UNIVERSITY OF CALIFORNIA, MERCED

OTUD6 deubiquitinates the 40S ribosomal protein RPS7 to increase protein translation and resistance to cellular stress

A dissertation submitted in partial satisfaction of the requirements for the degree Doctor of Philosophy

in

Quantitative and Systems Biology

by

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Committee in charge:

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Professor Fred Wolf

2022

The Dissertation of is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

X

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X

Professor Fred Wolf

University of California, Merced 2022

Dedication

I’d like to dedicate this to my wife, Josephine Sami, family, and friends. I would also like to dedicate it to my pups Pora, Penny, and Allie.

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Acknowledgements

I would like to acknowledge Dr Wolf and the rest of the Wolf lab for their insights and help in my project. I would also like to acknowledge Anika Padala in her help with fly maintenance and data collection.

I would like to acknowledge Aron Stahl and Seth Thomchik for generating learning and memory data for my project.

I would like to acknowledge Pankaj Dwivedi, Chris Rose, Don Kirkpatrick, and Vishva Dixit at Genentech for their invaluable contribution to assisting in designing, running, and analyzing mass spectrometry data for our proteomics experiments.

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Curriculum Vitae

Sammy Villa
Ph.D. Candidate
Quantitative and Systems Biology (QSB) | School of Natural Sciences
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Education

University of California, Merced CA **2022**
Ph.D. Candidate in Quantitative & Systems Biology
M.S. in Quantitative & Systems Biology **2019**

California State University, Sacramento **2015**
B.S. in Cell and Molecular Biology
Minor in Chemistry

Research Experience

University of California, Merced CA **03/2018-08/2022**
Graduate Researcher
My Ph.D. project seeks to determine the molecular mechanism of the deubiquitinase OTUD6 using the model organism Drosophila melanogaster. Using CRISPR Cas9, I created an OTUD6 null, two catalytically dead mutants, and a wild type and catalytically dead endogenously tagged fly lines. Using these mutants, I have found that OTUD6 plays a catalytic role in regulating the normal response to oxidative and alkylative stressors. I collaborated with Genentech and utilized their mass spectrometer to identify OTUD6 interacting proteins. I found that OTUD6 physically binds the 40S ribosome and several other proteins of interest. I have further discovered that OTUD6 deubiquitinates key ribosome subunits to regulate protein translation. Additionally, OTUD6 mutants display phenotypes consistent with impaired protein translation. My results have been presented at several conferences and a manuscript is in progress.

University of California, Merced CA **08/2018-05/2019**
NRT ICGE interdisciplinary research Project
As a part of the NSF funded Interdisciplinary Computational Graduate Education Program (NRT ICGE), I worked on a collaborative computational research project. I worked in a group of four graduate students from differing academic backgrounds to computationally track pollution in relation to wildfires. We gathered publicly available data from various government and private sources. We used several computational approaches to better understand the relationship between air quality and weather data, such as temperature, humidity, and wind speed. We presented our findings to a group of interdisciplinary graduate students and faculty.

University of California, Merced CA **12/2017-03/2018**
Xuecai Ge lab rotation
During my rotation in the Ge lab I designed, cloned, and transfected a CRISPR construct to knock out the Phosphodiesterase 4D interacting protein (Pde4dip) in two embryonic cell lines. Several loss of function mutants were

recovered, and the cell lines were used to characterize the role of Pde4dip in the Hedgehog signal transduction pathway. This research has been published (below).

California State University, Sacramento **06/2013-12/2015**
Independent Undergraduate Research
I started my undergraduate research as part of The California State University Louis Stokes Alliance for Minority Participation (LSAMP). ApoA-1 is the major protein component of the good cholesterol, High Density Lipoprotein. I expressed and nickel purified recombinant ApoA-1 and exposed wild type and mutant proteins to various detergents or pH conditions to measure the effect of protein aggregation. I presented my findings at several conferences (below).

Honors, Fellowships, and Awards

UC Merced USDA Scholar Award	2021-2022
QSB retreat student seminar award	2021
MCB Award for Excellence in Graduate Research	2021
2020-2021 QSB Summer Research Fellowship	2021
2020-2021 UC President's HSI Pre-Professoriate Fellowship	2020-2021
NSF Graduate Research Fellowship Program - Honorable Mention	2019
NSF NRT ICGE travel award	2018
UC Merced Graduate Dean's Relocation Award	2017
UC Merced Competitive Summer Bridge Program	2017
Hearst Minority Fellowship (University of Virginia) (Declined)	2017
Fall Academic Year CSU-LSAMP	2015
CSU-LSAMP	2013 and 2014
Summer CSUS Introduction to Scientific Research	2012

Publications

Villa, S., Wolf, F., The Drosophila deubiquitinase, OTUD6, regulates RPS7 ubiquitination during development and in response to cellular stressors. In draft

Peng, H., Zhang, J., Ya, A., Ma, W., **Villa, S**., Sukenik, S., & Ge, X. (2021). Myomegalin regulates Hedgehog pathway by controlling PDE4D at the centrosome. *Molecular Biology of the Cell*, *32*(19), 1807–1817. https://doi.org/10.1091/mbc.E21-02-0064

Presentations

The Drosophila Deubiquitinase, OTUD6, Regulates Ribosomal Ubiquitination **Villa, S.**, Wolf, F.W. UC Merced 2022 QSB retreat. Talk

The OTUD6 deubiquitinase associates with 40S ribosomes to regulate translation and responses to stressors in Drosophila. **Villa, S.**, Padala, A., Wolf, F. 2022 Ubiquitin Biology. Scheduled Talk.

The OTUD6 deubiquitinase catalytically regulates ribosomes in response to cellular stress. **Villa, S.**, Wolf, F.W. UC Merced 2021 QSB retreat. Short Talk.

The OTUD6 deubiquitinase catalytically regulates ribosomes in response to cellular stress. **Villa, S.,** Padala, A.,Wolf, F. 62nd Annual Drosophila Research Conference. 2021. Poster.

An OTU deubiquitinase plays a catalytic role in ribosome regulation in response to cellular stress. **Villa, S.,** Wolf, F. UC Merced Molecular and Cell Biology Seminar. November 2020. Talk.

An OTU deubiquitinase plays a catalytic role in the cellular response to DNA damage. **Villa, S.,** Wolf.,F. The Keystone Symposia Conference: Ubiquitin Biology, Snowbird, UT. 2020. Abstract accepted.

OTUD6 Plays a Catalytic Role in DNA Alkylation Repair Response. Padala, A., **Villa, S**., Wolf. F. The SACNAS National Diversity in STEM Conference, Honolulu, HI. 2019. Poster.

Tracking Wildfire Pollution. Davalos, O., Perez-Lopez, E., **Villa, S.**, Vargas, S. Interdisciplinary Computational Graduate Education Program Final Project Presentations, Spring 2019. Talk.

Expression and Purification of Recombinant Enterokinase for Laboratory Use**. Villa, S.,** LiWang, P. Competitive Summer Bridge Capstone presentations. Merced, CA. 2017. Talk.

Acidic pH Induces Oligomerization of the Fibrillogenic G26R Mutant of Human Apolipoprotein A-I. **Villa, S**., Ng, S., Rutaganira, A., Roberts, L.M. Annual CSUPERB meeting, Anaheim, CA. 2016. Poster.CSUS NSM research symposium, Sacramento, CA. 2015. Poster

Lysophosphatidylcholine Stimulates Aggregation in Amyloidogenic L178H Human Apolipoprotein A-I. Bhakta, M., **Villa, S.**, Villarruel, S., Gutierrez, C., Roberts,
L.M. Annual CSUPERB meeting, Santa Clara, CA. 2015. Northern California Undergraduate ACS Meeting, Sacramento, CA. 2014. Poster

The Effect of TritonX-100 on Aggregation in Wild-Type ApoA-I. **Villa, S.**, Roberts, L.M. CSUS NSM research symposium, Sacramento, CA. 2013. Poster.

Effects of Coffee Arabica and Citrus Paradisi on Breast Cancer Cell Growth. **Villa, S**. The SEE Undergraduate Student Research Symposium, Sacramento, CA. 2012. Talk.

Teaching Experience

Assistant instructor to QSB 296 Fall 2019 and Fall 2021

I assisted in teaching a graduate course in Professional Skills Development at UC Merced. The goal of the class is to teach graduate students' professional skills such as writing for fellowships/grants and effectively presenting their own research to an educated audience. I led several class discussions and helped come up with teaching material.

TA to Introduction to Nutrition Science Fall 2017 and Spring 2018

I led 3 50-minute discussions per week on topics pertaining to nutrients, human physiology, microbiology, biochemistry, and the psychology of wellness. I assisted in creating and implementing active learning models to engage the students.

SES English Tutor 2013-2014

As an SES tutor, I worked with low-income students at a school with a large concentration of low-income students (Title 1). I tutored 12 students in English as a part of an afterschool program through the Twin Rivers Unified School District. I developed and implemented learning strategies to engage and motivate the students to learn. Most of the students had learned English as a second language and had difficulty in their English coursework. By the end of the school year all the students significantly increased proficiency in English, as assessed by standardized testing.

Private Tutor 2011-2015

I tutored a variety of subjects from elementary to college level courses. This included one-on-one and group settings. I primarily tutored chemistry and biology to high school and college students.

Professional Experiences

Ampac Fine Chemicals 06/2016-07/2017

Lab Technician IV

Rancho Cordova, CA 95742

- Provide analytical chemistry support to multiple projects in accordance with company SOPs and protocols following GMP guidelines to support plant operations.
- Utilize Analytical chemistry work including: HPLC, FTIR, NMR, Coulometric KF, Titrimetric KF, LOD, Moisture analyzer, Densitometer, and SMB HPLC
- Preparation of chemical reagents and standards used for the chemical analysis of samples.
- Test and analyze plant and R&D samples within the specified turn-a-round time

Active Treatment Systems 11/2013-06/2016

Water Treatment Technician

Active treatment systems: 6301 Angelo Ct #9, Loomis, CA 95650

- Operated and maintained water treatment systems for construction stormwater, groundwater, and industrial water.
- Performed quality control analysis of effluent water to ensure discharge is within state determined specifications
- Perform site maintenance and calibrated necessary equipment for use in testing water
- Maintained accurate operational logs and prepared operational reports

Chemistry Stockroom Assistant 06/2012-06/2013

American River College, Fair Oaks, CA

- Assisted in preparing reagents and standards for use in chemistry labs
- Performed routine maintenance and calibration of precision lab equipment
- Managed accurate records of stock inventory
- Ensured that chemical labeling and storage met the appropriate requirements

Leadership and Mentoring

2021 QSB Retreat Planning committee 2021

I and one other graduate student organized the 2021 QSB retreat. This included planning out academic activities, inviting speakers, moderating student talks, and organizing networking events.

QSB Diversity Equity and Inclusion Committee Member 2020-2021

I served as a member of the QSB-DEI committee. The QSB-DEI is charged with developing and making recommendations to the QSB Executive Committee for promoting DEI initiatives in the QSB program.

QSB Graduate Student Representative (UC Merced) 2019-2021

I am the Graduate Student Representative for Quantitative and Systems Biology graduate group. I participate in several meetings, including the Graduate Student Association and the QSB Executive Committee. I solicit concerns, feedback, and perspectives of the QSB graduate student community to QSB Faculty, SNS Staff, Graduate Division, and the Graduate Student Association.

SACNAS graduate mentor (UC Merced) 2018-2022

I am the graduate mentor to the UC Merced SACNAS chapter. I assist undergraduates with issues and help facilitate collaborative projects with other programs on campus.

UC Merced Competitive Summer Bridge Graduate Mentor 2018

I worked with incoming graduate students individually and in groups to assist in the transition to graduate school. I created weekly workshops designed to expose students to graduate-level reading and presentation skills.

Graduate mentor to UROC Scholar (UC Merced) 2019-2022

I am mentoring a UROC scholar in scientific research. With my guidance she was able to gather enough data to present at several conferences.

Clifton Strengths Leadership Retreat 2018

I participated in a two-day retreat designed to identify and learn individual leadership skills using the Clifton Strengths assessment tool.

President/Treasurer CSUS MOSS 2013-2015

I served as the Treasurer and President of MOSS. The program provides a supportive system for students of diverse ethnic heritage, who are interested in pursuing careers in the health professions, math and science research, and teaching.

Abstract

OTUD6 deubiquitinates the 40S ribosomal protein RPS7 to increase protein translation and resistance to cellular stress

by Sammy L Villa

Doctor of Philosophy in Quantitative and Systems Biology

University of California, Merced

Professor Fred Wolf, Dissertation Advisor

Ribosome ubiquitylation is a highly regulated process that is essential for maintaining proper protein translation. While much is known about the role of ubiquitin ligases in these pathways, less is known about the function of deubiquitinases. OTUD6 is an ovarian tumor (OTU) family deubiquitinase that is conserved from yeast (OTU2) to humans (OTUD6A & B). To study OTUD6 in Drosophila, CRISPR/Cas9 was used to create a null mutant, two mutants with abolished catalytic activity, and epitope-tagged wild-type and catalytically dead (DUB-dead) endogenous OTUD6 fly lines. DUB-dead and loss-of-function OTUD6 mutants are markedly sensitive to oxidizing (paraquat) and alkylating (mms) agents that impact protein translation and ribosome function. Co-immunoprecipitation coupled with mass spectrometry in OTUD6.DUB-dead brains, where OTUD6 is highly expressed, identified 40S ribosome and RNA exosome proteins. The most significantly enriched 40S proteins, RACK1 and RPS3, play key roles in ribosome quality control and turnover under cellular stress. Mutation of RACK1 rescued the mms sensitivity of OTUD6 mutants, indicating that OTUD6 is likely a negative regulator of RACK1. RACK1 regulates ubiquitylation of several 40S ribosome subunits: a genetic screen of E3 ligases that ubiquitylate the 40S ribosome in a RACK1 dependent manner revealed that OTUD6.DUB-dead interacts epistatically with three E3 ligases. Two of the E3 ligases, RNF10 and RNF123, are implicated in RPS3 ubiquitylation in ribosome quality control. The additional E3 ligase, NOT4, is implicated in RPS7 ubiquitination. In support of OTUD6 regulating ribosome quality control or turnover, OTUD6 mutants have decreased protein translation, delayed development, and dramatically extended lifespan. Thus, OTUD6 deubiquitinates the ribosomal subunit RPS7, to regulate protein translation and the response to alkylation damage. Additionally, I show for the first time that RACK1 and RNF10 can regulate RPS7 ubiquitination.

