin and examined by Mass-Spec machine (LTQ-Orbitrap XL, Thermo Fisher Scientific). The indicated protein had been detected with heavy (H) and light (L) peptides. H/L ratio provided the relative amount of indicted protein between different protein sources [21].

3. Results

3.1. Isolation of leon mutants

To address the developmental role of the *Drosophila* USP5 homolog, we isolated mutant alleles by P-element-mediated excision of *P[EPgy2]BtbVII*^{EY07802}, inserted in the first intron of *BtbVII* next to *leon* (CG12082, Fig. 1A). About 100 excision alleles were examined by PCR. Shorter PCR products indicate imprecise excision events, and were sequenced to uncover the deleted region. The *19-2* allele was truncated for a 1 kb region including the shared promoter region, and the predicted ATG codon of *leon* (Fig. 1A and B). Homozygotes for the *19-2* allele were lethal, which could be due to loss of *leon*, *BtbVII* or both. To examine the cause of the lethality of *19-2*, the genomic rescue transgene *GFP-leon-GR* was constructed by fusing *GFP* to *leon* cDNA driven by the genomic sequence between the translational start sites of *BtbVII* and *leon*. The *GFP-leon-GR* (Fig. 1A) was introduced into *19-2* homozygotes and the resultant flies survived to adulthood with normal appearance (Fig 1D). Thus, the lethality of *19-2* is due to the lack of *leon* gene activity.

To identify specific *leon* alleles, ~4500 EMS-mutagenized alleles were tested for failure to complement the lethality of *19-2*. The alleles failed to complement *19-2* were further sequenced in the *leon* and *BtbVII* coding regions. Two lethal alleles carrying single amino acid substitutions in the ZnF UBP domain of Leon were isolated in the screen. The *leon*^{1} allele carries a substitution at Cys224 by Tyr, and the *leon*^{2} Gly225 by Arg (Fig. 1C). No amino acid substitutions in *BtbVII* were identified in *leon*^{1} and *leon*^{2}. We then examined the lethal phase of mutants with combinations of different *leon* alleles. Animals homozygous or transheterozygous for *19-2* and *leon*^{1} died at the late third-instar larval stage, whereas those homozygous for *leon*^{2} and transheterozygous carrying one *leon*^{2} allele survived to early pupal stages. These results indicate that *19-2* and *leon*^{1} are either null or close-to-null allele while *leon*^{2} is



alleles	*leon*^{2}	*leon*^{1}	*19-2*	*19-2,GR-leon-GR*
leon^2	pupae	pupae	pupae	Adult
leon^1		3rd instars	3rd instars	Adult
19-2			3rd instars	Adult

Fig. 1. Characterization of *leon* mutants. (A) Diagram shows *CG12082/leon* locus next to *BtbVII*, *P[EPgy2]BtbVII*^{EY07802} insertion, *19-2* deletion and the *GFP-leon-GR* construct which is the fusion of promoter (P), *leon* and *GFP* (G) as shown. (B) PCR detection of deleted regions in excision alleles. The pair of primers, as represented by red arrows in (A) was used to amplify 2.6 kb genomic sequence. The downshifted band of PCR product shown in *19-2* (lane 9) was sequenced to confirm the deletion. (C) Diagram shows Leon protein (827 a.a.) structure, including one zinc-finger ubiquitin binding domain (Z) in which mutations in *leon*^{1} and *leon*^{2} alleles were indicated, two ubiquitin-associated domains, U1 and U2, and one peptidase C19 domain that is split into a cysteine (C) and a histidine box (H). (D) Chart shows complementary results of *leon* mutants by lethal stages. The allelic strength is *19-2* = *leon*^{1} > *loen*^{2}. Introducing of *GFP-leon-GR* in *leon* mutants rescued the lethality of all allelic combinations to adult stage. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

a hypomorphic allele. Consistent with the rescue of *19-2*, the *GFP-leon-GR* transgene also rescued *leon*^{1} and *leon*^{2} mutants to adulthood, confirming that *leon* is essential for developmental processes.

