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Characterization of RNase X25 and Lamp1 in Drosophila melanogaster

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Characterization of RNase X25 and Lamp1 in Drosophila melanogaster

by

Ayesha Riaz

A thesis submitted to the graduate faculty
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

Major: Genetics

Program of Study Committee:
Gustavo MacIntosh, Major Professor
Linda Ambrosio
Diane Bassham

Iowa State University
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ABSTRACT

RNases T2 have been shown to be evolutionarily conserved in plants, deuto stomes and protostomes indicating that this ancient enzyme may be playing a conserved housekeeping role in diverse organisms. Indeed, evidence from studies in Arabidopsis, zebrafish and human suggests that RNases T2 are involved in rRNA recycling under normal conditions. In order to further elucidate the function of these enzymes in animals, we characterized RNase X25, the only member of the RNase T2 family present in Drosophila melanogaster. In this study, we have shown that RNase X25 is ubiquitously expressed throughout the life cycle, is a major contributor of ribonuclease activity in Drosophila and is upregulated under conditions of nutritional stress accompanied by a concomitant upregulation of Atg5, an autophagy marker. These findings suggest that RNase X25 has functions similar to those of RNase T2 in other organisms.

In order to further clarify the mechanism of rRNA uptake by lysosomes for degradation by RNase X25 in Drosophila melanogaster, we characterized the lysosomal associated membrane glycoprotein, Lamp1. Although it has been used extensively as a lysosomal marker in Drosophila, the actual function of this protein within the lysosomal membrane remains elusive in flies. LAMP2, an ortholog of Lamp1 in humans and mice has three splice variants with conserved luminal regions but different cytoplasmic and transmembrane domains. DmelLamp1 shows the strongest homology with LAMP2C among the three splice variants. Recently, the C-terminal regions of DmelLamp1 and LAMP2C were shown to have very high affinity for RNA isolated from mouse brain. LAMP2A has been implicated in chaperone-mediated autophagy, and LAMP2B deficiency has been shown to cause Danon disease, characterized by cardiomyopathy
and myopathy, in humans. In the current study, we have shown that, just as in the case of RNase X25, LAMP1 expression is upregulated under nutritional stress. We have also characterized a loss of function mutant for Lamp1 in Drosophila melanogaster. Lamp1 depletion leads to accumulation of autophagic vacuoles in fat body tissue as evidenced by Lysotracker-red (LTR) staining and upregulation of Atg8 expression. These results warrant further investigation into the putative role of Lamp1 in rRNA binding and transport to the lumen of lysosomes for degradation by RNase X25 in Drosophila.
CHAPTER 1
INTRODUCTION

Ribonucleases T2 and rRNA degradation

Enzymes in the Ribonuclease T2 (RNase T2) family belong to a general class of transferase--type endorbonucleases along with RNase A and RNase T1 families (Deshpande and Shankar, 2002). RNases T2 have been found in a large number of organisms, as diverse as bacteria and viruses to fungi, plants and animals (Hillwig et al., 2009; Irie, 1999; MacIntosh et al., 2010). At least one gene encoding these enzymes has been found in every eukaryotic genome that has been sequenced so far, the only exception being trypanosomes (Garcia-Silva et al., 2010). Although, classically defined as “Acid Ribonucleases” exhibiting a pH preference between 4 -5.5 (Deshpande and Shankar, 2002; Irie, 1999; Luhtala and Parker, 2010), many RNases T2 show a near neutral or basic pH preference (Reviewed in MacIntosh, 2011).

A conserved overall structure has emerged for T2 proteins from crystal structures of RNases T2 from plants (Gan et al., 2004; Ida et al., 2001; Kawano et al., 2002; Kawano et al., 2006; Matsuura et al., 2001; Nakagawa et al., 1999; Tanaka et al., 2000), fungi (de Leeuw et al., 2012; Kurihara et al., 1996), bacteria (Rodriguez et al., 2008), virus (Krey et al., 2012) and humans (Thorn et al., 2012). All of the characterized RNases have a conserved structure consisting of a β-sheet comprising of four anti-parallel β-strands, a smaller two-stranded anti-parallel β-sheet and three α-helices. The two active site motifs for RNases T2, namely CASI and CASII, reside within these conserved motifs (Kurihara et al., 1996; Rodriguez et al., 2008; Tanaka et al., 2000). Even though multiple alignments for RNase T2 proteins do not show very high overall amino acid sequence homologies, the amino acids present within the active sites
have been shown to be highly conserved (Ambrosio et al., 2014; Irie, 1999; MacIntosh et al., 2010; Rodriguez et al., 2008).

RNases T2 have been shown to perform a variety of functions in different organisms, including phosphate scavenging, self-incompatibility in plants, senescence, defense against pathogens, and rRNA degradation as well as catalysis independent functions like tumor suppression (Deshpande and Shankar, 2002; Irie, 1999; Luhtala and Parker, 2010; MacIntosh, 2011). Recently, it was reported that these RNases might play an important role in carnivory in carnivorous plants (Nishimura et al., 2014).

Phylogenetic analyses of plant T2 proteins indicate the existence of variable numbers of genes in different species as a result of extensive expansion and high rates of gene duplication and gene loss (MacIntosh et al., 2010). Whereas, in animals they have been maintained as a single copy gene in most species, with the exception of bony fishes expressing two copies, one of which is conserved among fishes (Hillwig et al., 2009). Phylogenetic analyses of insects with sequenced genomes also revealed the presence of a single gene, with the exception of *Nasonia vitripennis* expressing eight genes (Ambrosio et al., 2014). In plants, most Class II RNases T2 have been shown to be constitutively expressed suggesting that they may be performing a housekeeping role (MacIntosh et al., 2010). The conserved RNase T2 from zebrafish has been shown to be expressed in all tissues throughout development (Hillwig et al., 2009) similar to RNASET2 in humans (Henneke et al., 2009). Together, these findings suggest that RNase T2 enzymes in animals perform an evolutionarily conserved housekeeping role and may be acting as counterparts of Class II RNases in plants (Hillwig et al., 2009).

A number of studies involving characterization of RNases T2 in Arabidopsis, zebrafish and human indicate that these enzymes are likely involved in rRNA degradation in normal cells
Arabidopsis RNase T2 mutants accumulate RNA in the vacuole, display constitutive autophagy and have increased rRNA half-life (Hillwig et al., 2011). Zebrafish mutant for rnaset2 also exhibit rRNA accumulation in neuronal lysosomes in addition to brain lesions similar to those observed in human leukencephalopathies linked to defects in the RNASEt2 gene (Haud et al., 2011; Henneke et al., 2009). Thus, enzymes belonging to the RNase T2 family participate in the normal recycling of rRNA, an essential housekeeping gene for maintaining cellular homeostasis.

**Ribophagy: A possible path for rRNA degradation**

Ribosome synthesis within a cell is a major metabolic activity comprising a large number of reactions. Since hundreds of reactions are involved in this process, errors in ribosome assembly are likely to occur either in *cis* or in *trans*. Errors in *cis* can occur during RNA synthesis or due to exposure to genotoxic stress resulting in mutations. Errors in *trans* may occur during any of the numerous steps involved in the assembly of the ribosome such as failure to bind to, or loss of, an assembly factor or ribosomal protein. It is imperative that such defective molecules be gotten rid of, which could otherwise accumulate leading to pathological conditions. Most of the common neurological diseases in humans, in particular, Alzheimer disease, Parkinson disease, dementia with Lewy bodies and amyotrophic lateral sclerosis have been shown to be linked to oxidative RNA damage (Nunomura et al., 2009). Indeed, defects in ribosome biogenesis lead to riobosomopathies (Narla and Ebert, 2010).

A number of studies have indicated that surveillance pathways exist at every step of ribosome biogenesis (Doma and Parker, 2007; Lafontaine, 2010). For mutations occurring in *cis*, a number of surveillance pathways like the ‘no-go’ decay pathway (NGD) for eradicating
defective mRNAs (Doma and Parker, 2006) and ‘non functional rRNA decay’ or NRD pathway (LaRiviere et al., 2006) for degrading defective components of mature ribosomes have been described.

Under conditions of nutritional stress, *de novo* synthesis of ribosomes is shut down, and pre-ribosomes and mature ribosomes are targeted to bulk degradative pathways, to be recycled into essential building blocks to be used by the cell. Entire organelles such as ribosomes and mitochondria, as well as bulk portions of the cytoplasm including protein aggregates are degraded through macroautophagy. Macroautophagy, characterized by sequestration of cytoplasmic components destined for degradation by double membrane vesicles called autophagosomes involves fusion of autophagosomes with lysosomes to form hybrid-like organelles called autolysosomes (Luzio et al., 2007). A selective form of macroautophagy termed “ribophagy” has been described in yeast under conditions of prolonged nitrogen starvation which preferentially targets ribosomes to the vacuole (Kraft et al., 2008). This process requires the action of Ubp3, an ubiquitin protease, and its cofactor Bre5.

**LAMPS: Lysosome associated membrane glycoproteins**

Lysosomes are membrane bound organelles containing acid hydrolases, found in animal cells. They constitute as much as 5% of the total intracellular volume, are heterogeneous with respect to their size and morphology and act as the terminal degradative compartment for endocytosis as well as autophagy (Luzio et al., 2007).

The lysosomal membrane consists of a single phospholipid bilayer, 7-10 nm in thickness (Saftig et al., 2010). The membrane not only serves as a barrier between the cytoplasm and the hydrolytic enzymes enclosed within the lysosome, but is also responsible for other functions
such as maintaining the acidic pH within the lysosomal lumen and fusion with endosomes, autophagosomes and other organelles (Eskelinen et al., 2003; Saftig and Klumperman, 2009).

Existence of more than 20 lysosomal membrane transporters has been demonstrated (Sagne and Gasnier, 2008).

LAMP1 and LAMP2 proteins constitute a major fraction, ~50%, of all proteins within the lysosomal membrane. Both of these are characterized as type 1 membrane proteins with a short cytoplasmic tail, a transmembrane domain and a luminal domain that is heavily glycosylated. The human LAMP1 and LAMP2 have a molecular mass of 40-45 kDa sans glycosylation and approximately 120 kDa after glycosylation (Carlsson et al., 1988; Mane et al., 1989). Due to their abundance within the lysosomal membrane, LAMP1 and LAMP2 are also chief constituents of the glycoconjugate coat on the luminal side. It had been postulated that the proportion of the two LAMPs was high enough to form a nearly continuous layer on the luminal surface of the lysosome (Granger et al., 1990).

Studies in LAMP2 knockout mice show accumulation of autophagic bodies in heart and skeletal muscle, leading to fatal cardiomyopathy and myopathy, similar to the symptoms exhibited by patients suffering from Danon disease (Saftig et al., 2001). Another study involving LAMP-2 knockout mice reported impaired fusion between lysosomes and phagosomes leading to periodontitis (Beertsen et al., 2008). Depletion of both LAMPs in embryonic fibroblasts showed accumulation of autophagic vesicles, altered appearance of lysosomes and disruption of cholesterol metabolism without affecting protein degradation (Schneede et al., 2011). In mammalian cells, RNA induced silencing of LAMP2 showed a marked reduction in fusion between autophagosomes and autolysosomes (Gonzalez-Polo et al., 2005). LAMP2 has also been
shown to play a critical role in enodosomal/lysosomal cholesterol transport (Eskelinen et al., 2004; Schneede et al., 2011).