Introduction

Protein ubiquitination is a reversible post translational modification in which a small polypeptide, ubiquitin, is added to a protein. Ubiquitination is a three-step enzymatic reaction that starts when ubiquitin is added to ubiquitin conjugating enzymes (E1), a reaction requiring ATP. The E1 enzyme then transfers the ubiquitin molecule to one of approximately 40 ubiquitin conjugating enzymes (E2). The ubiquitin charged E2 enzyme then interacts with a ubiquitin ligase (E3), which is responsible for substrate specificity and ligation from the E2 to substrate (*Rape,* 2018). Deubiquitinases (DUBs) are proteases that remove ubiquitin molecules from substrates and regulate ubiquitin dependent pathways. Many of the ~100 human DUBs have been implicated in diseases such as inflammation, neurodegeneration, cancer, and developmental impairments (*Clague,* 2012).

The mammalian deubiquitinase OTUD6B is highly conserved in yeast, OTU2, and Drosophila CG7857 (OTUD6), suggesting it has an essential cellular function. Patients with homozygous loss of function mutations for their OTUD6B gene are afflicted with intellectual disability syndrome, seizures, and dysmorphic features (*Santiago-sim*, 2017) (*Straniero*, 2018) (*Abdel-Salam*, 2021). It has been shown that OTUD6B physically interacts with translation initiation machinery and can regulate protein synthesis through its deubiquitination activity (*Sobol,* 2017). While OTUD6B has been shown to be a functional deubiquitinase (*Xu,* 2011), its linkage specificity and substrates remain unknown (*Mevissen*, 2013). Currently, there is no known mechanism of OTUD6 in regulating nervous system development or function. My project seeks to determine the substrates and mechanism of OTUD6 in the nervous system, using *Drosophila melanogaster*.

Protein translation is an essential cellular pathway in which new proteins are translated from messenger RNA (mRNA) into proteins by the ribosomes. This is a highly regulated process to prevent aberrant protein synthesis, which can impair cellular function (*Rubio,* 2022). There is increasing evidence outlining the importance of ribosomal ubiquitination events in regulating protein translation. For instance, during ribosomal collisions the 40S ribosomal subunit, RACK1 and the E3 ligase, ZNF598 regulate the ubiquitination of several 40S ribosomal subunits, to induce ribosome quality control pathways (RQC) (*Sundaramoorthy,* 2017) (*Juszkiewicz*, 2018). In this pathway the ASC-1 complex is recruited to the stalled ribosome to split the two subunits, dissociate the mRNA, and target stalled peptides for degradation by the proteasome (*Sitron*, 2017) (*Juszkiewicz,* 2020). There are additional E3 ligases and deubiquitinases that regulate the ubiquitination of specific subunits to regulate specific ribosomal outcomes.

RNF10 is an E3 ligase that is responsible for the monoubiquitination of RPS2 and RPS3 in several impaired translation pathways, including non-functional 18S rRNA decay (18s NRD) (*Sugiyama*, 2019), RQC (*Garzia,* 2021), and initiation RQC (iRQC) (*Garshott*, 2021). In 18s NRD, RNF10 monoubiquitinates RPS3, then polyubiquitinated by ZNF598 in the event of impaired 18s rRNA (*Sugiyama*, 2019). In RQC and iRQC, RNF10 monoubiquitinates RPS2 and RPS3 in response to blocked scanning or elongation of ribosomes. Persistent ubiquitination of RPS3 results in specific degradation of the 40S ribosome. USP10 is a deubiquitinase that can prevent the degradation of the 40S ribosome by deubiquitinating RPS2 and RPS3 (*Meyer*, 2020).

NOT4 is the E3 ligase subunit of the CCR4-NOT complex and is responsible for ubiquitinating RPS7 during translation to regulate the stability of mRNA with non-optimal codons (*Buschauer*, 2020) (*Allen*, 2021). Additionally, NOT4 has been shown to ubiquitinate RPS7 in response to endoplasmic reticulum (ER) stress to regulate the translation of specific mRNAs (*Matsuki*, 2020). NOT4 additionally ubiquitinates RPS7 during ribosome collisions, in which RPS7 is then polyubiquitinated by ZNF598 to regulate no go decay of mRNA (*Ikeuchi,* 2019). Indicating that RPS7 ubiquitination is dynamically regulated to allow transcript specific translation or mRNA degradation.

While a few deubiquitinases have been characterized to deubiquitinated ribosomal subunits, there are current gaps in the literature for other DUBs and how they may regulate these pathways. It's also not known if these pathways are differentially regulated in different tissues or if they impact development. My research implicates OTUD6 as a regulator of RPS7 ubiquitination in response to alkylation and to regulate protein translation.

Chapter 1 OTUD6 Catalytically Regulates Protein Translation and Response to Oxidative and Alkylative Stress

Summary

In this chapter I use CRISPR-Cas9 to create two catalytically dead, a true null, and a wild-type and catalytically dead endogenously tagged OTUD6. These alleles were used to find that OTUD6 regulates the response to oxidative and alkylative stress independent of DNA damage. We additionally find that OTUD6 physically interacts with the 40S ribosome and the RNA exosome. Heterozygous mutations for the 40S subunit, RACK1 rescues OTUD6 mutant sensitivity to the alkylation stressor mms. Rack1 facilitates the ubiquitination of ribosomal subunits by E3 ligases. A genetic screen of E3 ligases found three that rescue OTUD6 mms sensitivity. Suggesting OTUD6 opposes ribosomal ubiquitination of one or several ribosomal proteins. In support of OTUD6 regulating ribosome quality control or turnover, OTUD6 mutants have decreased protein translation, delayed development, impaired learning and memory, and dramatically extended lifespan. Taken together, these findings suggest that OTUD6 physically interacts with the ribosome and opposes ribosomal ubiquitination to regulate translation and response to oxidative and alkylative stress.

Results

OTUD6 catalytic activity is essential for normal response to oxidative and alkylation stress

To study OTUD6 in Drosophila, I utilized CRISPR-Cas9 to create two catalytically inactive, a complete null, and a wild-type and catalytically inactive endogenously tagged OTUD6 lines (**Figure 1A**). Both catalytically inactive mutants are homozygous viable, whereas the null is homozygous lethal. The null is viable when crossed to either catalytically dead mutant but isn't viable when crossed to an OTUD6 containing deficiency. Indicating that OTUD6 has an essential function independent of its catalytic activity. I also utilized a transposable element insertion line, OTUD6(EP95), that has an EP insertion located in the 5'UTR; it results in a significant depletion of OTUD6 mRNA levels (**Figure S1a**). Previously, OTUD6 transcripts have been shown to be increased in response to oxidative stressors, paraquat (PQ) and cadmium (*Brown*, 2014). To determine if OTUD6 plays a role in regulating oxidative stress, I exposed adult flies to PQ for 72 hours and measured survival. I found that OTUD6 mutants are significantly sensitive to exposure to oxidative stress (**Figure 1B**). Oxidative stressors can damage a wide range of cellular pathways. In order to narrow down the pathway OTUD6 that may play a role, I exposed OTUD6 mutants to the alkylating agent methyl methanesulfonate (mms). I found that both catalytic dead OTUD6(C183R) and loss of function OTUD6(EP95) mutants are homozygous sensitive to mms exposure (**Figure 1C**). Interestingly, the OTUD6(C183A) mutant has both a homozygous and heterozygous sensitivity the mms. Cysteine to alanine substitutions of deubiquitinases can form dominant-negative interactions due to increasing the affinity of binding ubiquitinated substrates (*Morrow*, 2018). We believe that OTUD6(C183A) is a dominant negative specifically in response to mms exposure due to heterozygous OTUD6(C183A) not having any sensitivity to oxidative stress.

Oxidative and alkylative stressors can damage DNA and RNA, which can impair protein translation (*Yan*, 2019). To determine if OTUD6 regulates DNA damage repair, Drosophila larvae were exposed to increasing doses of DNA damaging x-ray irradiation. There was no impact on survival compared to control, suggesting that OTUD6 mutants don't regulate x-ray induced DNA damage (**Figure 1D**). I next characterized OTUD6 protein in response to mms using the wild-type OTUD6-FLAG.HA line. I found that OTUD6 increases in global abundance in response to 24-hour exposure to 0.05% mms (**Figure 1E**). To determine OTUD6 localization, I performed immunohistochemistry in the Drosophila brain and ovary using OTUD6.FLAG.HA. I chose these tissues because OTUD6 mRNA levels are significantly enriched there (*Brown*, 2014). I find that OTUD6 has both nuclear and cytoplasmic localization in Drosophila nurse cells and has pan-neuronal localization in the Drosophila brain.

OTUD6 opposes 40S ribosome ubiquitination

To identify potential OTUD6 substrates I used the endogenously tagged catalytically inactive OTUD6 and performed co-immunoprecipitation followed by mass spectrometry (MS). This was done in collaboration with Genentech. We found that OTUD6 primarily interacts with the 40S Ribosome and RNA exosome (**Figure 2A**). I confirmed that OTUD6(C183A) physically interacts with the 40S ribosome by co-immunoprecipitation followed by western for RACK1 detection (**Figure 2B**). The most abundant hits for the 40S

ribosome are RACK1 and RPS3, two ribosome components that have been implicated in ribosome quality control. RACK1 is necessary to facilitate the ubiquitination of several ribosome components, including RPS3, following ribosome collisions (*Sundaramoorthy*, 2017). To test if OTUD6 regulates RACK1 dependent ubiquitination of the ribosome I performed a genetic test using heterozygous OTUD6(C183A) sensitivity to mms. Two heterozygous mutants for RACK1 were able to rescue OTUD6 sensitivity to mms (**Figure 2C**). This may indicate that OTUD6 opposes RACK1 dependent ubiquitination of the 40S ribosome. RACK1 is thought to act upstream of E3 ligases to facilitate the ubiquitination of the 40S ribosome. To find the E3 ligase(s) that OTUD6 opposes, I performed a genetic screen using E2 conjugating enzymes and E3 ligases that are responsible for ubiquitinating the 40S ribosome during stress or in a RACK1 dependent manner (**Figure 2D**). Genetic interactors were determined if the heterozygous double was significantly rescued compared to heterozygous OTUD6(C183A) but not significantly different from the other heterozygous mutant. Three E3 ligases, RNF10, NOT4, and RNF123, were able to rescue OTUD6(C183A) sensitivity to mms (**Figures 2E-2G**). RNF10 is responsible for ubiquitinating RPS2 and RPS3, whereas RNF123 is less characterized but has been shown to be able to ubiquitinate RPS3 (*Jung*, 2017). NOT4 has previously been characterized to ubiquitinate RPS7 to regulate translation. This data taken together implies that OTUD6 regulates the ubiquitination of one or multiple 40S ribosomal subunits.

OTUD6 regulates protein translation

Now that we know that OTUD6 may oppose 40S ribosome ubiquitination, I decided to look to see if OTUD6 mutants have phenotypes characteristic of impaired ribosome abundance or function. To determine if OTUD6 regulates protein translation I performed a puromycin incorporation assay in the Drosophila head, where OTUD6 is enriched. Puromycin was fed to the flies overnight and the flies were decapitated for western blotting. Both catalytically inactive OTUD6 mutants have significantly reduced puromycin incorporation into the Drosophila head (**Figure 3A**). To determine if OTUD6 is necessary for regulating translation in neurons, I performed an OTUD6 knock down and overexpression in neurons, using the pan neuronal elav(c155)-Gal4 line, and assessed protein translation. OTUD6 knock down by RNAi also reduces puromycin incorporation, whereas overexpression of OTUD6 increases it (**Figure 3B**). The OTUD6 RNAi significantly reduces OTUD6 protein levels in Drosophila neurons (**Figures S1I**). The bi-directional regulation by OTUD6 is likely due to changes in ubiquitination state of OTUD6 substrate(s). Further showing that OTUD6 catalytic dependent regulation of the ribosome is necessary to regulate neural protein translation.

Previous research has shown that global reduction in protein synthesis can extend longevity (*Syntichaki*, 2007) (*Kapahi*, 2004). I assayed longevity in OTUD6 mutants and found catalytic and loss of function mutants have extended lifespan in the absence of cellular stressors (**Figure 3C**). Drosophila mutants with impaired ribosome function or abundance are typically afflicted with the Minute phenotype, a class of phenotypes that include extended development, short and thin bristles, and poor fertility (*Marygold*, 2007). There is no obvious impact of OTUD6 mutations on Drosophila size, bristle formation, or fertility. However, I tested the time to eclosion and found that OTUD6 mutants have delayed eclosion compared to control (**Figure 3B**). Additionally, there is no effect on

survival from egg to adulthood (**Figure 3C**). This would suggest that the change in eclosion is due to developmental timing and not due to changes in survival.

