Lysosomal RNASET2 is Required for Metabolic Stress-Mediated ROS Generation and Cell Death

George Caputa

Washington University in St. Louis

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Lysosomal RNASET2 is Required for Metabolic Stress-Mediated ROS Generation and Cell Death

by

George Caputa

A dissertation presented to the Graduate School of Arts and Sciences of Washington University in partial fulfillment of the requirements for the degree of Doctor of Philosophy

December 2015

Saint Louis, Missouri
Table of Contents

List of Figures and Tables……………………………………………………………………..v
List of Abbreviations…………………………………………………………………………..vii
Acknowledgements…………………………………………………………………………….ix
Abstract…………………………………………………………………………………………xi

CHAPTER ONE: Introduction to Lipotoxicity

Obesity and diabetes are global health concerns………………………………………1
Lipotoxicity underlies complications to metabolic diseases…………………………2
Molecular mechanisms linking metabolic excess to cell death……………………….5
Novel insights into lipotoxicity through a forward genetic screen…………………..7
The Rpl13a snoRNAs mediate lipotoxicity and general oxidative stress……………8
A novel role for RNASET2 in lipotoxicity………………………………………………9

CHAPTER TWO: RNASET2 mediates lipotoxic cell death

Introduction………………………………………………………………………………….10
Results……………………………………………………………………………………..11
  2B1 cells are resistant to lipotoxic cell death………………………………………..11
  The gene for RNASET2 is disrupted in 2B1 cells…………………………………..11
  Loss of RNASET2 protects cells from lipotoxic cell death………………………..13
  Catalytic activity of RNASET2 is required for lipotoxic cell death………………13
Conclusions…………………………………………………………………………………14
Figures…………………………………………………………………………………………17

CHAPTER THREE: RNASET2 regulates cellular redox tone
CHAPTER FOUR: Substrates of RNASET2

Introduction .................................................................................................................37

Results .........................................................................................................................37

Loss of RNASET2 does not alter rRNA, tRNA, or bulk RNA degradation........37

2B1 cells have differential changes in gene expression during lipotoxicity......38

RNASET2 influences XIAP mRNA and protein levels...............................39

XIAP does not mediate ROS generation.................................................................40

Haploinsufficiency for RNASET2 increases the half-life of XIAP mRNA......40

Loss of RNASET2 affects lysosomal homeostasis during lipotoxicity.........41

Neutralization of lysosomal pH stabilizes XIAP mRNA..............................43

XIAP mRNA associates with the lysosome-containing fraction of 2B1 cells....43

XIAP mRNA is enriched in the lysosomes of 2B1 cells.................................44

Conclusions .................................................................................................................45

Figures ........................................................................................................................49
CHAPTER FIVE: *In vivo* role of RNase X25 in oxidative stress

Introduction ..................................................................................................................65

Results..........................................................................................................................66

- Fat body RNaseX25 knockdown flies are fertile and have normal bodyweight.66
- Knockdown of RNaseX25 in fat bodies protects flies from paraquat toxicity….66
- Knockdown of RNaseX25 in fat bodies protects flies from oxidative stress…..67

Conclusions..................................................................................................................67

Figures ..........................................................................................................................70

CHAPTER SIX: Summary and Discussion

Summary ......................................................................................................................73

- RNASET2, ROS, and the *Rpl13a* snoRNAs. ...............................................................74
- RNASET2-mediated lysosomal degradation of RNA..................................................77
  *In vivo* role of RNASET2 during oxidative and metabolic stress.........................80

Figure ..........................................................................................................................83

CHAPTER SEVEN: Materials and Methods...................................................................84

References...................................................................................................................91
List of Figures and Tables

CHAPTER TWO

Figure 2.1 Loss of function screen produced palmitate resistant mutants.
Figure 2.2 2B1 cells are resistant to lipotoxic cell death.
Figure 2.3 Locus encoding RNASET2 is disrupted in 2B1 cells.
Figure 2.4 Loss of RNASET2 protects cells from lipotoxic cell death.
Figure 2.5 Catalytic activity of RNASET2 is required for lipotoxic cell death.

CHAPTER THREE

Figure 3.1 RNASET2 acts upstream of cytoplasmic accumulation of Rpl13a snoRNAs.
Figure 3.2 RNASET2 haploinsufficiency does not prevent production of Rpl13a snoRNAs.
Figure 3.3 Loss of RNASET2 protects cells from ROS accumulation during lipotoxicity.
Figure 3.4 2B1 cells have increased antioxidant capacity during lipotoxicity.
Figure 3.5 Loss of RNASET2 protects cells from ROS accumulation during oxidative stress.
Figure 3.6 Loss of RNASET2 protects cells from oxidative stress-induced cell death.
Figure 3.7 Transient overexpression of RNASET2 does not increase ROS or cell death.

CHAPTER FOUR

Figure 4.1 rRNA degradation occurs late in the lipotoxic response.
Figure 4.2 Loss of RNASET2 is associated with changes in gene expression
Table 4.1 Up-regulated genes in 2B1 cells
Figure 4.3 RNASET2 influences XIAP mRNA levels.
Figure 4.4 XIAP does not mediate ROS accumulation.
Figure 4.5 RNASET2 haploinsufficiency increases the half life of XIAP mRNA.
Figure 4.6 RNASET2 haploinsufficiency alters lysosomal homeostasis.
Figure 4.7  Disruption of lysosomal integrity influences XIAP mRNA levels.

Figure 4.8  XIAP mRNA associates with membrane bound organelles.

Figure 4.9  2B1 cells have an enlarged lysosomal compartment.

Figure 4.10  XIAP is enriched in 2B1 cells.

Figure 4.11  XIAP is enriched in the lysosomal compartment of 2B1 cells.

Table 4.1  Up-regulated genes in 2B1 cells

CHAPTER FIVE

Figure 5.1  Fat body RNaseX25 knockdown flies are fertile and have normal bodyweight.

Figure 5.2  KD of RNaseX25 in fat bodies protects flies from paraquat toxicity.

Figure 5.3  KD of RNaseX25 in fat bodies protects from oxidative stress.

CHAPTER SIX

Figure 6.1  RNASET2 degrades lysosomally-targeted XIAP mRNA.
## Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
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<tbody>
<tr>
<td>ABTS</td>
<td>2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)</td>
</tr>
<tr>
<td>DNPH</td>
<td>2,4-dinitrophenylhydrazine</td>
</tr>
<tr>
<td>DCF</td>
<td>2′,7′-dichlorodihydrofluorescein diacetate</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>CI</td>
<td>catalytically inactive</td>
</tr>
<tr>
<td>CHO</td>
<td>Chinese hamster ovary</td>
</tr>
<tr>
<td>Cyto</td>
<td>cytoplasm</td>
</tr>
<tr>
<td>DR5</td>
<td>death receptor 5</td>
</tr>
<tr>
<td>DHE</td>
<td>dihydroethidium</td>
</tr>
<tr>
<td>EV</td>
<td>empty vector</td>
</tr>
<tr>
<td>ER</td>
<td>endoplasmic reticulum</td>
</tr>
<tr>
<td>EtBr</td>
<td>ethidium bromide</td>
</tr>
<tr>
<td>gadd7</td>
<td>growth arrested DNA-damage inducible gene 7</td>
</tr>
<tr>
<td>H$_2$O$_2$</td>
<td>hydrogen peroxide</td>
</tr>
<tr>
<td>Lyso</td>
<td>lysosome</td>
</tr>
<tr>
<td>mdn</td>
<td>menadione</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger RNA</td>
</tr>
<tr>
<td>palm</td>
<td>palmitate</td>
</tr>
<tr>
<td>paraq</td>
<td>paraquat</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>P-body</td>
<td>processing body</td>
</tr>
<tr>
<td>PI</td>
<td>propidium iodide</td>
</tr>
<tr>
<td>qRT-PCR</td>
<td>quantitative reverse transcription and polymerase chain reaction</td>
</tr>
<tr>
<td>RACE</td>
<td>rapid amplification of cDNA ends</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
</tr>
<tr>
<td>---------</td>
<td>-------------</td>
</tr>
<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
</tr>
<tr>
<td>rRNA</td>
<td>ribosomal RNA</td>
</tr>
<tr>
<td>Rpl13a</td>
<td>ribosomal protein L13a</td>
</tr>
<tr>
<td>RST2</td>
<td>RNASET2</td>
</tr>
<tr>
<td>shRNA</td>
<td>short hairpin RNA</td>
</tr>
<tr>
<td>siRNA</td>
<td>small interfering RNA</td>
</tr>
<tr>
<td>snRNA</td>
<td>small nuclear RNA</td>
</tr>
<tr>
<td>snoRNA</td>
<td>small nucleolar RNA</td>
</tr>
<tr>
<td>tRNA</td>
<td>transfer RNA</td>
</tr>
<tr>
<td>TEAC</td>
<td>Trolox equivalent antioxidant capacity</td>
</tr>
<tr>
<td>TRAIL</td>
<td>tumor necrosis factor-related apoptosis-inducing ligand</td>
</tr>
<tr>
<td>UT</td>
<td>untreated</td>
</tr>
<tr>
<td>WT</td>
<td>wild-type</td>
</tr>
<tr>
<td>XIAP</td>
<td>X-linked inhibitor of apoptosis</td>
</tr>
<tr>
<td>ZDF</td>
<td>Zucker diabetic fatty</td>
</tr>
</tbody>
</table>
Acknowledgements

I would like to thank my thesis advisor, Dr. Jean Schaffer, for her constant support and guidance over the years. Together we have accomplished things unimaginable to me at the onset of my PhD. Her critical eye and attention to detail have elevated my appreciation for scientific experimentation. I will be forever indebted to her for instilling within me immense passion for the pursuit of science and fearlessness of the unknown.

I would like to thank the members of my thesis committee, Dr. Phyllis Hanson, my committee chair, Dr. Kerry Kornfeld, Dr. Eric Galburt, and Dr. Jennifer Duncan. Their suggestions and guidance helped to shape this body of work. I would like to thank Dr. Kerry Kornfeld for his advice and for challenging me to hone my scientific communication skills. I would like to especially thank Dr. Jennifer Duncan for giving me the opportunity to train in her lab and for mentoring me in Drosophila husbandry and metabolism. My time in your lab added a new dimension to my understanding of RNASET2, for which I am grateful.

Words are inadequate to express my gratitude to the members of Schaffer and Ory labs, both past and present. I would like to thank Dr. Dan Ory, for his advice and support. Dan is always able to answer any inquiry with a seemingly boundless familiarity for reagents, cell lines, professors, and papers, all just across the hall. Thank you to my fellow graduate students, Katrina Brandis, Ben Scruggs, Aggie Bielska, Maria Praggastis, Sarah Jinn, Mia Henderson, Melissa Li, and McKenna Feltes. Aside from the day-to-day help with experiments and advice, they have been my support system over the past several years and the completion of this dissertation is a testament to the community that we were able to create together. I would like to thank Dr. Jiyeon Lee, Dr. Christopher Holley, and Dr. Jamie Rimer, whose skill and wisdom can only be matched by their patience for answering all of my queries and quelling all my anxieties about lab, science, and the future. I would like to thank Dr. Linda Zhao, Josh Langmade, Hideji Fujiwara, Rohini Sidhu, and Jessi Zhang for all their help with the RNASET2 project. Thank you
to David Scherrer for all his help and his patience with a young whippersnapper like me. I will be forever grateful to Sarah Gale, who is the rock that the Ory and Schaffer labs are built upon. No matter how busy were are or how seemingly ridiculous the query, you always drop everything to help. Your selflessness is the foundation for all of our successes.

I would like to thank Dr. Rita Brookheart and Christina Wilson for their help and patience in teaching me about Drosophila husbandry. Thank you to Renate Lewis for her help with designing recombination vectors and making the complementation studies of RNASET2 possible. Special thanks to Dr. Ana Maria Cuervo and Dr. Susmita Kaushik for their collaboration in the isolation of lysosomes.

I would like to thank my family for their unwavering love, support, and understanding over the years. Thank you for always pushing me to fulfill my potential.

I would like to thank my friends, Elle, Shelby, Harrison, Reeny, Shannon, Liza, Amy, Mary Beth, Cody, Kristin, Brooke, Lauren, Decatur and so many others. You are the family that found me. Through my ups and downs, in life and in lab, you have always been there for me. We must love and support one another. We have nothing to lose but our chains.

To my husband, Kyle: We embarked on our adventure together shortly after I started graduate school. In every replicate of every experiment, successful or unsuccessful, your support, love, patience, and understanding are tacit constants. This thesis is as much yours as it is mine. I love you.
ABSTRACT OF THE DISSERTATION

Lysosomal RNASET2 is Required for Metabolic Stress-Mediated ROS Generation and Cell Death

by

George Caputa

Doctor of Philosophy in Biology and Biomedical Sciences
(Molecular Cell Biology)

Washington University in St. Louis, 2015

Professor Jean E. Schaffer, Chairperson

Ectopic lipid deposition in non-adipose tissues is a hallmark of metabolic diseases, such as obesity and diabetes, and associated with cell dysfunction, cell death, and organ dysfunction. However, the molecular mechanisms linking excess free fatty acid to cell death are poorly understood. In order to identify genes critical for lipotoxicity, our lab performed a genetic screen in Chinese hamster ovary cells and isolated mutants that were resistant to lipotoxic death. In one of the lines, mutant 2B1, an allele encoding RNASET2, a ribonuclease of the T2 family, was disrupted by proviral insertion. We found that RNASET2 is induced during lipotoxicity and is required for palmitate-induced oxidative stress and cell death. RNASET2 functions upstream of cytoplasmic accumulation of the non-canonical small nucleolar RNAs (snoRNAs), which have previously been shown to be required for amplification of lipotoxic reactive oxygen species (ROS). These functions are critically dependent on its catalytic activity.

Haploinsufficiency of RNASET2 confers increased antioxidant capacity and generalized resistance to ROS production and oxidative stress-mediated cell death in cultured cells. Furthermore, knockdown of RNASET2 in the Drosophila fat body confers increased survival and
protection from oxidative damage in the setting of oxidative stress inducers. Together, these findings demonstrate that RNASET2 regulates antioxidant tone and is required for pathological ROS responses.

Our observation that enzymatic activity of RNASET2 is required for its function in lipotoxic and oxidative stress suggested that RNASET2 functions by degrading RNA in these stress response pathways. Through microarray analysis, we identified XIAP mRNA as a potential substrate of RNASET2. Levels of this mRNA are inversely correlated with RNASET2 levels and dependent on lysosomal function. Both haploinsufficiency for RNASET2 and lysosomal perturbation prolong the half-life of this mRNA. Moreover, XIAP mRNA associates with the lysosomal fraction of wild type cells and increased lysosomal XIAP is found in the RNASET2 haploinsuffient 2B1 cells.