LAMP2 has 3 splice variants, namely LAMP2A, LAMP2B and LAMP2C, with identical luminal domains but different cytoplasmic and transmembrane domains (Hatem et al., 1995). The three isoforms exhibit characteristic subcellular localization and tissue distribution patterns (Cuervo and Dice, 2000; Gough and Fambrough, 1997; Konecki et al., 1995; Lichter-Konecki et al., 1999). LMAP2A has been implicated in chaperone-mediated autophagy (Cuervo and Dice, 2000; Zhou et al., 2005). Human LAMP2B is abundantly present in muscle and its deficiency leads to Danon disease (Nishino et al., 2000). The C-terminal region of LAMP2C has recently been shown to bind RNA with very high affinity (Fujiwara et al., 2013).

The genome of *Drosophila melanogaster* encodes for only one lysosomal associated membrane glycoprotein, Lamp1. The C-terminal sequence of fly Lamp1 shows the strongest homology (50%) with LAMP2C among the three variants for human LAMP2, and also exhibits similarly high levels of affinity for RNA (Fujiwara et al., 2013).

Fujiwara et al. (2013) have proposed a novel type of selective autophagy pathway for RNA degradation, which they dubbed “RNautophagy”. In RNautophagy, RNA is taken up directly by lysosomes for degradation by LAMP2C in an ATP-dependent manner, but unlike chaperone-mediated autophagy, it does not require involvement of HSP8/Hsc70. LAMP2C, a splice variant of LAMP2 was shown to act as a receptor for this pathway, its cytosolic tail having a very high specificity for almost all total RNA isolated from mouse brain. The cytosolic regions of LAMP2C are conserved among human, mouse and chicken (Eskelinen et al., 2005) whereas the C-terminal sequences of LAMP2 orthologs in *Drosophila melanogaster* (Lamp1) and *Caenorhabditis elegans* (LMP-1) show strongest homology to LAMP2C among the three
variants for human LAMP2 (Fujiwara et al., 2013). It has been suggested that direct uptake of RNA for degradation by lysosomes (RNautophagy) contributes to around 10%-20% of the total RNA being degraded in a cell, the rest being degraded by some LAMP2-independent pathway (Fujiwara et al., 2013) such as ribophagy or general autophagy.

In this study, we have explored the possibility of using Drosophila melanogaster as a model for dissecting rRNA degradation mechanisms at play. As a first step, we have characterized RNase X25, the only RNase T2 enzyme found in Drosophila. Further, we have also characterized Lamp1, the only LAMP family lysosomal membrane associated protein in the fly in an effort to shed light on the mechanism involved in transport of rRNA to the lysosomal lumen for degradation by RNase X25.

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tRNA-derived small RNAs is actively produced in Trypanosoma cruzi and recruited to specific cytoplasmic granules: Molecular and Biochemical Parasitology, v. 171, p. 64-73.


This chapter is part of the following publication,


Only the portions of the work that was conducted by me have been added to the chapter.

**Introduction**

RNase T2 enzymes are found ubiquitously, with RNase T2 genes in genomes of most eukaryotes, many bacteria, as well as some viruses (Irie, 1999; MacIntosh, 2011). These enzymes catalyze endonucleolytic RNA cleavage via a 2′-3′-cyclic phosphate intermediate (Irie, 1999). Although primary sequence identity between eukaryotic and prokaryotic enzymes is low, there are conserved secondary structures that contain key core hydrophobic residues associated with the RNase T2 active site (Deshpande and Shankar, 2002; MacIntosh, 2011).

These ancient ribonucleases are secreted or targeted to membrane-bound intracellular compartments (lysosomes and vacuoles) where they degrade single stranded RNAs. Long known for their function in gametophytic self-incompatibility, and as part of the response to phosphate starvation in plants (Luhtala and Parker, 2010; MacIntosh et al., 2010), the RNase T2 family has been recently shown to play distinctly different developmental and physiological roles in plants and animals. Recent insights from Arabidopsis thaliana and zebrafish indicate that conservation of the RNase T2 family in all eukaryotes may be related to an important housekeeping function.
carried out by these enzymes, which includes recycling of ribosomal RNAs (Haud et al., 2011; Hillwig et al., 2011). A ribophagy-like pathway is thought to mediate this turnover of rRNAs in normal, non-stressed cells (MacIntosh and Bassham, 2011), which is essential to maintain cellular homeostasis. Additionally, in *Saccharomyces cerevisiae* and *Tetrahymena thermophila*, the enzymatic activities of RNase T2 proteins have been associated with cleavage of mature tRNAs to produce tRNA halves in response to starvation and oxidative stress (Andersen and Collins, 2012; Thompson and Parker, 2009). The significance of the accumulation of these degradation intermediates is unknown, although it has been suggested that they may play a signaling role in the maintenance of cellular homeostasis (Luhtala and Parker, 2010). Alternatively, they may accumulate as a consequence of targeted degradation of the translation machinery during stress conditions that leads to suppression of cell division (Andersen and Collins, 2012). Interestingly, a different ribonuclease carries out tRNA cleavage function in response to stress in vertebrate cells. In this case angiogenin, a member of the vertebrate-specific RNase A family, is responsible for the accumulation of tRNA fragments (Yamasaki et al., 2009).

At least one member of the RNase T2 family has been found in every eukaryotic genome that has been sequenced, with Trypanosomatids as the only exception (MacIntosh, 2011). High frequency of gene duplication and extensive divergence of the T2 RNases has occurred in plants (Igic and Kohn, 2001; MacIntosh et al., 2010). On the other hand, only one, well-conserved gene, is found in most vertebrate genomes (Hillwig et al., 2009); and it has been proposed that RNase A members have replaced RNase T2 in several biological roles in these organisms (Hillwig et al., 2009; MacIntosh, 2011). Thus, characterization of the biological role played by RNase T2 enzymes in multicellular organisms is complicated in plants and vertebrates due to the presence of potentially redundant enzymatic activities. In contrast, the *Drosophila*
*melanogaster* genome contains only one RNase T2 gene, *RNase X25* (also known as *DmRNase-66B*), and no RNase A homolog; thus, this organism could be used as a simpler system to demonstrate the conserved function(s) of this enzyme family in animals. *RNase X25* (CG8194), located at 66A21 on chromosome 3, is 1658 nucleotides in length and encodes a single form of mRNA transcript with a 325 amino acid open reading frame (Hime et al., 1995). A signal peptide cleavage site is anticipated between residues 21 and 22 suggesting transport of the predicted polypeptide chain to the secretory pathway. In addition, two asparagine residues (positions 214 and 231) and a threonine (residue 34) may serve as N- and O-glycosylation sites, respectively. N-glycosylation is the most common modification found for the RNase T2 family, while a few cases of O-glycosylation have been observed for fungal enzymes (reviewed by MacIntosh, 2011).

As a first step towards understanding the role of RNases T2 in animals, biochemical analyses and gene expression studies were initiated in the fruit fly *D. melanogaster*. RNase T2 activity was detected in all *Drosophila* life cycle stages examined, and this correlated well with *RNase X25* gene expression patterns. Furthermore, *RNase X25* gene expression levels were responsive to nutritional stress as determined by the accumulation of *RNase X25* mRNAs in larvae starved for nutrients. A correlation between induction of autophagy and increased RNase X25 expression and activity was also observed in response to starvation. These analyses suggest that RNase X25 carries out a conserved housekeeping function as proposed for other RNases T2 in plants and animals, and that *Drosophila*, with a single RNase T2 gene, is a good eukaryotic model system in which to investigate the role of RNases T2 in the process of ribophagy.
Material and Methods

D. melanogaster strains and culture

In this study the Drosophila melanogaster strain $w^{1118}/w^{1118}$ with two wild type genes and $w^{1118}/w^{1118};Df(3L)Excel6279/+ \text{(denoted } Df(3L)Excel6279/+ \text{ in the text)},$ with one wild type gene were raised at 25°C on standard cornmeal media.

For staged embryo collections, females were placed in collecting bottles and eggs were gathered after aging from molasses-agar plates dusted with yeast.

Stress treatments

To provide standardized non-crowded growing conditions prior to stress treatments, 43 $w^{1118}/w^{1118}$ embryos (0–2 hrs) were gently transferred onto Formula 4–24 instant blue D. melanogaster diet (363.6 mg/1.625 ml H2O; Carolina Biological Supply, Burlington, NC, USA), that had been placed into a small petri dish (60 x 15 mm). Baker’s yeast was not sprinkled on this medium. Petri plates were placed in an incubator at 22°C and 80% humidity for 128 hours. Then 25 young, newly molted third instar larvae were gently transferred from each plate to either control D. melanogaster diet, or experimental media consisting of D. melanogaster diet containing 1% unconjugated wheat germ agglutinin (Vector Laboratories, Burlingame, CA, USA), or hydrogen peroxide at 0.1% [w/w] or 0.5% [w/w]. For starvation conditions larvae were placed onto PBS-saturated Whatman 1 filter paper. After 14 hours, larvae were collected, frozen at -80°C and stored for further processing. For detection of autophagy in fat body cells of starved and fed control larvae, embryos were placed onto Bloomington’s Drosophila Stock Center cornmeal/molasses/yeast soft media, sprinkled with Baker’s yeast, and subsequently processed as described above.
Fluorescence Microscopy

Fat body tissue was dissected in PBS followed by staining. LysoTracker Red DND-99 (Life Technologies, Carlsbad, CA, USA) staining of lysosomes and autolysosomes, and Hoeschst 33342 (Thermo Fisher Scientific Inc, Rockford, IL, USA) staining of DNA was performed as described previously (Juhasz and Neufeld, 2008; Scott et al., 2004). Stained fat body lobes were imaged in PBS using a Zeiss Axio Imager.Z2 microscope equipped with AxioCam HR digital camera using an LD Plan-Neofluar 40x/0.6 objective lens and ZEN imaging software.