I collaborated with the Seth Thomchik lab to test if OTUD6 mutants have impaired learning and memory due dysregulated ribosome function. Short term memory was assayed using a t-maze (*Tully*, 1985). We found that catalytically inactive OTUD6(C183A) mutant have significantly reduced learning and memory compared to our control flies. The loss of function OTUD6(EP95) flies had reduced learning that was trending but not significant. Difference in phenotypes can be due to differences in strength of mutations or type. Our data taken togethers shows that OTUD6 catalytic dependent regulation of the ribosome is necessary to regulate neural protein translation.

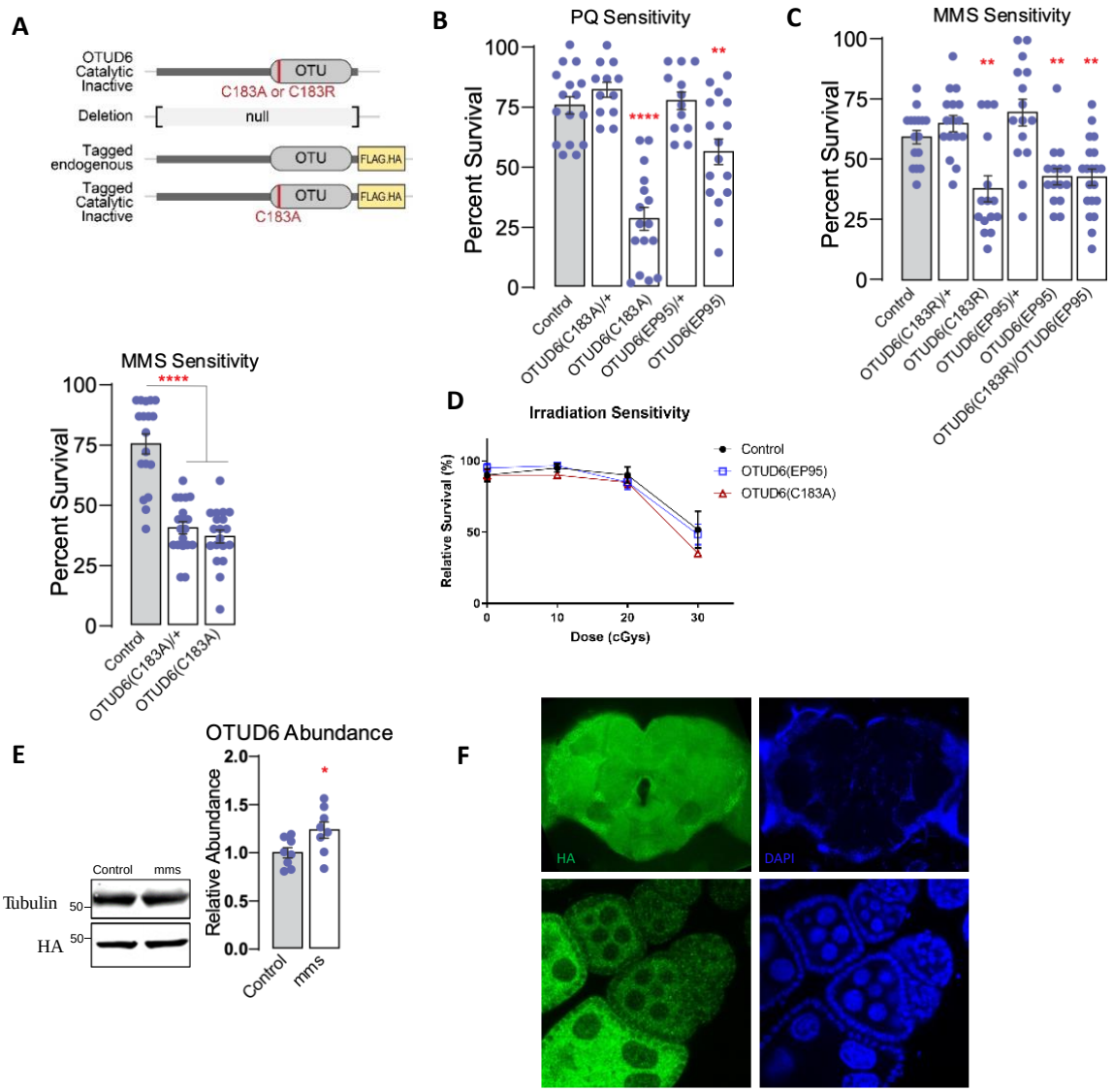
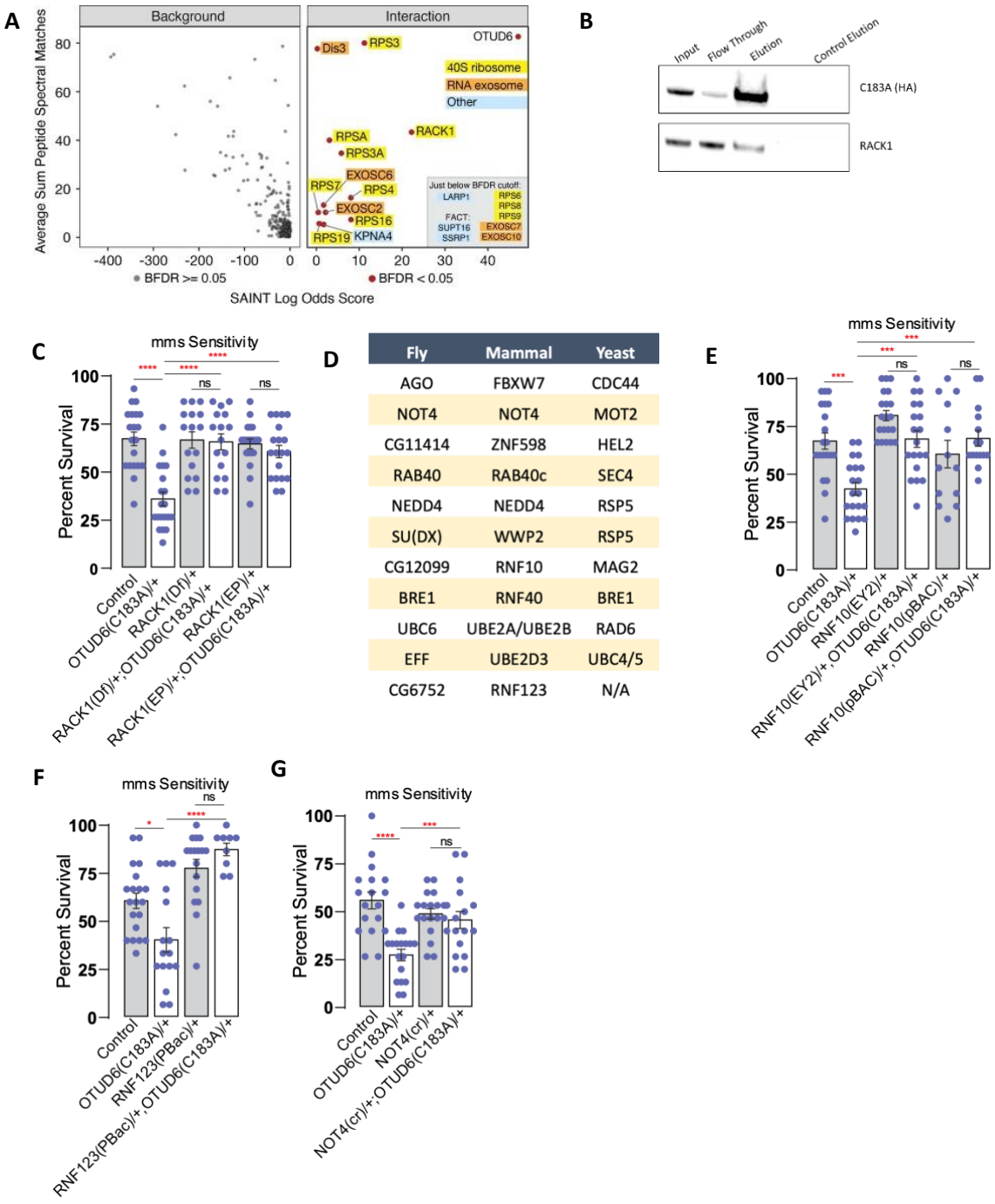


Figure 1. OTUD6 Plays a catalytic role in regulating the response to oxidative and alkylative stress. A. Drosophila OTUD6 mutant alleles and tagged endogenous forms. **B-C.** Adult OTUD6 mutants have decreased survival when exposed **B.** to 72 hours of 10 mM PQ and **C.** 32 hours of mms. Each data point represents the survival of a vial of 15 flies. One-way ANOVA/Dunnett's, compared to control. **D.** Relative survival of third instar larvae exposed to x-ray irradiation. **E.** Western blot analysis of whole fly lysate for OTUD6 levels using endogenously tagged OTUD6 following 24-hour mms treatment. OTUD6 levels normalized to tubulin. Two-tailed t-test. **F.** IHC of OTUD6 localization using OTUD6.FLAG.HA Drosophila. Top row is an adult Drosophila brain and the bottom row is a Drosophila ovary.



Fly	Mammal	Yeast
AGO	FBXW7	CDC44
NOT4	NOT4	MOT2
CG11414	ZNF598	HEL2
RAB40	RAB40c	SEC4
NEDD4	NEDD4	RSP5
SU(DX)	WWP2	RSP5
CG12099	RNF10	MAG2
BRE1	RNF40	BRE1
UBC6	UBE2A/UBE2B	RAD6
EFF	UBE2D3	UBC4/5
CG6752	RNF123	N/A

Figure 2: OTUD6 physically interacts with the 40S ribosome and opposes 40S ribosomal ubiquitination. A. Volcano plot of OTUD6 physical interactors. **B.** OTUD6 physically interacts with RACK1 as shown by FLAG co-immunoprecipitation followed by western blotting for RACK1 and C183A.FLAG.HA (HA). **C.** Genetic interaction analysis of heterozygous mutations of OTUD6 and RACK1 no mms sensitivity. **D.** List of E2 conjugating enzymes and E3 ligases tested for genetic interactions on mms sensitivity. **E-G.** Significant hits from genetic interaction screen. ANOVA/Tukey.