Taken together, our data provide evidence of a previously unsuspected role for RNASET2 in the response to metabolic stress and suggest a novel mechanism for selective degradation of an RNA transcript in the lysosome.
CHAPTER ONE

Introduction to Lipotoxicity

Obesity and diabetes are global health concerns.
Metabolic diseases, such as obesity and diabetes, are increasingly prevalent worldwide. In the past 35 years, the global rate of obesity among adults has doubled, affecting high, middle, and low-income countries (WHO Obesity and overweight fact sheet 2015, online). This swell in cases of obesity has been accompanied by a worldwide increase in cases of Type II diabetes (WHO Diabetes fact sheet 2015, online). In particular, global rates of childhood obesity have paralleled adult populations, and once rare cases of childhood Type II diabetes have now become more common. This growing prevalence of metabolic diseases has coincided with a global flux in lifestyle habits and dietary structure, due in part to accelerated urbanization, with disruption of traditional diets by access to industrially produced food and increasingly sedentary behaviors at work and leisure contributing to over-nutrition.¹

Complications are common in obesity and diabetes and contribute to increased morbidity.² Cardiovascular complications, including heart disease and stroke, account for half of deaths among individuals with diabetes.³ In addition to macrovascular complications, diabetic individuals have an increased prevalence of microvascular complications—diabetic neuropathy accounts for 60% of all non-traumatic lower-limb amputations, and diabetic retinopathy accounts for 4.8% of all incidents of visual impairment (National Diabetes Statistics Report, 2014; WHO report on blindness and visual impairment, online). Elevated BMI is associated with higher rates of cardiovascular disease, diabetes, osteoarthritis, and cancer (WHO Obesity and overweight fact sheet 2015, online). Not only do these complications greatly affect the quality of life of individuals, they also present an added burden on health systems, particularly in developing
countries, which are increasingly forced to address the effects over-nutrition as well as under-nutrition. In 2010 alone, diabetes amounted to 12% of global health expenditures. Therefore, understanding the pathogenesis of these diseases is essential to developing interventions to decrease morbidity and mortality, and reduce the growing financial burden on already strained global health systems.

**Lipotoxicity underlies complications to metabolic diseases.**

Metabolic diseases are linked to excess delivery of nutrients to tissues where these substrates lead to pathophysiological metabolic fluxes, signaling cascades, and cell death. Normally, glucose homeostasis is maintained by insulin signaling, which lowers serum glucose by activating glucose import and metabolism, hepatic lipid synthesis, and glycogen synthesis. One of the earlier events in development of hyperglycemia in Type II diabetes is decreased sensitivity of peripheral tissues to insulin signaling. While the pancreas initially responds by increased secretion of insulin, hyperglycemia occurs when this hyperinsulinemia is insufficient to maintain normoglycemia. Elevated or prolonged exposure of the vascular milieu to excess glucose has been implicated in the pathogenesis of diabetic microvascular complications, including edema, ischemia, retinopathy, and glomerulopathy through a process called glucotoxicity. Activation of protein kinase C signaling, non-enzymatic formation of advanced glycan end products, and increased reactive oxygen species generation and oxidative stress within vascular tissue underlie glucose-mediated tissue damage and cell death. Clinical studies have shown that tight glycemic control decreases the rate and extent of microvascular complications.

However, longitudinal studies suggest that amelioration of obesity- and diabetes-related complications through glucose-lowering interventions alone is insufficient to prevent
macrovascular complications, such as cardiovascular disease and stroke. This may be in part because in addition to hyperglycemia, excess serum lipid in the form of free fatty acids and triglycerides is common in metabolic diseases and remains unaddressed by glucose-lowering treatments. Physiologic lipid homeostasis is maintained by adipose tissue, which regulates serum lipid levels by sequestering excess lipid as triglycerides in neutral lipid droplets within adipocytes. Lipid sequestration in adipose depots is a protective mechanism that prevents the exposure of non-adipose tissues to high levels of lipid. However, chronic and prolonged elevated lipid levels may overwhelm the inherent storage capacity of adipose tissues, and insulin resistance may result in a failure to regulate adipose tissue lipolysis and hepatic de novo lipogenesis, all contributing to dyslipidemia. Elevated serum lipids correlate with increased adiposity, and pre-obese individuals have a greater degree of elevated serum cholesterol and triglycerides compared to normal- and under-weight individuals. Hyperlipidemia contributes to dysregulation of glucose metabolism—in one study, overfeeding of non-obese individuals for 28 days was sufficient to induce insulin resistance in the absence of elevated glucose.

Hyperlipidemia leads to ectopic lipid accumulation in the liver, skeletal muscle, pancreatic islets, kidney, and heart, which is associated with cellular dysfunction and organ dysfunction, including non-alcoholic steatohepatitis, insulin resistance, dysregulation of insulin secretion, glomerulopathy, and cardiomyopathy, respectively. Moreover, humans with congenital lipodystrophies resulting in either greatly diminished or absent adipose tissue exhibit hepatic steatosis, hypertrophic cardiomyopathy, insulin resistance, and pancreatitis. The similarities between acquired metabolic disease and inherited lipodystrophic syndromes further underscore the deleterious effects of excess lipid in non-adipose tissues.
Animal models of obesity and diabetes have complemented human studies of the pathogenesis of metabolic complications. Leptin-deficient (ob/ob) and leptin receptor-deficient (db/db) mice, genetic models of obesity, exhibit myocardial triglyceride accumulation, cardiac hypertrophy, cardiomyocyte apoptosis, and increased mortality. Zuckers diabetic fatty (ZDF) rats contain a mutation in the leptin receptor (fa/fa) and develop increased body weight, elevated serum lipids, and pancreatic islet lipid accumulation prior to development of hyperglycemia and insulin resistance. Myocardial triglyceride content of ZDF rats was three times their normal weight counterparts, and development of cardiac dysfunction and hypertrophy parallels what is observed in humans with chronic dyslipidemia.

While animal models of genetic obesity confirm the association of lipid accumulation with non-adipose tissue dysfunction, additional genetic models of tissue-specific lipid accumulation, in the absence of abnormal glucose and insulin levels, have confirmed a causative role of lipid as a driver of cellular dysfunction and death. Cardiac-specific overexpression of fatty acid transporter protein 1 or long chain acyl-CoA synthase 1, proteins that contribute to vectorial import and esterification of long chain fatty acids, is sufficient to drive increased ectopic lipid deposition and cardiomyopathy independent of elevated serum lipid levels. Lipoprotein lipase is the rate-limiting step for the hydrolysis of triglycerides contained within circulating lipoproteins, and tissue-specific overexpression results in increased delivery of lipid to those tissues without an increase in systemic lipid levels. Overexpression in skeletal muscle led to insulin resistance, while overexpression in the heart caused lipid deposition and cardiomyopathy.

Conversely, depletion of lipid stores is protective against tissue dysfunction. Mice overexpressing apolipoprotein B in cardiomyocytes have increased lipid efflux, reduced intracellular triglyceride levels, and are protected from diabetic cardiomyopathy. In addition
to removing lipid, tissues overexpressing enzymes that promote fatty acid utilization for energy production are protected from dyslipidemia and do not exhibit dysfunction.\textsuperscript{32,33} In humans, reductions in serum lipids levels of obese individuals through behavioral interventions, such as increased exercise and alterations in diet, is sufficient to ameliorate ectopic lipid accumulation and improve insulin resistance.\textsuperscript{34}

**Molecular mechanisms linking metabolic excess to cell death.**

Beyond lowering serum lipid levels, strategies to prevent lipotoxicity are hindered by our incomplete knowledge of the cellular pathways engaged by these metabolites when they are present in excess. \textit{In vivo} and \textit{in vitro} studies have revealed that accumulation of excess lipids in non-adipose cells precipitates changes in gene expression, activation of cell signaling, and initiation of cellular stress responses. Pancreatic $\beta$-cell lines treated with excess fatty acid up-regulate genes involving $\beta$-oxidation of fatty acids and down-regulate genes involved in glucose transport and metabolism in an attempt to cope with excess lipid substrate.\textsuperscript{35} Human islets treated with lipotoxic levels of palmitate and islet preparations from individuals with Type II diabetes both exhibit increased abundance of mRNAs encoding fatty acid metabolism genes, inflammatory cytokines, and stress pathway components, and a transcriptional decrease of cell growth machinery, general transcription factors, and pro-survival genes.\textsuperscript{36} Individuals with nonalcoholic seatohepatitis have changes in the abundance of microRNAs involved in cell proliferation, protein translation, apoptosis, inflammation, oxidative stress, and metabolism.\textsuperscript{37} As our understanding of the complex function of non-coding RNAs (ncRNAs) in regulation of transcription and translation deepens, we may find additional novel ncRNA mediators of lipotoxicity.\textsuperscript{38,39}
Compensatory incorporation of lipids into new membrane synthesis or triglyceride stores are likely to be initially protective,\textsuperscript{40,41} but ultimately prove maladaptive due to the deleterious consequences of altered membrane composition on organelle function,\textsuperscript{42} and because lipids may ultimately be mobilized from inert pools during prolonged exposure.\textsuperscript{43} Remodeling of membrane phospholipids to increase saturated fatty acid residues precipitates dramatic changes in endoplasmic reticulum (ER) membrane homeostasis by altering membrane fluidity and permeability. Furthermore, signaling through the unfolded protein response pathway is initiated.\textsuperscript{44} Engagement of ER stress machinery is initially adaptive and serves to maintain proper folding and maturation of luminal and membrane proteins, which are translated into the ER. Left unresolved, prolonged ER stress leads to translational arrest through the phosphorylation of eukaryotic initiation factor-2a, calcium leakage from the ER lumen, and activation of apoptosis.\textsuperscript{44,45}

Lipid overload also induces generation of reactive oxygen species (ROS). ROS is a byproduct of the electron transport chain, and typically scavenged by mitochondrial antioxidants, such as superoxide dismutase. While increased β-oxidation of excess lipids can be cytoprotective, excessive ROS is generated by the electron transport chain in the setting of excessive cycles of oxidative phosphorylation. Furthermore, excess lipids activate NADPH oxidase enzymes, leading to the generation of superoxide. Both pathways can overwhelm endogenous antioxidant defenses, leading to an accumulation of damaging free radicals and oxidation of lipids, proteins, carbohydrates, and nucleic acids.\textsuperscript{46-50} Like the ER, alterations in mitochondrial membrane fatty acid composition contribute to mitochondrial dysfunction by causing decreased membrane potential and proton leakage. The importance of oxidative stress in the pathophysiological response to substrate excess is underscored by the observation that treatment with chemical
antioxidants and overexpression of ROS-scavenging enzymes mitigate against lipotoxic cell death and against diabetic complications in animal models.\textsuperscript{51-55}

These stress responses precede and are thought to contribute to lipid-induced cell death following exposure of non-adipose cells to high concentrations of free-fatty acids. Caspase-3 cleavage, DNA laddering, and phosphatidylyserine externalization suggest that apoptosis pathways are involved at least in part during lipotoxicity, while extreme and prolonged lipotoxicity is associated with features of necrosis.\textsuperscript{14,51} In addition, fatty acids also up-regulate and activate tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) death receptor 5 (DR5) during metabolic stress.\textsuperscript{56}

**Novel insights into lipotoxicity through a forward genetic screen.**

To identify novel mediators of lipotoxic cell death, our laboratory has characterized genes identified through a loss-of-function genetic screen in mammalian fibroblasts. Fibroblasts were mutagenized using the ROSA\(\beta\)-geo retroviral promoter trap, and then selected for survival under lipotoxic conditions. This screen has identified several genes not previously associated with lipotoxicity and uncovered new mechanisms by which cells respond to metabolic stress. Eukaryotic elongation factor 1a-1, a translation elongation factor, is critical in mediating activation of cell death in response to lipotoxic and general ER stress possibly by altering actin cytoskeletal organization.\textsuperscript{57} Gadd7, a long non-coding RNA, was identified as a ROS responsive gene and as necessary for amplification of ROS in response to lipid overload and ROS-generating stimuli.\textsuperscript{58}

**The \textit{Rpl13a} snoRNAs mediate lipotoxicity and general oxidative stress.**

A particularly intriguing finding was that the 6F2 mutant contained a disruption in one allele of
the ribosomal protein L13a (Rpl13a) locus. Complementation and targeted knockdown experiments demonstrated that failure to produce four small non-coding RNAs, U32a, U33, U34, and U35a, encoded within the introns of the Rpl13a locus resulted in resistance to death from lipotoxic and generalized oxidative stress.\textsuperscript{59} These non-coding RNAs belong to the box C/D family of small nucleolar RNAs (snoRNAs), are \textasciitilde80 nucleotides in length, and form ribonucleoproteins that serve to covalently modify other cellular RNAs.\textsuperscript{60} These intronically-encoded snoRNAs are generated by RNA pol II transcription, and are processed from the intron lariat during splicing of the pre-mRNA. Another mutant isolated from the screen contained a disruption in one allele of SmD3, a core component of the spliceosome that was shown to be essential for processing of snoRNAs and other non-coding RNAs from intron lariats.\textsuperscript{61}

Canonically, box C/D snoRNAs reside and function in the nucleolus. They are part of a ribonucleoprotein complex that includes the methyltransferase, fibrillarin. An exposed 21-nucleotide tract on the snoRNA, the antisense element, acts as a zip code to guide the 2’-O-methylation of specific nucleotides on target RNAs. These modifications are thought to be important for the structure and function of the ribosome.\textsuperscript{62,63} Studies from the Schaffer lab suggest that the Rpl13a snoRNAs also have non-canonical functions. During metabolic and oxidative stress, the Rpl13a snoRNAs accumulate in the cytoplasm through a yet unknown mechanism, where they are critical for the amplification of ROS.\textsuperscript{59,64} Haploinsufficiency of the Rpl13a snoRNAs does not affect the methylation of their predicted target rRNAs, yet protects from lipotoxic and oxidative stress. The mechanism of action of these non-coding RNAs is an area of active investigation.

A novel role for RNASET2 in lipotoxicity.

Herein, we describe findings from a completely independent mutant isolated from the above
mentioned genetic screen in which an allele encoding RNASET2 was disrupted. RNASET2 is a lysosomal endoribonuclease of the T2 family that has been implicated in mediating the cellular response to oxidative stress.\textsuperscript{65,66} Mechanistically, this ribonuclease was initially of interest to us because of a potential link to production of the \textit{rpl13a} snoRNAs. However, our studies reveal that RNASET2 acts upstream of these non-coding RNAs by influencing cellular and organismal susceptibility to oxidative stress.
CHAPTER TWO

RNASET2 mediates lipotoxic cell death

INTRODUCTION

While the general pathways activated during lipotoxicity are understood, efforts to characterize the molecular underpinnings of lipotoxicity can provide us with a better understanding of metabolic stress and new potential therapeutic targets. Work from our lab over the past several years has focused on the central events surrounding lipotoxicity, ROS generation, and ER stress, and how these processes interact to activate cell death cascades. Much of the progress we have made has been through the use of loss-of-function forward genetic screens, which have proven to be a powerful tool to identify unknown and unexpected molecular components of complex genetic pathways like lipotoxicity.

Chinese hamster ovary (CHO) cells have been used extensively to elucidate mammalian genetic pathways, and are amenable to genetic screens as they are biochemically diploid and functionally haploid at many loci. While there exists a variety of chemical and targeted genetic methods to mutagenize DNA, ideal mutagens are random in order to prevent bias and to ensure a broad scope of affected genes. The generation of DNA mutations through chemical means is generally accepted as the most unbiased method of generating mutations, but identification of mutated region in mammalian cells through sequencing or complementation cloning is labor intensive. Our lab carried out insertional mutagenesis using the ROSAβgeo retroviral promoter trap. Following transduction with ROSAβgeo, the provirus inserts at relatively random locations within the genome and contains a promoterless β-galactosidase-neomycin cassette. This provirus confers antibiotic resistance only when it integrates
downstream of an actively transcribed RNA polymerase II promoter, resulting in a fusion transcript that contains host and viral neomycin phosphotransferase sequences, disrupting the endogenous gene at that site. CHO mutants were selected by growth in G418 and subsequently challenged by growth in media supplemented with 500 μM palmitate for 48 hours, which has been shown to cause lipotoxic cell death in wild type (WT) cells (Figure 2.1). The goal of this screen was to identify pro-death genes that participate in the cellular response to lipid overload. Herein, we describe an independently isolated, palmitate-resistant mutant, the 2B1 cell line.