Protein extracts and RNase activity assays

Protein was prepared from flies at different stages or collected from stress experiments, using approximately 100 mg of each sample. The material was homogenized in 1.5 ml eppendorf tubes and protein extractions were performed as described by Hillwig et al. (Hillwig et al., 2009), using the protease inhibitor cocktail Complete Mini EDTA Free (Roche Diagnostics, Indianapolis, IN, USA) or Protease Inhibitor Cocktail P8340 (Sigma-Aldrich, St. Louis, MO, USA). In gel RNase activity assays were performed following the protocol used by Yen and Green (Yen and Green, 1991) using high molecular weight Torula Yeast RNA (Sigma-Aldrich) as substrate, loading 20–80 mg of protein per lane. After running and washes, gels were incubated at pH 7.0. SDS-PAGE was run in parallel for each sample as loading and quality control, also using 20 mg of protein per lane, and then stained with Coomassie Brilliant Blue. Experiments were repeated at least 3 times.
Gene Expression Analysis

RNA was extracted from 100 mg of sample using Trizol (Fisher) according to manufacturers’ instructions. RNA was DNase-treated using Turbo DNA-free (Ambion), and cDNA was synthesized using the iScript Select cDNA Synthesis kit (Bio-Rad), also following manufacturers’ instructions for each procedure. qPCR was completed on a Stratagene MX4000 using the Absolute qPCR with SYBR Green + Rox kit (Fisher Scientific) according to manufacturers’ instructions. The transcript of ribosomal protein L32 gene (RPL-32) was used as the control for data normalization, using the Pfaffl method [19]. Primers used for RNase X25 were: Forward (5’-3’): TCCACGCCCTCTACACCTGCT, and Reverse (5’-3’): ACGCCAAAGTGAGCCCCTGCT; for RPL-32: Forward (5’-3’): TGGGACACCTCTCAAGAT, and Reverse (5’-3’): CAGGCGACCGTTGAGGT; for Atg5: Forward (5’-3’): ATCTGGGAGGGCCAGATAGG, and Reverse (5’-3’): TAGCTCCTGGAGTGAGCTTG; for Amyrel: Forward (5’-3’): GATCTAGATACATCTACAGCAGCC, and Reverse (5’-3’): ACTTGTAGGTCAGCAGGCA; and for Lip3: Forward (5’-3’): GCCTATTCTGTGCGGTAGT, and Reverse (5’-3’): AGTACTTGTGCAGCCCTGGAG.

Experiments were performed using triplicates, and repeated 3 times using independent samples. Statistical significance of the differences between treatments was determined using t-test. Graphs show averages of each sample normalized using the average value of the control sample. One star = P<0.05, two stars = P<0.01.
Results

**RNase X25 transcription is not stage specific**

In order to determine the pattern of RNase X25 transcription during development, embryos at 0–2, 2–6, and 0–16 hr after egg deposition, as well as wandering third instar larvae, white prepupae, pupae, adult males, females, and isolated ovaries were collected. Total RNA was isolated from each developmental sample, and RNase X25 expression was analyzed using quantitative real time PCR (qRT-PCR) (Figure 1). RNase X25 transcripts were found to be present in all of the stages analyzed, showing constitutive expression throughout Drosophila development. These results correspond well with those obtained from the in gel ribonuclease activity assay for the same developmental stages performed previously (Figure 2). Even though protein samples isolated from early 0-2 hr embryos and third instar larvae exhibited lower RNase activity compared to other stages, no significant stage-specific differences in mRNA accumulation were apparent. The two samples with low RNase activity had at least as much expression of RNase X25 as samples with high activity. This discrepancy between enzymatic activity and mRNA accumulation could suggest that RNase X25 is posttranscriptionally or posttranslationally regulated. Our gene expression analyses correspond well with expression data obtained from genome-wide transcriptome analyses deposited in FlyBase (http://flybase.org). Moreover, data obtained from the modENCODE (Celniker et al., 2009) and FlyAtlas (Robinson et al., 2013) databases indicated that RNase X25 expression is constitutive for all tissues of the fly at the 3rd instar larval and adult developmental stages with tissue specific expression ranging from very low to high levels (Figures S1 and S2).
Reduced expression of RNase X25 in Df(3L)Excel6279

The Df(3L)Excel6279 line with deficiency break points mapped to 66A17 and 66B5 was chosen for further analysis of RNase X25. This is the smallest known deletion in Drosophila that removes the RNase X25 gene located at position 66A21. Importantly, RNase X25 is the only ribonuclease encoding gene that lies between the breakpoints of the Df(3L)Excel6279 chromosome. RNA and protein extracts were produced from ovarian tissue from flies with either a wild type (+/+ ) genetic background with two RNase X25 gene copies or the Df(3L)Excel6279/+ background with one RNase X25 gene copy. A homozygous mutant for this line could not be obtained since homozygous deletions of this region are lethal. qRT-PCR analysis indicated a reduction in RNase X25 transcript levels by approximately one half for the Df(3L)Excel6279/+ ovaries compared to the wild type (Figure 3C). Furthermore, a corresponding decrease in RNase activity was observed for the 25–30 kDa bands in Df(3L)Excel6279/+ protein extracts (Figure 3A), when similar amounts of protein were examined for wild-type and heterozygous deletion mutants (Figure 3B). These results strongly suggest that the enzymatic activity observed by our in gel analysis was, in fact, RNase T2 activity, encoded by the Drosophila RNase X25 gene.

RNase X25 expression is altered by stress

In addition to a general housekeeping function in rRNA recycling, the RNase T2 family of enzymes is thought to play specialized roles in unicellular and multicellular eukaryotes. In yeast and Tetrahymena, RNase T2 activity is responsible for the cleavage of mature tRNAs to produce tRNA halves during the response to oxidative stress or amino acid starvation (Andersen and Collins, 2012; Thompson and Parker, 2009). Several microarray and RNAseq reports on
transcriptional responses to a variety of stresses in *Drosophila* are available in the literature and public databases. However, results related to the effect of starvation on *RNase X25* are not clear. A study (Li et al., 2009) reported that accumulation of *RNase X25* mRNAs was altered in *Drosophila* larval midgut tissue and starvation-like effect was observed in animals that were fed a diet supplemented with wheat germ agglutinin (WGA). To explore the possibility of RNase T2 playing a role in the fruit fly’s response to nutritional and oxidative stress, we determined whether *RNase X25* gene expression levels were altered after exposure to these pressures.

Whole animal extracts were prepared for molecular analysis from third instar larvae fed a control diet or subjected to starvation for 14 h (see Materials and Methods). As shown in Figure 4A, the accumulation of *RNase X25* mRNA transcripts increased approximately 80% for animals starved for nutrients (P<0.05) when compared to fed control larvae. Previously, experiments were carried out in our lab to determine if a change in *RNase X25* expression levels could be detected in whole animal extracts after larval ingestion of WGA (1% w/w). Consistent with a starvation-like effect, the WGA supplemented diet had resulted in a significant increase in *RNase X25* expression (P<0.01), similar to that observed in starved flies (Figure 4A, make a note of who did the experiment in caption).

An increase in *RNase X25* mRNA was also observed when larvae were fed a diet supplemented with hydrogen peroxide, an oxidative stressor (Figure 4B). It was reported/found that the normalized *RNase X25* expression level for whole animals exposed to 0.5% hydrogen peroxide was 50% higher (P<0.05) than control animals. At a lower dosage of 0.1%, an increase of 20% in *RNase X25* mRNA was observed, although this change was not statistically significant. Thus, analysis of whole larval extracts indicated that the *RNase X25* gene is responsive to various stressors including starvation, and treatments with 1% WGA or 0.5%
hydrogen peroxide. Data from microarray experiments performed by other laboratories suggest that a few other stress conditions and chemical treatments can also alter the expression of RNase X25 (Ambrosio et al., 2014).

**Starvation, RNase X25 expression and autophagy**

Since starvation induces autophagy and autophagy mediated RNA degradation, we also tested whether expression of Atg5, which encodes a protein that participates in an ubiquitin-like protein conjugation system essential for autophagy (Chang and Neufeld, 2010), was altered in our starved larvae. We found a small but significant (P<0.05) increase in the expression of this autophagy marker in starved, as compared with fed control larval samples (Figure 5A left F 4–24 panel). Since only a low level of induced Atg5 expression was detected in our starved samples, we used two gene markers, Amyrel (α-amylase related) (Li et al., 2009) and Lip3 (lipase) (Zinke et al., 2002) to monitor the starvation response for these animals grown on Formula 4-24 instant blue *D. melanogaster* diet. As shown in Figure 5B (F 4–24 panels) significant (P<0.01) increases in the level of Amyrel (2.5 fold) and Lip3 (14 fold) were apparent, indicating that the starved animals were indeed nutritionally stressed.

Next, we followed autophagy by examining the formation of Lysotracker red (LTR)-positive vesicles in larval fat body, as described previously (Jimenez-Sanchez et al., 2012). For animals fed a Formula 4–24 instant *D. melanogaster* diet, very high, but diffuse accumulation of LTR was observed for both fed control and starved larval fat body, complicating the interpretation of results (data not shown). The diet of these animals was not supplemented with Baker’s yeast, an important and major source of nutrients for Drosophila larvae (Rubin, 1990). However, for 3rd instar larvae growing on rich media containing yeast (see Materials and
Methods), characteristic LTR-positive vesicles were observed in fat body cells (Figure 6B, D and F) after a 14-hour starvation period while little to no puncta were visible for fed control animals (Figure 6A, C and E). Importantly, qRT-PCR analysis demonstrated that a significantly (P<0.01) higher level of Atg5 mRNA transcripts was present after 14 hours of starvation, as compared with those from non-starved control animals in rich media-fed larval samples (Figure 5A), confirming the induction of autophagy in these nutritionally stressed animals. The starved state of these animals was verified by the presence of significantly (P<0.01) higher levels of both Amyrel and Lip3 mRNA transcripts, as compared with fed-control larvae (Figure 5B). Finally, the level of RNase X25 gene expression and enzymatic activity were probed and found to be at higher levels for starved compared with fed-control animals (Figure 5C and D). Together, these results suggest that the autophagy process is concomitantly induced with an increase in RNase X25 mRNA expression and enzymatic activity after starvation.

Discussion

In this study we performed an initial characterization of Drosophila RNase X25, the only member of the RNase T2 family present in this insect. We found constitutive expression of RNase X25 mRNA during Drosophila development, and were able to show a correlation between the main RNase activity detected in zymograms and expression of this gene in wild-type and deletion mutants, indicating that RNase X25 is a major contributor of endonuclease activity in Drosophila extracts. This activity has a pH optimum in the acidic range (data not shown), a common characteristic of animal RNase T2 enzymes, which indicates that the active enzyme may normally be sequestered in an acidic compartment within cells to carry out its function.
Animal RNase T2 proteins have been found to be localized to lysosomes in zebrafish and humans (Campomenosi et al., 2006; Haud et al., 2011), and prediction of subcellular localization for RNase X25 indicated that this protein is also targeted to the secretory pathway (Hime et al., 1995).

Based on our results and data extracted from databases, RNase X25 seems to be active at all stages of development and in all larval and adult tissues investigated. This result is in agreement with studies that have characterized the RNase T2 family in other eukaryotes (MacIntosh, 2011), and suggests that, RNase X25 may perform a housekeeping function as has been proposed for other constitutively expressed eukaryotic RNase T2 enzymes. Absence of this constitutive RNase activity in Arabidopsis thaliana and zebrafish leads to accumulation of rRNA in vacuoles or lysosomes (Haud et al., 2011; Hillwig et al., 2011). Additionally, Arabidopsis plants lacking expression of RNS2, the housekeeping RNase T2 in this organism, show constitutive autophagy (Hillwig et al., 2011). Thus, it has been proposed that the role of these RNases is to maintain normal cellular homeostasis by recycling rRNA.

