Regulation of Metabolic Stress-Induced snoRNAs

Benjamin Steel Scruggs

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Regulation of Metabolic Stress-Induced snoRNAs

by

Benjamin Steel Scruggs

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 2012

Saint Louis, Missouri
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Abbreviations

7-keto 7-ketocholesterol
ASO anti-sense oligo
BSA bovine serum albumin
CHO Chinese hamster ovary
CM-H$_2$DCFDA 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein
diacetate, acetyl ester
CYT cytosolic
DETC diethyldithiocarbamate
DHE dihydroethidium
DPI diphenylene iodium
eEF1A-1 eukaryotic elongation factor 1A-1
FA fatty acid
FFA free fatty acids
gadd7 growth arrested DNA-damage inducible gene 7
GFP green fluorescent protein
H$_2$O$_2$ hydrogen peroxide
KD knockdown
LNA locked nucleic acid
LPS lipopolysaccharide
MCLA 2-methyl-6-(4-methoxyphenyl)-3,7-dihydroimidazo[1,2-A]pyrazin-3-one, hydrochloride
mRNA messenger RNA
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>miRNA</td>
<td>microRNA</td>
</tr>
<tr>
<td>MnTBAP</td>
<td>manganese(III) tetrakis(4-benzoic acid)porphyrin</td>
</tr>
<tr>
<td>NADPH</td>
<td>nicotinamide adenine dinucleotide phosphate</td>
</tr>
<tr>
<td>ncRNA</td>
<td>non-coding RNA</td>
</tr>
<tr>
<td>NUC</td>
<td>nuclear</td>
</tr>
<tr>
<td>O$_2^-$</td>
<td>superoxide</td>
</tr>
<tr>
<td>Palm</td>
<td>palmitate</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>PI</td>
<td>propidium iodide</td>
</tr>
<tr>
<td>PAGE</td>
<td>polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>PKC</td>
<td>protein kinase C</td>
</tr>
<tr>
<td>qPCR</td>
<td>quantitative polymerase chain reaction</td>
</tr>
<tr>
<td>RACE</td>
<td>rapid amplification of cDNA ends</td>
</tr>
<tr>
<td>rpl13a</td>
<td>ribosomal protein L13a</td>
</tr>
<tr>
<td>RNPs</td>
<td>ribonucleoproteins</td>
</tr>
<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
</tr>
<tr>
<td>rRNA</td>
<td>ribosomal RNA</td>
</tr>
<tr>
<td>scaRNA</td>
<td>small Cajal body RNA</td>
</tr>
<tr>
<td>shRNA</td>
<td>short hairpin RNA</td>
</tr>
<tr>
<td>siRNA</td>
<td>small interfering RNA</td>
</tr>
<tr>
<td>SMN</td>
<td>survival of motor neurons</td>
</tr>
<tr>
<td>snRNA</td>
<td>small nuclear RNA</td>
</tr>
<tr>
<td>snRNP</td>
<td>small nuclear ribonucleoprotein</td>
</tr>
<tr>
<td>Term</td>
<td>Definition</td>
</tr>
<tr>
<td>------------</td>
<td>------------------------------------------------</td>
</tr>
<tr>
<td>snoRNA</td>
<td>small nucleolar RNA</td>
</tr>
<tr>
<td>snoRNP</td>
<td>small nucleolar ribonucleoprotein</td>
</tr>
<tr>
<td>snrpD3</td>
<td>small nuclear ribonucleoprotein D3</td>
</tr>
<tr>
<td>SOD</td>
<td>superoxide dismutase</td>
</tr>
<tr>
<td>triol</td>
<td>3β,5α,6β-cholestantriol</td>
</tr>
<tr>
<td>tRNA</td>
<td>transfer RNA</td>
</tr>
<tr>
<td>WT</td>
<td>wild-type</td>
</tr>
</tbody>
</table>
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ABSTRACT OF THE DISSERTATION

Regulation of metabolic stress-induced snoRNAs

by

Benjamin Steel Scruggs

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

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Professor Jean E. Schaffer, Chairperson

Accumulation of excess lipid in non-adipose tissues is associated with oxidative stress and organ dysfunction and plays an important role in diabetic complications. While a number of stress responses have been implicated, the precise molecular mechanisms linking lipid accumulation and cellular dysfunction are not fully understood. To elucidate molecular events critical for lipotoxicity, we used retroviral promoter trap mutagenesis to generate mutant Chinese hamster ovary cell lines resistant to lipotoxic and oxidative stress. This approach uncovered a previously unsuspected role for small nucleolar RNAs (snoRNAs) as critical mediators of lipotoxic cell death.

Herein we show that under lipotoxic conditions, intronic snoRNAs in the rpl13a gene accumulate in the cytosol during metabolic stress, suggesting that these non-coding RNAs function non-canonically to target cytosolic RNAs. Moreover, we demonstrate that the rpl13a snoRNAs play a critical role in vivo in amplification of reactive oxygen species and downstream oxidative stress-mediated tissue injury.

Study of independent mutants from our genetic screen not only identified a role for snoRNAs in lipotoxicity, but also provided new insights into the cellular machinery for
production of these non-coding RNAs. We demonstrate that the spliceosomal protein SmD3 plays a critical role in expression of intronic non-coding RNAs, including the \textit{rpL13a} snoRNAs, by maintaining the abundance of snoRNA-containing intron lariats from which they are processed. Our findings indicate that this function may involve effects of SmD3 on small nuclear RNAs (snRNAs) U4 and U5.

Finally, we demonstrate a link between superoxide induction and cytosolic snoRNA accumulation. Under lipotoxic conditions, superoxide induction precedes cytosolic snoRNA accumulation. Other chemical inducers of superoxide also cause the \textit{rpL13a} snoRNAs to localize to the cytosol, and manipulation of superoxide dismutase demonstrates that superoxide levels directly correlate with cytosolic snoRNA expression.

Together, our studies identify an important role for snoRNAs in metabolic stress responses. The \textit{rpL13a} snoRNAs are essential for cell death in response to lipid overload through functions that are distinct from their canonical role in ribosomal RNA modification in the nucleolus. Moreover, these non-coding RNAs respond to chemical inducers of reactive oxygen species and thereby may contribute more broadly to disease pathogenesis that involves oxidative stress.
CHAPTER 1

Introduction to Lipotoxicity

Type 2 diabetes is a growing worldwide health concern

Metabolic syndrome, obesity, and type 2 diabetes are growing in prevalence at an astounding rate in the United States and around the world. Occurrence of type 2 diabetes is increasing in both adult and juvenile populations. In 2011, the Centers for Disease Control estimated that 26 million Americans have type 2 diabetes and 79 million have some form of metabolic syndrome, and those numbers are expected to double or triple by 2050 (National Diabetes Fact sheet 2011, online). Type 2 diabetics have increased risks for heart disease, stroke, hypertension, nephropathy, neuropathy, and retinopathy. In the United States, diabetes costs $174 billion annually, including $116 billion in direct medical expenses. Therefore, it is becoming increasingly important to understand the underlying causes of complications associated with diabetes.

Lipotoxicity is a hallmark of metabolic disease

Diabetes complications are likely related to the systemic metabolic abnormalities in this disease. While it is well appreciated that hyperglycemia contributes to diabetes pathogenesis, elevations in serum triglycerides and free fatty acids also play an important role in the pathogenesis of diabetic complications. Under physiological conditions, mammalian adipose cells internalize and store large quantities of lipid. However, under pathophysiological conditions, accumulation of fatty acids in non-adipose tissues causes cell dysfunction and cell death that lead to impaired organ
function (112). This phenomenon, known as lipotoxicity, contributes to the pathogenesis of heart failure, renal dysfunction, steatohepatitis, and progressive pancreatic insufficiency (3, 46, 50, 96, 97).

**Mechanisms linking lipid overload to cell death**

In vitro models in which the media of cultured cells is supplemented with excess fatty acid have been used to probe metabolic and signaling pathways involved in the cellular response to lipid overload. In a time- and dose-dependent manner, long-chain saturated fatty acids induce apoptosis in a variety of cell types (11, 18, 61, 66, 124), and this response is enhanced by high glucose (20). Although lipid overload in non-adipose cells is initially buffered by cytoprotective triglyceride stores (60, 63), when the limited capacity for neutral lipid storage in non-adipose cells is exceeded, excess saturated fatty acids initiate several cellular stress response pathways. Fatty acid-induced endoplasmic reticulum stress can result in reactive oxygen species (ROS) generation (99). Independently, oxidative stress is induced in a variety of cell types through activation of NADPH oxidase, mitochondrial dysfunction due to remodeling of organelle membranes, and excessive cycles of oxidative phosphorylation (45, 82, 101). NADPH oxidase participates in palmitate-induced superoxide production (31) and is likely to spark a cascade of ROS production as ROS are rapidly interconverted (34). Dismutation of superoxide produces hydrogen peroxide. Subsequently, hydrogen peroxide will produce highly reactive hydroxyl radicals in the presence of metal ions through Fenton or Harber-Weiss reactions. Hydrogen peroxide can also be converted to hypochlorous acid in the presence of myeloperoxidase. Superoxide can also react with
nitric oxide to form another highly reactive molecule, peroxynitrite. Excessive ROS lead to detrimental consequences including protein carbonylation, lipid peroxidation, and DNA damage (14, 17, 76). Administration of antioxidants to cultured cells and animal models of lipotoxicity mitigates against lipotoxic cell death (8, 9, 56, 61), suggesting a central role for oxidative stress in lipotoxicity.