RESULTS

2B1 cells are resistant to lipotoxic cell death.

To characterize the 2B1 mutant, we directly compared cell death responses in wild type CHO and 2B1 cells. The 2B1 line was significantly resistant to palmitate-induced cell death and apoptosis, as assessed by propidium iodide and annexin V staining, respectively (Figure 2.2a and 2.2b). However, when treated with the general inducers of apoptosis, staurosporine and actinomycin D, 2B1 cells were indistinguishable from the parental CHO cells with respect to the extent of cell death. This indicated that the 2B1 cells are resistant to lipotoxicity, but not generally resistant to cell death. Resistance to lipotoxicity in 2B1 cells was not simply the result of failure to take up fatty acids, because uptake of a fluorescent palmitate analog was indistinguishable between 2B1 and WT cells (Figure 2.2c).

The gene for RNASET2 is disrupted in 2B1 cells.

Our screen was performed with viral transduction at low multiplicity of infection to facilitate single gene disruptions. Southern analysis of genomic DNA from CHO and 2B1 cells revealed a single band in 2B1 cells that hybridized to a radiolabeled probe against viral sequences (Figure 2.3a). This result is consistent with a single integration of the ROSAβgeo insert in 2B1 cells. We
utilized the proviral fusion transcript to identify the disrupted locus in 2B1 cells. mRNA isolated from 2B1 cells was used for 5’ rapid amplification of cDNA ends (RACE), and sequencing of the RACE product identified the gene for RNASET2 as the site of integration (Figure 2.3b). PCR analysis of cDNA from CHO and 2B1 cells confirmed that ROSAβgeo had inserted into the RNASET2 locus in 2B1 cells (Figure 2.3c). Reactions using a forward RNASET2 primer and a reverse ROSAβgeo primer produced a product corresponding to the fusion transcript in 2B1, but not CHO cells, consistent with proviral integration only in the mutant. Reactions using forward and reverse RNASET2 primers produced a product of the expected size in both CHO and 2B1 cells, indicating that the 2B1 cells retain an intact allele in addition to the disrupted allele. To evaluate the consequences of disruption of an allele for RNASET2, we quantified RNASET2 mRNA in CHO and 2B1 cells under basal growth conditions and following lipotoxic stress (Figure 2.3d). Expression of RNASET2 was indistinguishable between these cell lines under normal growth conditions. RNASET2 transcript was induced under lipotoxic conditions in both cell types, although induction was significantly blunted in the 2B1 mutant. Together our observations are consistent with a model in which integration of the ROSAβgeo provirus disrupted one of two alleles for RNASET2, and in which 2B1 cells represents a model of RNASET2 haploinsufficiency. In the 2B1 cells, the remaining allele for RNASET2 is apparently adequate to support basal expression, but it is not sufficient to support induction of expression to WT levels under stress conditions.

**Loss of RNASET2 protects cells from lipotoxic cell death.**

RNASET2 is an acidic endoribonuclease that belongs to the T2 family of RNases implicated in diverse cellular functions. To confirm the role of RNASET2 in lipotoxicity, we tested whether targeted loss-of-function in an independent cell type also conferred resistance to palmitate. We isolated stable C2C12 murine myoblast cell lines that expressed either control, scrambled (scr)
short hairpin RNAs (shRNAs) or shRNAs targeting RNASET2 and assayed for RNASET2 expression and cell death following palmitate treatment. Similar to our findings in CHO cells, RNASET2 was transcriptionally induced with palmitate treatment in C2C12 cells, and two independently isolated clonal cell lines showed 46% (sh1) and 40% (sh2) knockdown under palmitate-treated conditions relative to control (Figure 2.4a). RNASET2 protein production (doublet indicated by asterisks mark completely and incompletely glycosylated forms) was also induced with palmitate treatment, and this induction was blunted in the RNASET2 shRNA knockdown cell lines compared to the control cell line (Figure 2.4b). The lines with RNASET2 knockdown showed significantly less palmitate-induced cell death compared to the scr control cell line (Figure 2.4c). These data provide independent confirmation that diminished expression of RNASET2 confers resistance to lipotoxic cell death.

**Catalytic activity of RNASET2 is required for lipotoxic cell death.**

Several reports have described biological roles for T2 family members that are independent of catalytic activity. In order to determine whether catalytic activity is required for the role of RNASET2 in lipotoxicity, we carried out complementation studies. A catalytically inactive (CI) murine RNASET2 construct was generated through site directed mutagenesis of two of the three catalytic histidines. Transient overexpression of both WT and CI murine constructs in CHO cells produced glycosylated, mature RNASET2 protein (doublet indicated by asterisks mark completely and incompletely glycosylated forms), recognized by an antiserum specific for the murine protein (Figure 2.5a). Zymography confirmed that only the WT construct had functional RNase activity at the permissive pH of 5. Independent 2B1 clonal cell lines stably expressing varying levels of either WT or CI murine RNASET2 mRNA (Figure 2.5b) and protein (Figure 2.5c) were then isolated. 2B1 cells were protected from palmitate-induced cell death, and this was not affected by transfection with empty vector (Figure 2.5d). Only WT, but not CI,
RNASET2 overexpression increased palmitate-induced cell death when compared to empty vector, despite comparable or higher levels of expression of the CI construct. These results further confirm the role for RNASET2 in palmitate sensitivity, and indicate that this function is dependent on its catalytic activity. Expression of the transfected murine RNASET2 constructs was unaffected by palmitate, likely because the transfected sequences lacked control elements present in the endogenous RNASET2 locus.

CONCLUSIONS

Through a loss-of-function genetic screen, we have identified RNASET2 as a critical mediator of lipotoxic cell death. The blunting of RNASET2 induction under lipotoxic conditions that was observed in the haploinsufficient 2B1 cells is sufficient to protect cells from palmitate-induced cell death. Moreover, the enzymatic activity of RNASET2 is required for its role in lipotoxicity. Together, this data expands our understanding of the cellular response to metabolic stress and highlights a novel molecular player in the lipotoxic pathway.

RNASET2 has previously been implicated as a pro-death gene; however this study is the first to implicate RNASET2 in the response to metabolic stress. Previous reports have extensively described RNASET2 as a tumor suppressor, since decreased levels of RNASET2 expression in lymphoma, ovarian cancer, and melanoma correlate with increased tumorogenicity.\textsuperscript{74-76} A loss-of-function model of RNASET2 zebrafish and association of RNASET2 mutation with human disease indicate that RNASET2 plays a critical role in neurons, and its absence leads to neurodegeneration.\textsuperscript{77,78} Our cell culture model of RNASET2 haploinsufficiency provides a new tool with which to examine the mechanism by which RNASET2 mediates cell death.
Our finding that RNASET2 mRNA is induced by lipotoxic stimuli mirrors similar observations made with other stress-inducing stimuli, such as UV radiation and ROS. On the other hand, cellular levels of RNASET2 protein are not significantly induced during lipotoxicity (Figure 2.4). The induction of RNASET2 mRNA may be compensatory in order to maintain steady state levels of RNASET2 protein, which may be lost through either non-canonical secretion of lysosomal contents, or through proteolysis. Further studies examining secreted RNASET2 protein species or steady state levels of intercellular RNASET2 protein may elucidate the fate of RNASET2 during stress conditions.

We provide evidence that the catalytic activity of RNASET2 is sufficient to restore sensitivity to metabolic stress. Previous studies have found both catalytic-dependent and catalytic-independent functions for RNASET2 and other T2 family members. Differences between these studies may relate to organism, cell type, and stimulus-specific biology. The catalytic requirement of RNASET2 during metabolic stress raises the question of which RNA it degrades. In zebrafish, RNASET2 has been implicated in degradation of lysosomally-targeted rRNA. Co-localization of human RNASET2 with P-bodies has suggested a possible role for degradation of mRNAs. However, functional evidence will be necessary to confirm either of these potential roles in lipotoxicity. Regardless, this data suggests a model in which pro-survival RNAs are degraded by RNASET2 during metabolic stress. Identification of these RNA substrates may provide further insight into the cellular response to metabolic stress, and may reveal a novel mechanism by which cells target and degrade RNAs.
Figure 2.1 Loss of function screen produced palmitate resistant mutants.

Wild type (CHO) cells were mutagenized by transduction with the ROSAβgeo retroviral promoter trap, and mutants were selected by treatment with neomycin. Lipotoxicity-resistant mutants were subsequently selected by growth in high glucose (glu) media supplemented with 500 μM palmitate (palm) for 48h.
Figure 2.2 2B1 cells are resistant to lipotoxic cell death.

(a and b) CHO (open bars) and 2B1 mutant (filled bars) cells were treated with 500 μM palm for 48h or with 80 nM staurosporine (staur) or 2 μM actinomycin D (actD) for 24h. Cell death was assayed by propidium iodide (PI) staining (a) and apoptosis was assessed by simultaneous annexin V and PI staining (b) with flow cytometric analysis of $10^4$ cells/sample and a minimum of $n = 3$ samples for each condition. Data is expressed as mean fluorescence + standard error (SE).

(c) CHO and 2B1 mutant cells were incubated with BODIPY-labeled fatty acid (BODIPY FA) for 1 min. Uptake was measured by flow cytometric analysis of $10^4$ cells/sample, with $n = 3$ samples for each cell type. Data is expressed as mean fluorescence + SE (relative [rel] units).

*, $p < 0.05$, **, $p < 0.005$ for 2B1 vs. CHO
Figure 2.3 Locus encoding RNASET2 is disrupted in 2B1 cells.

(a) CHO and 2B1 mutant genomic DNA was digested with restriction enzymes Bgl II, Xba I, or Nco I, and Southern blot was hybridized with a $^{32}$P-labeled probe against the integrated ROSAβgeo retroviral sequence.

(b) Diagram shows intact RNASET2 transcript in CHO cells, fusion transcript in 2B1 cells, and forward (F) and reverse (R) primers for PCR amplification of each (arrows). ROSAβgeo sequences are white and RNASET2 sequences are shaded.

(c) PCR analysis of cDNA from CHO and 2B1 cells or no cDNA (H$\text{2}O$ as control) used primers as indicated in (b). Products were analyzed by agarose gel electrophoresis and EtBr staining.

(d) RNA was isolated from CHO (open bars) and 2B1 (filled bars) cells grown in media supplemented with 500 μM palm for 0, 24, or 48h. RNASET2 expression relative to β-actin was determined by qRT-PCR.

All data is reported as mean + SE for a minimum of n = 3 samples per condition/cell type.

*, p < 0.05 for 2B1 vs. CHO
Figure 2.4 Loss of RNASET2 protects cells from lipotoxic cell death.

C2C12 murine myoblasts were transfected with short hairpin RNA (sh) sequences targeting RNASET2 or a scrambled (scr) control, and independent stable clonal lines were isolated. Cells were maintained in growth media (untreated, UT, filled bars) or supplemented with 500 μM palm for 16h (hatched bars) prior to analysis.

(a) RNA was isolated and analyzed by qRT-PCR for RNASET2 relative to β-actin.

(b) RNASET2 protein levels were analyzed by western blotting of total cell lysates. Representative blots for RNASET2 (glycosylated species indicated by asterisks) and hsp90 are shown below. Graph shows mean densitometry of maturely glysocylated RNASET2 species normalized to hsp90 as a loading control for each condition/cell type.

(c) Cell death was quantified by PI staining and flow cytometric analysis. Graph shows mean fluorescence + SE for 10^4 cells/sample.

All data is reported as mean + SE for a minimum of n = 3 samples per condition/cell type.

*, p < 0.05 for palm-treated sh vs. scr
Figure 2.5

(a) CHO cells were transiently transfected with empty vector (EV), wild type (WT), or catalytically inactive mutant (CI) murine RNASET2 sequences. Cells were analyzed by western blotting for expression of murine RNASET2 (antibody does not recognize hamster species) and hsp90 as a loading control, and by in-gel zymography (zym) for ribonuclease activity at pH 5 and pH 7. For zymography panels, bands represent areas of enzymatic activity in RNA impregnated gel. Images are representative of 3 independent experiments.

(b, c, and d) Mutant 2B1 cells were transfected with EV or plasmids encoding WT or CI murine RNASET2. Independent stable clonal cell lines were isolated for each construct and analyzed following growth in normal media (untreated, UT, black bars) or following supplementation with 500 μM palm for 48h (hatched bars). RNA was isolated and analyzed by qRT-PCR for RNASET2 expression relative to 36B4 using primers specific for the murine RNASET2 transcript (b). RNASET2 protein levels were analyzed by western blotting of total cell lysates. Representative blots for RNASET2 (glycosylated species indicated by asterisks) and hsp90 are shown below. (c) Graph shows mean densitometry of glycosylated RNASET2 species normalized to hsp90 as a loading control for each condition/cell type. Cell death was assayed by PI staining and flow cytometric
analysis on $10^4$ cells/sample (d).

Data is expressed as mean + SE for a minimum of $n = 3$ samples per condition/cell type.

*, p < 0.05 for comparisons indicated
CHAPTER THREE

RNASET2 regulates cellular redox tone

INTRODUCTION

Reactive oxygen species (ROS), such as superoxide and hydrogen peroxide, are potent second messengers for numerous physiological and pathophysiological processes. Superoxide is formed either enzymatically by NADPH oxidases, peroxidases, and xanthine oxidase or non-enzymatically as a by-product of respiration by the mitochondrial electron transport chain complex. Superoxide radicals can dismutate spontaneously or can be enzymatically dismutated by superoxide dismutatase into hydrogen peroxide, a longer lived and highly damaging ROS molecule.

Cellular oxidative tone is an interplay between ROS generation and scavenging through either enzymatic or antioxidant defenses. Minor fluctuations in ROS homeostasis can act as a sensor of physiological cellular by activating signaling cascades to effect cellular growth and proliferation, adhesion, neurotransmission, and immune responses. However, major or chronic disruptions in cellular oxidative tone, termed oxidative stress, can have pathophysiological consequences by activating stress pathways and leading to irreversible damage to essential macromolecules like proteins, lipids, and nucleic acids. Unchecked, excessive ROS can result in cell death. Oxidative stress has been implicated in a diverse array of pathologies, such as aging, hypertension, cancer, and cardiomyopathy.

Our lab uncovered a critical role for the Rpl13a C/D box snoRNAs in mediating cell death from oxidative stress. These snoRNAs are encoded within introns of the Rpl1ra locus, exonucleolytically processed from excised intron lariats, and reside in the nucleus under
homeostatic conditions.\textsuperscript{61} Canonically, snoRNAs guide the enzymatic 2'-O-methylation of rRNA and snRNA. However under lipotoxic and oxidative stress conditions, the $RpL13a$ snoRNAs accumulate in the cytoplasm and are critical for the amplification of ROS. Recent studies have implicated superoxide radicals and NOX4D, a nuclear isoform of the NADPH oxidase, as a potent inducers of the nucleo-cytoplasmic flux of these snoRNAs.\textsuperscript{64} Based on the requirement of RNase activity of RNASET2 for complementation of the 2B1 palmitate resistance phenotype, we hypothesized that the biochemical function of RNASET2 might contribute to the biogenesis of the $RpL13a$ snoRNAs.