RNase T2 enzymes may also play a role in rRNA recycling and cellular homeostasis in cells under nutritional stress conditions, likely through a specialized autophagy process known as ribophagy. Ribophagy, the targeted degradation of ribosomes through a mechanism that uses the autophagy machinery, has been described for yeast cells undergoing starvation (Kraft et al., 2008). Rny1, the only RNase T2 enzyme in yeast (MacIntosh et al., 2001), may mediate rRNA degradation under stress conditions (Thompson and Parker, 2009). While a direct role for RNase T2 enzymes in ribophagy has not been established, their participation in this process has been suggested for plants, animals, and unicellular eukaryotes (Andersen and Collins, 2012; Haud et al., 2011; MacIntosh and Bassham, 2011). Moreover, several plant RNase T2 genes are induced
under conditions of phosphate starvation, probably as a mechanism to scavenge nutrients (MacIntosh, 2011), and at least two Tetrahymena RNase T2 genes are also induced by starvation conditions (Miao et al., 2009). We observed that expression of RNase X25 is significantly induced in fly larvae when subjected to starvation or fed a WGA supplemented diet. Concomitant with this response we also observed an increase in the expression of Atg5, which encodes one of the core components of the autophagy machinery that has been previously shown to be induced by starvation in Drosophila ovaries (Barth et al., 2011), and the robust appearance of Lysotracker-positive vesicles in larval fat body cells, marking lysosomes and autolysosomes participating in the autophagy process (Jimenez-Sanchez et al., 2012; Scott et al., 2004). Induction of RNase X25 by nutritional stresses and evidence of autophagy may indicate that this enzyme also has a role in cellular homeostasis through recycling of cellular RNAs.

Data from a genome-wide microarray analysis of mRNA expression had previously identified RNase X25 as one of 61 transcripts differentially expressed when animals were fed a 1% WGA diet (Li et al., 2009). In that study, a 9-fold increase in RNase X25 transcript levels was observed for midgut tissue dissected from third instar larvae. Since whole animals were harvested for our analysis, it is conceivable that the modest ~2 fold increase in RNase X25 mRNA levels we observed reflects a tissue specific differential response to WGA. Higher levels of RNase X25 mRNAs may accumulate in tissues of the gut, with stable expression levels in remaining tissues. This effect could also explain the discrepancies in results observed in several high throughput analyses of starved Drosophila larvae or adults. This “dilution effect” may also explain the difference between the Atg5 levels of expression observed in our experiment and the experiments of Barth et al. (Barth et al., 2011), who isolated ovaries for their analyses.
In addition to a housekeeping role, RNase T2 enzymes have acquired novel functions during eukaryote evolution. In some cases, novel functions appeared after gene duplications. This seems to be the case for plant RNase T2 enzymes that participate in defense mechanisms, and also for S-RNases, specialized T2 enzymes that determine gametophytic self-incompatibility in several plant species (Igic and Kohn, 2001; MacIntosh et al., 2010). In other cases, a single protein can have multiple roles. For example, both RNase activity-dependent and -independent functions have been proposed for human RNASET2. Lack of RNASET2 causes cystic leukoencephalopathy in humans and a similar phenotype in zebrafish (Haud et al., 2011; Henneke et al., 2009). This neurological disorder is likely caused by lysosomal malfunction due to the high levels of rRNA that accumulate in these organelles in the absence of the enzyme (Haud et al., 2011). In addition, human RNASET2 has been shown to have anti-metastatic properties independent of its catalytic activity (Acquati et al., 2005). Another enzyme with more than one function is yeast Rny1. This protein may work in rRNA recycling during ribophagy-like processes, given its localization in vacuoles under normal growth conditions (Thompson and Parker, 2009). Additionally, Rny1 is responsible for tRNA cleavage during the cell's response to oxidative stress, after the enzyme is likely released from the vacuole into the cytoplasm. The accumulation of stable tRNA halves is thought to act as a signal during the stress response (Andersen and Collins, 2012; Thompson and Parker, 2009). Moreover, during the oxidative stress response, Rny1 is able to promote cell death through an unknown mechanism that is independent of its RNase activity (Thompson and Parker, 2009). Cleavage of tRNAs in stress conditions that inhibit cell growth is a response conserved in plants and animals (Andersen and Collins, 2012; Thompson et al., 2008), and there is some evidence that tRNA fragments also accumulate in Drosophila (Aravin et al., 2003; Brennecke et al., 2007). We showed here
that RNase X25 expression is increased in flies subjected to oxidative stress, making this enzyme the logical candidate for a role in tRNA cleavage in these insects, and suggesting that RNase X25 may have dual function, as shown for other members of the RNase T2 family.

Recent insights from Arabidopsis thaliana, zebrafish, and human indicate that the RNase T2 enzymes carry out an important housekeeping function in normal cells (Haud et al., 2011; Hillwig et al., 2011). Arabidopsis mutants lacking this conserved RNase T2 activity accumulate RNA, mainly in the vacuole, have an increased rRNA half-life, and exhibit constitutive autophagy (Hillwig et al., 2011); while rnaset2 mutant zebrafish show aberrant accumulation of undigested rRNA in neuronal lysosomes and present brain lesions similar to those observed in leukocephalopathies associated with deficiencies in RNASET2 in humans (Haud et al., 2011; Henneke et al., 2009). Thus, RNase T2 enzymes participate in the normal recycling of rRNA, and this housekeeping function seems to be essential for cellular homeostasis. Duplication and divergence of the RNase T2 gene family has occurred in the evolution of plants and fishes. Secreted RNases of the RNase A family seem to have acquired in vertebrates, including humans, some of the biological roles carried out by RNase T2 enzymes in other systems (reviewed by MacIntosh (MacIntosh, 2011)). Use of the Drosophila model, whose genome encodes only one RNase T2 gene and lacks RNase A homologs or other evident secretory RNases with similar activity, is likely to provide insight into the ancestral physiological function of this gene family in multicellular animals during normal growth and development, and also under stress conditions. Analyses of Drosophila mutants with reduced RNase X25 activity may lead to identification of phenotypic characteristics that could be the basis of genetic modifier screens to identify other key genes that participate in RNase T2 function. Since attempts at RNA interference mediated gene silencing proved to be unsuccessful for this gene (See Appendix A),
a CRISPR/Cas9 strategy might be employed in the future for introducing deletions in the gene
(See Appendix B). Such mutants shall prove to be essential for our understanding of the
underlying function of RNase X25 and may also contribute to our understanding of how RNA
degradation systems interface with other cellular processes.

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Figure 1. Developmental profile of RNase X25 transcript. RNA was isolated from embryos at 0–2 h, 2–6 h, and 0–16 h after egg deposition and from animals at 3rd instar larval (L3), white prepupal (WPP), pupal (P), and adult male (M) or female (F) stages of development. Ovarian tissue (O) was prepared from 3–5 day old females. qPCR quantification of the relative level of RNase X25 mRNAs in these samples was carried out using the ribosomal protein L32 (RpL32) transcript as internal standard control for normalization. RNase X25 expression was detected at all stages analyzed. Data are representative of 3 independent experiments and are means and S.E. of triplicates.
Figure 2. **Developmental profile of Drosophila RNase activities.** Protein extracts were produced from embryos at 0–2 hours (h), 2–6 h, and 0–16 h after egg deposition and from animals at 3rd instar larval (L3), white prepupal (WPP), pupal (P), and adult male (M) or female (F) stages of development. Ovarian tissue (ovary) was prepared from 3–5 day old females. (Upper panel) Protein was fractionated by electrophoresis through a 12% polyacrylamide gel containing 3 mg/ml Torula yeast RNA, washed to remove SDS, incubated in 100 mM Tris-HCl at pH 6.0 and stained with toluidine blue to visualize regions of nuclease activity. Low molecular weight (~25–30 kD) activities in the size range of the RNase T2 family were detected at all developmental stages assayed. High molecular weight (~200 kD) activities were also apparent (arrow), but absent from embryos. (Lower panel) Protein extracts were analyzed by SDS/PAGE and stained with Coomassie Blue R-250 to control for equal loading and protein integrity. Each lane in both gels contains 20 mg of protein.
Figure 3. **Reduced RNase activity and expression correlates with reduced RNase X25 gene dose.** Ovarian extracts were prepared from wild type control (+/+), or deletion mutant Df(3L)Excel6279/+ females (+/-), carrying two or one copy of the RNase X25 gene, respectively. Protein samples were analyzed using (A) in gel RNase activity assay, or (B) standard SDS/PAGE analysis. Compared to the control (+/+), RNase activity was reduced in ovaries dissected from females with one copy of the RNase X25 gene (+/-). Each lane in both gels contains 20 mg of protein. (C) RNA was isolated from ovaries and qPCR quantification of the relative level of RNase X25 mRNAs in these samples was carried out using the ribosomal protein L3 (RpL3) transcript as internal standard control for normalization. RNase X25 expression levels were reduced in tissue samples from mutant Df(3L)Excel6279/+ females (+/-), compared to control females (+/+). Data are representative of 3 independent experiments and are means and S.E. of triplicates. **, P<0.01 (t-test).
Figure 4. *RNase X25* gene expression is regulated by nutritional and oxidative stress. RNA was isolated from whole 3rd instar larvae, 14 h after transfer to control or experimental media (see Materials and methods). qPCR quantification of the relative level of *RNase X25* mRNAs in these samples was carried out using the ribosomal protein L3 (RpL3) transcript as internal standard control for normalization. Increased levels of *RNase X25* transcripts were apparent in samples after (A) starvation and treatments with 1% [w/w] wheat germ agglutinin (WGA), and (B) 0.1% [w/w] or 0.5% [w/w] hydrogen peroxide. Data are representative of 3 independent experiments and are means and S.E. of triplicates. *, P<0.05; **, P<0.01 (t-test).
Figure 6. Starvation induces expression of the autophagy marker, *Atg5* and *Amyrel*, *Lip3* and *RNase X25* in larvae. RNA was isolated from whole 3rd instar larvae, 14 h after transfer to control (C) or starvation (S) conditions (see Materials and methods). qPCR quantification of the relative level of (A) autophagy marker *Atg5*, (B) starvation markers *Amyrel*, and *Lip3*, and (C) *RNase X25* mRNAs in these samples was carried out using the ribosomal protein L3 (RpL3) transcript as internal standard control for normalization. Increased levels of *Atg5*, *Amyrel*, *Lip3*, and *RNase X25* transcripts were apparent in samples after starvation as compared with fed-control animals. Data are representative of 3 independent experiments and are means and S.E. of triplicates. *, P<0.05; **, P<0.01 (t-test). (D) Protein extracts from 14 h starved (S) and fed-control (C) whole 3rd instar larvae were analyzed using RNase in gel activity assays as described in Figure 1. RNase activity in the size range corresponding to RNase T2 enzymes was evident in starved as compared with fed-control animals. Each lane in both gels contains 80 µg of protein. “F 4–24” denotes extracts from animals nourished with Formula 4–24 instant D. melanogaster diet without yeast; “Rich” denotes extracts from animals fed a yeast rich diet (see Materials and methods).
Figure 6. **Effect of starvation on the accumulation of Lysotracker-positive vesicles in larval fat body.** (A and B) A high level of bright red Lysotracker-positive vesicles accumulate in fat body cells isolated from (B) 14 h starved 3rd instar larvae, with few observed for (A) fed-control larvae. (C and D) Hoescht 33342 staining of DNA, and (E and F) merged images. Scale bar = 20 µm.
**Supplemental Figure S1.** Expression profile of RNase X25 in different adult tissues, obtained from the modENCODE database through a query in FlyBase (http://flybase.org). Data were obtained through RNA-Seq analysis of the tissues described in the figure, and were reported as RPKM (Reads per kilo base per million).
Supplemental Figure S2. Expression profile of RNase X25 in different adult and larval tissues, obtained from the FlyAtlas database (http://flyatlas.org). Data were obtained through Affymetrix microarray chip analysis of the tissues described in the figure, and were reported as average normalized signal intensity (4 chips per sample) +/- standard error.
Appendix A: Attempts to induce RNAi targeted gene silencing of \textit{RNase X25} lead to overexpression