3.2. Abnormality in brain lobes and imaginal disks in leon mutants

To study Leon expression, the mouse anti-Leon antibodies were generated against purified GST-Leon fusion protein. The Leon antibodies recognized a protein of slightly larger than 100 kDa, although the expected Leon size is about 91 kDa (Fig. 2A). The signal was completely absent in *19-2,* suggesting that *19-2* is a protein null allele of *leon*. The Leon expressions were normal in the protein size and levels in *leon*^{1} and *leon*^{2} homozygotes and the levels were reduced in *leon*^{1}/*19-2* and *leon*^{2}/*19-2* transheterozygotes. Immunostaining of larval tissues by Leon antibodies showed a ubiquitous expression pattern of Leon in all tissues. Leon expression was completely absent in *19-2* homozygous larvae (Fig. 2B).

The ubiquitous expression pattern of Leon suggests that Leon might function in several tissues. Consistently, null or close-to-null *leon* mutants survived to the late third-instar larval stage. While *leon* larvae appeared normal in body size, brain lobes and wing disks became smaller in *leon*^{1}/*19-2* mutants (Fig. 2C). The hypomorphic *leon*^{2}/*19-2* larvae that survived to early pupal stages displayed no such phenotypes. While there were differential tissue growth rates in hypomorphic and null mutants, both of them were slow in larval growth as compared to heterozygous siblings.

We then tested whether these defects were caused by the lack of *leon* and whether the *leon* enzymatic activity is involved. The ubiquitous expression of wild type Leon (*UAS-leon*) by *tubP-GAL4* rescued *leon*^{1}/*19-2* lethality and restored tissue development (Fig. 2C). We also generated the enzyme-dead Leon (*UAS-ED-leon*) with Cys341 substituted by Ala in the catalytic site. Ubiquitous expression of ED-Leon, however, failed to rescue animal lethality and tissue growth in *leon*^{1}/*19-2*. These results indicate that the deubiquitinating activity of Leon is essential for its role in development.

3.3. Leon is a deubiquitinase

Protein sequence alignment suggests that Leon is homologous to human USP5, *A. thaliana* UBP14 and yeast UBP14, which process polyubiquitin chains to mono-ubiquitin [14–16]. We tested whether Leon also functions in the same process. Purified GST-Leon effectively deconjugated Lys48-linked polyubiquitin chains to mono-ubiquitin, while GST had no such effect (Fig. 3A, left panel). When incubated with Lys63-linked polyubiquitin chains, the deubiquitinating activity of GST-Leon was less effective (Fig. 3A, right panel). We also purified the enzyme-dead ED-Leon, which failed to deconjugate both Lys48- and Lys63-linked polyubiquitin chains (Fig. 3A).

We next examined whether the ubiquitin levels are altered in *leon* mutants. In all *leon* mutants we examined, free polyubiquitin chains were increased, exhibiting a ladder pattern corresponding to Lys48-linked polyubiquitin chains (Fig. 3B). Whereas moderate increases were detected in hypomorphic *leon*^{2} and *leon*^{2}/*19-2* mutants, large increases were found in null or close-to-null mutants, *leon*^{1}, *19-2*, and *leon*^{1}/*19-2*. Interestingly, mono-ubiquitin levels were also increased in all mutants, which were more in hypomorphic mutants. In addition, smeared signals at high molecular weights representing substrate-conjugated polyubiquitin chains were also detected in null and close-to-null mutants. The levels of free polyubiquitin chains, mono-ubiquitin and substrate-conjugated polyubiquitin chains were differentially elevated in *leon* mutants, suggesting the complex regulation of ubiquitin homeostasis by Leon.

We then tested whether the enzymatic activity of Leon is required to suppress the levels of polyubiquitin chains *in vivo*. Ubiquitous expression of wild-type *UAS-leon* by the ubiquitous driver *tubP-GAL4* suppressed the formation of free and conjugated polyubiquitin chains (Fig. 3C, lane 3). However, ubiquitous expression of *UAS-ED-leon* failed to suppress polyubiquitin chain formation (Fig. 3C, lane 4). These results suggest that the accumulations of polyubiquitin chains were caused by the lack of Leon deubiquitinating activity.