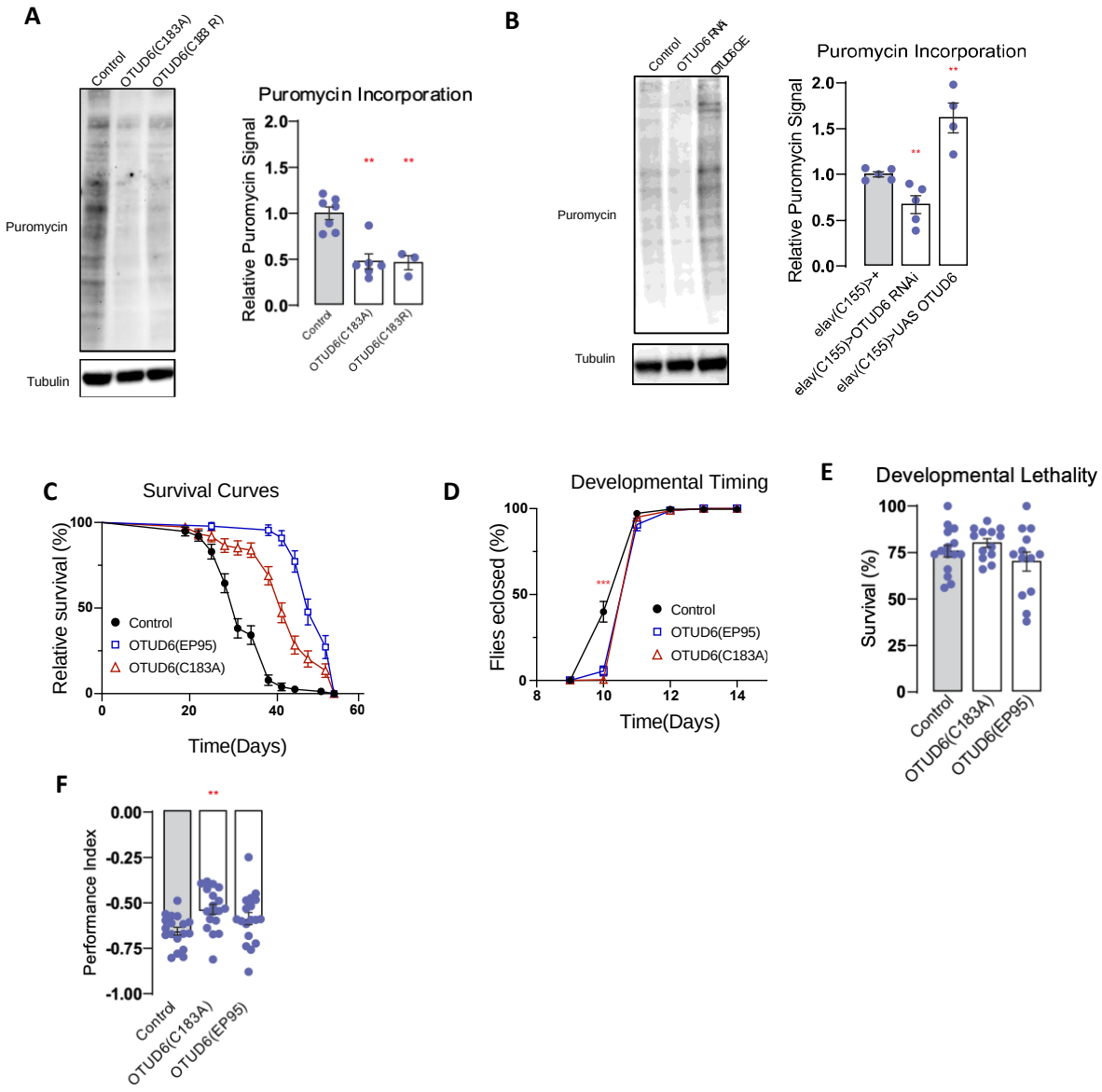
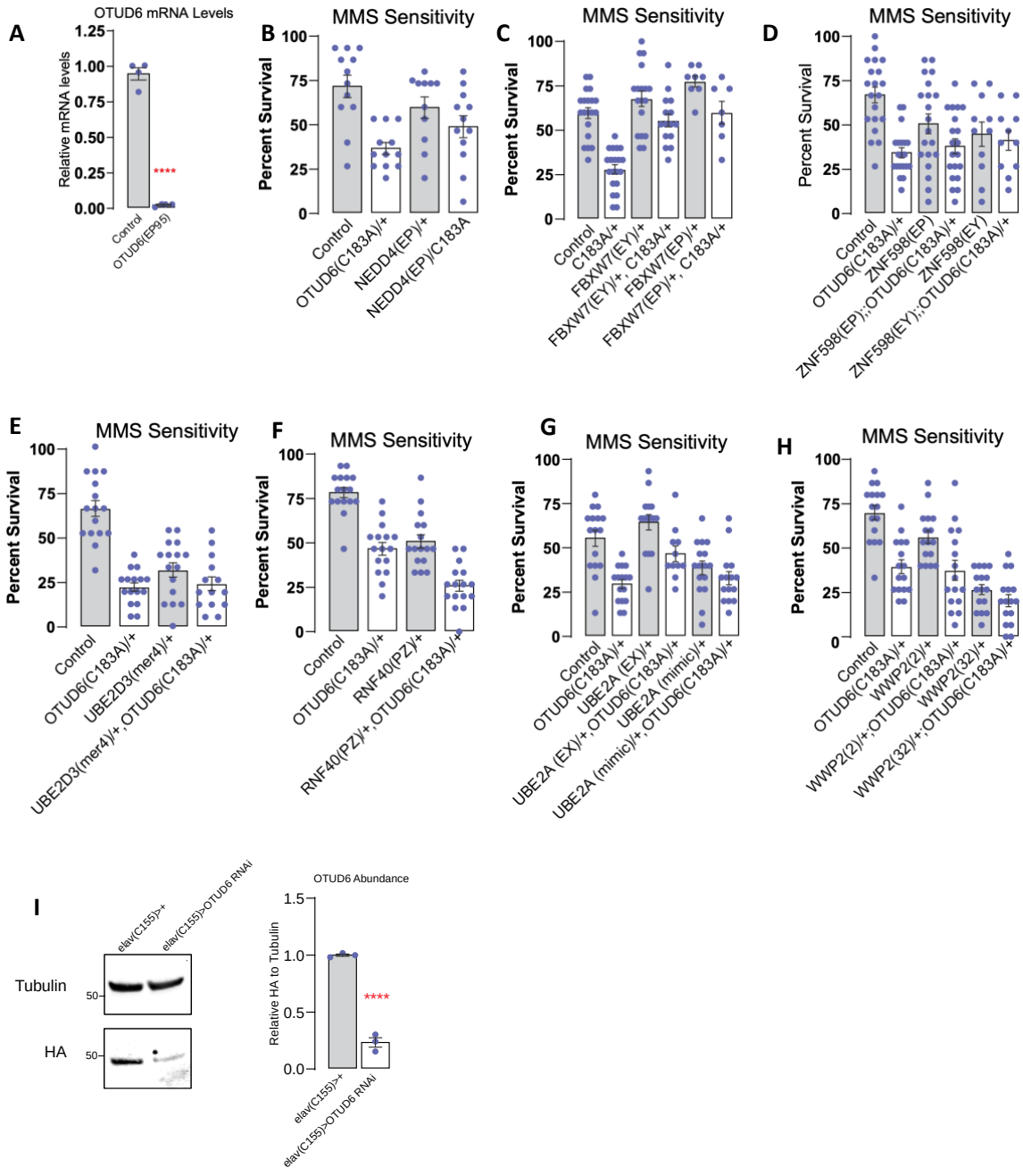


Figure 3. OTUD6 catalytic activity regulates protein translation. A-B. Western blot and quantification of puromycin incorporation assay using Drosophila head lysate. Puromycin signal was normalized to tubulin. One-way ANOVA/Dunnett's, compared to control. **C.** OTUD6 mutants have extended longevity as assayed by survival over time. Representative graph of three independent experiments. Curves compared using log-rank (Mantel-Cox). **D.** Developmental timing, from egg to adulthood, is extended in OTUD6 mutants. Graph displays cumulative eclosion rate. One-way ANOVA/Dunnett's, compared to control. **E.** OTUD6 mutants have no impact on survival from egg to adulthood. Graph represents percentage of eggs that made it to adulthood out of total eggs seeded. **F.** OTUD6 mutants have reduced short-term learning compared to control as assayed by T-maze. One-way ANOVA/Dunnett's, compared to control.



Supplemental Figure 1. A. qPCR analysis showing a significant decrease in OTUD6 mRNA levels in homozygous OTUD6(EP95) flies. Two-tailed t-test. **B-H.** Non-significant hits from E2 conjugating enzyme and E3 ligase screen. **I.** Western blot analysis showing OTUD6 RNAi reduces OTUD6 protein levels when expressed pan neuronally. Two-tailed t-test.

Discussion

Regulation of protein translation is an essential to prevent aberrant protein synthesis that can result in cellular stress or disease development (*Rubio*, 2022). Here I find that loss of function and catalytically dead OTUD6 mutants are significantly sensitive to oxidative and alkylative stressors. These stressors are known to damage a wide range of pathways, such as damaging mRNA and DNA (*Yan*, 2019). OTUD6 mutants have no sensitivity to the DNA damaging induced x-ray irradiation, indicating that OTUD6 mutants play a catalytic role in stress response independent of DNA damage.

OTUD6 is upregulated in response to alkylative stress and physically interacts with components of the 40S ribosome and RNA exosome. We currently don't know if the physical interaction of the exosome and ribosome are dependent on each other. If so, OTUD6 could be a direct regulator of mRNA decay pathways. Furthermore, OTUD6 genetically interacts with RACK1 in response to mms. RACK1 facilitates the ubiquitination of several 40S ribosomal subunits in response to ribosome collisions, or impaired ribosome function (*Sundaramoorthy,* 2017) (*Sugiyama*, 2019). OTUD6 opposes ribosome ubiquitination of several E3 ligases, suggesting that OTUD6 opposes ribosomal ubiquitination. However, it isn't known which ribosomal subunit(s) are regulated by OTUD6.

An interesting result from the genetic interactor screen is that a mutant for RNF40 enhanced the sensitivity of OTUD6(C183A) to mms (Figure S1C). RNF40 is responsible for K63 ubiquitination of the ribosome in response to oxidative stress (*Silva*, 2015). With OTUD6 mutants having a sensitivity to oxidative stress it would be interesting to see if OTUD6 and RNF40 act on oxidative stress in the same pathway. This could potentially expand the ribosomal oxidative stress response pathway.

OTUD6 additionally regulates protein translation independent of stressors. Indicating that OTUD6 regulation of the ribosome extends beyond just a role in stress response. Overexpression increased the amount of puromycin incorporation, whereas knockdown or loss of catalytic activity significantly reduced incorporation. Suggesting, that OTUD6 is normally a positive regulator of translation. This regulation of translation appears to be important during development due to OTUD6 mutants having impaired development compared to control. Additionally, the impaired short-term learning and memory can be due to impaired neural development.

Our model is that OTUD6 levels are constitutively upregulated in response to mms in order to deubiquitinate specific ribosomal subunit(s). Additionally, OTUD6 deubiquitinates ribosomal subunit(s) as a positive regulator of protein translation. My next chapter seeks to identify the specific substrates and characterize them in OTUD6 mutants in the context of alkylation stress.

Chapter 2 OTUD6 Regulates Free 40S RPS7 Deubiquitination

Summary

In this chapter I take a proteomics approach to identify OTUD6 substrates by selectively isolating ubiquitinated proteins and detecting changes by mass spec. I find that OTUD6(C183A) mutants have significantly increased levels of RPS7 ubiquitination by mass spec. I validate the hit by performing serial monoubiquitin isolation followed by western blotting for RPS7. I find that there is constitutive increase in ubiquitinated RPS7 in both catalytically dead mutants. Additionally, RPS7 is deubiquitinated in response to mms exposure and RNF10 and RACK1 regulate RPS7 ubiquitination. Expanding the role of RpS7 ubiquitination in regulating response to stress and adding additional mechanisms that regulate Rps7 ubiquitination levels.

Results

OTUD6 regulates RPS7 deubiquitination

To determine what substrate(s) OTUD6 is responsible for deubiquitinating, I performed a serial monoubiquitin isolation using Drosophila heads. Drosophila head extracts were incubated with beads that selectively isolate polyubiquitinated substrates. The extract that was incubated the beads was then incubated with beads that enrich for monoubiquitinated substrates. (**Figure 1A**). This allows for monoubiquitinated substrates to be selectively purified. This approach was chosen due to ribosomal subunits primarily being monoubiquitinated. Serial monoubiquitination enrichment was validated by probing the eluted substrates with an antibody that specifically recognizes polyubiquitinated substrates (FK1) and an antibody that recognizes total ubiquitin (p4d1) (**Figure S1A**). The polyubiquitin and monoubiquitin beads were washed and substrates were digested on the beads and ran on mass spec to identify differences in ubiquitinated proteins between control and OTUD6(C183A) flies. The 40S ribosomal protein, RPS7, is the only ribosomal protein that is significantly enriched in OTUD6(C183A) mono and polyubiquitin isolations (**Figure 1B and Figure S1B**).

Serial monoubiquitination enrichment in the Drosophila head followed by western blotting was performed in OTUD6(C183A) and OTUD6(C183R), to validate if RPS7 is a real hit. Both catalytically inactive OTUD6 mutants have a significant size shifted RPS7 band in the input, that is indicative of monoubiquitinated RPS7. This band is enriched in the elution suggesting it is indeed ubiquitinated (**Figure 1C**). To further validate this, OTUD6(C183A) head lysate was treated with recombinant USP2 or buffer and probed for RPS7 and ubiquitin. There is a significant decrease in total ubiquitin and loss of ubiquitinated RPS7 (**Figure S1C**). Additionally, driving an OTUD6 RNAi in neurons results in a significant increase in RPS7 ubiquitination, whereas overexpression significantly reduces it.

OTUD6 regulates RPS7 monoubiquitination in response to mms

OTUD6 co-immunoprecipitation in the presence of cycloheximide was performed to determine what state of the ribosome OTUD6 physically interacts with and if it's dependent on RPS7 ubiquitination. Cycloheximide blocks translation and the dissociation of the 80S into the 60S and 40S ribosome subunits. Catalytic dead OTUD6 exclusively interacted with ubiquitinated RPS7 independent of the 60s ribosome (**Figure 2A**). Thus, OTUD6 binds the free 40S when RPS7 is ubiquitinated. Next, to determine if neural RPS7 ubiquitination levels change in response to mms exposure, control and OTUD6(C183A) flies exposed to mms for 24 hours. Control flies show a significant decrease in RPS7 ubiquitination in response to mms (**Figure 2B**). Interestingly there is no difference in global ubiquitin levels of control flies exposed to mms (**Figure S2C**), suggesting changes in ubiquitination in response to mms is at specific proteins and not a global response. OTUD6(C183A) mms exposed flies also have significantly reduced RPS7 ubiquitination compared to the untreated OTUD6(C183A), however there is still significantly more than either control condition (**Figure 2B**). This decrease in ubiquitinated RPS7 in mms exposed OTUD6(C183A) could be due to redundancy in deubiquitinases or due to decreased RPS7 ubiquitination by E3 ligases.

The E3 ligase NOT4 monoubiquitinates RPS7, but the other OTUD6 genetic interactors have been implicated in ubiquitinating other ribosomal subunits. To determine if the

genetic interactions are due to RPS7 ubiquitination, RPS7 was probed for in mutants that rescued OTUD6(C183A) sensitivity to mms, in a homozygous OTUD6(C183A) background. There was a significant reduction in RPS7 monoubiquitination in both NOT4 and RACK1 heterozygous mutants and a homozygous RNF10 mutant compared to homozygous OTUD6(C183A) (**Figure 2C**). We additionally performed single step monoubiquitin isolation with heterozygous mutants in a heterozygous OTUD6(C183A) background and found the same effect on ubiquitinated RPS7 (**Figure S2A**). Thus, the rescuing effect of genetic interactors on mms sensitivity is likely due to the reduction of ubiquitinated RPS7. The RACK1 dependent decrease in RPS7 was additionally recapitulated in a homozygous OTUD6(C183R) background by western blot analysis (**Figure S2B**). This data, taken together, shows that OTUD6 dependent deubiquitination of the free 40S ribosome is necessary during alkylation stress. I additionally find that RACK1 and RNF10 can regulate RPS7 mono ubiquitination, although the context remains unknown.