In order to pinpoint the enzymatic activity observed in our \textit{in gel} assays to \textit{RNase X25}, we attempted to knock down expression of \textit{RNase X25} using RNA interference. We first used a \textit{MTD-Gal4} driver to direct the expression of \textit{UAS-RNaseX25} in the ovaries of female flies. The same driver was used to direct the expression of \textit{UAS-arl} in the ovaries of flies used as control for RNAi. Total RNA was extracted form isolated ovaries as described in the text. Levels of mRNA transcripts for \textit{RNase X25} were measured using qPCR in these flies as well as the wild type, \textit{UAS-RNaseX 25} and \textit{UAS-arl} lines without the drivers as additional controls. \textit{UAS-RNase X25} was analyzed to see if any leaky expression of RNAi in the absence of driver was evident. \textit{UAS-RNaseX 25}, \textit{UAS-arl} and \textit{UAS-arl} with ovary-specific drivers were expected to exhibit similar levels of \textit{RNase X25} mRNA as the wild type. As expected, no significant difference in expression was found between the wild type and \textit{UAS-arl} \textit{(}\textit{p} \textit{=} 0.52\textit{)} but the normalized level of \textit{RNase X25} transcript was found to be elevated more than three times in \textit{UAS-arl} with drivers compared to wild type (Figure 1A). Significantly higher levels of transcription were also shown by the \textit{UAS-RNaseX 25} and \textit{UAS-RNaseX 25} line with driver compared to the wild type. Instead of an expected reduction in the levels of \textit{RNase X25} transcripts, four times higher levels were detected in the RNAi line compared to the wild type for the gene being targeted. \textit{In gel} enzyme activity confirmed that the gene is being transcribed at levels similar to the wild type if not more (Figure 1B).

We decided to direct the expression in the somatic tissue instead of ovaries by using \textit{da-Gal4} driver and looked at \textit{RNase X25} expression in third instar larvae. \textit{UAS-Dicer} was also added to enhance the effect of RNAi in these animals. Total RNA was extracted as described and
analyzed using qPCR for \textit{RNase X25} expression. Flies transfected with an empty vector (\textit{att2/att2}) and \textit{da-Gal4/att2}, were used as controls. Other samples analyzed included \textit{UAS-RNaseX 25} (without driver), \textit{UAS-RNase X25; UAS-Dicer} (without driver), \textit{UAS-RNaseX 25/da-Gal4} and \textit{UAS-RNaseX 25; UAS-Dicer/da-Gal4}. We expected to see same levels of \textit{RNase X25} expression in the \textit{UAS-RNaseX 25/da-Gal4} and \textit{UAS-RNaseX 25} lines without drivers compared to controls. Although there was a slight increase in expression for both of these lines with respect to the controls, the difference was not statistically significant. Intriguingly, we saw increase in \textit{RNase X25} expression in \textit{UAS-RNaseX 25/da-Gal4} and \textit{UAS-RNaseX 25} lines without drivers compared to controls. Although there was a slight increase in expression for both of these lines with respect to the controls, the difference was not statistically significant. Intriguingly, we saw increase in \textit{RNase X25} expression in \textit{UAS-RNaseX 25/da-Gal4} and \textit{UAS-RNaseX 25; UAS-Dicer/da-Gal4} as well which again is contrary to expectations (Figure 2). Even though the increase is not as dramatic as seen in the ovaries, about two-fold for \textit{UAS-RNaseX 25/da-Gal4} and about 1.5 fold for \textit{UAS-RNaseX 25; UAS-Dicer/da-Gal4}, nonetheless it is interesting to see overexpression/increased expression where a knock down was expected.

It seemed like whenever there was expression of RNAi involved, leaky or otherwise, a dramatic increase in levels of \textit{RNase X25} was induced. The same phenomenon was observed in both cases, irrespective of whether the expression was directed to the ovarian or somatic tissue. One possible explanation might be, that the presence of shRNA in the organism induces a stress response resulting in overexpression of \textit{RNase X25}, which we have shown to be upregulated in response to stress in wild type flies.
Figure A1. **Attempts at silencing RNase X25 using RNAi result in overexpression instead of knockdown.** (A) qRT-PCR analysis for RNase X25 mRNA transcripts in controls and lines used for MTD-Gal4 driven expression of RNA induced silencing in ovaries. Leaky expression of shRNA in iX25 and complete expression of shRNA in iX25x31777 and iarlx31777 result in overexpression of RNase X25 instead of an expected knockdown in activity. (B) *In gel* analysis of RNase activity revealed presence of RNase X25 in all of the RNAi lines. A coomassie gel was run as a control for protein quality and loading.

Figure A2. **Attempts at silencing RNase X25 by directing expression in somatic tissue also result in overexpression.** (A) qRT-PCR analysis for RNase X25 mRNA transcripts in controls and lines used for att2-Gal4 driven expression of RNA induced silencing in somatic tissue with and without Dicer proved unsuccessful as was the case with MTD-Gal4 driven expression of RNA induced silencing in ovaries.
Appendix B: Use of CRISPR mediated generation of loss of function mutant for RNase X25

Since RNAi induced silencing did not effectively knock down the expression of RNase X25 (See Appendix A), we decided to employ a CRISPR mediated approach. Our approach consisted of designing two gRNAs (guide RNAs) within the coding sequence of the gene so that two double stranded cuts induced by the Cas9 nuclease guided by the gRNAs would result into deletion of a large region within the coding sequence. For designing the gRNAs, we used the CRISPR2 tool made available online by the Drosophila RNAi Screening Center at Harvard Medical School (http://www.flyrnai.org/crispr2/). The location of the two gRNAs chosen can be seen in Figure B1 below (Red Circles). As can be seen, the deletion induced thus would span a large region of the coding sequence and should result into a complete knock out or expression of defective protein product. Importantly, the region destined to be deleted contains both CASI and CASII regions, ensuring that any protein being successfully expressed remains catalytically inactive.
Figure B1. CRISPR strategy for generating RNase X25 loss of function mutants. CRISPR2 tool available online was used for generating two gRNA sequences (red circles) to be used for making two double stranded cuts within the RNase X25 gene. A deletion induced thus should result in complete knock out of expression or expression of a catalytically inactive protein since both of the conserved active sites for RNAse X25 activity are included within the region destined to be deleted.
CHAPTER 3
CHARACTERIZATION OF LYSOSOMAL ASSOCIATED MEMBRANE GLYCOPROTEIN-1 (LAMP1) IN DROSOPHILA MELANOGASTER

Introduction

Lysosomes are highly acidic, membrane bound organelles found in animal cells that enclose a variety of acid hydrolases and act as the terminal degradative compartment for endocytosis as well as autophagy. They constitute as much as 5% of the total intracellular volume, and are heterogeneous with respect to their size and morphology. (Luzio et al., 2007).

The lysosomal membrane, a 7-10 nm thick single phospholipid bilayer (Saftig et al., 2010), not only serves as a barrier between the cytoplasm and the hydrolytic enzymes enclosed within the lysosome, but is also responsible for other functions such as maintaining the acidic pH within the lysosomal lumen and fusion with endosomes, autophagosomes and other organelles (Eskelinen et al., 2003; Saftig and Klumperman, 2009). More than twenty membrane transporters have been shown to exist within the lysosomal membrane (Sagne and Gasnier, 2008).

Lysosome associated membrane glycoprotein-1 (LAMP1) and LAMP2 are major protein components of the lysosomal membrane, constituting ~50%, of all proteins within the lysosomal membrane. Characterized as type 1 membrane proteins, both LAMPs possess a short cytoplasmic tail, a transmembrane domain a heavily glycosylated luminal domain. The cytosolic tails of LAMP-1 and LAMP-2 are conserved, and required for intracellular targeting (Hunziker and Geuze, 1996). Despite many similarities and conservation in sequences, LAMP1 and LAMP2 are distinct proteins (Fukuda, 1991). The human LAMP1 and LAMP2 have a molecular mass of 40-45 kDa sans glycosylation and approximately 120 kDa after glycosylation (Carlsson et al., 1988; Mane et al., 1989).
A study showed that LAMP2 knockout mice exhibited symptoms similar to Danon disease in humans (Saftig et al., 2001). These animals exhibited accumulation of autophagic bodies in heart and skeletal muscle, leading to fatal cardiomyopathy and myopathy. Another study in LAMP2 knockout mice reported impaired fusion between lysosomes and phagosomes leading to early onset of periodontitis in these animals (Beertsen et al., 2008).

Embryonic fibroblasts that were depleted of both LAMPs displayed accumulation of autophagic vesicles, alteration in the appearance of lysosomes and disruption of cholesterol metabolism without any apparent effects on protein degradation (Schneede et al., 2011). RNA induced silencing of LAMP2 in mammalian cells, showed a noticeable reduction in fusion between autophagosomes and autolysosomes (Gonzalez-Polo et al., 2005). LAMP2 has also been implicated in endosomal/lysosomal cholesterol transport (Eskelinen et al., 2004; Schneede et al., 2011).

LAMP2 has three isoforms, namely LAMP2A, LAMP2B and LAMP2C, possessing identical luminal domains but have different cytoplasmic and transmembrane domains (Hatem et al., 1995). The splice variants exhibit distinguishing subcellular localization and have different tissue distribution patterns (Cuervo and Dice, 2000; Gough and Fambrough, 1997; Konecki et al., 1995; Lichter-Konecki et al., 1999). LMAP2A has been implicated in chaperone-mediated autophagy (Cuervo and Dice, 2000; Zhou et al., 2005), whereas LAMP2B is found abundantly in the muscle where its deficiency leads to Danon disease in humans (Nishino et al., 2000). The C-terminal region of LAMP2C has only recently been shown to be capable of binding RNA with very high affinity in a study carried out in mice (Fujiwara et al., 2013).