3.4. Proteasomal degradation of substrates is interfered in leon mutants

Accumulation of polyubiquitin chains in *leon* mutants might block proteasomal degradation of ubiquitinated substrates, leading to their accumulations [14]. This interpretation could explain the accumulation of polyubiquitinated proteins in *leon* mutants on western blot analysis (Fig. 3B and C). To test whether proteasomal

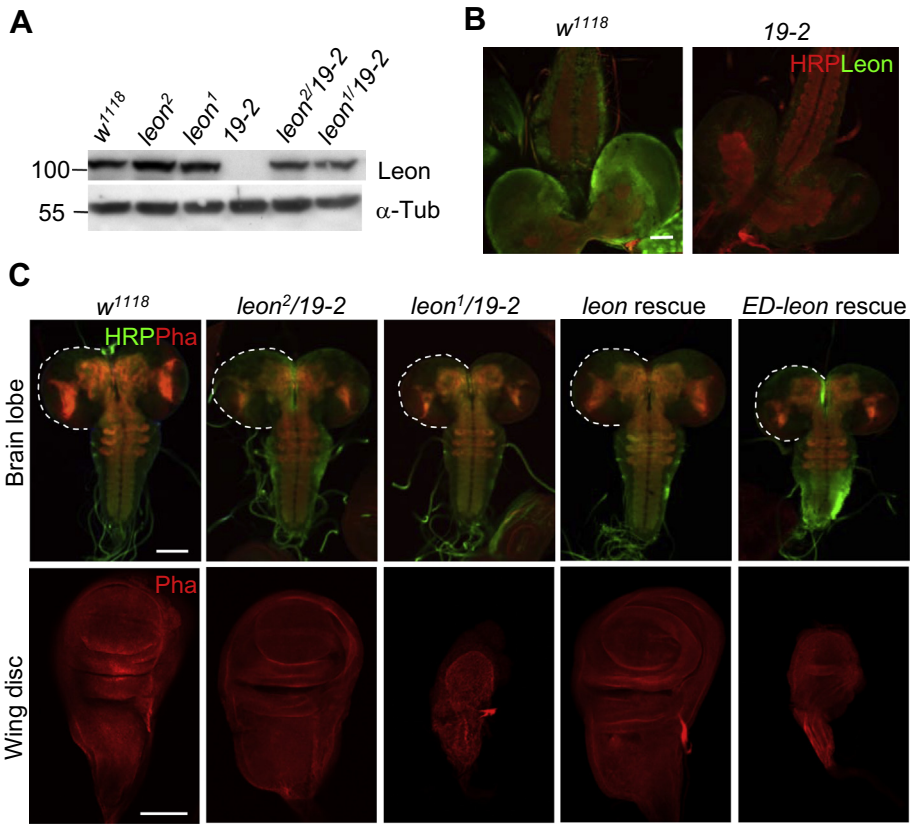


Fig. 2. The *leon* phenotypes. (A) Western blots of larval lysates of w^{1118}, homozygotes for $leon^2$, $leon^1$, and *19-2*, and transheterozygotes for $leon^1$/*19-2* and $leon^2$/*19-2* using antibodies against Leon and α-Tubulin (α-Tub). (B) Immunostaining of brain lobes shows ubiquitous expression of Leon (green) in w^{1118}, which is absent in *19-2* costained with HRP (red). Scale bar, 50 μm. (C) The morphology of brain and wing disks in w^{1118}, $leon^2$/*19-2*, $leon^1$/*19-2*, *tubP-GAL4 UAS-leon;* $leon^1$/*19-2* (*leon* rescue) and *tubP-GAL4 UAS-ED-leon;* $leon^1$/*19-2* (*ED-leon* rescue). Brains are costained with HRP (green) and phalloidin (Pha, red) with one brain lobe depicted. Wing disks are stained with Pha. Scale bar, 100 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