RESULTS

RNASET2 is a mediator of $RpL13a$ snoRNA cytoplasmic accumulation.

We compared RNASET2-deficient 2B1 and WT CHO cells for cytosolic accumulation of the $RpL13a$ snoRNAs in response to palmitate treatment. Cytosolic extracts were isolated by differential detergent extraction, and snoRNAs were quantified by qRT-PCR (Figure 3.1a). In contrast to the parental CHO cells, RNASET2-deficient 2B1 cells have blunted and delayed accumulation of $RpL13a$ snoRNAs in the cytoplasm after palmitate treatment. To test whether cytoplasmic accumulation of the snoRNAs requires RNASET2 activity as well as protein, we quantified cytosolic snoRNAs in the 2B1 complemented clones expressing either WT or CI RNASET2 following palmitate treatment (Figure 3.1b). Only 2B1 clones complemented with WT RNASET2, but not CI RNASET2, demonstrated cytoplasmic snoRNA accumulation, indicating that RNase activity is required for this function.

RNASET2 does not participate in the processing of the $RpL13a$ snoRNAs.

To probe whether differences in cytoplasmic snoRNA accumulation reflect altered expression from the $RpL13a$ locus or a defect in snoRNA processing, we quantified the precursor pre-
mRNA and intron lariats from which the snoRNAs are processed, and levels of the snoRNAs in the nucleus (Figure 3.2). As judged by each of these measures, the 2B1 cells were indistinguishable from parental CHO cells, indicating that the mutant is capable of snoRNA production, but fails to accumulate WT levels of these non-coding RNAs in the cytosol under stress conditions.

**RNASET2 is a mediator of ROS generation during lipotoxicity.**

ROS is an important signaling intermediate during lipotoxicity, given that pre-treatment with antioxidants decreases palmitate-induced ROS and cell death.\(^{45,58}\) The observation that treatment of cells with more direct inducers of oxidative stress, such as hydrogen peroxide, also causes cytoplasmic snoRNA accumulation suggests that ROS may be an upstream signal for the redistribution of snoRNAs.\(^ {59}\) Because cells haploinsufficient for RNASET2 fail to accumulate the \(RpL13a\) snoRNAs in the cytoplasm, but have no apparent defect in the generation of these snoRNAs (i.e., nuclear levels are unchanged), we tested whether haploinsufficiency of RNASET2 modulated the generation of ROS in response to palmitate. Following supplementation of the media with palmitate, we quantified cellular superoxide by dihydroethidium (DHE) staining, and cellular hydrogen peroxide by 2',7'-dichlorodihydrofluorescein diacetate (DCF) staining. In WT CHO cells, levels of superoxide increased as early as 2 hours following palmitate exposure, and this ROS further increased with longer incubations (Figure 3.3a). Increased hydrogen peroxide was detected later, following 12 and 16 hours of palmitate exposure (Figure 3.3b). By contrast, 2B1 cells failed to increase superoxide in response to palmitate and had markedly blunted increases in hydrogen peroxide at the later time points. Similarly, palmitate failed to induce increases in hydrogen peroxide in C2C12 cells with RNASET2 knockdown (Figure 3.3c). Given that cytoplasmic accumulation of \(RpL13a\) snoRNAs in CHO cells was detected by six hours of palmitate exposure, we
hypothesize that early palmitate-induced superoxide (DHE signal) is likely to be a trigger for cytoplasmic snoRNA accumulation and subsequent ROS amplification (hydrogen peroxide generation as indicated by DCF signal). To test whether the RNase activity of RNASET2 is necessary for ROS production during lipotoxicity, we measured the level of hydrogen peroxide species in the 2B1-complemented clones following palmitate treatment (Figure 3.3d). Only complementation with WT RNASET2 was sufficient to restore ROS production to WT levels. Our data is consistent with a model in which catalytically active RNASET2 is necessary for generation of ROS in response to lipotoxic stress.

2B1 cells have increased antioxidant capacity during lipotoxicity. Cells maintain their ROS tone by balancing generation and scavenging of ROS. To test whether the failure of 2B1 mutant cells to increase ROS in the face of a lipotoxic challenge was related to an altered ability to scavenge free radicals, we compared the Trolox equivalent antioxidant capacity (TEAC) of lysates from CHO and 2B1 cells. Under basal growth conditions, there was no difference in antioxidant capacity between these cell types. However, lysates from 2B1 cells treated with palmitate demonstrated a nearly 50% increase in antioxidant capacity compared to WT CHO (Figure 3.4). This suggests that haploinsufficiency of RNASET2 in 2B1 cells mitigates against lipotoxicity by enhancing the cellular antioxidant capacity.

Loss of RNASET2 protects cells from ROS accumulation during general oxidative stress. To test whether 2B1 cells were resistant to generalized ROS, we treated CHO and 2B1 cells with several non-lipid ROS inducers. Following exposure to hydrogen peroxide, 2B1 mutant cells failed to accumulate Rpl13a snoRNAs in the cytoplasm, similar to our findings with lipotoxic stimuli (Figure 3.5a). We were also unable to detect amplification of ROS in 2B1 cells following exposure to hydrogen peroxide (Figure 3.5b). Additionally, 2B1 cells were resistant to
ROS following treatment with menadione, a potent inducer of superoxide (Figure 3.5c). Although prolonged treatment with hydrogen peroxide or menadione caused CHO cells to die, 2B1 cells were relatively protected (Figure 3.6). Nonetheless, overexpression of RNASET2 in CHO cells in the absence of metabolic stress did not alter cellular ROS or cell death (Figure 3.7).

CONCLUSIONS

Our data show that RNASET2 is required for propagation of ROS, accumulation of snoRNAs in the cytoplasm, and progression to cell death in response to pathophysiological levels of the saturated fatty acid palmitate and in response to general inducers of oxidative stress. Moreover, we demonstrate that RNASET2 is necessary for the early amplification of ROS during lipotoxicity and that haploinsufficiency of RNASET2 confers increased antioxidant capacity in cultured cells. Taken together, this data establishes RNASET2 as a mediator of general cellular redox tone and supports a novel role for RNASET2 in snoRNA biology and maintenance of antioxidant capacity.

The T2 family has previously been associated with ROS and oxidative stress. Thompson and Parker established a link between the yeast family member, Rny1p, and oxidative stress by demonstrating that gain-of-function decreased viability in the setting of exposure to hydrogen peroxide. Wang et al. extended these findings to human RNASET2 in a vitiligo cell culture model, in which they found that overexpression of RNASET2 sensitized cells to oxidative stimuli. Moreover, we found that overexpression of RNASET2 was not sufficient in our system to cause oxidative stress and cell death in absentia of stress stimuli. These differences further underscore the potential cell type, tissue, and organism specific functions of T2 family members.
Our results suggest that RNASET2 influences the oxidative tone of the cell by altering the cellular antioxidant capacity in response to metabolic stress. Cells haploinsufficient for RNASET2 potentially lack the ROS stimuli required for the cytosolic accumulation of \textit{RpL13a} snoRNAs, making RNASET2 an early mediator of signaling events during lipotoxicity. The blunted cytoplasmic accumulation of snoRNAs in RNASET2-deficient cells could be explained by either a failure to generate superoxide or increased antioxidant scavenging of superoxide radicals, which, in conjunction with our recent data that nuclear NADPH oxidase-generated superoxide is a robust and rapid inducer of cytosolic snoRNA accumulation, underscores the influence of early ROS signaling in snoRNA biology.\textsuperscript{59,64} Gross antioxidant capacity is the sum of both enzymatic and non-enzymatic antioxidants. Future studies that measure expression of genetically encoded antioxidants (i.e. superoxide dismutase, catalase) and proteins with redox-sensitive cysteines (e.g., thioredoxins, glutaredoxins, peroxiredoxins) will help elucidate the pathways influenced by RNASET2.
Figure 3.1

**Figure 3.1** RNASET2 acts upstream of cytoplasmic accumulation of *Rpl13a* snoRNAs.

(a and b) CHO and 2B1 cells were treated with 500 μM palm (a) or 1 mM H₂O₂ (b) for the indicated times. Cytoplasmic RNA was isolated by sequential detergent extraction, reverse transcribed, and analyzed by qRT-PCR for *Rpl13a* snoRNAs relative to β-actin. Graph (left) shows *Rpl13a* snoRNA expression, and blots (right) show representative western analysis of fractions for hsp90 and histone H3 in cytosolic and nuclear fractions at 8h.

(c) CHO, 2B1 cells, and 2B1 cells stably transfected with EV or plasmids encoding WT or CI murine RNASET2 (as in Figure 2.5) were treated with 500 μM palm for 10h. Cytoplasmic RNA was isolated by sequential detergent extraction, reverse transcribed, and analyzed by qRT-PCR for *Rpl13a* snoRNAs relative to actin.

Graphs report mean values + SE for n = 3 independent experiments. *, p < 0.05 for treated vs. untreated; #, p < 0.05 for 2B1 vs. CHO.
Figure 3.2 RNASET2 haploinsufficiency does not prevent production of Rpl13a snoRNAs.

CHO (open bars) and 2B1 (filled bars) cells were untreated (-) or treated with palmitate for 9h (+) and then fractionated by sequential detergent extraction. RNA from nuclear fractions was reverse transcribed and quantified by qPCR for pre-mRNA (a), intron lariats (b), and nuclear snoRNAs (c), each relative to β-actin.

Graphs report mean values + SE for n = 3 independent experiments.
Figure 3.3 Loss of RNASET2 protects cells from ROS accumulation during lipotoxicity.

(a and b) CHO (open bars) and 2B1 (filled bars) cells were treated with palmitate for the indicated times. ROS generation was quantified by dihydroethidium (DHE) staining and fluorescence microscopy (a) or by 2',7'-dichlorodihydrofluorescein diacetate (DCF) staining with flow cytometric analysis of 10⁴ cells/sample (b). Insets show controls for detection of superoxide (a, DHE staining of menadione-treated CHO cells) and H₂O₂ (b, DCF staining of H₂O₂-treated CHO cells).

(c) C2C12 clonal lines transfected with shRNA sequences targeting RNASET2 or with scrambled (scr) control shRNA were treated with palm for 16h. ROS was quantified by DCF staining and flow cytometric analysis in untreated (filled bars) and palm-treated (hatched bars) treated cells.

(d) Mutant 2B1 clonal lines transfected with EV or plasmids encoding WT or CI murine RNASET2 were treated with 500 μM palm for 12h. ROS was quantified by DCF staining and flow cytometric analysis.

All graphs report mean (+ SE) fluorescence of 10⁴ cells/sample for n = 3 independent experiments.
*, p < 0.05 for comparisons indicated
Figure 3.4 2B1 cells have increased antioxidant capacity during lipotoxicity.

CHO (open bars) and 2B1 (filled bars) cells were untreated or treated with 500 μM palm for 8h. Antioxidant capacity of cell lysates (50 μg protein) was quantified by Trolox equivalent antioxidant capacity (TEAC) assay.

Graph reports mean (+ SE) for n = 3 independent experiments. *, p < 0.05 for 2B1 vs. CHO; ns, non-significant.
Figure 3.5 Loss of RNASET2 protects cells from ROS accumulation during oxidative stress.

(a) CHO and 2B1 cells were treated with 1 mM H$_2$O$_2$ for the indicated times. ROS generation in CHO (open bars) and 2B1 (filled bars) cells was quantified by DCF staining and flow cytometric analysis.

(b) CHO (open bars) and 2B1 (filled bars) cells were treated with 50 μM menadione (mdn) for 2h. ROS generation was quantified by DHE staining and flow cytometric analysis.

All graphs report mean (+ SE) fluorescence of 10$^4$ cells/sample for n = 3 independent experiments.

**, p < 0.05 for 2B1 vs. CHO; ns, non-significant
Figure 3.6 Loss of RNASET2 protects cells from oxidative stress-induced cell death. 

(a and b) CHO (open bars) and 2B1 mutant (filled bars) cells were treated with 2.5 mM H$_2$O$_2$ for 24h (a) or with 25 µM menadione (mdn) for 6h (b). Cell death was assayed by PI staining and flow cytometric analysis.

All graphs report mean (+ SE) fluorescence of $10^4$ cells/sample for n = 3 independent experiments.

**, p < 0.05 for 2B1 vs. CHO; ns, non-significant
Figure 3.7

(a and b) CHO cells were transfected with empty vector (EV, open bars) or expression plasmid encoding RNASET2 (filled bars). Cells were assessed 24h later for ROS by DCF staining (a) or for cell death by PI staining (b) and flow cytometry.

All graphs report mean (+ SE) fluorescence of $10^4$ cells/sample for $n = 3$ independent experiments.

**, p < 0.05 for 2B1 vs. CHO; ns, non-significant
CHAPTER FOUR
Substrates of RNASET2

INTRODUCTION
RNA degradation is a tightly controlled cellular process. In addition to the clearance of damaged or dysfunctional RNAs, pathways of RNA degradation are essential during both homeostatic and stress conditions in order to regulate the abundance and availability of RNAs. Transfer RNAs (tRNAs) undergo endonucleolytic cleavage during stress conditions, and tRNA fragments can influence both translation efficiency and cell fate. Damaged and dysfunctional ribosomes are selectively sequestered and degraded under stress conditions through a specialized form of autophagy, termed ribophagy. Additionally, during cellular stress, mRNAs are sequestered in P-bodies and stress granules, leading to translation arrest, mRNA decapping, and degradation.

While the substrates of mammalian RNASET2 are unknown, T2 family members have been implicated in degrading ribosomal RNAs in zebrafish and cleaving tRNAs in yeast in the setting of oxidative stress. Our goal was to determine the substrates of mammalian RNASET2 during metabolic stress, and to discover how RNASET2, a lysosomal RNase, contributes to cellular stress and cell death.

RESULTS
Loss of RNASET2 does not alter rRNA, tRNA, or bulk RNA degradation.
To test whether lipotoxic conditions induce rRNA or tRNA degradation in an RNASET2-dependent manner, we analyzed total RNA from CHO and 2B1 cells following palmitate treatment. While the abundance of 18S rRNA was unchanged by palmitate treatment, 28S rRNA
decreased in WT CHO and increased in 2B1 cells 16 h following palmitate treatment (Figure 4.1a). These late changes in 28S rRNA levels between CHO and 2B1 cells occurred substantially after ROS and snoRNA differences were detected, both of which were complemented by expression of enzymatically active RNASET2 (Figures 3.1 and 3.3). We also quantified tRNAs in the setting of lipotoxic stress. However, palmitate treatment did not cause depletion of several tRNA species, and we observed no difference in tRNA abundance between WT CHO and 2B1 cells (Figure 4.1b). In addition, palmitate treatment did not cause significant changes in total RNA content of WT CHO or 2B1 cells (Figure 4.1c). Together, these findings suggest that the failure to produce early ROS during lipotoxicity in 2B1 cells is not attributable to loss-of-function of RNASET2 in rRNA, tRNA, or bulk RNA degradation pathways.

2B1 cells have differential changes in gene expression during lipotoxicity.