Only one lysosomal associated protein, Lamp1 has been reported in the genome of Drosophila melanogaster. The C-terminal sequence of this protein shows a strong homology
(50%) with LAMP2C, one of the three variants for human LAMP2, and has also been shown to exhibit similarly high levels of affinity for RNA (Fujiwara et al., 2013). Lamp1 has been extensively used as a lysosomal marker in *Drosophila*, but the actual function of this protein in the lysosomal membrane remains unknown.

The aim of present study was to elucidate the role of Lamp1 in *Drosophila melanogaster*. Initial characterization of this gene points to a possible role in autophagy under normal and starvation conditions in the fat body tissue.

**Material and Methods**

*D. melanogaster* strains and culture conditions

*w*^11* was obtained from the Bloomington Drosophila Stock Center at Indiana University ([http://flystocks.bio.indiana.edu](http://flystocks.bio.indiana.edu)) and Lamp1*^e00879* (PBac{RB}Lamp1*^e00879*) was obtained from Exelixis at Harvard Medical School ([https://drosophila.med.harvard.edu/](https://drosophila.med.harvard.edu/)). All flies were maintained on standard cornmeal medium at 25ºC unless otherwise noted.

For starvation experiments, eggs were collected onto Bloomington’s Drosophila Stock Center cornmeal/molasses/yeast soft media sprinkled with Baker’s yeast. Early and late third instar lavae were starved by being placed onto PBS-saturated Whatman 1 filter paper at 25ºC for the indicated amount of time. At the end of this period, the larvae were either dissected for fluorescence microscopy or frozen at -80ºC for molecular analysis. A group of fed larvae was also collected and processed from the same plates used for picking larvae for starvation and was included as control for all experiments.
Inverse PCR

Genomic DNA was extracted from five *Lamp1<sup>e00879</sup>* flies as described on the Exelexis website (http://flystocks.bio.indiana.edu/pdfs/Exel_links/5_Fly_Prep_for_iPCR_pub.pdf). Inverse PCR (iPCR) was performed to confirm the location of P-element insertion within the gene of interest by following the procedure provided by the Exelexis website (http://flystocks.bio.indiana.edu/pdfs/Exel_links/5__fly_iPCR_piggyBac_pub.pdf).

Gene Expression Analysis

Total RNA was extracted from 100 mg of sample using Trizol (Fisher) according to manufacturers’ instructions. RNA was DNase-treated using Turbo DNA-free (Ambion), and cDNA was synthesized using the qScript Flex cDNA kit (Quanta Biosciences), also following manufacturers’ instructions for each procedure. Quantitative real time PCR (qRT-PCR) was performed on a Stratagene MX4000 system using the Absolute qPCR with SYBR Green + Rox kit (Fisher Scientific) according to manufacturers’ instructions. The transcript of ribosomal protein L32 gene (RPL-32) was used as the control for data normalization, using the Pfaffl method (Pfaffl, 2001). The primers used in this study are listed in Table 1.

Experiments were performed using triplicates, and repeated 3 times using independent samples. Statistical significance of the differences between treatments was determined using t-test. Graphs show averages of each sample normalized using the average value of the control sample. One star = P<0.05, two stars = P<0.01.
**Fluorescence Microscopy**

Fat body tissue was dissected in PBS followed by staining with LysoTracker Red DND-99 (Life Technologies, Carlsbad, CA, USA) and Hoeschst 33342 (Thermo Fisher Scientific Inc, Rockford, IL, USA) as described previously (Juhasz and Neufeld, 2008; Scott et al., 2004). Stained fat body lobes were imaged in PBS using a Zeiss Axio Imager.Z2 microscope equipped with AxioCam HR digital camera using an LD Plan-Neofluar 40x/0.6 objective lens and ZEN imaging software. Quantitative analysis was carried out manually using ZEN imaging software by randomly choosing a region of interest of defined size. Six images were analyzed for each treatment and the number/average area data were normalized to control. Statistical difference was determined by performing two-tailed t-tests.

**Results and Discussion**

*Lamp1 is expressed during all stages of the Drosophila life cycle*

As a first step towards characterization of *Lamp1* in *Drosophila*, gene expression was determined at various stages of the life cycle, viz. larvae, white pupae, brown pupae, adult males, adult females as well as isolated ovaries using qRT-PCR. *Lamp1* transcript was found to be highly expressed during the larval and pupal stages in addition to adult male flies, the highest expression being during the brown pupae stage (Figure 1). Even though, the expression varied greatly among the sexes, approximately same amounts of transcripts were found for adult females and isolated ovaries.
**Lamp1 expression is upregulated upon induction of autophagy**

In *Drosophila*, autophagy is induced under nutritional stress as well as a normal physiological response to hormones during development. During the fly developmental cycle, metamorphosis takes place during the pupal stage in response to a high titer pulse of the steroid hormone, ecdysone (20-hydroxyecdysone), released at the end of the third instar in the wandering larva (Riddiford and Truman, 1993). Total RNA was isolated from *w*^1118^ early and late third instar larvae reared under standard feeding conditions and *Lamp1* expression was analyzed using qRT-PCR. *Lamp1* expression was found to be significantly increased (P<0.01) in late compared to early *w*^1118^ third instar larvae (Figure 2A). Accumulation of approximately two-fold more *Lamp1* transcript in the later stage indicated that *Lamp1* expression is upregulated upon induction of developmental autophagy in larvae prior to pupation. qRT-PCR analysis of *Atg8*, an autophagy marker, showed a non-significant increase (P=0.064) in expression in late compared to early *w*^1118^ third instar larvae (Figure 2B). This may be explained by the fact that even though a high titer pulse of ecdysone is released at the end of the third instar stage, not all tissues seem to respond to the hormone at the same time. Midgut histolysis initiates immediately following pupation whereas degeneration of salivary glands begins 12-14 hours later (Jiang et al., 1997). The fat body appears to be the only tissue where histolysis begins before pupation (Butterworth and Forrest, 1984), which may be the reason why levels of *Atg8* from whole larval extracts were not found to be significantly high as would be expected upon induction of autophagy.

In order to see whether the expression of *Lamp1* is altered upon induction of autophagy under nutritional stress, *w*^1118^ larvae were starved for 14 hours as described in materials and methods followed by qRT-PCR analysis. Larvae that were fed a standard diet comprised the
control group. Significantly higher (P<0.01) levels of Lamp1 transcripts were found to be accumulated in starved compared to fed w^{1118} larvae (Figure 2C) indicating upregulation of Lamp1 upon induction of autophagy under conditions of nutritional stress. The magnitude of increase in Lamp1 mRNA was similar to that observed in late 3i larvae.

**Lamp1 and RNase X25 are concomitantly induced under stress**

RNase X25 is the only RNase T2 present in *Drosophila*. This enzyme is localized to lysosomes and has been shown to be induced under nutritional stress (Ambrosio et al., 2014). We found a concomitant increase in Lamp1 and RNaseX25 mRNA transcripts in w^{1118} larvae that were subjected to starvation for 14 hours compared to fed control animals (Figure 3).

**Depletion of Lamp1 results in larger autophagic vacuoles under normal conditions**

An insertional loss of function mutant for Lamp1, Lamp1^{e00879}, was obtained from Exelixis at Harvard Medical School. In order to confirm the location of the insertion within the gene of interest, iPCR was performed on genomic DNA extracted from flies as described. The recovered flanking sequences located the insertion in the first intron of the gene (Figure 4A).

RT-PCR for Lamp1 revealed that the gene had been successfully knocked out with no amplification in the loss of function mutant (Figure 4B). Further analysis using qRT-PCR in the wild type (w^{1118}) and Lamp1^{e00879} third instar larvae confirmed that gene expression had indeed been interrupted (P<0.01; Figure 4C). We chose fat body tissue as a model system to study the effect of Lamp1 depletion on the process of autophagy because of its extensive use as a model for understanding autophagy in *Drosophila* (Rusten et al., 2004; Scott et al., 2004; Wang et al., 2012). Using Lysotracker red (LTR) staining, we generated a developmental profile in w^{1118} to
use as a control to compare against $Lamp1^{e00879}$. Fat body tissue was dissected from third instar larvae under standard feeding conditions at three developmental stages, viz., early (E) stage when the larvae are actively feeding, late and wandering on food (LF) stage when the larvae are feeding only occasionally and are wandering on the surface of the food, and late and wandering on the lid (LL) stage, when the larvae have lost interest in feeding and are wandering on the lid of the petri plate containing food, in preparation to pupate. LTR staining showed high background fluorescence and few to no puncta in E, whereas a marked increase in the number of LTR-positive puncta could be seen in LF (Figure 5A-C) consistent with onset of developmental autophagy. LTR staining in LL showed a dramatic increase in size of LTR-positive puncta compared to LF.

We then proceeded to analyze the fat body tissue of $Lamp1^{e00879}$ using similar technique to see if $Lamp1$ depletion has any effect in this context. Fat body tissue was dissected from $Lamp1^{e00879}$ larvae reared under standard feeding conditions at the same three developmental stages as $w^{1118}$, as described above. Numerous punctate LTR-positive vesicles were visible in $Lamp1^{e00879}$ fat body tissue in E compared to wild type (Figure 5D). The size of the LTR-positive vesicles was also seen to increase gradually from E to LF to LL in $Lamp1^{e00879}$ just as in the wild type but the overall size of these vesicles appeared to be much larger in $Lamp1^{e00879}$ comparatively (Figure 5E and F). Quantitative analysis of the number and size of LTR-positive vesicles in the LL stages for both treatments indicated that even though there was no significant difference with respect to the total number of vesicles among the two, the average area of the vesicles was significantly greater in $Lamp1^{e00879}$ compared to $w^{1118}$ (Figure 5I). Moreover, these larger vesicles seemed to be forming aggregates (Figure 5H, white arrows). Quantitative analysis for comparing the average count and average size of LTR-positive vesicles was conducted for
LL stage only since overexposure of images from E stages resulted in high background levels making it difficult to select LTR-positive puncta for analysis with certainty (See Appendix).

**Atg8 is upregulated in Lamp1 mutant under normal conditions**

LAMP2 depletion has been shown to lead to accumulation of autophagic vacuoles in embryonic fibroblasts and heart and skeletal muscle in mice (Saftig et al., 2001; Schneede et al., 2011). A number of studies have shown that absence of LAMP2 leads to defects in phagosome-lysosome fusion and autophagosome-lysosome fusion, resulting in accumulation of autophagosomes within the cells (Beertsen et al., 2008; Gonzalez-Polo et al., 2005). Mechanistically, autophagy can be divided into three major steps: induction, nucleation and membrane expansion/completion (Xie and Klionsky, 2007). Atg1 complex is responsible for induction of autophagy in eukaryotes. A complex containing the Vps34 (class III PI3K) is involved in autophagosomal membrane nucleation. The last step, i.e. vesicle expansion, requires two distinct sets of ubiquitin-like protein conjugation systems, viz., Atg5-Atg12-Atg16 and Atg8, both of which have been found to be highly conserved (Ohsumi, 2001). Two Atg8 genes are present in *Drosophila*, both of which have been shown to be localized to autophagosomes (Juhasz et al., 2008; Rusten et al., 2004; Scott et al., 2004).