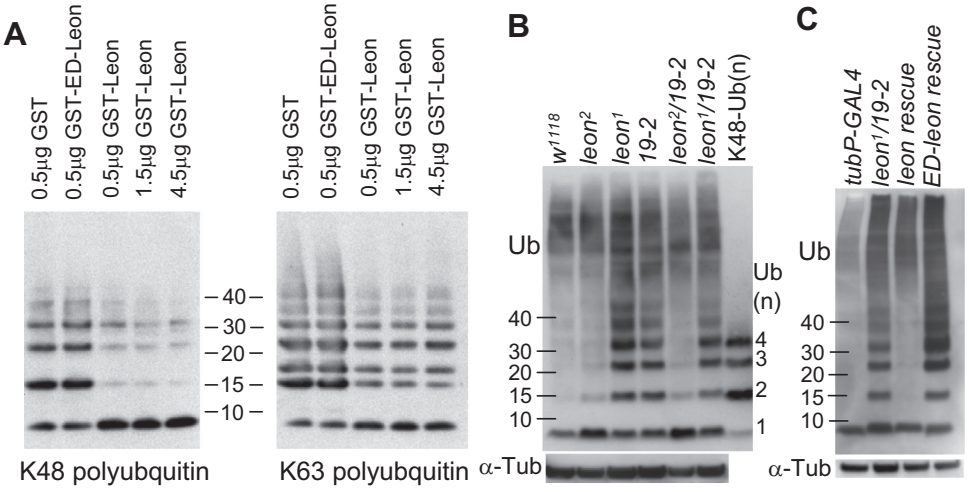


Fig. 3. Leon deubiquitinating activity. (A) Deconjugation of K48- (left panel) and K63- (right panel) linked polyubiquitin chains by GST-Leon (lanes 3–5 in left panel), but not GST (first lanes in both panels) and GST-ED-Leon (second lanes). (B) Western blots of w^{1118}, $leon^2$, $leon^1$, *19-2*, $leon^1$/*19-2*, and $leon^2$/*19-2* larval lysates with antibodies for ubiquitin (Ub) show monomers, dimers, trimers and tetramers of ubiquitin in parallel to K48-linked polyubiquitin K48-Ub(n) chains (rightmost lane). (C) Western blot of *tubP-GAL4* (lane 1), *tubP-GAL4;* $leon^1$/*19-2* (lane 2), *tubP-GAL4 UAS-leon;* $leon^1$/*19-2* (lane 3) and *tubP-GAL4 UAS-ED-leon;* $leon^1$/*19-2* (lane 4) using ubiquitin antibodies shows suppression of ubiquitin chain formation in *leon* mutants by wild-type *leon* but not enzyme-dead *ED-leon*. Anti-α-Tub antibodies are control in (B, C).

degradation is interfered in *leon* mutants, we examined the level of the proteasomal substrate CL1-GFP [18]. When expressed by *24B-GAL4* in muscles, CL1-GFP was almost undetectable (Fig. 4A). In hypomorphic $leon^1$/*19-2*, the levels of CL1-GFP were also almost undetectable. Strikingly, CL1-GFP accumulated at high levels in close-to-null $leon^1$/*19-2* mutants (Fig. 4A). Quantification of GFP intensities at muscles showed significant increases of CL1-GFP signals in $leon^1$/*19-2* mutants (Fig. 4B). These results, taken together