Overexpressed human RNASET2 co-localizes with P-bodies, post-translational processing bodies where mRNAs are sequestered and degraded during some cell stresses. The regulation of mRNA levels can have important consequences for cell adaptation and survival. Therefore, we hypothesized that loss of RNASET2 could cause an increase or persistence of specific mRNAs during metabolic stress, which could lead to a failure to produce ROS or increased cell survival. To test this hypothesis, we performed a microarray on total RNA from WT CHO and 2B1 cells under basal growth conditions and following lipotoxic stress. We found that 76 genes had 1.75-fold higher expression in 2B1 cells compared to WT CHO cells (p < 0.05) (Figure 4.2a and Table 4.1). 22 genes were more highly expressed in 2B1 cells than WT CHO cells under basal growth conditions, while 36 genes were more highly expressed under palmitate-treated conditions. 18 genes were more highly expressed in 2B1 cells under both conditions. Thus, haploinsufficiency of RNASET2 causes significant changes in gene expression.
We hypothesized that antioxidant or ROS scavenging genes would be overrepresented in 2B1 cells given their inability to amplify ROS in response to oxidative and metabolic stress. Our microarray analysis revealed several genes, known to function in oxidative stress responses, with increased expression in 2B1 cells—two subunits of the mitochondrial electron transport chain complexes (Ndul5b, Cox7c) and several antioxidant genes (Gstm1, Prdx4, Prdx3). We validated increased expression of Cox7c and Prdx3 by RT-qPCR (Figure 4.2b). Increased expression of these mitochondrial proteins could contribute to the increased antioxidant capacity of 2B1 cells (Figure 3.4).

**RNASET2 influences XIAP mRNA and protein levels.**

X-linked inhibitor of apoptosis (XIAP) was among the genes with the highest expression in 2B1 cells under both basal growth and lipotoxic conditions (Table 4.1b). This E3 ubiquitin-ligase was first identified as a master regulator of cell death and has been shown to bind and inhibit the caspase 3/7/9 apoptosome complex. To confirm our microarray findings, we quantified XIAP mRNA from WT CHO or 2B1 cells under basal growth or lipotoxic conditions using RT-qPCR (Figure 4.3a). In 2B1 cells, XIAP mRNA was increased 2.2-fold under basal conditions and 2.9-fold under palmitate-treated conditions compared to in WT CHO, in which XIAP mRNA levels were unchanged by palmitate treatment. To confirm that the increase in XIAP mRNA was due to loss of RNASET2, we transiently transfected NIH 3T3 cells with siRNA targeting RNASET2 (or scrambled siRNA [siSCR] as control) and quantified XIAP mRNA levels. Cells transfected with siRNA targeting RNASET2 had a 52% decrease in RNASET2 mRNA levels that was accompanied by an increase in the levels of both XIAP mRNA and protein (Figure 4.3b and 4.3c). To further confirm the relationship between RNASET2 and XIAP, we examined the effect of re-expressing RNASET2 in the 2B1 mutant. We hypothesized that if RNASET2 regulates
XIAP levels, restoration of RNASET2 expression should normalize XIAP expression. We transiently transfected 2B1 cells with RNASET2 (or empty vector [EV] as control) and quantified levels of XIAP mRNA (Figure 4.3d and 4.3e). Overexpression of RNASET2 in 2B1 cells caused a 53% decrease in XIAP mRNA. Together, these data indicate that RNASET2 levels regulate the amount of cellular XIAP mRNA and protein.

**XIAP does not mediate ROS generation.**

XIAP has been implicated in the generation of ROS through modulation of antioxidant levels. Since 2B1 cells have altered ROS tone and fail to amplify ROS during lipotoxicity and following treatment with hydrogen peroxide, it was possible that increased levels of XIAP led to these aspects of the phenotype in RNASET2 haploinsufficient cells. We tested whether sensitivity of 2B1 cells to hydrogen peroxide was modulated by transient knockdown of XIAP (Figure 4.4a and 4.4b) and by chemical inhibition of the E3 ligase activity of XIAP with embelin (Figure 4.4c). Neither knockdown of XIAP nor treatment with embelin altered ROS amplification in 2B1 cells. These observations suggest that XIAP does not directly regulate ROS levels in 2B1 cells, but rather functions through an alternate mechanism.

**Haploinsufficiency for RNASET2 increases the half-life of XIAP mRNA.**

Steady state mRNA levels reflect rates of synthesis and degradation of individual transcripts. Our previous data established that the RNase activity of RNASET2 is critical for the function of RNASET2 in lipotoxic and oxidative stress. Since XIAP mRNA is increased when RNASET2 levels are low, we hypothesized that XIAP mRNA might be a bona fide substrate for RNASET2. A corollary of this hypothesis is that XIAP mRNA would be expected to have a longer half-life in the setting of RNASET2 haploinsufficiency. To test this hypothesis, we metabolically labeled cellular RNA with 5-ethynyl uridine. This uridine analogue is taken up by cells and incorporated
into newly synthesized RNAs, which can be recovered by biotinylation of cellular RNA using click chemistry reagents and streptavidin affinity adsorption. We pulsed WT CHO and 2B1 cells with 5-ethyl-5-uridine, chased in 5-ethynyl uridine-free media, and recovered and quantified labeled mRNAs by qRT-PCR. XIAP mRNA half-life was twice as long as in WT CHO cells compared to haploinsufficient 2B1 cells (Figure 4.5). This indicates that RNASET2 affects the half-life of XIAP mRNA and suggests that XIAP could be a direct substrate of the enzyme.

**Loss of RNASET2 affects lysosomal homeostasis during lipotoxicity.**

RNASET2 is a lysosomally-targeted acidic hydrolase, and it is unclear how this enzyme would come into contact with mRNA transcripts such as XIAP that reside in the cytoplasm. Some previous studies have provided evidence for stress-induced release of T2 RNase enzymes from lysosomes and vacuoles. Therefore, we tested whether lipotoxic stress induced the release of RNASET2 from the lysosome into the cytosol. We fractionated cytosol and membrane-bound organelles from NIH 3T3 fibroblasts under basal growth and palmitate-treated conditions. Western analysis for marker proteins indicated that the cytoplasmic fraction was relatively enriched for soluble proteins (i.e., tubulin) and depleted for proteins that reside within membrane-bound organelles (i.e., calnexin, LAMP1) (Figure 4.6a). RNASET2 was associated with membrane-bound organelles under both basal growth conditions and palmitate-treated conditions and did not accumulate in the cytoplasmic fraction under lipotoxic stress. Our data support a model in which lysosomally-resident RNASET2 functions in the propagation of ROS during lipotoxicity.

Mutation or loss of lysosomal hydrolases is known to cause lysosomal dysfunction. Thus, we wanted to determine whether loss of RNASET2 was associated with lysosomal dysfunction in the setting of lipotoxicity. To assess lysosomal function, we incubated WT CHO and 2B1 cells
under basal growth and palmitate-treated conditions with LysoTracker, a pH dependent lysosomal stain, (Figure 4.6b) There was a trend for lower LysoTracker staining under basal growth conditions, although this was not significant. However, 2B1 cells had significantly decreased LysoTracker staining following palmitate treatment. This suggests that the mutant cells either fail to acidify lysosomes or fail to generate new lysosomes during metabolic stress. On the other hand, we observed no difference between WT CHO and 2B1 cells in the cleavage of Magic Red, an artificial cathepsin substrate (Figure 4.6c). This indicates that despite the differences in LysoTracker staining, the ability to degrade protein substrates is not significantly compromised in 2B1 cells.

Both metabolic and oxidative stress are potent inducers of autophagy. Lipid-induced autophagy has been shown to either promote cell death or be cytoprotective in the setting of nutrient excess in some tissues and cell types. We hypothesized that haploinsufficiency of RNASET2 may blunt maladaptive lipid-induced autophagy or autophagic flux. To test this, we examined the accumulation of p62 and the lipidation of LC3-I to LC3-II in WT CHO and 2B1 cells under basal growth and palmitate-treated conditions (Figure 4.6d). 2B1 cells had more p62 than WT CHO cells under basal growth and palmitate-treated conditions, but upon inhibition of autophagic flux by treatment with the lysosomal-autophagosome fusion inhibitor bafilomycin A1, WT CHO and 2B1 cells appeared to have equivalent levels of cellular p62. WT CHO and 2B1 cells have similar levels of LC3-I and LC3-II. Taken together, these data indicate that haploinsufficiency for RNASET2 does not have a major effect on both induction autophagy under basal growth and lipotoxic conditions and flux of lysosomally-targeted substrates. Future studies will be necessary to further assess the contribution of RNASET2 to autophagy.

Neutralization of lysosomal pH stabilizes XIAP mRNA.
If XIAP mRNA levels are regulated by the function of RNASET2, then in addition to genetic depletion of RNASET2, chemical inhibition of the enzyme’s function would be expected to increase XIAP mRNA levels. Since RNASET2 functions at a pH optimum of 4-5, we tested whether neutralization of lysosomal pH was sufficient to cause an accumulation of XIAP mRNA. WT CHO cells were treated with chloroquine or ammonium chloride, agents that increase lysosomal pH. Although these treatments increased lysosomal pH, as measured by LysoTracker staining, they did not induce cell death as assessed by propidium iodide staining (Figure 4.7a). Alkalinization of lysosomal pH led to increased steady-state levels of XIAP mRNA and increased XIAP mRNA half-life (Figure 4.7b and c). These results further support a model in which XIAP mRNA levels are regulated, in part, through functions within the lysosome.

**XIAP mRNA associates with the lysosome-containing fraction of 2B1 cells.**

XIAP is a soluble, cytoplasmic protein that is translated on free ribosomes. Our results suggest that under lipotoxic conditions, XIAP mRNA is targeted to the lysosome for RNASET2-mediated degradation. To test whether XIAP mRNA is in part associated with membrane-bound organelles, we isolated RNA from both the cytoplasm and a crude mitochondrial light pellet (MLP), which contains membrane bound organelles, including mitochondria, ER, Golgi, and lysosomes (Figure 4.8a). Using RT-qPCR, we measured the levels of 36B4, a representative cytoplasmic transcript, and NPC1, a membrane-associated transcript (Figure 4.8b). Transcript for the cytoplasmic 36B4 was enriched in the cytoplasmic fraction of both WT and 2B1 cells, and the transcript for the lysosomally-targeted NPC1 was enriched in the MLP fraction. Interestingly, XIAP mRNA was enriched in the MLP in both WT CHO and 2B1 cells. XIAP mRNA is also enriched in both the cytoplasm and MLP of 2B1 cells compared to WT CHO cells. These data suggest that the XIAP mRNA can associate with membrane-bound organelles, and
that haploinsufficiency for RNASET2 leads to a build-up of XIAP mRNA in both the cytoplasmic and in membrane-bound compartments.

**XIAP mRNA is enriched in the lysosomes of 2B1 cells.**

It is well appreciated that cellular components destined for degradation can be targeted to the lysosome, however, lysosomal targeting and degradation of RNA remains poorly understood. In order to explore potential targeting of XIAP to the lysosome for degradation by RNASET2, we isolated cytoplasm, ER, and lysosomes from WT CHO and 2B1 cells. We were able to quantify both protein and RNA from the lysosomal fraction, indicating that RNA is present within lysosomes. The lysosomal fraction from 2B1 cells had more protein and RNA compared to WT CHO cells (Figure 4.9a). However, the ratio of protein to RNA in WT CHO and 2B1 lysosomal fractions was similar, suggesting that 2B1 cells have more or bigger lysosomes than WT CHO cells, but that the composition of their lysosomes are similar (Figure 4.9b).

Using RT-qPCR, we measured the levels of actin as a representative cytoplasmic transcript, NPC1 as a representative ER-associated transcript, 28S rRNA, and XIAP (Figure 4.10a-d). Since there are no established “housekeeping” mRNAs against which to compare specific mRNA abundance in the ER and lysosomal compartments, we initially analyzed these RNAs based simply on the cycles required for amplification of these species alone. Compared to WT CHO cells, 2B1 cells had increased XIAP mRNA in total RNA, cytoplasmic RNA, ER, and the lysosomal fraction. By contrast, actin and NPC1 were more abundant in the cytosol and NPC1 was also more abundant in the ER of 2B1 cells. 28S RNA was similar in total RNA, cytoplasmic RNA and the ER, but decreased in the lysosomes of 2B1 cells. Furthermore, we compared the amount of each RNA to the amount present in the crude cell lysate (total, Figure 4.11a) or the
cytoplasmic fraction (Figure 4.11b). XIAP mRNA is increased in the lysosome fraction of 2B1 cells compared to WT CHO cells using either of these measures, while actin, NPC1, and 28S RNAs are decreased. We next wanted to determine whether this increase in XIAP mRNA in 2B1 cells was due to more or larger lysosomes in 2B1 cells compared to WT CHO cells. When we normalized the amount of XIAP mRNA to the amount of lysosomal protein in WT CHO and 2B1 cells, the lysosomes of 2B1 cells had more XIAP mRNA than WT CHO cells. These data indicate that XIAP mRNA specifically associates with the lysosomal fraction, and that cells that are haploinsufficiency for RNASET2 have an enrichment of XIAP mRNA in their lysosomes.

CONCLUSIONS

Herein, we establish XIAP mRNA as a potential substrate for RNASET2-mediated mRNA degradation. Our data show XIAP mRNA and protein levels are dynamically influenced by RNASET2, and that both genetic depletion of RNASET2 and chemical inhibition of lysosomal function increase XIAP mRNA levels by stabilizing its half-life. Additionally our data suggest that in the setting of metabolic stress, lysosomally-resident RNASET2 may be necessary for proper lysosomal function. Finally, we provide evidence that XIAP mRNA is associated with lysosomes and is enriched in the lysosomes of cells that are haploinsufficient for RNASET2. These findings suggest a novel pathway of translational control of XIAP mRNA by lysosomal targeting and degradation by RNASET2.

The T2 family of RNases has been implicated in many pathways of RNA degradation. It cannot be ruled out that RNASET2 participates in bulk degradation of lysosomally-targeted RNA. Nitrogen starvation of the yeast Rny1p knockout strain exhibits substantial accumulation of RNA.113 Haud et al. also observed a build-up of 28S rRNA in the CNS, but this was not extended to other tissues, and knockdown of RNASET2 in HEK 293 cells did not replicate this
phenomenon. We did not observe bulk degradation of RNA at early time points during metabolic stress during which we observed the effects of RNASET2 on ROS generation and cytoplasmic snoRNA accumulation. It should be noted that in addition to RNASET2, two RNase A family members, RNASET1 and RNASE6, were detected in proteomic screens for M6P-modified and secreted proteins. While it remains to be firmly established that these RNases are resident in lysosome, multiple lysosomal RNA hydrolases could be required for bulk RNA degradation, with RNASET2 having specific RNA substrates. Thompson et al. observed robust tRNA cleavage in response to peroxide treatment in mammalian cells, but only at late time-points immediately preceding cell death, whereas our studies of RNASET2 focus on its function upstream of ROS generation during metabolic stress. While the yeast Rny1p mediates tRNA cleavage in response to oxidative stress, studies in mammalian cells have implicated angiogenin, a secreted RNase A family member, in this function. tRNA cleavage could be redundantly mediated in mammalian cells by RNASET2 and angiogenin, or possibly RNASET2 has lost this function through evolution.