**Elucidating molecular mechanisms of lipotoxicity**

Despite identification of stress pathways involved in lipotoxicity, the precise molecular responses following lipid overload have yet to be elucidated. To identify genes critical for the cellular lipotoxic response, we performed a genetic screen in Chinese hamster ovary (CHO) cells, with mutagenesis by transduction with ROSAβgeo retrovirus at low multiplicity of infection to achieve, on average, one insertion per ten genomes. Although the integrated provirus contains a cDNA cassette for a β-galactosidase-neomycin phosphotransferase fusion protein, it lacks its own promoter, and thus its transcript is expressed only if the retrovirus inserts downstream of an active promoter and splice donor site. Mutagenized cells that survived a round of neomycin selection were then treated for 48 h in media supplemented with a lipotoxic concentration of palmitate (500 µM) to model pathophysiological states. Under these conditions, wild type (WT) cells were killed, but mutant cells, each with a single disrupted gene critical for lipotoxicity, survived.

This genetic screen led to the identification of many unpredicted genes as essential for the lipotoxic response. The eukaryotic elongation factor (eEF) 1A-1 was shown to have a role in ROS and ER stress induced death (7). The non-coding RNA
gadd7 was shown to be induced by lipotoxic stress in a ROS-dependent fashion and drive ROS propagation following palmitate treatment, demonstrating that gadd7 functions as a feed-forward regulator of lipid-induced and ROS-induced cell death (9). In another mutant cell line (6F2), the promoter trap disrupted the locus for *ribosomal protein L13a* (*rpL13a*) (72). Studies of this mutant revealed that the portions of this gene essential for lipotoxicity are three highly conserved small nucleolar RNAs (snoRNAs) embedded within the *rpL13a* introns, rather than the protein-coding exonic sequences. These findings suggest a previously unsuspected role for snoRNAs in the regulation of metabolic stress in mammalian cells.

**Conserved functions of snoRNAs**

SnoRNAs are typically nucleolar-localized RNAs that guide the modification of other non-coding RNAs (ncRNAs). Eukaryotic cells contain more than 200 unique snoRNAs comprised of two families—the box C/D snoRNAs and box H/ACA snoRNAs (57). The vast majority of snoRNAs transiently base pair with complementary target RNA to guide either 2'-O-methylation (C/D snoRNAs) or pseudouridylation (H/ACA snoRNAs) of target RNA (69). These modifications are the most common covalent modifications found in ribosomal RNA (rRNA) and are found in key regions of rRNA including the peptidyl transferase center and the mRNA-decoding center. Both types of modifications are essential for ribosome function, and the importance of these modifications is emphasized by the evolutionary conservation of modification locations. Other snoRNA modification targets include snRNAs in eukaryotes, transfer RNAs in archaea, and spliced leader RNAs in trypanosomes (16, 19, 111). Spliceosome function
also depends on the modification of snRNAs by C/D and H/ACA snoRNAs. Modification of snRNAs takes place in Cajal bodies providing evidence for extranucleolar roles for snoRNAs (16). The precise function of modified nucleotides in rRNAs and snRNAs remains unknown, but they are hypothesized to have a critical function, since complete loss of modification causes lethality and specific mutations can cause disease (15).

Both snoRNA families are structurally and functionally conserved from humans to Archaea (77, 104, 105). Box C/D snoRNAs contain the conserved sequence motifs UGAUGA (C box) and CUGA (D box) near the 5' and 3' ends of the snoRNA, respectively. The two boxes are separated by ~60nt and internal C' and D' boxes are often present in that region. Base-pairing to target RNAs typically takes place in the region directly upstream of the D box or D' box, involving a sequence known as the antisense element (Figure 1.2) Box C/D snoRNAs are associated with four box C/D snoRNP proteins, fibrillarin, Nop56, Nop58, and 15.5K/NHPX (55, 74, 93, 107, 123, 127). Fibrillarin catalyzes the 2'-O-methyl transfer at the site determined by the box C/D snoRNA guide (118)

Similarly, H/ACA snoRNAs are characterized by a conserved secondary structure. Each H/ACA snoRNA is defined by a hairpin–hinge–hairpin–tail structure with two short conserved sequences called boxes H and ACA. One or both hairpins have an internal loop with two short sequences that are complementary to the rRNA substrate. These pseudouridylation pockets enable anti-sense base-pairing with target RNAs in the region where they direct modifications (Figure 1.2). The box H/ACA snoRNAs form snoRNP complexes with dyskerin, Nhp2, Nop10, and Gar1 proteins (4, 29, 35, 65, 120). The enzyme responsible for uridine-to-pseudouridine isomerization is dyskerin (39).
Palmitate treatment induces *rpL13a* snoRNA accumulation in CHO cells.

Identification of the snoRNA encoding *rpL13a* locus through the genetic screen conducted in the Schaffer Lab suggested snoRNAs have a role in lipotoxicity. All mammalian loci for *rpL13a* contain four highly conserved intronic box C/D snoRNAs that are processed during splicing of the *rpL13a* pre-mRNA transcript (Figure 1.3A) (75). These snoRNAs, U32a, U33, U34, and U35a, are encoded within introns 2, 4, 5 and 6, respectively, and range in size from 61-82 nucleotides. U32a, U33, U34, and U35a are highly conserved across species.

Lipotoxic conditions induce the pre-mRNA expression of many genes including *rpL13a*. Consistent with up-regulation of the pre-mRNA, *rpL13a* snoRNAs also accumulate under stress conditions as assessed by RNase protection assays with ³²P-labeled probes specific for the snoRNA sequences (Figure 1.3B & C). In comparison, levels of the microRNA miR-16 do not change under these conditions. In WT CHO cells, U32a, U33, and U35a snoRNAs are expressed at low levels under basal conditions and increased following palmitate treatment, whereas U34 is not detected under basal or lipotoxic conditions. In mutant 6F2 cells, induction of U32a, U33, and U35a is markedly attenuated, consistent with disruption of the *rpL13a* locus (Figure 1.3B & C). Palmitate induction of U32a, U33, and U35a snoRNAs is also observed in WT C2C12 murine myoblasts, which demonstrate similar sensitivity to lipotoxic conditions (72). Moreover, these snoRNAs are induced by saturated fatty acids known to cause lipotoxicity (myristic, palmitic, and stearic acids), but not by unsaturated palmitoleic acid and oleic acid, which are well tolerated by cells. These findings suggest a conserved role for snoRNAs as mediators of lipotoxicity.
Confirmation of the role for \textit{rpL13a} snoRNAs in lipotoxicity.

Demonstration of \textit{rpL13a} snoRNAs as mediators of metabolic stress was confirmed in two ways (72). First, stable cell lines generated in the mutant 6F2 background showed that snoRNA sequences within the \textit{rpL13a} genomic locus are required for complementation of 6F2 cells. Mutant cells transfected with a plasmid containing 4.3 kb from the murine \textit{rpL13a} genomic locus, including all eight exons and intervening introns, restored palmitate-induced snoRNA expression, palmitate-induced ROS, and palmitate-induced cell death. By contrast, when mutant cells were transfected with a similar construct in which all four snoRNAs were removed, but promoter and exon-intron structure was otherwise intact, complementation was lost. In an alternate approach to genetic confirmation, simultaneous knock-down of U32a, U33, and U35a in wild-type murine myoblasts by nucleofection with phosphorothioate-modified anti-sense oligos (ASOs) rendered these cells resistant to palmitate-induced ROS and cell death. Since loss of a single snoRNA alone did not recapitulate lipotoxicity resistance, these data support a model in which the three snoRNAs function in concert to promote palmitate-induced oxidative stress.

Biogenesis of box C/D and H/ACA snoRNPs

The ability of the \textit{rpL13a} locus to regulate oxidative stress is dependent on expression of the encoded snoRNAs, which are presumed to form small nucleolar ribonucleoproteins (snoRNPs). Biogenesis of box C/D and H/ACA snoRNPs is a highly regulated, multi-step process initiated during the transcription of a snoRNA host gene.
In vertebrates, the vast majority of box C/D and H/ACA snoRNAs are encoded within introns of pre-mRNAs (25, 52, 58, 108, 109). All host introns for vertebrate snoRNAs encode only a single intronic snoRNA. During splicing, snoRNAs are excised from the pre-mRNA as part of the intron lariat. Mammalian pre-mRNA intron lariats are rapidly debranched and degraded after splicing (83). 5’ to 3’ and 3’ to 5’ exonucleases recycle the linearized intron lariat (79). Active recruitment of snoRNP proteins to the nascent snoRNA during synthesis and/or splicing of the host pre-mRNA is required for efficient intronic snoRNP production. The associated snoRNP proteins define the termini of the snoRNA by protecting them from the processing exonucleases (13, 51, 108, 122). Following trimming of the flanking intron, the mature snoRNP is released.