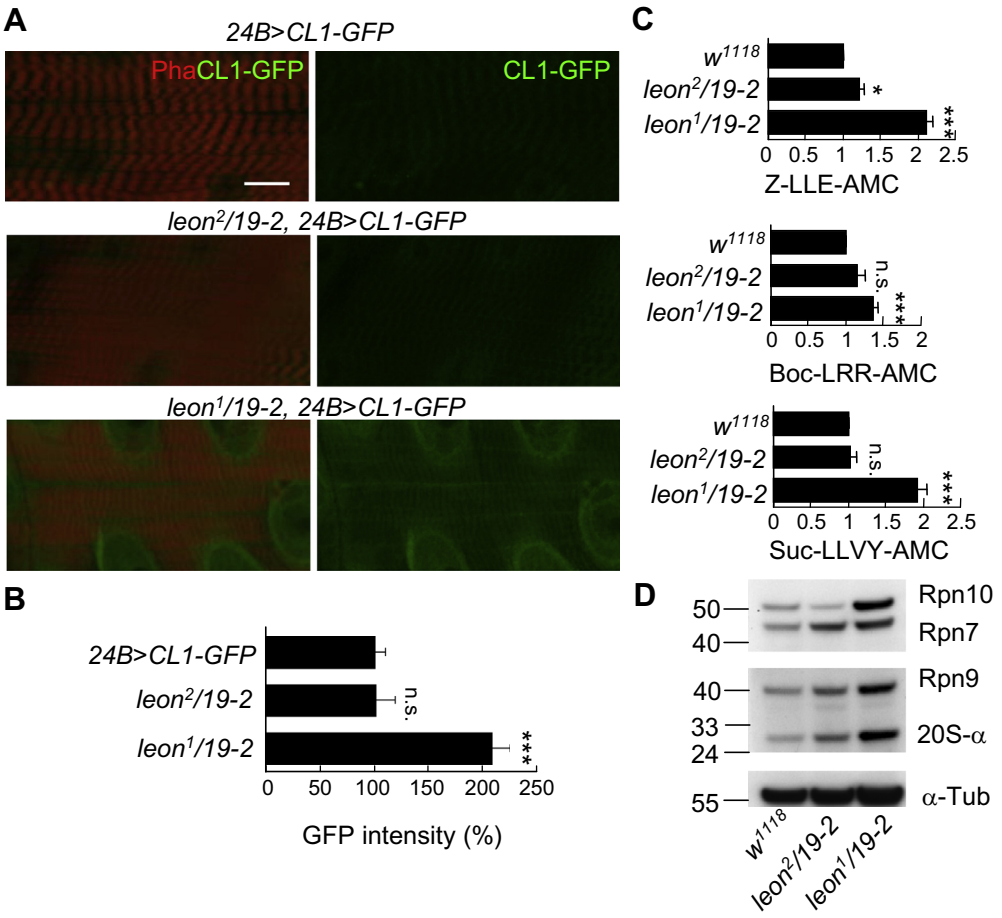


Fig. 4. Compromised protein degradation in *leon* mutants. (A) Images show expression of CL1-GFP by postsynaptic *24B-GAL4* (*24B > CL1-GFP*) that is elevated in $leon^1/19\text{-}2$ mutants. GFP (green) and Pha (red) double (left panels) and GFP single (right panels) channels are shown. Scale bar, 20 μm. (B) Quantification of CL1-GFP intensity at muscles as percentages to control. (C) Quantification shows elevations of proteasomal caspase-like, trypsin-like and chymotrypsin-like activities in $leon^1/19\text{-}2$, and normal activities in $leon^2/19\text{-}2$, compared to w^{1118}. n.s., no significance. $^{*}p < 0.05$ and $^{***}p < 0.005$ by Student's *t* test. (D) Western blots of w^{1118}, $leon^1/19\text{-}2$, and $leon^2/19\text{-}2$ larval lysates with antibodies for 20S-α, Rpn10, Rpn7 and Rpn9. Anti-α-Tub antibody as control. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

with western blot analysis (Fig. 3B and C), suggest that Leon is required for normal proteasomal degradation of polyubiquitinated substrates.

With the blockade of proteasomal degradation, we examined if the enzymatic activities of the proteasome were compromised. Fluorogenic substrates, Z-LLE-AMC, Boc-LRR-AMC and Suc-LLVY-AMC, were used to detect the caspase-like, trypsin-like and chymotrypsin-like activities of the proteasome, respectively [20]. Surprisingly, the enzymatic activities of *leon* mutants when compared to wild-type control were either normal in hypomorphic $leon^2/19\text{-}2$ or higher in close-to-null $leon^1/19\text{-}2$. While the proteasomal degradation of protein substrates were hindered, the results suggest that the accumulation of polyubiquitinated proteins in *leon* mutants is not caused by inactivation of enzymatic activities of the proteasome (Fig. 4C).

We have preformed a stable isotope labeling by amino acids in cell culture (SILAC) method for *Drosophila* [21], to compare proteomic expression profiles between wild-type and $leon^1/19\text{-}2$ mutant larvae. Among the differentially expressed proteins, we focused on the levels of proteasomal subunits in this analysis and found that all proteasomal subunits, including lid and core subunits, were detected in this analysis, consistent with the idea that all proteosomal subunits are abundant in cells [22]. Surprisingly, all proteasomal subunits were increased in protein levels to almost two folds in the $leon^1/19\text{-}2$ mutant (Table 1). To confirm the increase in the levels of proteasomal subunits, a collection of antibodies that recognize Rpn7 (P42A), Rpn9 (P39A) and Rpn10 (P54) of the 19S complexes and α subunit of the proteasomal core complexes were used in western blot analysis. Those four proteasomal subunits were all increased in $leon^1/19\text{-}2$ mutants. In $leon^2/19\text{-}2$, Rpn7, Rpn9 and α subunits were slightly increased and Rpn10 was slightly decreased (Fig. 4D). Combined with the fluorogenic substrate assay for enzymatic activities, higher proteasomal activity in $leon^1/19\text{-}2$ mutants is likely attributed to higher levels of the proteasome (Fig. 4C). The elevation of the proteasomal levels is likely to compensate for the interfered proteasome, whose capacity to degrade substrates is completely blocked in $leon^1/19\text{-}2$ mutants.