In order to determine whether Lamp1 deficiency in *Lamp1<sup>e00879</sup>* resulted in accumulation of autophagic vesicles, qRT-PCR analysis of *Atg8a* was performed. Significantly higher levels of *Atg8* mRNA transcripts in the *Lamp1* loss of function mutant, *Lamp1<sup>e00879</sup>* were present compared to wild type larvae under normal feeding conditions (Figure 6). This upregulation in Atg8 expression correlated well with the appearance of LTR-positive vesicles at E stage in *Lamp1<sup>e00879</sup>* larvae (Figure 5D).
**RNaseX25 and Acph-1 expression is not altered by stress in Lamp1**

Ribonuclease X25 (RNaseX25) belongs to the RNase T2 family, has an acidic pH preference and is likely localized within lysosomes in *Drosophila* (Ambrosio et al., 2014). Acid phosphatase 1 (Acph-1) is known to be localized within lysosomes and shares a number of important amino acid motifs with its human counterpart (Chung et al., 1996). The modENCODE Temporal Expression Data available in FlyBase (http://flybase.org) for both RNaseX25 and Acph-1 show very similar patterns of expression during the third instar stage. qRT-PCR analysis for both of these genes in larvae reared under standard feeding conditions revealed no significant differences in terms of expression between *Lamp1** and *w** (Figure 6) indicating that the induction of autophagy in *Lamp1** fed larvae corresponded to accumulation of autophagic vacuoles rather than lysosomes.

**Lamp1 deficiency causes LTR-positive puncta to become bigger and less numerous under nutritional stress**

Since our RT-qPCR analysis had revealed an upregulation of *Lamp1* expression under conditions of nutrient stress, we decided to look at the behavior of putative autophagic vacuoles in the *Lamp1* mutant *Lamp1** under similar conditions. Lysotracker red staining was performed for fat bodies dissected from early (E), late wandering on food (LF) and late wandering on lid (LL) larvae subjected to nutritional stress for 3-4 h. It had been reported previously that autophagosomes appear in the fat body of *Drosophila* within one hour of starvation (Scott et al., 2004).

In *w** nutritional stress resulted in accumulation of a large number of LTR-positive vesicles at E stage (Figure 7A) in contrast to fed *w** at the same stage (compare to Figure5A).
A gradual increase in number and size of vesicles was seen at LF and LL stages just as in fed larvae, but the effect seemed more intense in the starved larvae (Figure 7B and C). Quantitative analysis confirmed that the number of LTR-positive vesicles was indeed significantly higher (P<0.01) in \( w^{1118} \) starved larvae at LL stage compared to fed larvae at the same stage (Figure 8C). In \( \text{Lamp1}^{e00879} \), there appear to be at least as many as, if not more punctate LTR-positive vesicles at E stage as starved \( w^{1118} \) larvae (Figure 7D, compare to A). The LF stage (Figure 7E) exhibited a clear increase in size and reduction in number of puncta compared to E and numerous, large vesicles could be seen at the LL stage (Figure 7F, compare to C). At the LL stage, a significant decrease was observed in the number of LTR-positive puncta in starved \( \text{Lamp1}^{e00879} \) compared to starved \( w^{1118} \) (P<0.05) accompanied by a significant increase in average area (P<0.01; Figure 7I). The enlargement of vesicles seemed due to the aggregation of multiple relatively smaller vesicles (Figure 7H).

**Conclusions**

In this study, we performed an initial characterization of Lysosomal associated membrane glycoprotein-1 (Lamp1). Even though, Lamp1 has been extensively used in cell biology as a lysosomal marker, the actual function of this gene remains anonymous in *Drosophila*. We have shown that *Lamp1* is highly expressed during the early developmental stages of *Drosophila* life cycle as well as in adult males, although the level of expression plummeted in adult females. Moreover, the expression seemed to be very similar among adult females and isolated ovaries which could be explained by the fact that a large proportion of the female body is occupied by ovaries.
A significant upregulation of \textit{Lamp1} transcription during the later phase of wild type third instar coincided with the time of onset of developmental autophagy in flies, indicative of a putative role of Lamp1 in autophagy or macromolecular degradation within the fat body cells under normal conditions. In \textit{Drosophila}, autophagy is induced as a normal physiological response to ecdysone, a steroid hormone, in preparation for metamorphosis during the pupal stage (Riddiford and Truman, 1993). A similar upregulation in \textit{Lamp1} expression was shown for larvae under conditions of nutritional starvation which further strengthens the assumption that this protein is likely linked to autophagy.

In order to further deduce the role of this protein in the context of autophagy, we characterized a loss of function mutant for \textit{Lamp1}, \textit{Lamp1\textsuperscript{e00879}}, which showed accumulation of fewer but significantly larger LTR-positive vesicles in the late (LL) stage of third instar compared to wild type under both normal feeding and starvation conditions. Unlike its orthologs in human and mice, where two such genes, namely, \textit{LAMP1} and \textit{LAMP2} are found, only one such gene has been shown to exist in \textit{Drosophila}. Moreover, only one annotated transcript has been reported for this gene in the fly. The C-terminal domain of Lamp1 has been shown to be homologous to the C-terminal domain of LAMP2C of mice and humans (Fujiwara et al., 2013). In humans and mice, LAMP2 deficiencies have been correlated with anomalies in the autophagic pathway (Beertsen et al., 2008; Gonzalez-Polo et al., 2005; Saftig et al., 2001; Schneede et al., 2011). The presence of abnormal lysosomes/autophagic vacuoles aggregating in groups in \textit{Lamp1\textsuperscript{e00879}} seems akin to what has been observed in prior studies involving \textit{LAMP2} knockouts in other model systems. Furthermore, analysis of molecular markers associated with autophagosomes and lysosomes, (\textit{Atg8} for the former, \textit{RNaseX25} and \textit{Acph-1} for the latter) revealed significant upregulation of \textit{Atg8} but not for \textit{RNaseX25} and \textit{Acph-1} in \textit{Lamp1\textsuperscript{e00879}}.
compared to wild type under normal conditions. Analysis of these molecular markers in $Lamp1^{e00879}$ under conditions of starvation is underway in our lab and might shed more light on the nature of function in $Lamp1$ in flies.

In conclusion, initial characterization points to the possibility that $Lamp1$ might be involved in the successful culmination of the final step of the autophagic pathway, i.e. fusion of autophagic vacuoles with lysosomes. One other possible function for this protein that was recently suggested might be direct binding of RNA for uptake for degradation within the lysosomes (Fujiwara et al., 2013). For future experiments, further insight into the function of this gene might be gained by crossing $Lamp1^{e00879}$ with GFP-Lamp1 flies to rescue the phenotype; phenotypic characterization of $Lamp1^{e00879}$ such as determination of lifespan, metamorphosis time and sensitivity to stress; analysis of aggregates of LTR-positive vesicles seen at LL stage using electron microscopy and experiments to determine transport of RNA into lysosomes in a cell-free system.

REFERENCES


Figure 1. **Developmental profile of Lamp1 transcript.** RNA was isolated from animals at 3rd instar larval (Larvae), white prepupal (White pupae), pupal (Brown pupae), and adult male or female stages of development. Ovarian tissue was prepared from 3–5 day old females. qPCR quantification of the relative level of RNase X25 mRNAs in these samples was carried out using the ribosomal protein L32 (Rpl32) transcript as internal standard control for normalization. *Lamp1* expression was detected at all stages analyzed. Data are representative of 3 independent experiments and are means and S.E. of triplicates.
Figure 2. *Lamp1 gene expression and autophagy*. RNA was isolated from appropriately stages wild type (w1118) whole 3rd instar (3i) larvae fed standard diet or starved for 14 h (see Materials and methods). qPCR quantification of the relative level of *Lamp1* mRNAs in these samples was carried out using the ribosomal protein L32 (RpL32) transcript as internal standard control for normalization. (A) Significantly increased levels of *Lamp1* transcripts were apparent in late 3i larvae compared to early ones upon induction of developmental autophagy. (B) Accumulation of *Atg8* mRNA levels in late 3i larvae were not significantly different from early 3i larvae. (C) Significantly increased levels of Lamp1 transcripts were apparent in starved 3i larvae compared to the control fed ones. Data are representative of 3 independent experiments and are means and S.E. of triplicates. *, P<0.05; **, P<0.01 (t-test)
Figure 3. *Lamp1* and *RNase X25* are concomitantly induced under stress. RNA was isolated from appropriately staged wild type (w<sup>1118</sup>) whole 3rd instar (3i) larvae fed standard diet or starved for 14 h (see Materials and methods). qPCR quantification of the relative levels of *Lamp1* and *RNaseX25* mRNAs in these samples was carried out using the ribosomal protein L32 (RpL32) transcript as internal standard control for normalization. Significantly increased levels of both *Lamp1* and *RNaseX25* transcripts were apparent in starved 3i larvae compared to early ones upon induction of developmental autophagy. Data are representative of 3 independent experiments and are means and S.E. of triplicates. *, P<0.05; **, P<0.01 (t-test)
Figure 4. **Loss of function mutant for Lamp1.** (A) Schematic of the PiggyBac (PBac) insertion in the *Drosophila Lamp1* gene. The insertion was mapped by iPCR as described in materials and methods. Boxes represent exons, and lines represent introns (between exons). (B) Total RNA extracted from third instar larvae was subjected to RT-PCR using *Lamp1*-specific primers (indicated by purple arrows in A) in *w*1118 and *Lamp1*e00879. No amplification product was seen in *Lamp1*e00879 indicating successful loss of activity of *Lamp1* in the mutant. (C) qPCR quantification of the relative level of *Lamp1* in *w*1118 and *Lamp1*e00879 was carried out using the ribosomal protein L32 (RpL32) transcript as an internal standard for normalization using the same 3’ primer as for B with a 5’ prime primer designed to span the exon-exon junction between exons 3 and 4 (indicated by red in A). Data are representative of 3 independent experiments. *, P<0.05, **, P<0.01
Figure 5. **Accumulation of Lysotracker red (LTR)-positive vesicles in fat body during larval development.** Lysotracker red staining was performed for fat bodies dissected from early (E), late wandering on food (LF) and late wandering on lid (LL) larvae reared under standard feeding conditions. (A-C) In *w^{118}*, very few LTR-positive puncta are visible at the early stage, however a marked increase in number of LTR-positive puncta can be seen in (B) at LF stage, whereas LL stage (C) exhibits a noticeable increase in size as well. (D-F) In *Lamp1<sup>e00879</sup>* tissue, numerous punctate LTR-positive vesicles are visible at E stage (D) compared to (A), the LF stage (E) exhibits increase in size and reduction in number of puncta compared to (B) and numerous, huge vesicles can be seen at the LL stage (F, compare to C). (G-H) Enlarged regions of interest from E and F show preponderance of large LTR-positive vesicles in mutant LL stage tissue which seem to be aggregating (white arrows). (I) Quantitative analysis of LL stages in *w^{118}* and *Lamp1<sup>e00879</sup>* reveals that there are lesser but bigger vesicles present in the mutant compared to the wild type. Scale bar = 5 µm, *, P<0.05, **, P<0.01 (t-test)
Figure 6. *Atg8* is upregulated whereas *RNaseX25* and *Acph-1* expression levels remain unchanged under normal conditions in *Lamp1^{e00879}* . RNA was isolated from appropriately staged whole 3rd instar (3i) larvae fed standard diet. qPCR quantification of the relative levels of *Atg8*, *RNaseX25* and *Acph-1* mRNAs in these samples was carried out using the ribosomal protein L32 (RpL32) transcript as internal standard control for normalization. Significantly increased levels of *Atg8* in *Lamp1^{e00879}* compared to wild type indicated increase in autophagosomes whereas unchanged levels for both *RNaseX25* and *Acph-1* transcripts indicated similar numbers of lysosomes in *Lamp1^{e00879}* . Data are representative of 3 independent experiments and are means and S.E. of triplicates. *, P<0.05; **, P<0.01 (t-test)
Figure 7. *Lamp1* deficiency causes autophagic vacuoles to become bigger and lesser under nutritional stress* Lysotracker red staining was performed for fat bodies dissected from early (E), late wandering on food (LF) and late wandering on lid (LL) larvae subjected to nutritional stress for 3-4 h as described in text. (A-C) In *w*1118, nutritional stress results in accumulation of LTR-positive vesicles (A) which show a gradual increase in size at LF stage (B) and LL stage (C). (D-F) In *Lamp1*00879, there appear to be at least as many as, if not more than (A) punctate LTR-positive vesicles at E stage (D), the LF stage (E) exhibits increase in size and reduction in number of puncta compared to (B) and numerous, huge vesicles can be seen at the LL stage (F, compare to C). (G-H) Enlarged regions of interest from E and F show preponderance of large LTR-positive vesicles in mutant LL stage tissue which seem to be aggregating (white arrows). (I) Quantitative analysis of LL stages in *w*1118 and *Lamp1*00879 indicates a significant decrease in number accompanied by a significant increase in size of the LTR-positive vesicles in the mutant compared to the wild type. Scale bar = 5 µm, *, P<0.05, **, P<0.01(t-test)
Figure 8. **Summary of quantitative analysis of average number and average size of LTR-positive vesicles in wild type and mutant fat body.** (A) No significant difference with respect to number of LTR-positive puncta was seen between the two treatments under fed conditions, but a significant decrease in *Lamp1<sup>e00879</sup>* (e00879) was apparent under starved conditions. (B) On average, the size of LTR-positive puncta was greater in *Lamp1<sup>e00879</sup>* compared to wild type under both fed and starved conditions. (C) Significant increase in the number of LTR-positive puncta was observed in starved *w<sup>1118</sup>* compared to fed larvae.
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Table 1. **List of primers used in this study.**
Appendix: Limitations in quantitative analysis of LTR-positive puncta in Early (E) stage of third instar