Biosynthesis of functional box C/D snoRNPs requires the ordered recruitment of snoRNP proteins 15.5K/NHPX, Nop56, Nop58, and fibrillarin. To establish a scaffold for the 15.5K/NHPX snoRNP protein, a functional Kink-turn structure must be formed by the nascent snoRNA. The 5’ and 3’ box C and D terminal regions of the snoRNA form this Kink-turn by folding into a stem-internal loop-stem structure (54, 123). Conserved nucleotides in the C and D box motifs form non-canonical G-A, A-G, and U-U base pairs establishing the Kink-turn and docking site for the 15.5K/NHPX protein. Binding of 15.5K/NHPX induces a sharp bend in the phosphodiester backbone of the two contiguous RNA stems of the Kink-turn (102, 103, 115, 119). This conformational change provides the structural requirements for the subsequent binding of Nop58, Nop56, and two copies of fibrillarin (12). One fibrillarin and Nop58 bind to the box D and C sequences in the upper stem of the Kink-turn. Nop56 and another copy of fibrillarin bind to the internal C’ and D’ boxes.
Position within the intron is also critical for efficient processing of human box C/D snoRNAs. Most box C/D snoRNAs are located about 80-90 nucleotides upstream of the 3’ splice site (38). An optimal distance of ~50 nucleotides between the snoRNA coding region and the branch point of the host intron is required for efficient snoRNA processing. Increasing or decreasing the length between the snoRNA and the branch point significantly compromises snoRNA accumulation (37). The relationship between snoRNA location and the branch site suggests there is a synergy between intronic snoRNA processing and pre-mRNA splicing. Consistent with a splicing-depending mechanism for box C/D snoRNA processing, 15.5K/NHPX is recruited to box C/D intronic snoRNAs at the C1 splicing complex stage. A general splicing factor, intron binding protein 160 (IBP160), provides a molecular link between the spliceosome and the nascent box C/D snoRNA (36). IBP160 has putative helicase activity and is thought to trigger box C/D snoRNP assembly by interacting with the U2 spliceosomal small nuclear RNP (snRNP) or other splicing factors associated with the branch-point region in the C1 splicing complex.

Box H/ACA snoRNPs also require ordered recruitment of snoRNP proteins. However, in contrast to box C/D snoRNAs, human H/ACA snoRNAs have no preferential intronic location relative to the 5’ or 3’ splice sites of the host introns (88, 92). Human box H/ACA snoRNAs are commonly found in introns of longer than average length. H/ACA snoRNAs are also processed efficiently from artificial host pre-mRNAs regardless of position within the intron suggesting that box H/ACA snoRNAs are processed independent of relative splice site location (88).
Many class specific factors regulate the biogenesis of box C/D snoRNAs and box H/ACA snoRNAs, but the excision of all intronic snoRNPs requires splicing. The relationship between pre-mRNA splicing and snoRNA biogenesis suggests the spliceosomal machinery is critical for snoRNA expression. The spliceosome is a complex RNA machine comprised of its own set of ncRNAs, the small nuclear RNAs (snRNAs), and over 100 proteins (117). Each snRNA is bound to a unique set of proteins to form a small nuclear ribonucleoprotein (snRNP). In addition to snRNP-specific proteins, each of the major snRNAs (U1, U2, U4, and U5) is bound to a common set of seven proteins (SmB/B’, SmD1, SmD2, SmD3, SmE, SmF, and SmG). These proteins form a seven-membered ring around the short, highly conserved, uridine-rich sequence on each snRNA called the Sm site (1, 47, 100). Assembly of Sm cores onto snRNAs is regulated by the SMN complex (22, 71, 84). Following assembly of the Sm protein heptameric ring, the 5’ cap of the snRNA is hypermethylated to form the 2,2,7-trimethyl guanosine cap, and the mature snRNP is then imported into the nucleus by snurportin and importin β for final association with snRNP-specific proteins and utilization in mRNA splicing (23, 24, 42, 70, 86).

**Summary**

Herein, we describe work that provides new understanding of the function of the *rpL13a* snoRNAs in cells and in a rodent model of acute oxidative stress, identifies a role for the spliceosomal protein, SmD3, in regulating the biogenesis of intronic ncRNAs including the *rpL13a* snoRNAs, and uncovers a link between superoxide production and
induction of the \textit{rpL13a} snoRNAs. Together, our studies provide important new insights into the production and function of snoRNAs during metabolic stress.
Figure 1.1

**Figure 1.1 Genetic screen to isolate palmitate resistant CHO mutants.** Wild type (WT) CHO cells were transduced with the ROSAβgeo retrovirus, leading to integration of the provirus containing a splice acceptor, promoterless β-galactosidase-neomycin resistance cassette, and polyadenylation sequences. Promoter-trapping and gene disruption at the site of integration was selected for by growth in neomycin (NEO), and palmitate-resistant mutants were selected by growth in media with 500 µM palmitate (palm) for 48 h.
Figure 1.2 Secondary structure of box C/D and box H/ACA snoRNAs. A) Box C/D snoRNAs (green) are characterized by box C and D motifs. Sequences upstream of box D and D’ base pair (blue) with target RNAs (red) to guide the 2’-O-methylation of target RNA. B) Box H/ACA snoRNAs are characterized a hairpin–hinge–hairpin–tail structure and box H and ACA motifs. Pseudouridylation pockets allow for base pairing with target RNAs to guide pseudouridylation modifications.
Figure 1.3

(A) rpL13a gene organization showing location of ROSAβgeo promoter trap insertion and locations of intronic U32a, U33, U34, and U35a snoRNAs. (B, C) WT and 6F2 cells were untreated (UT) or supplemented with palmitate for 48 h. (B) Small RNA was harvested and used in RNase protection assay with $^{32}$P-labeled hamster rpL13a snoRNA probes or miR-16 probe as control. (C) Autoradiograms from RNase protection experiments as in (B) were quantified by densitometry. All data expressed as mean ± SE for 3 independent experiments. * p < 0.01 for palmitate treated vs. untreated.

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Figure 1.3 rpL13a-encoded box C/D snoRNAs are induced in palmitate treated CHO cells. (A) rpL13a gene organization showing location of ROSAβgeo promoter trap insertion and locations of intronic U32a, U33, U34, and U35a snoRNAs. (B, C) WT and 6F2 cells were untreated (UT) or supplemented with palmitate for 48 h. (B) Small RNA was harvested and used in RNase protection assay with $^{32}$P-labeled hamster rpL13a snoRNA probes or miR-16 probe as control. (C) Autoradiograms from RNase protection experiments as in (B) were quantified by densitometry. All data expressed as mean ± SE for 3 independent experiments. * p < 0.01 for palmitate treated vs. untreated.

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CHAPTER TWO

*rpl13a* snoRNAs function as non-canonical box C/D snoRNAs

INTRODUCTION

Box C/D snoRNAs U32a, U33, and U35a are critical for the propagation of ROS following lipid overload in cultured cells (72). The mechanisms through which *rpl13a* snoRNAs recognize metabolic stress and regulate the cellular stress response remain unclear. Typically, box C/D snoRNAs serve as guides for ribonucleoprotein particles during 2'-O-methylation of rRNA. These modifications occur within the nucleolus, where the vast majority of snoRNAs reside. Extranucleolar functions for snoRNAs have also been described. SnoRNAs are known to traffic to Cajal bodies where they can serve to guide the modification of snRNAs (16). Recent studies have suggested a number of additional non-canonical roles for snoRNAs. A brain specific snoRNA, HBII-52, has been shown to interact with a pre-mRNA in the nucleoplasm and play a role in alternative splicing (49). Biochemical and computational analyses from multiple groups show snoRNAs can be processed into miRNAs in a Dicer dependent fashion, suggesting snoRNAs may localize to the cytoplasm where processing and function would take place (21, 78, 90). Additional snoRNAs have been identified or computationally predicted to lack rRNA or snRNA antisense homology (43). These “orphan” snoRNAs could serve as guides for currently unidentified target RNAs in unknown locations.
Here, we extend the initial finding that \textit{rpL13a} snoRNAs are critical mediators of metabolic stress by examining their role \textit{in vivo}. We demonstrate that U32a, U33, and U35a contribute to oxidative stress and oxidative damage in a mouse model of inflammation and oxidative stress. Furthermore, we broaden our understanding of snoRNA function in the metabolic stress pathway in several ways. By examining predicted \textit{rpL13a} modification sites in 6F2 cells, we show that methylation of these rRNA nucleotides is not affected by altered levels of the snoRNAs during lipotoxicity. By characterizing the localization of \textit{rpL13a} snoRNAs in wild type cells, we show that they accumulate in the cytoplasm during lipotoxicity. This novel localization for intron-encoded snoRNAs provides insight into the potential mechanism through which \textit{rpL13a} snoRNAs regulate oxidative stress.

\textbf{RESULTS}

\textit{In vivo expression of \textit{rpL13a} snoRNAs.} Our studies of the \textit{rpL13a} snoRNAs show that these molecules function in the cellular response to lipotoxic and oxidative stress. To extend these findings to an \textit{in vivo} model of oxidative stress, we examined the expression of \textit{rpL13a} snoRNAs in a well-established model of lipopolysaccharide (LPS)-mediated liver injury that is characterized by inflammation, steatosis, and oxidative stress (5). Compared to saline-injected control mice, liver tissue from LPS-treated mice showed significant up-regulation of cytosolic U32a, U33, and U35a snoRNAs (Figure 2.1), demonstrating that the \textit{rpL13a} snoRNAs are induced \textit{in vivo} in response to metabolic stress.
**In vivo rpL13a snoRNA knockdown reduces oxidative damage.** Based on these findings, we analyzed the effects of loss-of-function of the *rpL13a* snoRNAs in the LPS-mediated liver injury model. To achieve specific knockdown of the snoRNAs *in vivo*, prior to LPS injection, mice were treated with three serial intraperitoneal injections of antisense locked nucleic acid oligonucleotides directed against each of the three snoRNAs or directed against GFP as a control. Antisense oligonucleotides directed against the snoRNAs achieved 72, 84, and 74% knockdown of U32a, U33, and U35a, respectively, in liver tissue following LPS injection without diminishing LPS-induced inflammation (Figure 2.2). Knockdown of the snoRNAs mitigated LPS-induced oxidative stress in the liver as demonstrated by dihydroethidium staining for superoxide and oxidative damage to liver tissue proteins and lipids (Figures 2.3 and 2.4). These findings indicate that *rpL13a* snoRNAs are required *in vivo* for propagation of oxidative stress.