4. Discussion

The molecular action of USP5 family members was clearly depicted in many species [14–16]. However, the role of USP5 in animal development is not clear. Our study in *leon* mutants for the *Drosophila* USP5 has revealed roles in animal development. Being an essential gene, null or close-to-null and hypomorphic *leon* mutants were lethal at larval and pupal stages, respectively, suggesting that Leon is required at least in two distinct developmental stages. Interestingly, null or close-to-null but not hypomorphic *leon* mutants displayed severe developmental disorder in larval stages. Similar to USP5, Leon also has the ability to convert free polyubiquitin chains into mono-ubiquitin. Consistent with allelic strength in different combinations of mutant alleles, the amounts

Table 1
Chart shows elevation of proteasomal subunits in *leon*^{1}*/19-2* by SILAC method.

20S			19S lid			19S base		
CG number	Protein name	Ratio (H/L)	CG number	Protein name	Ratio (H/L)	CG number	Protein name	Ratio (H/L)
CG18495	α-1	3.2	CG42641	Rpn3	1.6	CG7762	Rpn1	1.99
CG5266	α-2	1.95	CG1100	Rpn5	1.6	CG11888	Rpn2	1.94
CG9327	α-3	3.11	CG5289	Rpn6	2.45	CG7619	Rpn10	2.27
CG3422	α-4	2.5	CG5378	Rpn7	1.69	CG1341	Rpt1	2.24
CG10938	α-5	2.54	CG3416	Rpn8	2.62	CG5289	Rpt2	2.45
CG4904	α-6	2.3	CG10230	Rpn9	2.32	CG16916	Rpt3	1.95
CG1519	α-7	2.53	CG18174	Rpn11	2.24	CG3455	Rpt4	2.17
CG8392	β-1	2.91	CG4157	Rpn12	2.05	CG10370	Rpt5	2.15
CG3329	β-2	2.48				CG1489	Rpt6	2.19
CG11981	β-3	2.48						
CG17331	β-4	3.5						
CG12323	β-5	1.67						
CG4097	β-6	2.73						
CG12000	β-7	2.83						

of free polyubiquitin chains correlate with the animal lethal phases and tissue disorders. Meanwhile, loss of *Drosophila* USP5 has been shown to cause severe apoptosis in eye disks [23]. It is possible that small brain lobes and wing disks in close-to-null *leon*^{1}*/19-2* were due to severe apoptosis. Ultimately, severe disruption of ubiquitin homeostasis in close-to-null *leon*^{1}*/19-2* might induce apoptosis and finally lethality. Hypomorphic *leon* mutants might tolerate mild disruption of ubiquitin homeostasis and delay apoptosis until pupal stages. This result also suggests that the stage of *Drosophila* metamorphosis is vulnerable to deficient ubiquitin homeostasis.

Accumulation of free polyubiquitin chains severely disrupts ubiquitin homeostasis, leading to the inhibition of proteasomal degradation of protein substrates. Accumulations of ubiquitinated protein substrates are evident in western blots, showing smear signals at high molecular weights in null or close-to-null *leon* mutants (Fig. 3B). By examining the proteasomal function reporter CL1-GFP, we also showed the inhibition of proteasomal function in *leon* mutants (Fig. 4A and B). Taken together, Leon/USP5 is important to maintain ubiquitin homeostasis, and the unbalanced ubiquitin levels *in vivo* have an impact on protein degradation by the proteasome. We also found that the proteosomal level and enzymatic activities were elevated when the proteasomal degradation was hampered. Thus, when the ubiquitin homeostasis is unbalanced and the proteasomal degradation is interfered, the proteasomal levels are elevated, likely to compensate for the initial deficiency in degrading polyubiquitinated proteins. The compensation mechanism eventually is not adequate to remove the accumulated proteins, leading to cellular toxicity.

Mutations in DUBs are associated with diseases including cancers and neurodegeneration [6,7]. For example, USP7 destabilizes tumor suppressor p53 through stabilizing Mdm2, the E3 ubiquitin ligase for p53 [24]. Also, loss of USP5 in mammalian cells also causes p53 stabilization by accumulation of free polyubiquitin chains [15]. Thus, different DUBs may target the same substrate, providing a multi-facet regulation or a cell-context dependent regulation. Until now, USP5 has been shown to link two carcinomas: alterations of USP5 isoforms in glioblastoma tumorigenesis and USP5 linked-suppression of p53 in melanoma [25,26]. Treatment of DUBs inhibitor EOAI3402143, targeting to USP9X and USP5, succeed to suppress melanoma growth [26]. Thus, DUBs could be the important targets for disease therapy [27].

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