Our results suggest that RNASET2 functions within the lysosome during lipotoxicity. While both yeast Rny1p and mammalian RNASET2 have documented extralysosomal functions we failed to detect cytoplasmic accumulation of RNASET2 during lipotoxicity. Metabolic and oxidative stresses are potent inducers of macroautophagy, and RNASET2 could be playing an important role in degrading lysosomally-targeted RNA. Clearance of autophagic vesicles can be cytotoxic or cytoprotective depending on the duration of the stress and the cell type. In our experimental paradigm, we did not observe major changes in autophagic flux. Nonetheless, failure of 2B1 cells to produce early ROS in response to palmitate may be a necessary signal for selective autophagy and lysosomal function. Further experiments in CHO cells clarifying
whether lipotoxicity causes increased lysosomal acidification, using LysoSensor, or increased lysosome biogenesis will help to clarify the contribution of RNASET2 to lysosomal homeostasis.

XIAP is a potential novel substrate of RNASET2-mediated degradation. For the first time, we demonstrate a relationship between cellular levels of RNASET2 and the abundance of the XIAP mRNA; both haploinsufficiency and genetic knockdown of RNASET2 causes an accumulation of XIAP transcript and protein, while reintroduction of RNASET2 reduces the levels of XIAP transcript. Moreover, our hypothesis that RNASET2 degrades lysosomally-targeted XIAP mRNA is supported by increased steady state levels of XIAP transcript and increased transcript half-life in the setting of lysosomal perturbation. In addition, XIAP mRNA associates with lysosomes, an unexpected location for an mRNA that encodes a cytoplasmic protein. XIAP protein is an essential inhibitor of apoptosis, and is vital to cell survival during cell stress.95 Prior studies have revealed multiple cis- and trans-interacting factors that regulate XIAP mRNA translation and stability, including a 5’ internal ribosome entry site (IRES) and several RNA binding proteins, suggesting that levels of XIAP mRNA are key to cell survival during stress.120-125 Further studies using RNA-seq to identify which RNAs accumulate within the lysosomes of RNASET2 haploinsufficient cells and physical capture of XIAP mRNA through Cross-Linking and ImmunoPrecipitation (CLIP) of RNASET2 could suggest a novel mode of mRNA degradation, whereby mRNAs can be specifically targeted for lysosomal degradation by RNASET2.
4.1 rRNA degradation occurs late in the lipotoxic response.

(a and b) Total RNA was isolated from palm-treated CHO (open bars) and 2B1 (filled bars) cells. Reverse transcription with random hexamers (a) or with target-specific stem-loop primers (b) and qPCR was used to quantify rRNAs (a) and tRNAs (b).

(c) CHO and 2B1 mutant cells were treated with palm for 8h. Total RNA content was assessed by SYTO RNAselct staining and flow cytometric analysis. Graph shows mean fluorescence + SE for $10^4$ cells/sample.

All graphs report mean (+ SE) for n = 3 independent experiments. *, p < 0.05 for 2B1 vs. CHO; ns, non-significant.
Figure 4.2 Loss of RNASET2 is associated with changes in gene expression

(a) Venn diagram depicts the number of transcripts that were enriched in 2B1 cells relative to CHO under untreated, palm-treated, and both conditions.

(b) Total RNA was isolated from 8h palm-treated CHO (open bars) and 2B1 (filled bars) cells. Reverse transcription with random hexamers and qPCR was used to quantify select target mRNAs relative to 36B4. Graph reports mean (+ SE) for n = 3 independent experiments. *, p < 0.05 for 2B1 vs. CHO
Figure 4.3 RNASET2 influences XIAP mRNA levels.

(a) Total RNA was isolated from 8h palm-treated CHO (open bars) and 2B1 (filled bars) cells. Reverse transcription with random hexamers and qPCR was used to quantify XIAP mRNA relative to 36B4.

(b and c) NIH 3T3 cells were transiently transfected with either scrambled siRNAs (siSCR) or siRNAs targeting RNASET2. Total RNA was isolated, reverse transcribed with random hexamers, and qPCR was used to quantify RNASET2 and XIAP mRNA relative to 36B4 (b). RNASET2 and XIAP protein levels were analyzed by western blotting of total cell lysates. Representative blots for RNASET2, XIAP and hsp90 are shown below (c).
(d and e) 2B1 mutant cells were transiently transfected with either empty vector (EV) or a plasmid expressing RNASET2. Total RNA was isolated, reverse transcribed with random hexamers, and qPCR was used to quantify RNASET2 (d) and XIAP mRNA (e) relative to 36B4. All graphs report mean (+ SE) for n = 3 independent experiments. *, p < 0.05 for 2B1 vs. CHO; **, p < 0.05 for siRNASET2 vs. siSCR
Figure 4.4 XIAP does not mediate ROS accumulation.

(a and b) CHO (open bars) and 2B1 mutant (filled bars) cells were transiently transfected with siRNA targeting XIAP (siXIAP) or scrambled siRNA (siSCR) as control. Total RNA was isolated, reverse transcribed with random hexamers, and qPCR was used to quantify XIAP mRNA relative to 36B4 (a). ROS generation was quantified under normal growth conditions and following treatment with 1 mM H$_2$O$_2$ for 3h by DCF staining and flow cytometric analysis. Graph shows mean fluorescence (+ SE) for $10^4$ cells/sample (b).

(c) CHO and 2B1 mutant cells were pre-treated with 10 µM embelin for 3h and then co-treated with 10 µM embelin and 1 mM H$_2$O$_2$ for 3h. ROS generation was quantified by DCF staining and flow cytometric analysis. Graph shows mean fluorescence (+ SE) for $10^4$ cells/sample

All graphs report data from a single experiment.
Figure 4.5

RNASET2 haploinsufficiency increases the half life of XIAP mRNA.

CHO and 2B1 mutant cells were pulsed with 5-ethynyl uridine (5-EU) for 1h and then chased with in media without 5-EU. Total RNA was isolated at listed timepoints and click-biotin labeled. Biotinylated RNA was captured with streptavidin beads, reverse transcribed with random hexamers, and qPCR was used to quantify XIAP mRNA relative to β-actin.

Graphs reports mean (+ SE) for n = 3 independent experiments. *, p < 0.05 for 2B1 vs. CHO
Figure 4.6

RNASET2 haploinsufficiency alters lysosomal homeostasis.

(a) NIH 3T3 cells were maintained in growth media or supplemented with 500 μM palm for 8h. Cytoplasmic and membrane-associated fractions were isolated by sequential detergent extraction and RNASET2 localization was analyzed by western blotting. Blots show representative western analysis of fractions for cytosolic (tubulin), endoplasmic reticulum (calnexin), and lysosomal (LAMP1) compartments.

(b) CHO and 2B1 mutant cells were treated with 500 μM palm for 8h. Lysosomal acidification was quantified by Lysotracker Green staining (b) and protein degradation was quantified by Magic Red cleavage (c) with flow cytometric analysis. Graph shows mean fluorescence + SE for 10^4 cells/sample.

(d) CHO and 2B1 mutant cells were treated with 500 μM palm for 8h in the presence or absence of bafilomycin A. p62 and LC3 protein levels were analyzed by western blotting of total cell lysates.
Blots show representative western analysis of p62, LC3, and hsp90 are shown below.

Graphs reports mean (+ SE) for n = 3 independent experiments. *, p < 0.05 for 2B1 vs. CHO; ns, non-significant
Figure 4.7

Disruption of lysosomal integrity influences XIAP mRNA levels.

CHO and 2B1 mutant cells were treated with chloroquine or ammonium chloride for 16h

(a) Lysosomal acidification was quantified by Lysotracker Green (LTG) staining and cell death was quantified by PI staining with flow cytometric analysis. Graph shows mean fluorescence + SE for 10^4 cells/sample.

(b) Total RNA was isolated, reverse transcribed with random hexamers, and qPCR was used to quantify XIAP mRNA relative to 36B4.

Data is presented as the mean (+SE) for n = 3 independent experiments. *, p < 0.05 for comparisons indicated.

(c) CHO cells were treated with ammonium chloride for 4h, pulsed with 5-EU for 1hr and then chased in media without 5-EU for 1h. Total RNA was isolated at listed timepoints and click-biotin labeled. Biotinylated RNA was captured with streptavidin beads, reverse transcribed with random hexamers, and qPCR was used to quantify XIAP mRNA relative to β-actin. Data is presented as the mean (+SE) for n = 2 independent experiments
Figure 4.8 XIAP mRNA associates with membrane bound organelles.

Cytoplasmic and membrane-associated components (MLP) of CHO and 2B1 mutant cells were separated by differential centrifugation.

(a) RNA was isolated from each fraction, reverse transcribed with random hexamers, and qPCR was used to quantify mRNA.

(b) Blots show representative western analysis of fractions for cytosolic (tubulin), endoplasmic reticulum (calnexin), mitochondrial (prohibitin), and lysosomal (LAMP1 and cathepsin K) compartments.

All graphs report data from a single experiment.
Figure 4.9 2B1 cells have an enlarged lysosomal compartment.

Cytoplasmic, ER-enriched, and lysosome-enriched fractions, and total cell lysates were isolated from CHO and 2B1 mutant cells. Equal volumes from each cell type were used to isolate RNA and protein for each fraction.

(a) Graph shows the ratio of lysosomal RNA or protein isolated compared to total cellular RNA or protein.

(b) Graph shows the amount of RNA recovered from cytoplasmic, ER-enriched, and lysosome-enriched fractions, and total cell lysates of CHO and 2B1 mutant cells each normalized to the amount of recovered protein.

All graphs report data from a single experiment.
Figure 4.10 XIAP is enriched in 2B1 cells.

Cytoplasmic, ER-enriched, and lysosome-enriched fractions, and total cell lysates were isolated from CHO and 2B1 mutant cells. Equal micrograms of RNA from each cell type was reverse transcribed with random hexamers, and qPCR was used to quantify XIAP mRNA (a), representative cytoplasmic (actin, b) and ER (NPC1, c) transcripts, and 28S rRNA (d). Transcript amounts in 2B1 cells are normalized to the value from WT CHO for each cellular compartment. Note these quantifications are not normalized for a “housekeeping” gene.

All graphs report data from a single experiment.
**Figure 4.11**

RNA was isolated from total lysates, cytoplasm and lysosome-enriched fractions of CHO and 2B1 mutant cells. Equal micrograms of RNA from each cell type was reverse transcribed with random hexamers, and qPCR was used to quantify XIAP, actin, and NPC1 transcripts and 28S rRNA.

Amount of lysosomal RNA is normalized to amount of the same species in total cellular (a) or cytoplasmic RNA (b), or normalized to lysosomal protein recovered (c).

All graphs report data from a single experiment.
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CHAPTER FIVE

*In vivo* role of RNase X25 in oxidative stress

INTRODUCTION

Our studies demonstrate that mammalian RNASET2 mediates the propagation of reactive oxygen species in response to inducers of metabolic and oxidative stress. To extend our findings from cell culture to an *in vivo* model, we turned to the model organism *Drosophila melanogaster*, which has previously been used to study physiological and pathophysiological oxidative stress. We utilized the herbicide paraquat, an established inducer of toxic ROS in *Drosophila*. Unstable paraquat radicals are formed through the interaction of paraquat ions with complex III of the mitochondrial electron transport chain. These radicals react with molecular oxygen to form superoxide and peroxide radicals, leading to oxidative stress and cellular dysfunction.

The *Drosophila* genome contains one member of the T2 family, RNase X25, which is induced by ROS and promotes cell death when overexpressed. Whether loss-of-function of RNase X25 is sufficient to protect flies from oxidative stress-induced death is not known. Given that haploinsufficiency and knockdown of RNASET2 in cultured mammalian cells showed this gene to be critical for oxidative stress-induced cell death, in these studies we used the GAL4-UAS RNAi system to knockdown RNase X25 in flies. In this approach, selected promoter-specific expression of the transcription factor GAL4 drives transcription of a transgenic UAS-controlled siRNA against RNase X25. The goal of these studies was to determine whether deficiency for RNase X25 is sufficient to protect flies from oxidative stress-induced toxicity following paraquat exposure.
RESULTS

Fat body RNaseX25 knockdown flies are fertile and have normal bodyweight.

Initially, we attempted to generate flies with constitutive, whole body knockdown of RNase X25 by crossing cg8194 flies, which contain a siRNA against RNase X25, with Act-Gal4 flies, which express GAL4 under the control of an actin promoter. The progeny of this cross were not viable, underscoring the importance of RNase X25 in general housekeeping functions. We next turned to tissue specific expression of the siRNA in the fly fat body, an organ that plays a key role in metabolic physiology. Flies with fat body-specific expression of siRNA against RNase X25 demonstrated 53% knockdown of the RNase X25 transcript by qPCR of fat body-enriched tissue, while two predicted off-target genes showed no difference in transcript level. This indicated that knockdown was specific for RNase X25 (Figure 5.1a). Fat body-specific knockdown flies were born in Mendelian ratios, were viable, and had similar body weight to WT flies (Figure 5.1b). Therefore, this fat body-specific knockdown of RNase X25 provided an excellent system to test the effects of RNase X25 deficiency in the setting of oxidative stress.

Knockdown of RNaseX25 in fat bodies protects flies from paraquat toxicity.

Haploinsufficiency and stable knockdown of RNASET2 in cultured cells is protective against metabolic and oxidative stress-induced cell death. Therefore, we hypothesized that knockdown of RNase X25 in fat bodies, a highly metabolic signaling tissue in flies, would be sufficient to protect flies from exposure to inducers of oxidative stress. Following paraquat feeding, fat body RNase X25-knockdown flies had improved survival compared to WT controls (Figure 5.2). This indicated that deficiency of RNase X25 in fat bodies is sufficient to protect from systemic paraquat toxicity.

Knockdown of RNaseX25 in fat bodies protects flies from oxidative stress.
We hypothesized that increased survival of fat body RNase X25-knockdown flies following paraquat feeding was related to the effects of decreased ROS generation. In lieu of directly measuring in vivo ROS production, we quantified two reporters of non-enzymatic ROS damage to proteins and lipids.\textsuperscript{132,133} RNase X25 deficient flies demonstrated \textasciitilde35\% decreased levels of protein carbonylation under basal and stress conditions and 65\% decreased levels of 7-keto cholesterol under stress (Figure 5.3a and 5.3b). Our findings support the model that deficiency for RNase X25 in fat bodies is sufficient to protect from oxidative damage.

**CONCLUSIONS**

RNase X25 is an essential mediator of oxidative stress in *Drosophila*. Whole body knockdown of RNase X25 results in non-viable progeny, but fat body-specific knockdown protects flies from paraquat-induced oxidative stress and death. These findings underscore the importance of RNase X25 in *Drosophila* physiology and expand our understanding of the in vivo role of RNase X25 in mediating oxidative stress.