**rpL13a guided rRNA modifications are unaltered during lipotoxicity.** Canonical box C/D snoRNAs participate in ribonucleoproteins that localize to nucleoli. In *S. cerevisiae* and in *X. laevis*, box C/D snoRNAs serve as guides that target 2′-O-methylation of rRNAs with which they share short stretches of antisense homology (53). Although they lack some sequence features of canonical 2′-O-methylation guide snoRNAs (internal box C’ sequence not well-conserved, U33 lacks box D’, rRNA complementarity not upstream of box D in U35a), U32a, U33, and U35a each contain 10-12 nucleotide stretches of complementarity to rRNA sites of 2′-O-methylation (Figure 2.5), suggesting a potential role as guide RNAs for 2′-O-methylation of G1328 in 18S and A1511 in 28S (U32a), U1326 in 18S (U33), and C4506 in 28S (U35a) rRNAs (75). We reasoned that if the mechanism of action of snoRNAs U32a, U33, and U35a in lipotoxic and oxidative
stress involved 2’-O-methylation of these rRNAs, modifications of these rRNA sites should be diminished in 6F2 compared to WT cells under metabolic stress conditions when the snoRNAs are induced in WT cells. However, primer extension studies showed no differences in the extent of modification of these rRNA sites between WT and 6F2 cells under basal or palmitate-treated conditions (Figure 2.6). These data indicate that under basal and lipotoxic conditions, either residual expression of U32a, U33, and U35a in 6F2 cells is sufficient to support these modifications of rRNAs, or this function is subserved by other molecules in eukaryotic cells. Furthermore, at a point in the lipotoxic response at which absence of snoRNA induction is readily apparent and functionally correlates with resistance to lipotoxicity in 6F2 cells, there is no corresponding change in 2’-O-methylation of rRNAs.

rpL13a snoRNAs accumulate in the cytoplasm during lipotoxicity. We hypothesized that if U32a, U33, and U35a were involved in functions other than modification of ribosomal RNAs, then under lipotoxic stress conditions, they may have a subcellular distribution distinct from canonical box C/D snoRNAs, which co-localize with nascent rRNAs in the nucleolus. Following palmitate treatment of C2C12 cells, we isolated nuclear and cytosolic RNAs by sequential detergent extraction and quantified U32a, U33, and U35a by qRT-PCR. With palmitate treatment U32a, U33, and U35a increase in the cytoplasm, whereas levels of these snoRNAs remain unchanged in the nucleus (Figure 2.7A and B). Accumulation of rpL13a snoRNAs in the cytosol under lipotoxic conditions was confirmed by fluorescence in situ hybridization. As expected, anti-sense probe for snoRNA U3 demonstrated strong nucleolar localization, and this
was unaffected by lipotoxic stress (Figure 2.8A). Staining for the *rpL13a* snoRNAs was performed in cells nucleofected with control ASO (GFP) or with ASO targeting each of the *rpL13a* snoRNAs to ascertain the specificity of signal. Consistent with data from RNase protection and qPCR assays, in control nucleofected cells expression of U32a, U33, and U35a was low under normal growth conditions and increased under lipotoxic conditions (Figure 2.8B, GFP-nucleofected panels). Prominent staining for each of these snoRNAs was observed in the cytoplasm, but not nucleoli. The probe for U32a also stains non-nucleolar regions of the nucleus. Cytoplasmic staining for the *rpL13a* snoRNAs under lipotoxic conditions was markedly diminished when the snoRNAs were depleted by specific ASOs that target each snoRNA (Figure 2.8B, U32a, U33, and U35a ASO-nucleofected panels). Cytoplasmic staining for U32a, U33, and U35a was also distinct from the nuclear pattern observed under lipotoxic conditions using a probe specific for intron 1 (Figure 2.8A), indicating that the cytosolic distributions of the *rpL13a* snoRNAs do not simply reflect localization of the pre-mRNA. The nuclear staining for U32a resembled the staining for intron 1 and was not diminished with ASOs that target U32a, suggesting that this represents detection of the pre-mRNA, which is not targeted by U32a ASOs. Together our biochemical and *in situ* hybridization data support a model in which U32a, U33, and U35a snoRNAs act in non-canonical roles in the cytoplasm during lipotoxic stress.

**CONCLUSIONS**

SnoRNAs U32a, U33, and U35a are critical for the cellular response to metabolic stress. *In vivo* knockdown of these three snoRNAs further illustrates their importance to the pathophysiology of metabolic stress. The LPS-induced model of hepatic tissue injury
confirms that the \textit{rpL13a} snoRNAs are induced in the setting of oxidative stress \textit{in vivo}. Moreover, our \textit{in vivo} knockdown data show that these snoRNAs are required for amplification of oxidative stress and for propagation of oxidative stress-mediated damage to proteins and lipids. Because LPS has pleiotropic effects \textit{in vivo} and because ASO knockdown \textit{in vivo} is limited by liver toxicity, it is not surprising that \textit{in vivo} knockdown of these snoRNAs provided only a partial reduction in the oxidative stress response and did not blunt the release of serum transaminases into the circulation (not shown). In future studies, genetic approaches to loss of function and/or blunting of additional effector pathways (e.g., inflammatory signaling) may be required to block liver injury entirely. Nonetheless, our \textit{in vivo} knockdown studies show that the \textit{rpL13a} snoRNAs are required for the full induction of tissue oxidative stress in the murine LPS model, thus providing \textit{in vivo} evidence for a role of snoRNAs in metabolic stress.

We show here in a stable mutant cell line that decreased basal expression of U32a, U33, and U35a snoRNAs and loss of palmitate induction of these snoRNAs is not associated with changes in 2'-O-methylation of predicted rRNA targets, yet is associated with resistance to lipotoxicity. While it is possible that these snoRNAs contain more than one functional guide sequence to target multiple substrates including rRNAs (13, 110), their accumulation in the cytosol during lipotoxicity suggests a non-nucleolar function for these snoRNAs. Movement of snoRNAs to the cytosol is not without precedent, since the independently transcribed box C/D snoRNA, U8, has been shown to be exported from the nucleus (121). Here, we provide the first direct evidence that intronic snoRNAs can also localize to the cytosol. Our data are most consistent with a model in which the \textit{rpL13a} snoRNAs function in lipotoxicity and oxidative stress.
response pathways as non-canonical box C/D snoRNAs. Furthermore our findings suggest that the rpl13a snoRNAs affect targets in the cytoplasm.
Figure 2.1

**Figure 2.1 In vivo expression of rpL13a snoRNAs.** (A) Mice were injected intraperitoneally with lipopolysaccharide (LPS), or equivalent volume of phosphate-buffered saline (PBS) as control, and liver tissue was harvested 12 h later. Cytosolic RNA isolated from livers was used for quantification of rpL13a snoRNAs (relative to 36B4) or iNOS and Cox2 (relative to actin). Graph shows mean ± SE from a representative experiment with N = 3 (PBS) and N = 4 (LPS) animals per group. * p < 0.05 for LPS vs. PBS.
Figure 2.2 ASO knockdown of *rpL13a* snoRNAs *in vivo*. Mice were pretreated with three serial doses of ASOs targeting *rpL13a* snoRNAs or GFP as control prior to LPS injection and analysis of liver tissue. snoRNA, iNOS, and Cox2 expression in liver cytosol was quantified as in Figure 2.3. *n* = 4 to 6 per group.

* *p* < 0.05 for GFP vs. snoRNA knockdown.
Figure 2.3 *rpL13a* snoRNAs are required for oxidative stress *in vivo*. Mice were pretreated with three serial doses of ASOs targeting *rpL13a* snoRNAs or GFP as control prior to LPS injection and analysis of liver tissue. Representative images show frozen sections of liver tissue stained with DHE and parallel sections in which staining was performed in the presence of pegylated superoxide dismutase (SOD) as control. Scale bar, 100 µm. Graph shows quantification of fluorescence intensity. n = 4 to 6 per group. * p < 0.05 for GFP vs. snoRNA knockdown.
Figure 2.4 In vivo *rpL13a* snoRNA knockdown reduces oxidative damage. Mice were pretreated with three serial doses of ASOs targeting *rpL13a* snoRNAs or GFP as control prior to LPS injection and analysis of liver tissue. Quantification of (A) protein carbonylation by western blotting and (B) tissue oxysterols (7-ketocholesterol, 7-keto; 3β,5α,6β-cholestantriol, triol) 24 h following LPS. n = 4 to 6 per group. * p < 0.05 for GFP vs. snoRNA knockdown.
Figure 2.5 U32a, U33, and U35a contain antisense homology to rRNA. Comparison of *rpL13a* snoRNA sequences from mouse (*Mm*), hamster (*Cg*), and human (*Hs*) species. C, D, and D' box sequences are indicated in boxed bolded text. Regions with antisense homology are underlined. Conserved and non-conserved nucleotides are displayed in lower and upper case letters, respectively.