Ubiquitinated RPS7 is incorporated in the translating ribosome

OTUD6 interaction with the 40S ribosome is independent of the 60s and is dependent on the monoubiquitination of RPS7. However, it is unclear if the 40S ribosome can be reinitiated when RPS7 is ubiquitinated. To test this, I performed co-immunoprecipitation of the translating ribosome using RPL3.3xFLAG in a wild type and OTUD6(C183R) background. I found that there is an increase in ubiquitinated RPS7 that is incorporated into the translating ribosome in the OTUD6(C183R) mutant (**Figure 3A**). I also found that there is increased monoubiquitinated RPS7 associating with mRNA isolated by polyDT isolation (**Figure 3C**). Monoubiquitination of RPS7 by NOT4 is important in regulating the mRNA stability during ribosome pausing (*Buschauer*, 2020). The SKI complex, which is required for cytoplasmic exosome activity, predominantly acts in translation-associated surveillance during ribosome stalls (*Tuck*, 2020). Thus, to determine if there is increased RNA turnover I looked to see if there is any change in exosome localization to the translating ribosome. I found that there is a significant increase in the catalytic domain of the exosome, DIS3, localizing to the translating ribosome. This may indicate that there is more mRNA turnover and that ubiquitinated RPS7 may be necessary for the exosome recruitment to the ribosome.

To determine if there is a genetic interaction between DIS3 and OTUD6, two heterozygous mutants for DIS3 were tested to see the impact on OTUD6(C183A) sensitivity to mms. Surprisingly, both mutations to DIS3 rescue OTUD6(C183A) sensitivity, indicating opposing roles in response to mms (**Figure 3D**). This may make sense since decreasing or blocking RPS7 ubiquitination can increase the stability of mRNA containing non optimal codons (*Buschauer*, 2020). It's possible that there is excessive degradation of these mRNA in OTUD6 mutants and that decreasing mRNA turnover by the exosome could stabilize essential mRNA prior to or during mms exposure.

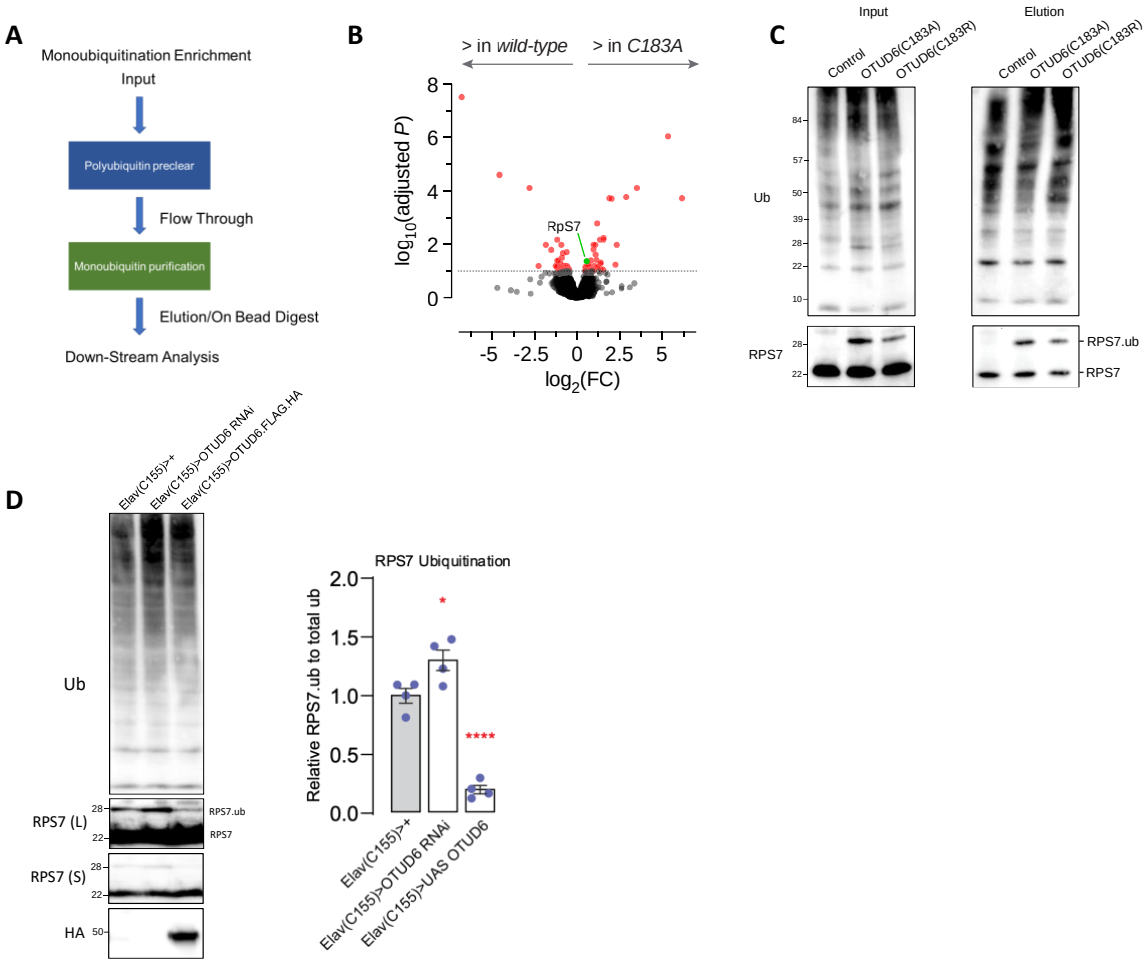


Figure 1. OTUD6 regulates RPS7 deubiquitination. A. Flow through of monoubiquitin enrichment followed by MS or western blotting. **B.** MS analysis showing differentially ubiquitinated substrates between OTUD6(C183A) and control. Red illustrates significant hits. **C.** OTUD6 catalytic mutants have increased RPS7 ubiquitination determined by monoubiquitin enrichment followed by western for RPS7 and total ubiquitin. **D.** Pan neuronal overexpression and knockdown of wild-type OTUD6 show opposing effects on RPS7 ubiquitination state by western. Ubiquitinated RPS7 normalized to total ubiquitin. One-way ANOVA/Dunnett's, compared to control.

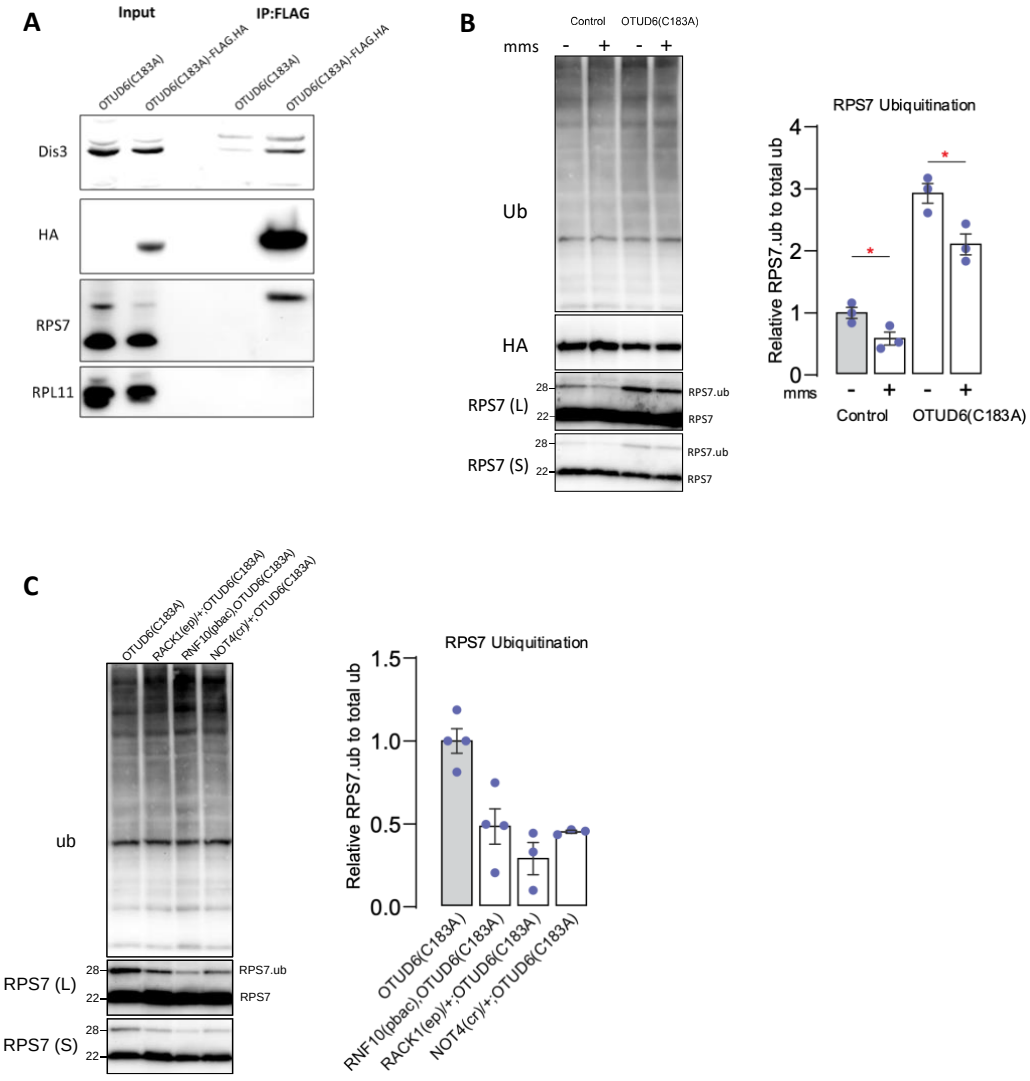


Figure 2 OTUD6 regulates RPS7 monoubiquitination in response to mms. A. FLAG co-immunoprecipitation followed by western blotting for RPS7 shows that catalytically inactive OTUD6 exclusively interacts with ubiquitinated RPS7 independent of the 60s. **B, C.** Western blot analysis of Drosophila head lysate and quantification of ubiquitinated RPS7 normalized to total ubiquitin. **B.** Wildtype and OTUD6(C183A) flies exposed to 0.05% mms for 24 hours. **C.** Head extracts of flies containing homozygous OTUD6(C183A) and doubles for genetic interactors to mms. Anova/Dunnet compared to control.