Previous studies of T2 family members have shown that mutations, deletions, and deficiencies of T2 RNases in humans and in zebrafish cause neuropathological defects.\textsuperscript{77,78} Therefore, it is not surprising that whole body knockdown of RNase X25 in flies is lethal. Fat bodies were chosen for tissue specific knockdown because of their high metabolic activity, their role as xenobiotic scavengers, and their metabolic signaling roles in *Drosophila*. While no gross differences were observed in body weight, further studies looking specifically at body composition, lipid levels (e.g., such as triacylglyceride and free fatty acids), and metabolic signaling (e.g Akt and insulin signaling), may uncover possible systemic effects of fat body-specific deficiency of RNase X25 under basal conditions (i.e., in the absence of paraquat).
Similar to our knockdown and haploinsufficiency studies in mammalian cell culture, genetic knockdown using the GAL4-UAS system in flies resulted in only a 50% knockdown of RNase X25 mRNA, underscoring the potential importance of this RNase for normal housekeeping functions in cells. Previous reports in larvae have shown that exposure to starvation, wheat germ agglutinin, and hydrogen peroxide cause an induction of the RNase X25 transcript, paralleling our observation of RNase X25 mRNA induction in fat bodies in response to paraquat feeding. Typically, T2 RNases are targeted to the lysosome, and under some circumstances they can be secreted. Our hypothesis that RNASET2 degrades lysosomally-targeted RNAs, resulting in the propagation of ROS and cell death, could be extended to RNase X25. Studies confirming the lysosomal localization of RNase X25, determining if previously verified targets of RNASET2 degradation are building up in RNase X25 deficient cells, and direct measurements of ROS generation in response to metabolic and oxidative stress inducers would help us better determine if the cellular mechanism of RNase X25 is similar to its mammalian counterpart, underscoring a previously unappreciated conserved stress response pathway.

We have demonstrated that knocking down RNase X25 solely in fat body tissue is sufficient to protect adult flies from paraquat toxicity. This phenomenon highlights an important role for fat bodies in the oxidative stress response pathway. Fat body tissue lacking RNase X25 has both decreased protein carbonylation and lipid oxidation, indicating that local oxidative damage is mitigated in the setting of RNase X25-deficiency. Fat bodies are responsible for fat storage and processing, and for metabolic signaling within the larval and adult fly. They are closely associated with oenocytes, which perform lipid processing and xenobiotic scavenging functions, similar to mammalian hepatocytes. RNase X25 may play a role in fat body signaling to or from oenocytes, possibly through ROS. Furthermore, our evidence that RNASET2 deficient mammalian cells maintain a higher antioxidant capacity could suggest that *Drosophila* fat bodies
have an increased ability to scavenge ROS generated by paraquat toxicity. Several genes overrepresented in our microarray of RNASET2 deficient CHO cells were involved in xenobiotic detoxification, suggesting that loss of RNase X25 may also impart increased ability to safely metabolize paraquat. Further studies looking at xenobiotic detoxification genes in RNase X25 deficient fat bodies, metabolites of paraquat detoxification, or general antioxidant capacity would shed light on the possible mechanism by which RNase X25 deficiency protects from cells from paraquat toxicity.
**Figure 5.1** Fat body RNaseX25 knockdown flies are fertile and have normal bodyweight.

(a) Flies of each genotype were fed 5% sucrose (-) or 5% sucrose with 15mM paraquat (+) for 20h. Total RNA was isolated from fat body-enriched tissue of 8 flies, oligo dT primed for cDNA synthesis, and expression of RNase X25, moi, and tgs1 was determined by qRT-PCR relative to tubulin.

(b) Cohorts of 6 female control (open bars, $w^{1118}$) and RNase X25 fat body knockdown (filled bars, $CG8194$) flies were weighed. Body weight is reported as mean weight per fly (+ SE) for $n = 3$ independent crosses.

Graph shows mean + SE for $n = 3$ independent crosses. *, $P < 0.05$ for comparisons indicated.
Figure 5.2 KD of RNaseX25 in fat bodies protects flies from paraquat toxicity.

Cohorts of control (open bars, w^{1118}) and RNase X25 fat body knockdown (filled bars, CG8194) flies were fed 5% sucrose (-) or 5% sucrose with 15mM paraquat (+) for 20h. Mean (+ SE) survival at 20h was quantified for 60 flies per genotype/condition in n = 3 independent crosses. *, p < 0.05 for comparisons indicated.
**Figure 5.3** KD of RNaseX25 in fat bodies protects from oxidative stress.

Cohorts of control (open bars, w^{1118}) and RNase X25 fat body knockdown (filled bars, CG8194) flies were fed 5% sucrose (-) or 5% sucrose with 15mM paraquat (+) for 20h. Extracts were prepared from fat body-enriched tissue pooled from 8 flies.

(a) Carbonylated proteins were derivitized using DNPH or control solution, and analyzed by western blotting using α-DNP antibody. Representative blot is shown (left) and graph (right) quantifies results from 4 independent crosses.

(b) 7-ketocholesterol was quantified in fat body lipid extracts by LC-MS/MS and is reported normalized to protein concentration (mean ± SE for n = 3 crosses).

*, p < 0.05 for comparisons indicated
CHAPTER SIX
Summary and Discussion

Summary.
Through characterization the 2B1 mutant cell line, we have identified RNASET2 as a novel mediator of lipotoxicity. RNASET2 is required for metabolic and oxidative stress-induced cytoplasmic accumulation of the Rpl13a snoRNAs, ROS generation, and cell death. Through complementation studies, we have determined that the catalytic activity of RNASET2 is necessary for its role in lipotoxicity. Taken together, our data suggests a model in which RNASET2 is critical for the early events of lipotoxicity, potentially through altering cellular antioxidant capacity during stress.

While RNASET2 and its family members have been implicated in oxidative stress, cell proliferation, and RNA degradation, one of the goals of this study was to uncover the molecular mechanism of RNASET2 during lipotoxicity.66,75,76,78 2B1 cells did not exhibit any differences in rRNA, tRNA, or bulk RNA degradation early in lipotoxicity. However, we found that 2B1 cells had differential abundance of many mRNAs under basal growth and lipotoxic conditions. One of the most highly regulated transcripts by RNASET2 was XIAP. We propose that XIAP transcripts are a novel target of RNASET2-mediated degradation. Levels of XIAP mRNA and protein are inversely correlated with the expression of RNASET2, and loss of RNASET2 increased the half-life of XIAP mRNA. RNASET2 is a lysosomal RNase, and loss of RNASET2 not only alters lysosomal homeostasis, but also results in increased association of XIAP mRNA with lysosomes. Disruption of lysosomal acidity, independent of alterations in RNASET2 expression, also increased the stability and abundance of XIAP mRNA. Our data suggests a novel pathway of lysosomally-targeted mRNA degradation.
In vivo studies of RNASET2 have been limited to observations of the effects of mutations in both humans and zebrafish.\textsuperscript{77,78} We present the first study of tissue-specific knockdown of a T2 family member. Fat body-specific knockdown of \emph{Drosophila} RNase X25 is sufficient to protect flies from paraquat-induced oxidative stress and toxicity. These findings highlight the importance of RNase X25 in mediating oxidative stress responses, and further expand our understanding of the contribution of T2 RNases to physiology.

**RNASET2, ROS, and the Rpl13a snoRNAs.**

RNASET2 was identified through a loss-of-function forward genetic screen in fibroblasts for genes that are critical for lipotoxicity. RNASET2, like many of the other genes identified from this screen, is required for generation or amplification of ROS during lipotoxicity, underscoring the importance of ROS as both a signaling component and as a critical contributor to cellular damage during metabolic stress.\textsuperscript{58,59,61,99} Moreover, two of these genes, SmD3, and RNASET2, were found to contribute to the biological function of the \emph{Rpl13a} snoRNAs.\textsuperscript{61,99} Identification and characterization of further mutants from this screen will continue to enrich our knowledge of the mechanisms of lipotoxicity and may reveal novel biological pathways.

Our initial observation that 2B1 cells fail to accumulate the \emph{Rpl13a} snoRNAs in the cytoplasm in response to metabolic and oxidative stress led us to hypothesize that this RNase could be participating in the production or maturation of nascent snoRNAs. A previously characterized gene from our loss-of-function screen, SmD3, is a component of the spliceosome and regulates snoRNA abundance through its role in splicing or stabilization of snoRNA-containing lariats.\textsuperscript{61} However, RNASET2 remains in the lysosome during basal growth and lipotoxicity, and the levels of mature nuclear snoRNAs in 2B1 cells as well as their precursor pre-mRNAs and intron
lariats are indistinguishable between WT and 2B1 mutant CHO cells. This not only disproved our initial hypothesis, but supported two potential corollaries concerning snoRNA biology.

First, 2B1 cells did not have a defect in processing or nuclear levels of mature snoRNAs, but lacked the molecular signal for their nucleo-cytoplasmic transport during stress. Work by Holley et al. specifically implicates superoxide radicals as a potent signal for the cytoplasmic accumulation of snoRNAs. Consistent with their work, we were able to detect production of superoxide preceding the cytoplasmic accumulation of the snoRNAs in wild type CHO cells in response to palmitate treatment. 2B1 cells failed to produce this early superoxide, which we hypothesize may be the necessary signal for cytoplasmic snoRNA accumulation during lipotoxicity. The source of this superoxide and exactly how loss of RNASET2 inhibits its production will help determine the significance of the early superoxide production during lipotoxicity.

Second, both 2B1 cells that are haploinsufficient for RNASET2 and 6F2 cells that are haploinsufficient for Rpl13a both fail to amplify ROS in response to palmitate. This failure of 2B1 cells to amplify ROS in response to palmitate may be due to increased antioxidant capacity or may be attributable to the failure to accumulate the snoRNAs in the cytoplasm, which themselves contribute to amplification of ROS. Over the course of this study, the question has been raised as to whether the cytoplasmic accumulation of the snoRNAs is sufficient to induce ROS generation. To address this would require a complementation scheme in 2B1 cells in which the snoRNAs were specifically overexpressed in the cytoplasm in the setting of stress. Through immunoprecipitation and proteomic analysis of the cytoplasmic U33 RNP, the Schaffer lab is beginning to understand the diversity of protein and RNA binding partners of the cytoplasmic snoRNAs. However, further understanding of mechanisms of snoRNA transport will
be necessary to determine the exact mechanism by which the cytoplasmic accumulation of the
*Rpl13a* snoRNAs promotes the amplification of ROS during cell stress.

Future studies will also be required to understand how RNASET2 influences cellular antioxidant
levels. The cell has multiple classes of antioxidants and the necessary machinery to maintain
them. Antioxidants, such as catalase and superoxide dismutase, scavenge specific ROS
species, whereas redox sensitive proteins, like peroxiredoxins, thoredoxins, and gluaredoxins,
reduce oxidative modifications. The expression of antioxidants is cell compartment, cell type,
and even tissue specific. This is appropriate, as organelles like mitochondria and peroxisomes
are exposed to high levels of ROS and require specific protections to function normally.
Moreover, ROS molecules are short-lived and likely to act locally. We were able to detect
increased levels of some components of the antioxidant defense system, however a more
detailed measurement of major antioxidants and antioxidant families in the setting of RNASET2
haploinsufficiency could be informative. First, RNASET2 may be influencing the antioxidant
capacity of specific cellular compartments, which may be critical for maintaining cellular
homeostasis during metabolic or oxidative stress, such as the mitochondria or the nucleus.
Second, up-regulation of specific antioxidants may point to a common transcriptional network
downstream of RNASET2. Nrf2 and HIF1α are noted master transcriptional regulators of
antioxidant genes, and activators of these networks could be targets of RNASET2-mediated
degradation. While XIAP has been implicated in mediating cellular antioxidant levels,96-98,121,134
our studies using genetic knockdown and chemical inhibition of XIAP suggest that other
mechanisms are at play.

**RNASET2-mediated lysosomal degradation of RNA.**
Our data supports a model of RNASET2-mediated degradation of XIAP mRNA. XIAP is a master inhibitor of apoptosis, and knockdown or deletion of XIAP renders cells sensitive to a wide array of cellular stressors. The abundance and translation of XIAP are governed by complex regulatory mechanisms. During stress, cells inhibit 5’ cap-dependent translation of mRNAs as a cellular survival mechanism. This enables cells to pause a majority of protein production in order to divert the energy and material required for translation towards the resolution of cellular insults. The mRNA of XIAP is 8.5 kb long, with a 6.7 kb 3’ UTR with many microRNA binding sites and a 120 bp 5’ UTR, which contains an internal ribosome entry site (IRES) which enables XIAP to be translated in the absence cap-dependent initiation machinery. IRESes were first discovered as components of viral RNAs that allow viral components to be translated and viral replication to continue despite cellular inhibition of translation, one of the first cellular responses to viral infection. Subsequently, IRESes have been discovered within dozens of eukaryotic transcripts that are vital for cellular homeostasis, such as BCL2, BiP, c-Myc, c-Jun, eIF4G, FGF1, Hsp70, Hsp90, IGF2, LamB1, Nrf1, p27, and p53 (IRESite, online). During stress, these genes are still required for basic cellular function, and only in settings of intense or prolonged insult, such as stressors that cause irreparable damage to DNA, mitochondria, or vital protein signaling complexes, would it be imperative that cells degrade these transcripts, thereby fully committing to apoptosis. Aberrant overexpression of many IRES-containing transcripts, especially XIAP, is observed in many cancers, in which failure to commit to apoptosis is deleterious.

Nascent RNA is subjected to intense exosomal surveillance. Improperly transcribed, spliced, or processed RNA is immediately degraded in the nucleus by the nuclear exosome complex. Cytoplasmic mRNAs undergo rounds of translation with incremental polyA tail shortening by deadenylases. Aberrant translation of mRNAs, such as ribosome stalling (no-go decay),
premature stop codons (nonsense mediated decay), or run-off into the polyA tail (non-stop decay), is detected by RNA surveillance factors, resulting in targeted exosomal degradation. Global arrest of translation, due to stress, viral infection, or genomic instability, leads to sequestration of mRNAs is stress granules or P-bodies, where they can be held until the stressor is resolved, or either degraded through either docking of decapping and exosomal nucleases, or autophagic delivery to the lysosome.

Lysosomal targeting and degradation of the XIAP transcript during an acute stress presents a novel pathway to control the abundance of transcripts that are otherwise unaffected by normal cellular pathways of translational regulation. Many of our observations of the differential regulation of XIAP mRNA in 2B1 cells has been under basal growth conditions, suggesting that under homeostatic conditions, some XIAP transcripts are targeted to the lysosome (Figure 6.1). In the setting of lipotoxicity, we hypothesize that XIAP mRNA is increasingly targeted to the lysosome, sensitizing cells to stress and increasing their apoptotic potential. Future studies tracking the level of XIAP protein during the progression of lipotoxicity, measuring the association of XIAP transcript on polysomes, and tracking the location of XIAP transcript in intact cells will add to our understanding of the dynamic regulation of XIAP transcript abundance and activity. We cannot rule out exosome-mediated degradation of XIAP transcript, and further studies measuring XIAP mRNA half-life in the setting of knockdown of exosome components will reveal the contribution of exosomal degradation to XIAP mRNA stability.

Biochemical studies have shown that purified RNASET2 has little specificity for substrate sequences. Thus, we hypothesize that specificity of RNASET2 is most likely mediated through lysosomal delivery of its substrates. Differential localization of degradation machinery for RNAs is not an uncommon cellular feature. The RNA exosomal machinery has the capacity
to degrade most forms of RNA, however it is composed of different nuclease with different substrate preferences in different cellular compartments. The same may be true for RNASET2. There are multiple specialized forms of autophagy and lysosomal targeting that allow for different types of substrates to be degraded, such as mitophagy, ribophagy, microautophagy, and chaperone-mediated autophagy.