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Figure 2.6 2’-O-methylation is unaffected in 6F2 cells. WT and 6F2 cells were treated with 500 µM palmitate and total RNA analyzed for pseudouridylation or 2’-O-methylation nucleotide modification of predicted sites using reverse transcriptase primer extension. Autoradiograms show primer extension assays and parallel sequencing for detection of (A) U32a and U33 target sites on 18S rRNA (G1328 and U1326, respectively); (B) U32a target site on 28S rRNA (A1511); (C) U35a target site on 28S rRNA (C5406); (D) unrelated snoRNA target sites on 18S rRNA as controls (snoRNAs Z17a & Z17b target U121; snoRNAs U45a & U45c target A159). For each panel, arrows point to bases in rRNA (numbered according to human rRNA sequence) that are modified and corresponding DNA sequence is shown in the left-most four lanes.
Figure 2.7

(A) C2C12 cells were untreated (UT) or treated with palmitate for 24 h. Cells were separated into cytosolic (CYT) and nuclear (NUC) fractions by sequential detergent solubilization. (A) Fractions were analyzed by western blotting for a cytosolic marker, hsp90, and for a nuclear marker, lamin B1. (B) Total RNA was prepared from the fractions and analyzed for rpL13a snoRNA abundance relative to 36B4 by qRT-PCR. Graphs show mean ± SE from a representative experiment (n = 3). * p < 0.05 for palmitate treated vs. UT.

Figure 2.7 rpL13a snoRNAs accumulate in the cytoplasm under lipotoxic conditions. (A, B) C2C12 cells were untreated (UT) or treated with palmitate for 24 h. Cells were separated into cytosolic (CYT) and nuclear (NUC) fractions by sequential detergent solubilization. (A) Fractions were analyzed by western blotting for a cytosolic marker, hsp90, and for a nuclear marker, lamin B1. (B) Total RNA was prepared from the fractions and analyzed for rpL13a snoRNA abundance relative to 36B4 by qRT-PCR. Graphs show mean ± SE from a representative experiment (n = 3). * p < 0.05 for palmitate treated vs. UT.
Figure 2.8

(A) C2C12 cells were analyzed by in situ hybridization under basal conditions (UT) and following 24 h treatment with palmitate (PALM) using specific snoRNA or control probes (red). Nuclei were stained with SYTOX Green. (A) Cells were probed with U3 antisense probe for known nucleolar snoRNA, control U3 sense probe, and control rpl13a intron 1 antisense probe. (B) Control GFP ASO-nucleofected and specific snoRNA ASO-nucleofected cells were examined by in situ hybridization with antisense probes for U32a, U33, and U35a. Bars, 10 µm.

Figure 2.8 rpL13a snoRNAs accumulate in the cytoplasm under lipotoxic conditions.
CHAPTER THREE
SmD3 regulates intronic snoRNA biogenesis

INTRODUCTION

Most vertebrate snoRNAs are encoded within introns and co-transcribed with their host genes (109). Intrinsic box C/D snoRNP protein assembly initiates during the C1 complex stage of splicing (37), with subsequent intron lariat formation at the C2 complex stage of splicing followed by debranching and exonucleolytic trimming of the mature snoRNP (51, 80, 85). The observations that rpL13a snoRNAs rapidly accumulate in the cytosol during metabolic stress and are required for lipotoxic cell death suggest that cytoplasmic RNAs may be their primary targets and that efficient processing of these intronic elements is important for the lipotoxic response. However, the precise molecular mechanisms through which snoRNAs are induced and regulated during lipotoxicity remain to be elucidated.

Our genetic screen led to isolation of a second, independent mutant cell line harboring a disruption in an RNA-related gene. This novel mutant cell line is haploinsufficient for SmD3, a core component of the spliceosome. We demonstrate that SmD3 participates in the lipotoxic response through regulation of intron lariat abundance and biogenesis of intron-encoded rpL13a snoRNAs. We also provide evidence linking the expression of SmD3 to the levels of critical snRNA components of the spliceosome and generalized production of intronic non-coding RNAs (ncRNAs). Our results extend the known function for SmD3 in splicing to a specific role within individual snRNPs essential for the biogenesis of intronic ncRNAs.
RESULTS

SmD3 haploinsufficiency confers resistance to palmitate-induced cell death. The 6H2 mutant cell line was isolated from our genetic screen for genes that are critical for the lipotoxic response. To quantify the degree of palmitate resistance in mutant 6H2, WT and 6H2 cells were treated with palmitate for 48 h, and cell death was quantified by propidium iodide (PI) staining and flow cytometric analysis. Compared to WT cells, 6H2 cells were significantly protected from palmitate-induced death (Figure 3.1A). By contrast, treatment of WT and 6H2 cells with the general apoptosis inducers camptothecin, staurosporine, and actinomycin D revealed no differences in sensitivity. Therefore, palmitate-resistant 6H2 cells are not generally resistant to cell death.

The promoter trap mutagenesis facilitated identification of the disrupted gene because of the unique fusion transcript produced upon a single productive integration. To confirm the presence of a single retroviral integration, Southern blot analysis of 6H2 genomic DNA was performed, probing for the ROSAβgeo sequence (Figure 3.2A). The presence of a single hybridizing band in DNA digested with multiple different restriction enzymes is consistent with a single retroviral integration. To identify the disrupted gene in the mutant 6H2 cells, mRNA was isolated and used for 5' rapid amplification of cDNA ends (5' RACE). Unique sequence from the RACE product was analyzed using NCBI BLAST, which revealed that the site of integration was the snrpD3 gene encoding the small nuclear ribonucleoprotein SmD3. SmD3 is a component of the spliceosome that, together with other Sm family proteins, forms a heteroheptameric ring around snRNAs U1, U2, U4, and U5 to generate small nuclear ribonucleoproteins (snRNPs) essential for
the removal of introns from pre-mRNA (125). PCR was performed to confirm integration of the ROSAβgeo sequence into snrpD3 (Figure 3.2B). Reactions with forward and reverse primers designed to snrpD3 resulted in PCR products for both WT and 6H2 cDNA, indicating that each cell type maintains at least one intact allele. As expected, no product was detectable in WT cells when snrpD3 forward and ROSAβgeo reverse primers were used, but this set of primers produced the expected PCR product from the fusion transcript in 6H2 cells. These PCR results confirm our 5′ RACE identification of the disrupted gene and suggest that 6H2 cells are haploinsufficient for snrpD3. Consistent with this model, quantitative real-time PCR (qRT-PCR) revealed a ~50% reduction in relative snrpD3 mRNA (Figure 3.2C) and western blotting revealed a corresponding ~50% reduction of SmD3 protein (doublet at 15 kDa and 18 kDa) in 6H2 relative to WT cells (Figure 3.2D). Thus, expression of snrpD3 in 6H2 cells is consistent with a model in which integration of the ROSAβgeo provirus disrupted one of two alleles for snrpD3.

**Targeted knockdown of SmD3 recapitulates 6H2 phenotype.** To confirm that the palmitate-resistant phenotype in 6H2 cells is due to diminished SmD3 protein expression, we used shRNA to knockdown SmD3 in WT CHO cells and tested for associated changes in palmitate sensitivity. SmD3 protein levels were measured by western blot following isolation of individual stable clonal knockdown lines. Two independently isolated clonal lines showed 47% (sh1) and 64% (sh2) knockdown relative to scrambled (contr) shRNA transfected cells (Figure 3.3A and 3.3B). Knockdown clones were protected from palmitate-induced death as measured by PI
staining, and the degree of protection was proportional to the degree of knockdown (Figure 3.3C). These data provide independent genetic evidence that loss-of-function of SmD3 protects against lipotoxicity.

SmD3 disruption protects cells from generalized oxidative stress induction. Lipotoxicity is known to involve fatty acid (FA) import and the generation of oxidative stress (8, 60). To test whether 6H2 cells acquired resistance through diminished capacity to take up palmitate, initial rates of FA uptake were quantified in WT and 6H2 cells. There was no significant difference between WT and 6H2 cells (Figure 3.4A), indicating that resistance to lipotoxicity in 6H2 cells did not result from failure to take up exogenous FA. To probe downstream aspects of the lipotoxic response, we quantified palmitate-induced ROS in WT and 6H2 cells by CM-H2DCFDA (DCF) staining and flow cytometric analysis. At 5 and 16 h following palmitate supplementation, ROS induction was significantly blunted in 6H2 cells (Figure 3.4B). SmD3 knockdown clones were also protected from palmitate-induced ROS (Figure 3.4C). More direct induction of oxidative stress following exposure to H2O2 or menadione also resulted in blunted ROS levels in 6H2 cells compared to WT (Figure 3.4D), indicating 6H2 cells are protected not only from palmitate-induced ROS but also from generalized oxidative stress induction or amplification.