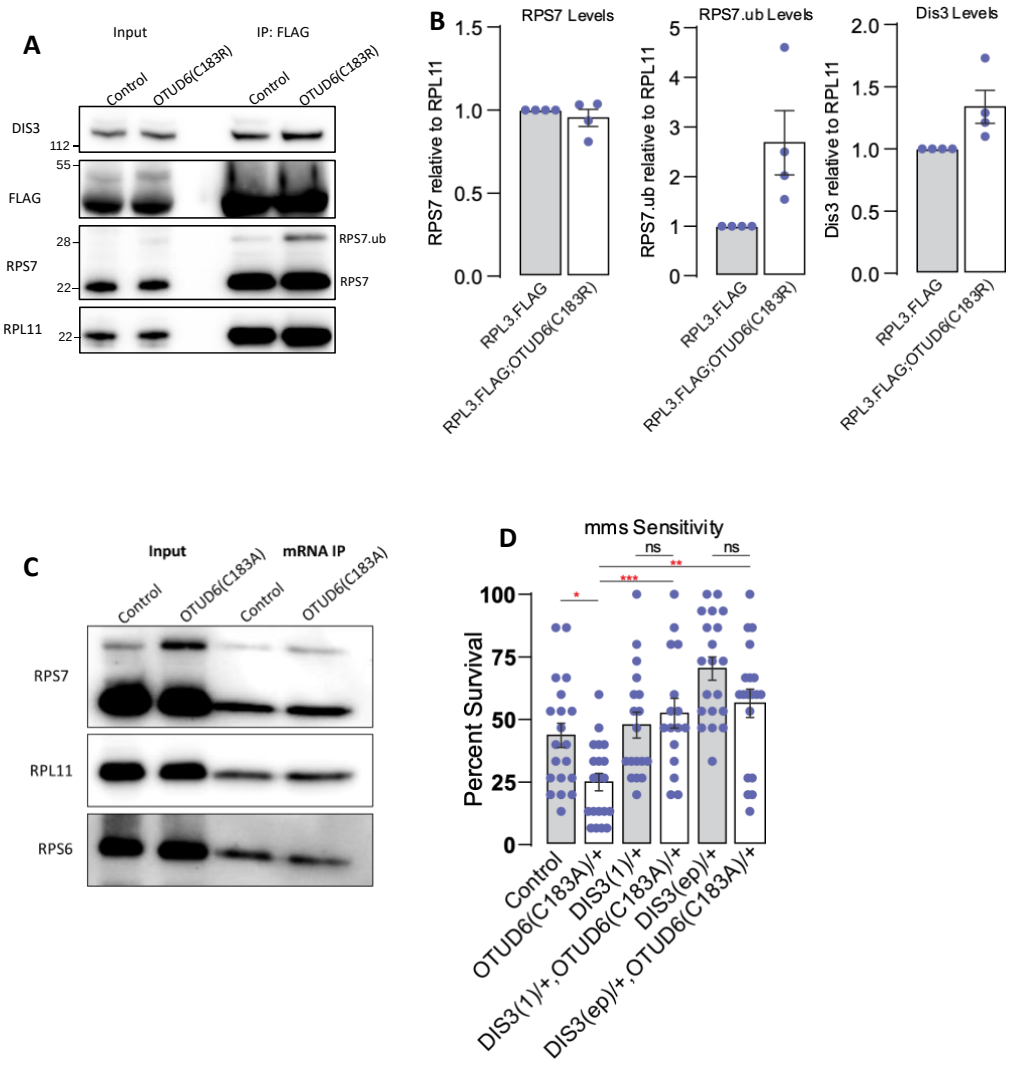
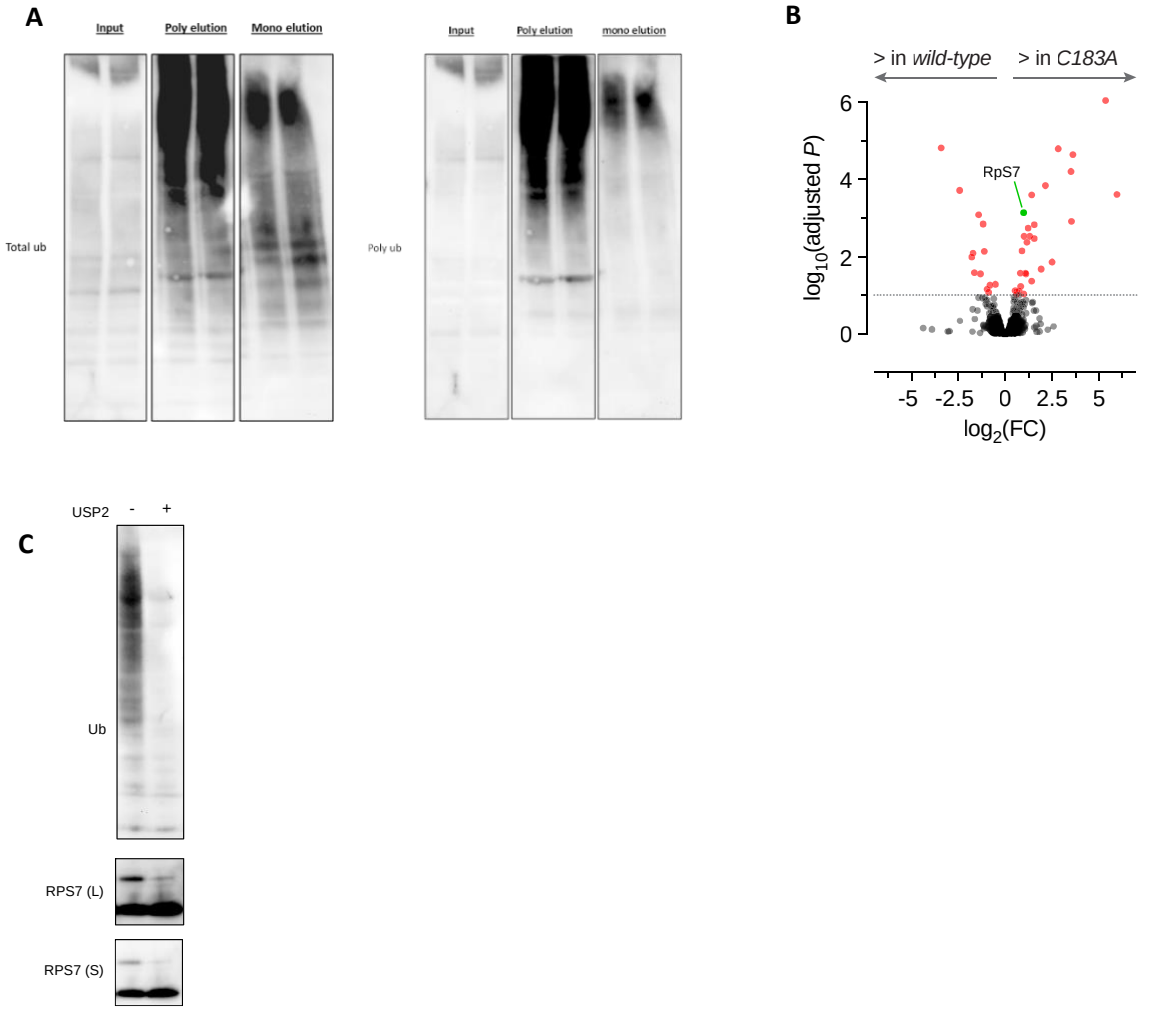
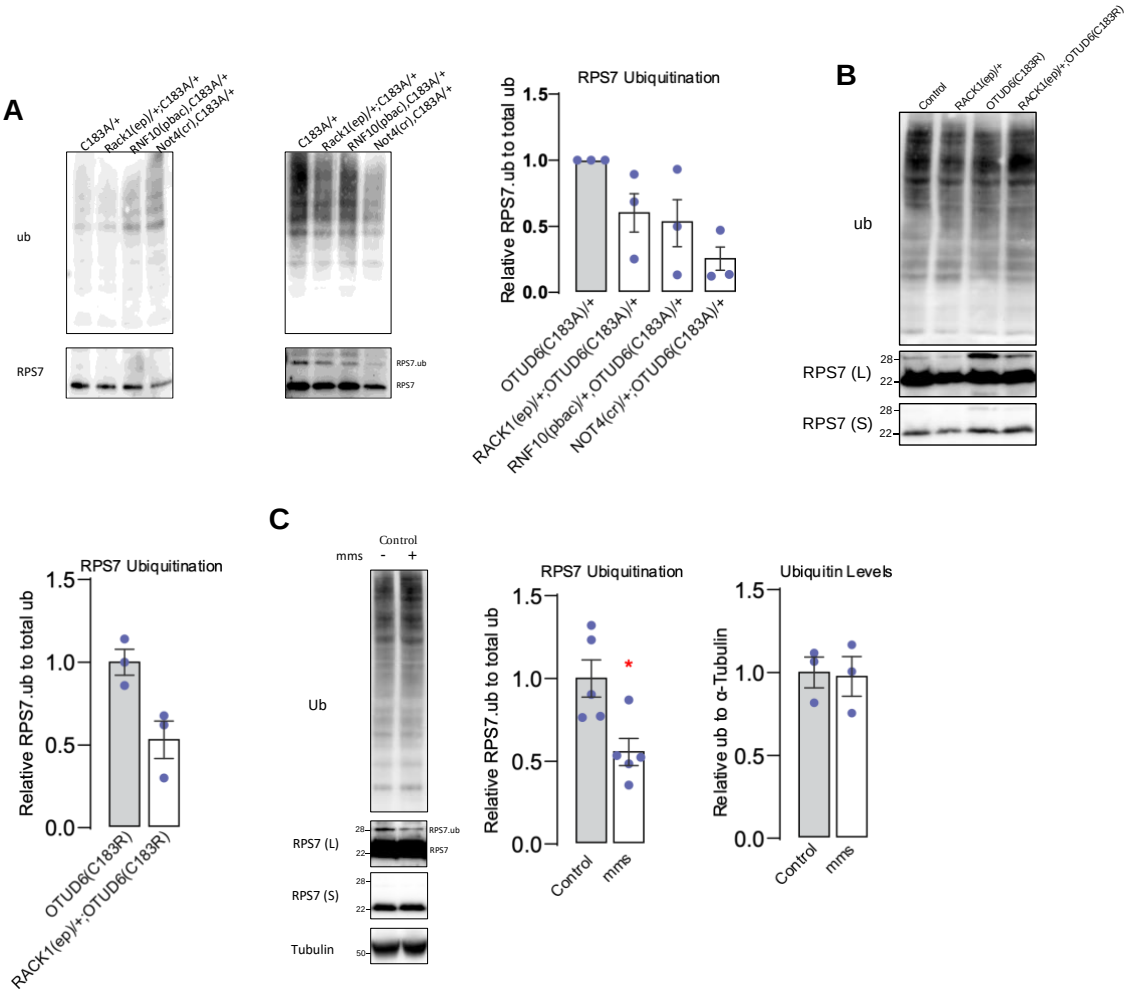


Figure 3. Ubiquitinated RPS7 is incorporated in the translating ribosome. A. FLAG co-immunoprecipitation of RPL3.3xFLAG in a wildtype and OTUD6(C183R) genetic background. **B.** Quantification of co-immunoprecipitation results across for blots. Two-tailed t-test. **C.** Western analysis of proteins physically interacting with mRNA following oligo(dT) capture. **D.** Genetic interaction analysis of heterozygous mutations of OTUD6 and DIS3 on mms sensitivity. Anova/Tukey.



Supplemental Figure 1. A. Western blotting for polyubiquitin (FK1) and total ubiquitin (p4d1) following serial monoubiquitin enrichment. **B.** Volcano plot of protein following polyubiquitin isolation and analysis by mass spectrometry. **C.** Western blot of OTUD6(C183A) lysate following treatment with recombinant USP2 or water.



Supplemental Figure 2. A. Monoubiquitin isolation of genetic interactors show a constitutive decrease in ubiquitinated RPS7 compared to heterozygous OTUD6(C183A) control. **B.** Western blot analysis shows a reduction in ubiquitinated RPS7 with the addition of a RACK1 mutant in homozygous OTUD6(C183R) background. Two-tailed t-test.

Discussion

OTUD6 physically interacts with the 40S ribosome specifically when RPS7 is monoubiquitated and independent of the 60s ribosome. This limits OTUD6 activity to regulating RPS7 ubiquitination to the free 40S ribosome, however we don't know at what step or for what particular purpose. This has recently been reported in yeast, where yeast OTU2 deubiquitinates RPS7 on the free 40S to release mRNA and enable ribosome recycling (*Takehara*, 2021). However, their data only showed that OTUD6 physically interacts with the free 40S that is bound to mRNA and didn't differentiate between translation termination or the scanning 40s ribosome. This could explain why there is increased incorporation of ubiquitinated RPS7 in the translating ribosome. There may be specific pathways in which RPS7 deubiquitination is essential but others where it is dispensable. Additionally in their paper there was no obvious impact on growth or translation *in-vivo* except with the addition of a second mutation for an additional deubiquitinase, USP10. This could suggest that there are differences in OTUD6 regulation between yeast and Drosophila.

RPS7 ubiquitination is reduced in response to mms exposure in both the control and OTUD6(C183A) flies. However, there is still a significant increase in ubiquitinated RPS7 in the OTUD6(C183A) compared to treated or untreated control, indicating the difference is due to OTUD6 specific deubiquitinase activity. Additionally, mutants for RACK1, RNF10, and NOT4 reduce ubiquitinated RPS7 in an OTUD6 mutant background, implicating RACK1 and RNF10 in regulating RPS7 ubiquitination for the first time. This also implies that preemptively reducing RPS7 ubiquitination prior to mms exposure rescues the mms sensitivity. Under normal conditions, OTUD6 deubiquitination of RPS7 is essential for normal response to mms.

Ubiquitinated RPS7 is increased in the translating ribosome even though OTUD6 appears to only interact with the free 40S. It's possible that ubiquitinated RPS7 containing free 40S ribosomes predispose the ribosomes to stall during translation. It's also possible that free 40S ribosomes containing ubiquitinated RPS7 may favor specific mRNA, such as low codon containing mRNA, resulting in more stalls and mRNA turnover. This could explain why there is consistent ubiquitination of RPS7 and increased exosome localization. It has been seen in yeast, where RPS7 is ubiquitinated in response to ER stress to selectively translate specific mRNA needed for stress resolution (*Matsuki*, 2020). It's possible that OTUD6 deubiquitination is important to prevent or reduce the translation or stability of specific transcripts.

OTUD6 physically interacts with components of the exosome and the 40S ribosome. There is an increase in exosome localization to the translating ribosome in OTUD6 mutants with increased RPS7 ubiquitination. Additionally, heterozygous mutations for DIS3 rescue OTUD6 sensitivity to mms. This strongly suggests there is a regulatory role of OTUD6 deubiquitination of RPS7 that regulates the exosome localization to the ribosome. It's yet to be seen if there is increased localization to free 40S during scanning or post translation termination, as OTUD6 specifically binds the free 40S. It's possible that OTUD6 binds ubiquitinated RPS7 containing 40S ribosome to regulate mRNA degradation by the exosome in some way. Future studies would need to further tease out the relationship between OTUD6 and the exosome and if specific mRNAs are affected.

Chapter 3 Conclusions

Conclusions

My data strongly implicates OTUD6 in regulating protein translation and response to alkylation stress through deubiquitination of RPS7. Ribosomepathies are class of developmental disorder that result from impaired ribosome biogenesis or function (*Hetman*, 2019). My data shows that Drosophila with compromised OTUD6 function have impaired neural protein translation, developmental delays, and extended longevity, which are consistent with impaired ribosome function. OTUD6 regulation of RPS7 ubiquitination could be a potential mechanism for patients that have homozygous OTUD6B mutations. Consistent with this hypothesis, there is evidence that OTUD6B can deubiquitinate components of the preinitiation complex and regulate translation (*Sobol*, 2017). Suggesting that this is a conserved pathway.

In the absence of stress, we know that OTUD6 acts on ubiquitinated RPS7 containing 40S ribosomes to regulate aspects of translation. However, it isn't known all the instances that would result in RPS7 ubiquitination during translation. RPS7 is ubiquitinated at translation initiation and in response to non-optimal codons in a NOT4 dependent manner. If non-optimal codon induced stalls aren't resolved, then the ribosome is split and mRNA is degraded (*Buschauer*, 2020). It's possible OTUD6 acts in these pathways but it's unclear how this would impact translation or allow for persistent RPS7 ubiquitination in the translating ribosome. Moving forward, it would be important to determine different 40S stages to determine how OTUD6 regulates recycling and at what.

RPS7 ubiquitination is important in regulating the response to ER stress where it ubiquitinated by NOT4 to regulate the translation of specific mRNA (*Matsuki*, 2020). Additionally, RPS7 is mono and then polyubiquitinated in response to collisions, in order to regulate no-go decay (*Ikeuchi*, 2019). I found RPS7 is deubiquitinated in response to mms, suggesting that RPS7 ubiquitination status is dynamically regulated in response to specific stressors. It's possible OTUD6 regulates RPS7 deubiquitination to prevent improper translation of stress specific mRNA. Additional studies would be needed to see if there

There have been attempts to determine OTUD6 physical interactors in high throughput screens, yet the interaction with the ribosome had been missed (*Sowa*, 2017). This is likely due to the fact that most of the high throughput proteomics rely on overexpression of tagged proteins. My data shows that the interactions with the endogenously tagged catalytic mutant is consistent and dependent on the ubiquitination of RPS7. Additionally, overexpression significantly decreased the amount of ubiquitinated RPS7, which is likely why it has previously not been seen in these assays.