Roy Parker’s group has provided evidence that P-bodies and stress granules are cleared by autophagy, and further work has identified Hsp70 and Hsp40 as potential chaperones for lysosomal delivery. Identifying the pathway by which mRNAs, either in stress granules/P-bodies or as single transcripts, are targeted to the lysosome during basal growth or stress will help in our understanding of lysosomal-targeted RNA degradation. While we provide evidence for XIAP transcript targeting to the lysosome, we believe that this may be a broader mechanism affecting many classes of transcripts. Future studies can leverage the 2B1 cells as a tool to identify lysosomally-targeted mRNAs. Both RNA-seq of lysosomal RNA from 2B1 cells and cross-linking and immunoprecipitation (CLIP) of RNAs that are in physical proximity to RNASET2 will reveal additional targets of degradation. In addition to XIAP, increased levels of all IRES-containing mRNAs in 2B1 lysosomes or physically linked to RNASET2 would hint that these mRNAs as a class are targeted to the lysosome for degradation.

**In vivo role of RNASET2 during oxidative and metabolic stress.**

Our studies of RNase X25 in *Drosophila* indicate that, in addition to contributing to oxidative stress-induced death, RNase X25 plays an important role in development or cell survival since whole body knockdown of RNase X25 is lethal. ROS is an highly important second messenger for many homeostatic cellular processes, such as cell proliferation, wound healing, and differentiation. Similar to RNASET2, RNASE L was identified as a cellular ribonuclease that
mediates cell death. RNASE L is activated in response to viral infection, where it non-specifically degrades RNAs, leading to the activation of apoptosis. However, recent studies have shown that RNASE L also regulates the steady state level of CHOP10 mRNA during adipocyte differentiation, and knockout of RNASE L leads to adipose tissue hyperplasia and impaired lipid storage. As a mediator of ROS, RNASET2 may play a critical role in mammalian development and future mouse models of RNASET2 loss-of-function will likely require temporal and tissue-specific control.

RNASET2 is a lysosomal enzyme and has multiple mannose-6-phosphate modifications. Lysosomal hydrolases are delivered to the lysosome by the mannose-6-phosphate receptors (M6PRs) directly or through secretion and reuptake. In some congenital lysosomal enzyme deficiencies, the latter extracellular pathway has been leveraged in therapies in which these proteins can be supplied exogenously and taken up by deficient cells. Other T2 family members are known to be secreted and can be endocytosed in trans, most likely through mannose-6-phosphate receptor mediated endocytosis. Administration of recombinant RNASET2 in trans alters tumor growth and inhibits the angiogenic effects of FGF and VEGF in vitro. While RNASET2 is secreted, the contribution of trans-endocytosed RNASET2 to metabolic and oxidative stress is an area that has not been explored.

RNASET2 is most highly expressed in hematopoietic cells, which are highly proliferative and employ ROS for many processes, including signaling and cellular defenses. Knockout of RNASET2 in individual lymphocyte and monocyte lineages may uncover novel roles for RNASET2 in immune function. Oxidative stress and tissue damage are critical components of metabolic dysfunction, and knockout of RNASET2 in other highly metabolic tissues, such as the
heart, skeletal muscle, liver, and in pancreatic β-cells will extend our understanding the contribution of RNASET2 to the pathogenesis of metabolic diseases. RNASET2 was identified as part of a network of transcriptionally elevated genes in Mexican and Finnish individuals with elevated serum triacylglyceride levels and its ability to be secreted makes RNASET2 mRNA and protein a novel potential biomarker for metabolic disease. However, because of its tumor suppressor functions, drugs or siRNA therapeutics targeting RNASET2 will need to be approached with caution.

The “Antioxidant Hypothesis” for treating diabetic complications was initially proposed in the late 1950s. However, many studies that employed dietary, exogenous, and intracellular antioxidants have failed to support treatment strategies centered around antioxidants or ROS scavenging. For example, in 2009, the HOPE study found that dietary or exogenous supplementation of vitamin E had no benefit in preventing atherosclerosis or cardiac events. Failed interventions such as this may reflect limitations to our understanding of ROS and the pharmacology of antioxidants. ROS is produced in specific cellular compartments, and therapies may need to be targeted to specific organelles or tissues in order to be effective. Different ROS species will require specific strategies, and pharmacological approaches targeting ROS-producing enzymes may provide the best precision to target harmful rather than homeostatic species of ROS. Beyond the well-appreciated enzymes in ROS production and ROS scavenging, our study has provided new insights regarding the contributions of a lysosomal RNase to oxidative stress biology.
Figure 6.1 RNASET2 degrades lysosomally-targeted XIAP mRNA.

A model for the lysosomal degradation of XIAP mRNA is shown. Under homeostatic conditions, XIAP transcripts are degraded through either exosomal targeting or lysosomal targeting and degradation by RNASET2. During metabolic stress, increased delivery of XIAP transcripts to the lysosome sensitizes cells to stress, increasing their apoptotic potential.
CHAPTER SEVEN

Materials and Methods

Materials. Palmitate was from Nu-Check Prep. Actinomycin D and OxyBlot Protein Oxidation Detection Kit were from Millipore. Staurosporine, hydrogen peroxide, Trolox, 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), paraquat, menadione, embelin, chloroquine diphosphate salt, and ammonium chloride were from Sigma-Aldrich. Fatty acid-free bovine serum albumin (BSA) was from SeraCare. Hygromycin B, BODIPY® FL C16 (4,4-Difluoro-5,7-Dimethyl-4-Bora-3a,4a-Diaza-s-Indacene-3-Hexadecanoic Acid) D-3821 (BODIPY FA), propidium iodide (PI), dihydroethidium (DHE), Trizol, 2′,7′-dichlorodihydrofluorescein diacetate (DCF), SYTO RNAselect, LysoTracker Green, Click-iT Nascent RNA Capture Kit and Lipofectamine LTX and RNAiMAX were from Life Technologies. Purified Torulla yeast RNA was from Ambion. All synthetic oligonucleotides were from Integrated DNA Technologies. Restriction enzymes were from New England BioLabs.

Cell culture. CHO K1 cells (American Type Culture Collection, ATCC) and CHO-derived cell lines were maintained in high-glucose (4.5 mg/ml Dulbecco’s modified Eagle’s medium and Ham’s F-12 nutrient mixture [1:1]) medium with 5% noninactivated fetal calf serum, 2 mM L-glutamine, 50 units/ml penicillin G sodium, 50 units/ml streptomycin sulfate, and 0.5 mM sodium pyruvate. C2C12 murine myoblast cells (ATCC) were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% (v/v) heat-inactivated fetal calf serum, 2 mM glutamine, 50 U/ml penicillin, and 50 μg/ml streptomycin. For lipotoxicity experiments, medium was supplemented with 500 μM palmitate complexed to BSA at a 2:1 molar ratio, as described previously.51 For ROS induction, medium was supplemented with the indicated concentrations of hydrogen peroxide or menadione.
**Genetic screen.** CHO cells were transduced with vesicular stomatitis virus G protein-pseudotyped murine retrovirus containing the ROSAβgeo retroviral promoter trap at a low multiplicity of infection, as previously described. Retroviral insertion into the genome was assessed by Southern blotting. The gene disrupted by retroviral insertion was identified by 5’ rapid amplification of cDNA ends (RACE) using an oligonucleotide tag and ROSAβgeo sequences (SMART RACE cDNA amplification kit; Clonetech). The 5’ RACE product was TA-cloned, sequenced, and analyzed by NCBI BLAST.

**Fatty acid uptake assay.** Fluorescent fatty acid uptake was performed as described. Briefly, cells were trypsinized, washed in media, and incubated at 37°C for 1 minute in fatty acid uptake solution (20 μM fatty acid-free bovine serum albumin and 6 μM BODIPY-3821). Cells were washed at 4°C, pelleted, resuspended in media, and mean fluorescence was determined by flow cytometry (10⁴ cells/sample).

**Transient transfections.** The murine RNASET2 cDNA sequence was cloned by PCR downstream of 2kb of the genomic murine RNASET2 promoter into pcDNA3.1(-)hygro. QuikChange II Site-directed Mutagenesis (Stratagene) was used to create the catalytically inactive construct (H117F/H122F). Cells were transfected with Lipofectamine Plus according to the manufacturer’s instructions and assayed 20h post-transfection.

**Stable cell lines.** siRNAs to murine RNASET2 were designed (Ambion’s siRNA Target Finder Program), tested in transient transfections, and corresponding shRNA oligonucleotides were designed and cloned into a pSilencer4.1-CMVneo (Ambion). Constructs were nucleofected into
C2C12 cells (Amaxa nucleofector kit V, Lonza) with plating at limiting dilution and selection in 500 μg/ml G418 for isolation of stable clonal lines. Full-length genomic or catalytically inactive murine \textit{RNASET2}, including 4.3 Kb upstream and 1.5 Kb downstream, was recombineered into pcDNA3.1(−)neo. Mutant 2B1 cells were stably transfected with Lipofectamine Plus reagent, plated at limiting dilutions in 300 μg/ml hygromycin, and clonal lines were isolated.

**Cell death.** Cell death was assessed by membrane permeability to 1 μM propidium iodide (PI) and flow cytometry analysis. Apoptosis was assessed by simultaneous annexinV-EGFP and PI staining with flow cytometry quantification annexinV+/PI− cells. For flow analyses, mean fluorescence was quantified on of 10⁴ cells/sample.

**Quantitative Real Time PCR (qRT-PCR).** RNA was isolated using TRIzol or TRIzol LS reagent and reverse transcribed to cDNA (SuperScript III First-Strand Synthesis System, Life Technologies) by priming with oligo(dT) to detect mRNA or random hexamers to detect pre-mRNA, intron lariats, and ribosomal RNA. For detection of snoRNAs and tRNAs, cDNA synthesis was primed with hairpin stem-loop oligonucleotides as previously described, with overhang complementarity to the 3’ end of the processed snoRNA or tRNA. For detection of rRNA, RNA was extracted and reverse transcribed using random hexamers, normalizing per cell number. For all RNA species, cDNA was amplified for 40 PCR cycles using SYBR green PCR master mix (Applied Biosystems) and 100nM template-specific primers in an ABI Prism 7500 Fast real-time PCR system. Relative quantification of gene expression was performed using the comparative threshold method.

**Generation of the RNASET2 antibody.** Affinity purified polyclonal rabbit anti-peptide antibody was generated against NH₂-DGPIFYPPPTKTQH-OH (murine sequence, amino acids 246-259).
Immunoblot Assays. Whole-cell protein lysates were prepared using RIPA buffer (50mMTris-Cl, 150mMNaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, and 5 mM EDTA) containing 1 mM phenylmethylsulfonyl fluoride and 1X Protease Complete inhibitor mixture (Roche). Subcellular fractions were isolated by sequential detergent solubilization as described previously. Proteins were resolved by SDS-PAGE, transferred to nitrocellulose membranes (Whatman), and probed with antibodies to RNASET2 (1:500), β-actin (A2066; 1:5,000; Sigma), and hsp90 (SPA-846; 1:2,000; Stressgen). Proteins were visualized using horseradish peroxidase-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories; 1:10,000) and chemiluminescence reagents (PerkinElmer Life Sciences). A representative blot is shown for each experiment.

Assessment of catalytic activity of RNASET2 constructs. Zymography was performed as described.

Detection of Reactive Oxygen Species. Following treatments as indicated, cells were rinsed with PBS and incubated at 37°C in the dark with PBS containing 0.5mM MgCl₂, 0.92mM CaCl₂, and either 3 μM DCF for 1h or 10 μM DHE for 15 min. The cells were recovered by trypsinization, and mean fluorescence was determined by flow cytometry (10⁴ cells/sample). For DHE detection by microscopy, cells were grown on gelatin-treated glass coverslips and stained for DHE as above. In each independent experiment, DHE fluorescence of 20 cells per field in 5 random fields was quantified using AxioVision software.

Trolox equivalent antioxidant colorometric (TEAC) assay. Antioxidant capacity was assessed by TEAC assay as previously described.
**RNA staining using SYTO RNaselect.** Following palmitate treatment, cells were incubated with 500 nM SYTO RNaselect in complete media for 30 mins. The cells were recovered by trypsinization, and mean fluorescence was determined by flow cytometry (10^4 cells/sample).

**Microarray.** Two independent sets of CHO and 2B1 cells were treated with BSA alone or BSA supplemented with 500 μM palmitic acid for 8h and RNA was isolated Trizol. Samples were prepared from total RNA using NuGen Ovation Pico amplification kit. Targets were labeled using NuGen Encore Biotin kit, and hybridized to the CHO Gene ST Array (Affymetrix). Analysis was carried out using Partek Genomics Suite.

**siRNA knockdown.** Cells were transfected with silencer select siRNAs against RNASET2 (Invitrogen, ID# s234054), XIAP (Invitrogen, ID# s62360), or scramble control using Liptofectamine RNAiMAX in Opti-MEM (Life) according to the manufacturer’s instructions and assayed either 24h or 48h post-transfection.

**Lysosomal Perturbation.** Cells were treated with 80mM ammonium chloride or 50uM chloroquine diphosphate for 16h at indicated concentrations. Lysosomal function was assessed by incubating treated cells with 500 nM Lysotracker for 45 min at 37°C. The cells were recovered by trypsinization, and mean fluorescence was determined by flow cytometry (10^4 cells/sample).

**mRNA transcript half-life measurement.** Cells were starved in DMEM for 30 min prior to labeling with 500 nM ethynyl uridine for 1h, washed, and chased with complete media. Total RNA was extracted, biotinylated, and captured according to manufacturer’s instructions.
Isolation of MLP-associated mRNAs. Isolation of MLP was modified from Boonen et al. 2009. Cells were trypsinized, washed with PBS, and then swelled and homogenized in a 0.25M sucrose solution. Nuclei were pelleted by spinning at 1,000xg for 10 min at 4°C. Membrane bound organelles (MLP) were pelleted by spinning the resulting post-nuclear supernatant at 35,000 rpm for 1h at 4°C using a Beckman SW55Ti rotor. The resulting pellet was resuspended in either Trizol or RIPA buffer for RNA and protein analysis, respectively.

Lysosome isolation. Lysosomes were isolated by Dr. Susmita Kaushik in the laboratory of Prof. Ana Maria Cuervo. Briefly, lysosomes were isolated from CHO and 2B1 cells after disruption of the plasma membrane by nitrogen cavitation and sequential centrifugation in percoll/metrizamide discontinuous density gradients.

Drosophila Studies. Flies were maintained at 25°C on molasses-based fly food. The following stocks were used: w¹¹¹⁸ (VRDC 60100), cgGal4 (Bloomington 7011), UAS-CG8194 RNAi (VRDC 13018). To generate fat body RNaseX25 knockdown, we crossed UAS-CG8194 RNAi males with cgGal4 virgin females. For paraquat treatment studies one-week-old female flies were maintained on agar supplemented with 5% sucrose in PBS for 4 hours. Then, flies were transferred to vials containing either agar with 5% sucrose or agar with 5% sucrose and 15 mM paraquat (20 flies/vial, 3 vials each condition for 3 independent crosses). Live flies were counted after 20h. Oxidative stress signatures were assessed after 20h of paraquat treatment. In order to obtain fat body-enriched tissue, we dissected flies and processed the abdominal cuticle with fat body still attached for RNA, protein extraction, and and lipid extraction. Carbonylated proteins were analyzed using OxyBlot.
Protein Oxidation Detection Kit per manufacturer’s instructions. 7-ketocholesterol was quantified by LC-MS/MS as previously described.\textsuperscript{133}

**Statistics.** Data were analyzed using ANOVA for multiple comparisons or t-test for pairwise comparisons. Statistical significance is indicated for $p < 0.05$. 
REFERENCES


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