SmD3 regulates intronic non-coding RNA expression. The ROS resistance phenotype observed in 6H2 cells is similar to the previously described 6F2 mutant. Given the related phenotypes of these two mutants, and the well-appreciated
interactions of SmD3 with RNA, we assayed for the expression of the *rpL13a* snoRNAs in 6H2 cells by RNase protection assay. Following palmitate treatment, WT cells show the expected increase in snoRNA expression by RNase protection (Figure 3.5A). Under the same conditions, snoRNA induction is blunted in 6H2 cells. Similarly, SmD3 knockdown clones were impaired in *rpL13a* snoRNA induction relative to control (Figure 3.5B). While snoRNAs are thought to be produced in the nucleus and canonical box C/D snoRNAs function in that location, our data demonstrates that *rpL13a* snoRNAs accumulate in the cytosol during metabolic stress (Chapter 2). Fractionation of WT and 6H2 cells by sequential detergent solubilization revealed that under basal conditions snoRNAs are detectable in the nucleus and the cytosol but are substantially more abundant in the nucleus (Figures 3.5C and 3.5D). qRT-PCR analysis of these fractions reveals reduced *rpL13a* snoRNAs in the cytosol in 6H2 cells under palmitate-treated conditions, and reduced levels of these snoRNAs in the nucleus under both basal and palmitate-treated conditions (Figures 3.5E and 3.5F). The observation that haploinsufficiency of SmD3 caused impairment of basal expression and lipotoxic cytosolic accumulation of the intronic *rpL13a* snoRNAs, a deficit known to cause resistance to lipotoxicity, is consistent with the ROS resistant phenotype observed in 6H2 cells. Furthermore, the observation that nuclear levels of the *rpL13a* snoRNAs are decreased in 6H2 cells under basal as well as lipotoxic conditions implicates a defect in the nuclear production of the snoRNAs.

To test whether 6H2 cells have a general defect in expression of intronic snoRNAs, we measured basal nuclear expression of intronic box C/D snoRNAs U50, U57, U60, and U21 and intronic box H/ACA snoRNAs U17b, U64, and ACA28 (Figure
Expression of each of these snoRNAs was reduced in the nuclei of 6H2 versus WT cells. Furthermore, we measured the expression of intronic splicing-dependent/Drosha-independent pre-miRNAs to probe an unrelated class of intronic ncRNAs (6, 89). Mirtrons miR-1224 and miR-1225 showed reduced expression in 6H2 cells. In contrast, independently transcribed, splicing-independent, non-intronic snoRNAs U8 and U13 and pre-miR-23a showed no difference in expression between WT and 6H2. Taken together, these data suggest that WT levels of SmD3 are generally required for effective expression of splicing-dependent intronic ncRNAs.

**SmD3 knockdown perturbs snRNP biogenesis.** Disruption of multiple components of the snRNP assembly pathway has previously been shown to alter snRNP expression (98, 130). To test whether reduced levels of SmD3 affect snRNA expression, we used a chimeric locked nucleic acid/DNA (LNA/DNA) oligonucleotide to specifically knockdown SmD3 in murine fibroblasts. Following knockdown of SmD3 to ~50% of control SmD3 levels, we observed reductions in rpL13a snoRNAs similar to our mutant, (Figures 3.6A and 3.6B). In SmD3 knockdown cells, qRT-PCR revealed that U4 and U5 snRNA expression was decreased, but levels of other snRNAs were indistinguishable from control (Figure 3.6C). Since unbound snRNAs are unstable compared to snRNAs in snRNP complexes, quantification of snRNA levels provides insight into snRNP integrity (91, 131). Consistent with this, U4 and U5 snRNAs were reduced following immunoprecipitation with the α-Sm protein Y12 antibody, indicating that U4 and U5 snRNPs are also diminished (Figure 3.6D). By contrast, 50% knockdown of SmB, another Sm protein family member, produced more modest decreases in snoRNA
production (Figure 3.6F). SmB knockdown cells remained sensitive to palmitate-induced death (Figure 3.6G) and displayed a different pattern of alteration of snRNA expression, characterized by decreases in U2 and U4 and increase in U4atac (Figure 3.6H). Our data suggest that a reduction in SmD3 expression disrupts a specific set of snRNPs. While there is some overlap with the effects of SmB knockdown on snRNA levels, the pattern with SmD3 is distinct.

6H2 cells display normal splicing efficiency. Given that SmD3 levels affect snRNP expression, we hypothesized that differences in intronic ncRNA expression caused by SmD3 haploinsufficiency in the 6H2 cells could be explained by a defect in splicing. To assess splicing related to the rpL13a snoRNAs, we measured the expression of endogenous rpL13a splicing precursors and spliced products. There was no difference in rpL13a pre-mRNA levels between WT and 6H2 cells under basal or palmitate-treated conditions, as assessed by amplifying random hexamer-primed cDNA with primer pairs designed across the junction between exon 3 and intron 3 (Ex3/Int3, Figures 3.7A and 3.7B). Quantification of the endogenous rpL13a mRNA, using oligo-dT-primed cDNA and primers designed across the splice junction between exons 7 and 8, showed similar expression between WT and 6H2 cells (Ex7/8, Figures 3.7A and 3.7B). Furthermore, priming across four splice junctions formed by the removal of snoRNA-encoding introns (Ex 2/3, 4/5, 5/6, and 6/7) displayed similar expression between WT and 6H2 cells (Figures 3.7A and 3.7B).

To assess splicing efficiency more broadly, we transfected WT and 6H2 cells with a previously validated reporter construct for expression of firefly luciferase from two
exons separated by a β-globin intron (128). Functional splicing results in the formation of a luciferase mRNA encoding a protein with measurable luminescence and a processed β-globin intron lariat. In the absence of splicing, a truncated luciferase protein without enzymatic activity is formed due to multiple in-frame stop codons. Following transfection of this construct, no difference in luciferase production was detected between WT and 6H2 under untreated or palmitate treated conditions or in the presence of clotrimazole, a known splicing inhibitor (128) (Figure 3.7C). To test whether the presence of an intronic snoRNA affected splicing of flanking exons in 6H2 cells, we replaced the intron in the split luciferase vector with the murine rpL13a intron 2 containing U32a. Following transfection with this construct, there was no difference in luciferase levels between WT and 6H2 cells, although expression of the exogenous murine U32a was reduced in 6H2 cells (Figures 3.7D and 3.7E). Similarly, we detected no difference in luminescence using a Δsno split luciferase splicing reporter containing mutated rpL13a intron 2 sequences lacking the entire 83 nucleotide U32a, and pre-mRNA from the reporters with the intact U32a intron and the Δsno intron were comparable (not shown). Furthermore, we quantified mRNA expression of the host genes containing the intronic non-coding elements quantified in Figure 3.5G. We detected no differences in mRNA levels from any of these host genes between WT and 6H2 cells (Figure 3.7F). Together, these data indicate that differences in intronic non-coding RNA levels are not attributable to defective splicing of exons in 6H2 cells.

**SmD3 knockdown does not alter alternative splicing.** Previously, it was reported that 85% or more knockdown of the survival of motor neuron (SMN) protein disrupts the
snRNP assembly pathway and leads to widespread differences in snRNA levels and alternative splicing in a number of tissues (130). To test whether reduced levels of SmD3 affected snoRNA processing through broad alterations in alternative splicing, exon utilization in control (GFP) and SmD3 knockdown cells was analyzed using the Affymetrix GeneChip mouse exon 1.0 ST microarray. Due to the lack of publically available exon microarrays containing hamster sequences, we used our LNA/DNA oligonucleotides to generate murine fibroblasts with haploinsufficiency of SmD3 to phenocopy the 6H2 cells that have 50% wild type CHO levels for this protein (Figures 3.6A and 3.6B). Following knockdown, RNA was harvested from three independent samples of GFP and SmD3 knockdown cells. Among the 266,200 probesets supported by putative full-length mRNA, ~190,000 probesets with significant signals above background and representing exons of ~16,000 out of ~30,000 genes in the mouse genome were included in the analysis. With a false discovery rate set at less than 0.1, no genes were identified as having potential splicing pattern changes (fold change ≥ 1.5). Plotting probeset intensity values from control (GFP LNA) samples against SmD3 knockdown samples revealed a highly linear relationship ($R^2 = 0.98475$), consistent with little variance between the two groups (Figure 3.8). These data show that a 50% reduction in SmD3 expression does not have global effects on alternative splicing.

**SmD3 controls intron lariat abundance.** Box C/D snoRNAs, box H/ACA snoRNAs, and mirtrons are each defined by unique consensus sequences, protein assembly factors, and position within the intron (6, 38, 69, 88, 89). Nonetheless, these intronic non-coding RNAs all require pre-mRNA splicing, intron lariat formation, debranching,
and exonucleolytic trimming prior to formation of a functional ribonucleoprotein. We used qRT-PCR to probe snoRNA precursors to determine the level of processing at which 6H2 cells are defective. Intron lariats from each \textit{rpL13a} snoRNA-containing intron were quantified by qPCR primers reading across the branch point with a sense primer designed to a 3’ region of the intron and an antisense primer to a 5’ region (116). This approach revealed a 38-63\% decrease in lariats from each of the snoRNA-containing \textit{rpL13a} introns under untreated and palmitate treated conditions in 6H2 cells (Figure 3.9A). Validity of this approach was confirmed by the observation of a single PCR product for each intron lariat (Figure 3.9B) and sequence analysis of the PCR products (Figure 3.9C). Each primer pair read across the branch point and allowed for mapping branch sites in \textit{rpL13a} introns, each defined by an adenosine and 6 of 7 nucleotides correlating with the consensus major spliceosome branch site. Interestingly, we were unable to detect PCR products for endogenous \textit{rpL13a} intron lariats lacking snoRNAs, a finding consistent with recent work from others showing that intronic sequences lacking snoRNAs are degraded more quickly than introns containing snoRNAs (126). On the other hand, we were able to quantify both snoRNA-containing and non-snoRNA-containing introns when they were overexpressed in cells transfected with the split luciferase vector construct containing the U32a intron and the snoRNA-deleted U32a sequence, respectively. In WT cells the presence of the intronic snoRNA was associated with increased lariat abundance, whereas this apparent increase was not observed in 6H2 cells (Figure 3.9D). These findings suggest that SmD3 contributes to expression of intronic snoRNAs by enhancing intron lariat formation or stability.
CONCLUSIONS