The fluorescent images were analyze in order to quantitate the number and size of LTR-positive puncta observed in fat body tissue in different fly strains under different treatments in order to perform statistical testing for significant differences. This was achieved successfully for images obtained from late, wandering on lid (LL) larvae but could not be done for images obtained from larvae at the early feeding stage (E). At this stage, a high background for Lysotracker red was present due to very long exposure times (set automatically by the imaging system used during microscopy) compared to other stages (see Figure A1). This was particularly true for the wild type larvae particularly under fed condition, and also to a lesser extent under starvation. The mutant also exhibited a higher background compared to mutant larvae subjected to starvation.
Figure A1. **Lysotracker red (LTR) to Hoechst ratio for Exposure time (ms).** For each treatment a ratio for exposure time was calculated for LTR and Hoechst from each image within a treatment and average was calculated. The plot shows the average ratio for each treatment and the error bars represent standard errors.
Conclusion

Lysosomes act as the terminal degradative compartment for a number of catabolic pathways within the cell, such as endocytosis and autophagy. Approximately fifty hydrolases are present within the lysosome, and deficiency in even any one of these can lead to a lysosomal storage disorder, resulting in accumulation of the deficient enzyme’s substrate to toxic levels. In the present study, we performed initial characterization of two genes, RNase X25 and Lamp1 in Drosophila melanogaster, the products of which could have direct consequences on the functioning of lysosomes. RNase X25 is an RNase T2 enzyme likely targeted to lysosomal lumen whereas Lamp1 is a lysosomal membrane associated protein which has recently been implicated as a receptor in a novel type of autophagy, termed “RNautophagy” (Fujiwara et al., 2013).

Initial characterization of RNase X25, the only member of the RNase T2 family present in Drosophila revealed that it is constitutively expressed during Drosophila development. In gel ribonuclease assays in wild-type and deficiency mutants confirmed that RNase X25 is a major contributor of endonuclease activity in Drosophila. Presence of a signal peptide cleavage site in the amino acid sequence (Hime et al., 1995) and a pH optimum in the acidic range for enzyme activity (Ambrosio et al., 2014) indicate that RNase X25 may be targeted to the secretory pathway and localized within an acidic vesicle such as lysosome. This is consistent with localization of other RNase T2 enzymes to lysosomes in zebrafish and humans (Campomenosi et al., 2006; Haud et al., 2011). Ubiquitous expression during development and adulthood in Drosophila suggests that RNase X25 may perform a housekeeping function as has been proposed for other constitutively expressed eukaryotic RNase T2 enzymes (MacIntosh, 2011). Studies aimed at elucidating the nature of this housekeeping function indicate that the role of
these RNases is to maintain normal cellular homeostasis by recycling rRNA since lack of these enzymes in Arabidopsis and zebrafish lead accumulation of rRNA in vacuoles or lysosomes (Haud et al., 2011; Hillwig et al., 2011). One possible route for achieving this function would be a specialized type of autophagy process known as ribophagy. Ribophagy has been described for yeast cells undergoing starvation, as the targeted degradation of ribosomes through a mechanism that uses the autophagy machinery of the cell (Kraft et al., 2008).

Even though RNase T2 enzymes have not been shown to play a direct role in ribophagy, they have been implicate as likely participants in this process (Andersen and Collins, 2012; Haud et al., 2011; MacIntosh and Bassham, 2011). We have shown that flies subjected to nutritional stress exhibited a significant induction of RNase X25 expression in the third instar larvae. We also observed an increase in the expression of Atg5, which encodes one of the core components of the autophagy machinery that has been previously shown to be induced by starvation in Drosophila ovaries (Barth et al., 2011), and the robust appearance of Lysotracker red-positive vesicles in larval fat body cells, marking lysosomes and autolysosomes participating in the autophagy process (Jimenez-Sanchez et al., 2012; Scott et al., 2004). Induction of RNase X25 by nutritional stresses and evidence of autophagy may indicate that this enzyme also has a role in cellular homeostasis through recycling of cellular RNAs. Induction of RNase X25 under oxidative stress (Ambrosio et al., 2014) indicates that this enzyme may have additional functions other stress responses as well.

Our interest in Lamp1 was piqued by a recent study that demonstrated that LAMP2C behaves as a receptor for RNA in a novel type of autophagy, where RNA is uptaken directly in an ATP-dependent manner for degradation by the lysosomes (Fujiwara et al., 2013). The C-terminal region of LAMP2C in mice and humans is totally conserved and exhibits a high level of
homology with the C-terminal region of Lamp1 in *Drosophila* and has been shown to bind RNA with the same affinity as LAMP2C (Fujiwara et al., 2013). Even though Lamp1 has been used as a lysosomal marker extensively in *Drosophila*, the actual function of this protein remains unknown. We hypothesized that rRNA might be transported to the lysosomal lumen through the action of Lamp1 as a transporter in an ATP-dependent manner where it is degraded by RNase X25. However, it was suggested that direct uptake of RNA for degradation by lysosomes (RNautophagy) contributed to around 10%-20% of the total RNA being degraded in a cell, the rest being degraded by some LAMP2-independent pathway (Fujiwara et al., 2013), most likely ribophagy or general autophagy pathways.

In this study, we have demonstrated that Lamp1 is highly expressed during the larva and pupal stages of development in *Drosophila*. A high level of expression was also observed in adult males, however the level of expression in adult females was much lower comparatively. In *Drosophila*, autophagy is not only induced as in response to nutritional stress, but also as a normal physiological response to a steroid hormone, ecdysone, during the pupal stage, in preparation for metamorphosis (Riddiford and Truman, 1993). We observed induction of Lamp1 transcription during the later phase of wild type third instar, coinciding with the time of onset of developmental autophagy in flies, indicative of a putative role of Lamp1 in autophagy or macromolecular degradation within the fat body cells under normal conditions. Similarly, Lamp1 expression was also found to be upregulated for larvae under conditions of nutritional starvation indicating that this gene is likely linked to autophagy.

A loss of function mutant, Lamp1e00879 was characterized to deduce the role of Lamp1 protein in the context of autophagy. Fluorescence microscopy of fat body tissue revealed accumulation of fewer but significantly larger LTR-positive vesicles in the late (LL) stage of
third instar compared to wild type under conditions of both standard feeding as well as starvation conditions. Humans and mice possess two LAMP genes, namely LAMP1 and LAMP2 unlike Drosophila, where only one such gene has been reported. Only one annotated transcript has been reported for Lamp1 in Drosophila with a C-terminal domain homologous to the C-terminal domain of LAMP2C of mice and humans (Fujiwara et al., 2013). LAMP2 deficiencies have been correlated with anomalies in the autophagic pathway in humans and mice (Beertsen et al., 2008; Gonzalez-Polo et al., 2005; Saftig et al., 2001; Schneede et al., 2011). The incidence of enlarged, atypical lysosomes/autophagic vacuoles aggregating in groups in Lamp1e00879 seems analogous to observations in previous studies involving LAMP2 knockouts in mice. Also, significant upregulation of Atg8, an autophagy marker was observed, but not for RNaseX25 and Acph-1, molecular markers of lysosomes, in Lamp1e00879 compared to wild type under normal feeding conditions. Analysis of these molecular markers in Lamp1e00879 under conditions of starvation might shed more light on the nature of function of Lamp1 in flies.

In conclusion, initial characterization of Lamp1 implies that this transmembrane protein may likely be involved in fusion of autophagic vacuoles with lysosomes in addition to being a receptor for RNA for uptake for degradation within the lysosomes as recently suggested (Fujiwara et al., 2013). Whereas initial characterization of RNase X25 suggests that this ancient enzyme may be playing a physiologically conserved role in the fly as demonstrated in other organisms. Use of Drosophila as a model, whose genome encodes only one gene each for Lamp1 and RNase T2 and lacks RNase A homologs or other evident secretory RNases with similar activity, it is likely to provide insight into the possible roles being played by the two in rRNA degradation during normal growth and development, and also under stress conditions.
REFERENCES