Future studies are to further determine the effect of OTUD6 on translation. We still don't know if OTUD6 is essential for one or multiple steps of ribosome recycling. It would be important to determine how this differs from the response to alkylation stress response. It would also be worth looking at how general of a response RPS7 ubiquitination and deubiquitination is in response to different types of stress conditions. Most studies look to see increases in specific ubiquitination ribosomal ubiquitination events that happen during stress response. However, deubiquitination events may be as important and may be necessary for the proper ubiquitination to occur.

Moving forward it would be important to investigate the role of RACK1 and RNF10 in ubiquitinating RPS7. At the moment there is no known mechanism or context for when they ubiquitinate RPS7 and if they are dependent on eachother. It's possible that RNF10 acts redundantly with NOT4 but it's possible it acts on separate ribosome states or distinct mRNAs. It would be interesting to see if the RPS7 ubiquitination by RNF10 is in a different pathway than RNF10 has been previously characterized in.

Materials and Methods

Fly husbandry and strains

All flies were reared on corn meal molasses food. All lines were outcrossed to the same genetic background (w^{1118}). Lines were reared at 25°C at 60% humidity.

Generation of OTUD6 Mutants

Generation of UAS-OTUD6.FLAG.HA

A plasmid containing a FLAG.HA tagged OTUD6 under control of a UAS promoter was obtained from the Drosophila genome resource center (UFO07783). The plasmid was injected into attP containing pupae on the second chromosome (BDSC:9736), by BestGene.

To generate the epitope and catalytic dead OTUD6 mutants, a single guide RNA (gRNA) was utilized near site of mutagenesis. A single stranded oligodeoxynucleotide (ssODNs) containing the desired mutations or FLAG.HA tag was used a template for integration of the desired mutations. All gRNA constructs were cloned into the pU6-BbsI-chiRNA plasmid and injected into the yw; attP40{nos-Cas9}/CyO fly line by BestGene. See reagents list for specific sequences of gRNA and ssODNs.

Paraquat and MMS exposure

Fifteen adult males 2-5 days post-eclosion, were fed 10 mM PQ (Sigma 856177) or 0.05% mms (Fisher Scientific AC156890050) on 1.5% low melt agarose containing 5% sucrose. For PQ, survival was assayed every 12 hours over 72 hours. For mms, survival was assayed every 2 hours from 24 to 36 hours. Each exposure was repeated across a minimum of 3 separate days for a minimum of 12 biological replicates of 15 males per genotype tested.

X-ray irradiation

A 6-hour timed collection was performed at 25°C using Drosophila apple juice agar plates (doi:10.1101/pdb.rec065672). 20 Third instar larvae were moved to dishes containing normal food. The larvae were exposed to various doses of irradiation at 110 rad/min. Survival was determined by the number of larvae that survived to adulthood.

Eclosion timing

To quantify developmental timing a timed collection was performed for 6hrs at 25°C. Following the collection 50 eggs per genotype and replicate were moved to vials containing normal food and kept at 25°C. The number of flies to eclose was counted daily at the same time each day. Survival from egg to adulthood was determined by taking percentage of eclosed adults against the total number of eggs seeded into the vials.

Western blotting

5-10 adult fly heads were homogenized in RIPA buffer (Sigma 20-188) that was supplemented with 1x Complete, Mini, EDTA-free protease inhibitor. The homogenate was centrifuged at 12,000 x g for 5 minutes at 4 °C. 30µLof the lysate was retained and recombined in 10µLof 4x NuPage LDS buffer (NP0007) containing 5% 2-mercaptoethanol

(sigma M6250). The samples were heated for either 3 minutes at 95°C or 10 minutes at 70°C. 20-40µgof lysate was ran on an Invitrogen 4-12% Bis-Tris gel (NP0322) and MOPS running buffer (NP0001). Proteins were transferred to a PVDF membrane (Millipore IPFL00010), blocked in either 5% non-fat dry milk or 5% BSA. Blots were probed using rabbit anti-HA (3724S, Cell Signaling) (1:1,000), mouse anti-FLAG (F3165, Sigma-Aldrich), rabbit anti-α-Tubulin (2125S, Cell Signaling) (1:10,000), mouse anti-α-Tubulin (T6074, Sigma-Aldrich)(1:10,000), mouse aniti-ubiquitin (P4D1)(3936, Cell Signaling)(1:2,000), mouse anti-polyubiquitin (FK1)(04-262, Sigma-Aldrich), mouse anti-puromycin (PMY-2A4)(1:1,000), rabbit anti-RPL11 (A303931AT, Bethyl laboratories)(1:1,000).

Translation quantification

To assay translation levels in the brain, 2–4-day old flies were starved for 6 hours before being fed 600 µM puromycin in a 5% sucrose solution containing 3% ethanol for 16-18 hours. Flies were decapitated and homogenized in RIPA buffer and 20µgof extract was used per condition. Puromycin incorporation was quantified by western blot using anti-puromycin and normalized to α-Tubulin.

Co-Immunoprecipitation

Antibody coupling

To make anti-FLAG beads 10 µg of mouse anti-FLAG (F3165; Sigma-Aldrich) antibody was bound per 1mg of beads Dynabeads M-270 epoxy (143-02D; Invitrogen). Antibody was coupled to the beads using the coupling kit protocol and the beads were suspended at 10 mg/mL.

Co-immunoprecipitation

To perform co-immunoprecipitation, 900 tagged OTUD6(C183A) and untagged OTUD6(C183A) flies were collected. Flies were decapitated using liquid nitrogen and collected using a sieve. The heads were homogenized in 1mL of lysis buffer (10mM hepes buffer PH7.4, 150 mM NaCl, 5mM MgCl2, and 0.5% Triton X-100). The lysate was centrifugation at 20,000 xg for 20 min at 4°C and filtered through a 0.45µm low protein binding PVDF syringe filter (Millipore, SLHVX13NL). 500µL of extract was incubated with 200µl of prewashed mouse anti-FLAG magnetic beads at 4°C for 30 min. The beads were washed four times in wash buffer (10mM hepes buffer PH7.4, 150 mM NaCl, 5mM MgCl2, and 0.1% Triton X-100) and two additional washes without detergent and protease inhibitor. For mass spec the beads were eluted in 30µL of 500 ng/µL 3xFLAG peptide (F4799, Sigma) at 4°C for 30 min with mixing.

For co-immunoprecipitation from chapter 2 figures, 100 µg/ml Cycloheximide and 100 mM iodoacetamide was added to the lysis and wash buffers. Additionally, the proteins were eluted in 30µL 1x LDS buffer at 95°C for 5 min.

For RPL3.3xFLAG co-IP experiments, 400x flies were used. The samples were prepped the same as above, with 100 µg/ml Cycloheximide and 100 mM iodoacetamide added to the lysis and wash buffers. The protein was eluted from the beads in 30µL 1x LDS buffer at 95°C for 5 min.

Serial monoubiquitin enrichment

To couple ubiquitin binding domains, purified recombinant protein was coupled to prewashed NiNTA agarose beads (30210, Qiagen) at 1 mg/mL for 1 hour at 4°C with rotation. The beads were washed three times with wash buffer (0.1% NP-40, 150 mM NaCl, and 50mM Tris pH 7.5) prior to use.

Approximately 400 flies were collected, and head extracted. The heads were homogenized in 1mL of denaturing lysis buffer (50mM Tris-HCL pH 7.5, 150mM NaCl, 1mM EDTA, 1% NP-40 and 10% glycerol) supplemented with protease inhibitor tablet and 50mM N-ethylmaleimide. The lysate was centrifuged at 20,000 xg at 4°C for 20min and filtered through a 0.45µm low protein binding PVDF syringe filter. 3 mg of protein in a final volume of 500 µLwas added to 50 µL1:1 slurry containing His-HALO-hPlic1UBA4x. The beads mix were incubated for 1hr at 4°C with rotation. The flow protein extract was removed from the beads and 400µLwas added to prewashed beads containing His-hP2-UBA and incubated at for 1hr at 4°C with rotation. The beads were washed 4x times with 1 mL wash buffer. The proteins were either eluted by on-bead digest or in 30 µL of 1x loading buffer.

RPS7 Antibody Generation

To create and RPS7 antibody we identified a region of antigenic sequence in the RPS7 peptide using: http://imed.med.ucm.es/Tools/antigenic.pl, which is determined using the method the method of Kolaskar and Tongaonkar (1990). The peptide was ordered from Sigma-Aldrich and the antibody was created by Pocono Rabbit Farm. The final bleed was affinity purified to the peptide.

Mass spectrometry

Co-Immunoprecipitation

In-gel digestion

For biological replicate 1 sample preparation, 50% of each sample (Control and Bait) was loaded onto a 10% Bis-Tris SDS-PAGE gel (Novex, Invitrogen) and separated approximately 1cm using the MES buffer system. The gel was stained with Commassie and the entire mobility region was excised into 5 segments. The gel segments were processed using a robot (ProGest, DigiLab) with the following protocol: (i) washed with 25 mM ammonium bicarbonate followed by acetonitrile; (ii) reduced with 10 mM dithiothreitol at 60 °C followed by alkylation with 50 mM iodoacetamide at room temperature; (iii) reduced and alkylated protein sample were digested with trypsin (Promega) at 37 °C for 4h; (iv) the digestion reaction was quenched with formic acid and supernatant was analyzed directly with LC-MS/MS. The biological replicate samples 2 and 3 (Control-2, 3 and Bait-2,3), protein lysates were run with 4-12% Bis-Tris SDS-PAGE gel (Novex, Invitrogen) at 125 V for 15 min and separated approximately 1.5 cm using MOPS buffer. The gel was stained with commassie and the entire mobility region was excised into small gel pieces. The in gel digestion was performed as following: (i) gel pieces were washed with 1x ddH2O followed by dehydration with 50% acetonitrile/50% H2O solution at room temperature for 15 min; (ii) gel pieces were dried in the speed vac for 10 min; (iii) trypsin

digestion was performed in 50 mM ammonium bicarbonate, pH 8.0; (iv) trypsin digestion reaction was performed at 37 °C for overnight; (v) the reaction was quenched with 1 µLof 1% acetic acid before peptide extraction with 50% acetonitrile/45% ddH2O/5% formic acid at room temperature for 15 min.

LC-MS/MS and data analysis

The extracted peptides from biological replicate-1 samples were analyzed by nano LC-MS/MS with a Waters M-class HPLC system interfaced to a ThermoFisher Fusion Lumos. Peptide were loaded on a trapping column and eluted over a 75 µm analytical column at 350 nL/min; both columns were packed with XSelect CSH C18 resin (Waters); the trapping column contained a 3.5 µm particle, the analytical column contained a 2.4 µm particle. The column was heated to 55°C using a column heater (Sonation). A 1h gradient was employed. The mass spectrometer was operated in data-dependent mode, with MS and MS/MS performed in the Oribitrap at 60,000 FWHM resolution and 15, 000 FWHM resolution, respectively. APD was turned on. The instrument was run with 3s cycle for MS and MS/MS. The peptides from biological replicates 2 and 3 were separated with Thermo UltiMate 3000 UHPLC system interfaced to a ThermoFisher Fusion Lumos. Peptides were loaded on a trapping column and eluted over a 75 µm analytical column at 400 nL/min. A 125 min gradient was employed for peptide separation. The mass spectrometer was operated in data-dependent mode with FAIMS source ion, with MS and MS/MS performed in the Orbitrap at resolution of 240000. APD was turned on. The instrument was run with 3s cycle for MS and MS/MS. Collected data for all of the 3 independent biological replicates were searched with following parameters: (i) enzyme: Trypsin; (ii) database: Uniprot Drosophila (concatenated forward and reverse plus common contaminants); (iii) fixed modification: carbamidomethyl (C); (iv) variable modifications: Oxidation (M); mass values: monoisotopic; (v) precursor mass tolerance: 25 ppm; (vi) fragment ion tolerance: 0.8 Da; (Vii) allowed missed-cleavages: 2. Data were further filtered at 2% FDR at peptide and protein level. SAINT (Significance Analysis of INTeractome) analysis (*Choi*, 2011) was performed on the filtered dataset to find protein-protein interaction. The analysis used peptide spectral match (PSM) scoring function to assign a probabilistic scoring to find out the potential protein-protein interacting partners based on the log Odd score values. The positive log Odd score values indicate higher confidence in the prey protein to the bait.

For serial monoubiquitin enrichment

On bead digestion (Reduction, Alkylation, and Trypsin Digestion)

Ubiquitinated proteins were enriched on beads for a total of 12 samples (WT and mutant replicates). Following this, protein digestion was performed on the beads for each sample as follows. The beads were washed with 1 mL of ice-cold PBS buffer before the beads were resuspended with 200 µLof 50 mM HEPES, pH 8.0 buffer. Reduction and alkylation were performed with 3 µLof 500 mM DTT at 37ºC for 45 mins and 11.8 µLof 380 mM IAA at RT in dark for 30 mins. To quench the overall reduction/alkylation reaction, an additional 3 µLof 500 mM of DTT was added for a period of 15 mins in the dark at RT. Then, 100 ng of trypsin was added to the beads for 1 hr on a shaker at 37ºC. Following the 1 hr digestion, the beads were briefly centrifuged and supernatant was collected in a fresh eppendorf tube. An additional 100 ng of trypsin was added to the supernatant and digestion was performed overnight on a shaker at 37ºC. The digested samples were acidified with

trifluoroacetic acid (TFA) (2% final) and cleaned with C18 stage tips (Pierce, cat# 87782). Peptide quantification was then performed using BCA colorimetric peptide kit (Pierce, cat# 23275) and 20 µgof peptide per sample was used for TMT-16 plex labeling.