Through the use of a genetic screen in CHO cells, our laboratory has identified loci involved in lipid-induced cell death, a process in which oxidative stress is a central feature. Lipotoxic and oxidative stress critically involve cytosolic expression of intronic snoRNAs from the *rpL13a* genomic locus (72). We provide additional insight into the underlying mechanisms of metabolic stress responses through the characterization of a mutant cell line with disruption of one allele of the gene encoding SmD3. Our data show that reduced cellular levels of SmD3 decrease the propagation of oxidative stress and protect cells from palmitate-induced death. Although mutant 6H2 cells are distinct from previously described palmitate-resistant mutants from our screen, mutation at the SmD3 locus confers a related molecular phenotype in that 6H2 cells fail to induce *rpL13a* snoRNAs under lipotoxic stress. We show that SmD3, beyond its known role in splicing, regulates expression of the intronic snoRNAs. Compared to WT levels of SmD3, reduced levels of SmD3 lead to decreased intron lariat abundance and decreases in U4 and U5 snRNPs necessary for intron lariat formation. These perturbations decrease the basal levels of the *rpL13a* intronic snoRNAs and subsequently blunt their induction during lipotoxicity.

SmD3 together with six other Sm proteins form a heptameric ring around the uridine-rich Sm-binding site found in snRNAs. These snRNPs form the major building blocks of the spliceosome. Sequential assembly of the hetero-oligomers of SmD1-D2, SmE-F-G, and SmB/B'-D3 is a highly regulated process (27, 84). Although these proteins are critical for proper snRNP assembly, our data indicate that haploinsufficiency of SmD3 is sufficient to maintain splicing of a specific endogenous...
pre-mRNA and an exogenously provided splicing reporter under normal growth conditions. Consistent with an ability to support overall wild type capacity for mRNA splicing, the growth of mutant 6H2 cells is indistinguishable from parental wild type cells. These findings suggest that excision of introns during the catalytic stage of splicing goes to completion and haploinsufficient levels of SmD3 in these cells is not limiting for production of mRNAs. Furthermore, ~50% knockdown of SmD3 does not cause global changes in exon utilization. These findings indicate that the phenotype of SmD3 haploinsufficiency does not relate to global defects in formation of mRNAs.

To our knowledge, this is the first report of the loss-of-function phenotype for SmD3. The finding of a second mutation that reduces rpL13a snoRNA expression and leads to resistance to lipotoxicity further highlights the important role of these ncRNAs in metabolic stress responses. More importantly, our study provides novel insights into the broader molecular cell biology of intronic non-coding RNA elements. Future studies of the precise molecular interactions of SmD3 within individual snRNPs are likely to elucidate mechanisms through which this protein regulates non-coding RNA biogenesis.
Figure 3.1

Figure 3.1 6H2 cells are resistant to palmitate-induced cell death. (A) WT and palm-resistant 6H2 mutant cells were incubated with 500 µM palm for 48 h; or 10 µM camptothecin (camp), 80 nM staurosporine (staur), or 2 µM actinomycin D (actD) for 24 h. Cell death was quantified by propidium iodide (PI) staining and flow cytometry. Data are expressed as mean fluorescence ± standard error (SE) for 3 independent experiments with $10^4$ cells/sample. *, p<0.005 for 6H2 versus WT.
Figure 3.2 6H2 cells are haploinsufficient for SmD3. (A) Autoradiogram shows Southern blot analysis of WT (lanes 1-3) and 6H2 (lanes 4-6) genomic DNA digested with restriction enzymes Bgl II, NcoI, or XbaI. Blot was probed with a $^{32}$P-labeled fragment corresponding to the ROSAβgeo sequence. (B) PCR was performed on cDNA from WT (lanes 1 and 2) and 6H2 (lanes 3 and 4) cells with reactions containing no cDNA as controls (lanes 5 and 6). Forward (F) and reverse (R) primers for snrpD3 were designed to detect endogenous snrpD3 (lanes 1, 3, and 5). Forward snrpD3 primer and reverse primer for the proviral sequence were used to detect fusion transcript (lanes 2, 4, and 6). (C) RNA was isolated from WT and 6H2 cells and reverse transcribed using...
either random hexamers (ran hex) to prime total RNA or oligo dT to prime mRNA. snrpD3 expression was determined by quantitative real-time PCR (qPCR) and normalized to β-actin expression. (D) Protein expression in WT and 6H2 cells was determined by western blotting and quantified by densitometry. Bands at 15 kDa and 18 kDa likely reflect known post-translational modification of SmD3. Representative blot shown for SmD3 and β-actin control. On bar graphs, data are expressed as mean ± SE for three independent experiments. *, p < 0.05 for 6H2 versus WT.
Figure 3.3 Targeted knockdown of SmD3 confers palmitate-resistance. Stable clonal cell lines were generated following transfection with a scrambled (contr) or snrpD3-targeting shRNA (sh1 and sh2). (A) SmD3 and β-actin protein expression was determined by western blot. Blot shows three independent protein samples from each respective cell line. (B) SmD3 expression relative to β-actin was quantified by densitometry of blots as shown in A. Graph shows mean ± SE for three independent samples. *, p < 0.05 for knockdown versus scrambled. (C) Scrambled and knockdown cells were treated with palm for 48 h and cell death was assessed by PI staining and flow cytometry. All data are expressed as mean fluorescence ± SE for three independent experiments with $10^4$ cells/sample. *, p<0.005 for knockdown versus scrambled.
Figure 3.4 SmD3 disruption protects cells from palmitate-induced and generalized oxidative stress induction. (A) WT and 6H2 cells were incubated with $^{14}$C-palmitate under lipotoxic conditions (500 µM palm). Mean initial rates of palmitate uptake are expressed per µg protein (± SE) for 3 independent experiments. (B,C,D) WT and 6H2 cells were incubated with palmitate (B, 5 hr and 16 h), scrambled (contr) and knockdown (sh1 and sh2) cells were treated with palmitate (C, 16 h), or WT and 6H2 cells were untreated (UT) or treated with menadione (mena) or H$_2$O$_2$ (D, 2h). ROS induction was assessed by CM-H$_2$DCFDA (DCF) labeling and flow cytometry. Graphs show mean fluorescence ± SE for 3 independent experiments with $10^4$ cells/sample. *,
p<0.05 for 6H2 versus WT, or for knockdown versus scrambled.
Figure 3.5 Intronic non-coding RNA expression is disrupted in 6H2 cells. (A,B) WT and 6H2 cells (A) and scrambled control and knockdown cells (B) were untreated or supplemented with palm for 48 h. Small RNA was harvested and used in RNase protection assay with $^{32}$P-labeled rPL13a snoRNA probes or miR-16 probe as control. Protected probe was analyzed by autoradiography. (C,D,E,F) WT and 6H2 cells were untreated or treated with palm for 9 h. Cells were separated into cytosolic (CYT) and nuclear (NUC) fractions by sequential detergent solubilization. (C) Fractions were analyzed by western blotting or PCR visualization for cytosolic markers, hsp90 and tRNAGlu, and for nuclear markers, fibrillarin and U6 RNA. (D) Molar quantities of
rpL13a snoRNAs from cytosolic and nuclear fractions were quantified relative to a standard curve. Plot represents relative molar ratio. (E,F) Total RNA was prepared from the cytosolic (E) and nuclear (F) fractions and analyzed for rpL13a snoRNA abundance relative to β-actin by qRT-PCR. (G) Total RNA was prepared from nuclear fractions of WT and 6H2 cells and analyzed for intronic and non-intronic box C/D snoRNAs; intronic box H/ACA snoRNAs; and splicing-dependent/Drosha-independent intronic and non-intronic miRNAs. All data are expressed as mean ± SE for three independent experiments. *, p < 0.05 for 6H2 versus WT.
Figure 3.6 SmD3 knockdown disrupts U4 and U5 snRNPs. (A,B,C,D) NIH 3T3 cells were transfected with LNA/DNA oligonucleotides specifically targeting GFP or SmD3. (A) Protein expression in GFP and SmD3 transfected cells was determined by western blotting and quantified by densitometry. Representative blot shown for SmD3 and β-actin control. (B) Nuclear RNA was isolated and analyzed for SmD3 mRNA and rpL13a snoRNA expression by qRT-PCR relative to 36B4. (C) Total RNA was isolated and
analyzed for snRNA expression relative to 36B4 by qRT-PCR. (D) Cell lysates were immunoprecipitated using α-Sm Y12 antibody or IgG control. RNA was isolated following immunoprecipitation and analyzed by qRT-PCR for α-Sm immunoprecipitated snRNA relative to control (IgG) precipitated. (E,F,G,H) NIH 3T3 cells were transfected with control (contr) siRNA or siRNA targeting SmB. (E) Protein expression in control and SmB siRNA cells was determined by western blotting and quantified by densitometry. Representative blot shown for SmB and β-actin control. (F) Nuclear RNA was isolated and analyzed for SmB and rpL13a snoRNA expression by qRT-PCR relative to 36B4. (G) Control and SmB siRNA cells were incubated with 500 µM palm for 24 h or 48 h. Cell death was quantified by PI staining and flow cytometry. (H) Total RNA was isolated and analyzed for snRNA expression by qRT-PCR relative to 36B4. Data are expressed as mean ± SE for three independent experiments. *, p < 0.05 SmD3 versus GFP or SmB versus control; ns, not significant.
Figure 3.7 Host gene expression is normal in 6H2 cells. (A) *rpL13a* locus is shown with non-coding region in black lines, exons in black boxes and snoRNAs in gray ovals. Location of primers for qPCR analysis of pre-mRNA and mRNA are noted. (B) WT and 6H2 cells were untreated (UT) or treated with palm for 9 h. For analysis of *rpL13a* pre-mRNA expression, total RNA was reverse transcribed using random hexamers, and amplified using primers that span the junction between exon 3 and intron 3. For analysis of *rpL13a* mRNA expression, total RNA was transcribed using oligo-dT and amplified using primers that span exon-exon junctions. Primer pairs are as indicated in (A). (C) WT and 6H2 cells were transfected with a split luciferase reporter containing a β-globin 52
intron. 24 h post-transfection, cells were untreated or treated with palm or clortrimazole (clor) for 4 h. Luminescence was measured and normalized to luciferase pre-mRNA expression by qPCR. (D,E) WT and 6H2 cells were transfected with a split luciferase construct containing the intact U32a intron (sno) or the U32a intron lacking the 83 nucleotide U32a snoRNA (Δsno). Total RNA was analyzed for luciferase pre-mRNA and U32a snoRNA expression. Luminescence (D) and U32a snoRNA (E) were normalized to luciferase pre-mRNA expression. Note that sequences differences between the murine intronic sequences in the reporter construct and endogenous hamster sequences enable discrimination between exogenous murine and endogenous hamster snoRNAs using species-specific PCR primers. (F) Total RNA was prepared from WT and 6H2 cells and analyzed for host genes of endogenous intron-encoded snoRNAs and mirtrons by qRT-PCR relative to β-actin mRNA. All data are expressed as mean ± SE for three independent experiments. *, p < 0.05; ns, not significant.
Figure 3.8