TMT-16plex labeling and High-pH fractionation

The dried peptides (20 µgpeptide per sample) were resuspended in 100 µLof 200 mM HEPES, pH 8.0 buffer. The TMT reagent was solubilized in 20 µLof anhydrous acetonitrile and mixed with the peptide solution. Samples were pooled together once the TMT labeling efficiency reached >98% in the label check experiment. Before pooling each labeling reaction was quenched with 5 µL5% hydroxylamine (Sigma-Aldrich, cat# 438227) 15 min at RT. The pooled sample was desalted with Sep-Pak C18 column (Waters, cat# WAT054955) and dried in the speed vac. The dried labeled peptide mixture was fractionated into 24 fractions using Thermo spin column kit (Pierce, cat # 84868) and combined into final 12 fractions. The combined fractions were cleaned up with C18 spin tips (Pierce, cat# 84850) and dried in speed vac prior to LC-MS analysis.

LC-MS and data analysis

A Dionex Ultimate 3000 RSLCnano system (Thermo Fisher Scientific, Inc) and an Orbitrap Eclipse Tribrid MS (Thermo Fisher Scientific, Inc) were used for analysis of the samples, which were reconstituted in LC buffer A (2% acetonitrile/ 0.1% formic acid). Peptide separation was performed on 25 cm length and 75 uM diameter AURORA series column packed with 1.6 uM C18 material with pore size of 120 Å (Ion Opticks, serial# IO2575011997). A linear LC gradient of 185 min with 2% to 30% buffer B (98% acetonitrile/0.1% formic acid) in buffer A (2% acetonitrile/0.1% formic acid) at flow rate of 300 nL/min. The sample analysis was performed using a multinotch MS3-TMT method and data dependent mode. The scan sequence started with FTMS1 spectra (resolution = 120,000; mass range (m/z) = 350-1350; maximum injection time = 50 milliseconds; normalized AGC target (%) = 250; dynamic exclusion = 35 sec with a +/- 10 ppm mass tolerance window. In data dependent scans, the time between master scans was set up as 1 sec with 2-6 as charge state filter. The selected peaks were fragmented via collision-induced dissociation (CID) in the ion trap (CID collision energy (%) = 35; maximum injection time = 100 milliseconds; isolation window = 0.5 Da; normalized AGC target (%) = 150). Following ITMS2 acquisition, an in-house developed real-time search (RTS) method was used to score peptides and select only the high scoring peptides for trigger synchronous-precursor-selection (SPS) MS3 quantitation. For the real-time search, carbamidomethyl on Cysteines (57.0215 Da) and TMTpro16plex (304.2071 Da) on Lysines were included as static modifications. For variable modifications, Oxidation on Methionine (15.9949 Da) and TMTpro16plex on Tyrosines (304.2071 Da), and gg-remnant (114 Da) were selected. The additional real-time search parameters were: max missed cleavages = 1; max variable mods/ peptide = 1; enable FDR filtering = True; precursor neutral loss (m/z) = 0.0; enable protein close-out = True; max peptides per protein = 3; max search time = 35 milliseconds. Up to 8 SPS precursors were further isolated and fragmented with high energy collision-induced dissociation (HCD) with Orbitrap analysis

(HCD collision energy (%) = 40; resolution = 50,000; max injection time = 350 milliseconds; normalized AGC target (%) = 250; isolation window = 1.2 Da).

The data was searched using comet (*Eng*, 2012) against a Drosophila database that included Uniprot *Drosophila melanogaster* protein sequences, contaminant sequences of proteins. The search parameters were: peptide mass tolerance = 25 ppm; enzyme specificity = fully digested; allowed missed cleavages = 2; variable modifications = oxidation of methionine (15.9949 Da), TMTpro (tyrosine) = 304.2071 Da; fragment ion tolerance = 0.4; static modifications = carbamidomethyl on Cysteines (57.0215 Da), TMTpro16plex (304.2071 Da) on N-terminal and Lysines residues, and gg-remnants on lysines (114 Da). Peptide and protein level data was passed through 2% false discovery rate (FDR) separately following the previously published algorithm (*Huttlin*, 2010) (*Elias*, 2007). The searched dataset was further processed for TMT reporter ion intensity-based quantitation using Mojave algorithm (*Zhuang*, 2013) with an isolation width of 0.5.

Quantification and statistical analysis

PSMs were filtered out from peptides with length less than 5; with isolation specificity less than 50%; with reporter ion intensity less than 2^8 noise estimate; from peptides shared by more than one protein; with summed reporter ion intensity (across all 12 channels) lower than 30,000. In the case of redundant PSMs (i.e. multiple PSMs in one MS run corresponding to the same peptide ion), only the single PSM with the least missing values or highest isolation specificity or highest maximal reporter ion intensity was retained for subsequent analysis. Quantification and statistical analysis were performed by MSstatsTMT v2.2.7, an open-source R/Bioconductor package (*Huang*, 2020). Multiple fractions from the same TMT mixture were combined in MSstatsTMT. If the same peptide ion was identified in multiple fractions, only the single fraction with the maximal summation of reporter ion intensity was kept. MSstatsTMT generated a normalized quantification report across all the samples at the protein level from the processed PSM report. Global median normalization equalized the median of the reporter ion intensities across all the channels and TMT mixtures, to reduce the systematic bias between channels. The normalized reporter ion intensities of all the peptide ions mapped to a protein were summarized into a single protein level intensity in each channel and TMT mixture. MSstatsTMT performed differential abundance analysis for the normalized protein intensities. MSstatsTMT estimated log2(fold change) and the standard error by linear mixed effect model for each protein. The inference procedure was adjusted by applying an empirical Bayes shrinkage. To test the two-sided null hypothesis of no changes in abundance, the model-based test statistics were compared with the Student t-test distribution with the degrees of freedom appropriate for each protein and each dataset. The resulting P values were adjusted to control the FDR with the method by Benjamini-Hochberg.

Reagent or Resource	Source	Identifier
Drosophila lines		
OTUD6 RNAi	Vienna Drosophila Resource Center (VDRC)	VDRC# 105469
OTUD6(C183A)	This study	N/A
OTUD6(C183R)	This study	N/A
OTUD6(null)	This study	N/A
UAS.OTUD6.FLAG.HA	This study	N/A
OTUD6(C183A).FLAG.HA	This study	N/A
OTUD6.FLAG.HA	This study	N/A
RPL3.3xFLAG	Bloomington Drosophila Stock Center (BDSC)	BDSC# 79221; RRID:BDSC_79221
UAS-RPS3.3xHA	FlyORF	F000813
UAS-RPL11.GFP	https://doi.org/10.1091/mbc.e08-06-0592	N/A
Rack1(EP)	BDSC	BDSC# 15000; RRID:BDSC_15000
Rack1 df	BDSC	BDSC# 24153; RRID:BDSC_24153
Dis3(ep)	BDSC	BDSC# 30112; RRID:BDSC_30112
Dis3(1)	BDSC	BDSC# 83000; RRID:BDSC_83000
Dis3 gfp	BDSC	BDSC# 83003; RRID:BDSC_83003
ZNF598(EP)	BDSC	22091; RRID:BDSC_22091
ZNF598(EY)	BDSC	24087; RRID:BDSC_24087
NEDD4(EP)	BDSC	15289; RRID:BDSC_15289
RNF10(EY)	BDSC	22641; RRID:BDSC_22641
RNF10(PBac)	BDSC	17910; RRID:BDSC_17910
RNF123(Pbac)	BDSC	17772; BDSC_17772
RNF40(PZ)	BDSC	11541; RRID:BDSC_11541
UBE2A(EX)	BDSC	15757; RRID:BDSC_15757
UBE2A(mimic)	BDSC	35975; RRID:BDSC_35975
WWP2(2)	BDSC	293; RRID:BDSC_293
WWP2(32)	BDSC	26660; RRID:BDSC_26660
UBE2D3(mer4)	BDSC	6401; RRID:BDSC_6401
Antibodies		
HA	Cell Signaling	3724S
FLAG	Sigma-Aldrich	F3165

RACK1	(*Kadrmas*, 2007)	N/A
ubiquitin (P4d1)	cell signaling	3936
Ubiquitinylated proteins,mAb clone FK1	Millipore Simga	04-262
Dis3	(*Snee*, 2016)	N/A
RPS2	Avia Systems Biology	ARP63572_P050
RPL11	Bethyl Laboratories	A303-931A
RPS7	This study	N/A
α-Tubulin (11H10)	Cell Signaling	2125S
α-Tubulin	Sigma-Aldrich	T6074
Puromycin	Developmental Studies Hybridoma Bank	PMY-2A4
Rabbit-HRP	Jackson ImmunoResearch	711-035-152; RRID: AB_10015282
Mouse-HRP	Jackson ImmunoResearch	715-035-150; RRID: AB_2340770
Chicken-HRP	Jackson ImmunoResearch	703-035-155; RRID: AB_10015283
Guinea pig-HRP	Invitrogen	A18769; RRID: AB_2535546
Recombinant DNA		
UAS-OTUD6.FLAG.HA (UFO07783)	Drosophila Genome Resource Center	1642336; RRID:DGRC_1642336
pU6-BbsI-chiRNA	Addgene	45946; RRID:Addgene_45946
Chemicals, Peptides, and Recombinant Proteins		
3XFLAG peptide	Sigma-Aldrich	F4799
methyl methanesulfonate	Thermo Fisher Scientific	156890050
methy viologen dichloridie hydrate	Sigma-Aldrich	856177
Dynabeads M-270 Epoxy beads	Invitrogen	14311D
His-HALO-hPlic1UBA4x	Gift from Eric Bennett	N/A
His-hP2-UBA	Gift from Eric Bennett	N/A
USP2core	Lifesensors	DB-0501-0025
Agarose	Sigma-Aldrich	A6013
RPS7peptide: KIIKPGGSDPDDFEKS	Sigma-Aldrich	N/A
Software		
ImageJ/Fiji	NIH	https://imagej.nih.gov/ij/

PRISM 8/9	GraphPad	https://www.graphpad.com/scientific-software/prism/
Excel	Microsoft	https://products.office.com/en-us/excel

Oligo Name	Sequence	Used for
C183A ssODN	CAGCAAAGCTATCCCAACGCCAATTATCCCTTCACAATATACCCTCCGATGGCGACGCCTTATATCAGTCCATCAGACATCAACTTATCGTAAATGCTCTTCCAGGTAATGCTTCACGATTCAGTCCACACAGCACCAAAACTAATCCATAATAATTGCACCAAAGGTCA	To create OTUD6(C183A)
C183R ssODN	CAGCAAAGCTATCCCAACGCCAATTATCCCTTCACAATATACCCTCCGATGGCGACCGCTTATATCAGTCCATCAGACATCAACTTATCGTAAATGCTCTTCCAGGTAATGCTTCACGATTCAGTCCACATAGCACCAAAACTAATCCATAATAATTGCACCAAAGGTCA	To create OTUD6(C183R)
OTUD6.FLAG.HA ssODN	GATCATCTGCTACCACCGCCACATATACCAATTGGGTGCCCACTACAATTCCACGGTGCCAGCAGCCgactacaaggatgacgatgacaagctcgatggaggatacccatacgatgttccagattacgctTAAACGGGAAATGCCCAATGGCATTTACCATCAACTCACTCGATTTTGTAACAGATTGTTTTATTACATA	Tagging wildtype OTUD6 and OTUD6(C183A)
C183A gRNA sense	CTTCGAAGAGCATTTACGATAAGT	Cloning gRNA for OTUD6(C183A)
C183A gRNA antisense	AAACACTTATCGTAAATGCTCTTC	Cloning gRNA for OTUD6(C183A)
OTUD6.FLAG.HA sense	CTTCGCATTTCCCGTTTAGGCTGC	Cloning gRNA for epitope tag
OTUD6.FLAG.HA antisense	AAACGCAGCCTAAACGGGAAATGC	Cloning gRNA for epitope tag
OTUD6-F	CTCCGATGGCGACCG	WT OTUD6 genotyping
OTUD6-R	CAACTCCTGAACACTGTG	WT OTUD6 genotyping

OTUD6(C183A)-F	CTCCGATGGCGACGC	OTUD6(C183A) genotyping
OTUD6(C183A)-R	GCACATAATTGGCAGTTTC	OTUD6(C183A) genotyping
OTUD6 (C183R)-F	CTCCGATGGCGACCG	OTUD6(C183R) genotyping
OTUD6 (C183R)-R	GGCCATCTCATCGCTACTC	OTUD6(C183R) genotyping
OTUD6 ORF-F	GTGACGATATTTCCGACAGCTTTC	Sequencing OTUD6 open reading frame
OTUD6 ORF-R	GCTTGAATTTACACTAAACCGCCAC	Sequencing OTUD6 open reading frame
OTUD6 tag-F	GGCCATCTCATCGCTACTCC	Genotyping endogenously tagged OTUD6
OTUD6 tag-R (same as ORF-R)	GCTTGAATTTACACTAAACCGCCAC	Genotyping endogenously tagged OTUD6
OTUD6 qPCR-F	CCAACGCCAATTATCCCTTCAC	qPCR of OTUD6
OTUD6 qPCR-R	TTAGCGAGTCCTTGTGAGCA	qPCR of OTUD6

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