**Figure 3.8 Alternative splicing is normal in 6H2 cells.** Exon array analysis was used to predict differences in alternative splicing. Relative probeset intensity values from exon array were plotted for SmD3 LNA versus GFP LNA transfected cells using data from three independent samples/arrays for each condition.
Figure 3.9 SnoRNA-containing intron lariats are decreased in 6H2 cells. (A) WT and 6H2 CHO cells were untreated or treated with palm for 9 h. Nuclear RNA was isolated and reverse transcribed using random hexamers. *rpL13a intron lariat abundance was determined by qRT-PCR using primers reading across branch points (red arrows in diagram). Quantification of lariat PCR product is normalized to β-actin mRNA. (B,C) PCR products generated by intron lariat primers in CHO WT cells were visualized on 2% agarose gel (B) or cloned and sequenced (C). (B) Lanes with cDNA (+) contain RT product as template. Lanes without cDNA (-) were negative control PCR reactions with no RT product provided as template. (C) Sequencing revealed the branch
site adenosine (red) for each intron. Branch site nucleotides showing conservation (uppercase) are indicated. (D) WT and 6H2 cells were transfected with a split luciferase construct containing the intact murine U32a intron (sno) or the U32a intron lacking the 83 nucleotide U32a snoRNA (Δsno). Intron lariat abundance was determined by qRT-PCR using primers specific for the murine lariat sequences and normalized to luciferase pre-mRNA expression. All data are expressed as mean ± SE for three independent experiments. *, p < 0.05; ns, not significant.
Figure 3.10

Figure 3.10 Role for SmD3 in snoRNA production. A model for SmD3 role in snoRNA production is shown. Open boxes denote exons; black lines denote intronic sequences; and snoRNA sequences are shown in yellow and blue. Colored balls represent snRNPs. In the presence of wild type levels of SmD3, the complement of snRNAs is sufficient to support splicing of pre-mRNAs into mature mRNAs and intron lariats that are sufficiently long-lived to produce snoRNAs. While haploinsufficiency of SmD3 is able to support wild type levels of mRNA production from splicing, associated decreases in the abundance of the of U4 and U5 snRNPs results in decreased intron lariat abundance and decreased levels of intronic snoRNAs (yellow, blue).
CHAPTER 4
Superoxide triggers cytosolic snoRNA accumulation

INTRODUCTION

Under physiological conditions, ROS serve a number of critical functions. Superoxide ($O_2^-$) and hydrogen peroxide ($H_2O_2$) are known to regulate gene expression by signaling through phosphatases, JAK/STATs, MAP kinases, and transcription factors including NF-κB (34). ROS levels are kept in check by antioxidant enzymes and scavengers including dismutases, peroxiredoxins, glutathione peroxidases, and catalase.

Under pathophysiological conditions, when ROS generation exceeds cellular antioxidant defenses, oxidative stress results. The $rpL13a$ snoRNAs are critical for ROS propagation during lipotoxic stress (72). NADPH oxidase activation is also important for FFA-induced oxidative stress, since inhibition of NADPH oxidase blocks lipotoxic cell death (11). Lipotoxic conditions can activate NADPH oxidase through ceramide synthesis or, in the absence of de novo ceramide synthesis, through protein kinase C (PKC) dependent pathways (45, 61).

Nox enzymes generate ROS by transferring electrons from NADPH to molecular oxygen. $O_2^-$ is the primary product of Nox enzymes, and Nox4 is also able to produce $H_2O_2$. In many cell types including cardiomyocytes and cardiac fibroblasts, Nox2 and Nox4 are the predominant NADPH oxidases (67). Nox2 is normally quiescent, and its activation requires stimulus-induced membrane translocation of cytosolic regulatory subunits, including $p47^{phox}$, $p67^{phox}$, $p40^{phox}$, and Rac1, a small GTPase. This
translocation is triggered by phosphorylation of p47phox by PKC (2). Nox4 is constitutively active when assembled as a heterodimer with p22phox. Nox4 ROS generation does not require association of cytosolic factors but is regulated at the gene expression level.

Given that NADPH oxidase activation is a critical contributor to ROS generation during lipotoxicity, we hypothesized that specific ROS (e.g. O$_2^-$) may be linked to ROS propagation driven by the rpL13a snoRNAs. Here, we demonstrate a link between O$_2^-$ induction and cytosolic rpL13a snoRNA accumulation. Our results extend the known relationship between ROS and rpL13a snoRNAs, suggesting a role for these snoRNAs in additional oxidative stress-related diseases.

RESULTS

**Palmitate-induced superoxide precedes cytosolic snoRNA accumulation.**

Following palmitate treatment, rpL13a snoRNAs accumulate in the cytosol in a time-dependent fashion in H9c2 cells (Figure 4.1A). By 6 hr following palmitate treatment, U32a, U33, and U34 are elevated ~2-fold in the cytosol, and U35a is elevated 1.5-fold. Since rpL13a snoRNAs are critical for ROS propagation during lipotoxicity, we hypothesized that rpL13a snoRNAs participate in a feed-forward ROS cascade initiated by ROS production following palmitate treatment. O$_2^-$ is produced during lipotoxicity through NADPH oxidase (31). To test how quickly O$_2^-$ is generated following palmitate treatment, we quantified O$_2^-$ production in cells treated with palmitate in the presence of the O$_2^-$ detector MCLA. Palmitate treatment rapidly raised O$_2^-$ levels, and levels remained high compared to untreated cells over the course of 1 hr (Figure 4.1B)
data indicate $O_2^-$ induction is an early response to lipotoxicity and precedes cytosolic snoRNA accumulation.

**Direct superoxide induction causes rapid cytosolic snoRNA accumulation.** To test whether $O_2^-$ generation can induce cytosolic snoRNA accumulation independent of metabolic stress, we treated H9c2 cells with chemical inducers of $O_2^-$. Since $O_2^-$ can be rapidly dismutated into $H_2O_2$, we initially examined the relative production of $H_2O_2$ following treatment with menadione and doxorubicin. We loaded cells with DCF to track $H_2O_2$ generation and observed a dose dependent increase in DCF signal following menadione treatment (Figure 4.2B). In comparison, doxorubicin generated less than half the $H_2O_2$ of menadione even at high concentrations, suggesting doxorubicin is not a potent inducer of $H_2O_2$.

We confirmed that doxorubicin generates $O_2^-$ in H9c2 cells by treating cells with doxorubicin for 1 hr followed by $O_2^-$ detection by MCLA. We detected a dose dependent increase in $O_2^-$ production (Figure 4.3A). Since doxorubicin generates $O_2^-$ while only producing modest levels of $H_2O_2$, we wanted to test whether doxorubicin could induce cytosolic snoRNA accumulation. Following treatment with doxorubicin, we observed rapid accumulation of $rpl13a$ snoRNAs in the cytosol (Figure 4.3B). U32a, U33, and U34 were up-regulated by at least 2.5 fold as early as 20 min following palmitate treatment. Additionally, the magnitude of cytosolic snoRNA expression was higher than observed following palmitate treatment in H9c2 cells or other cell types (72, 94). These data suggest that direct $O_2^-$ induction can rapidly and robustly induce cytosolic snoRNA accumulation.
Superoxide levels correlate with cytosolic snoRNA accumulation. Conversion of \( \text{O}_2^- \) to \( \text{H}_2\text{O}_2 \) is predominately regulated by superoxide dismutases (SOD). Isoforms of these antioxidant enzymes are located throughout the cell and extracellular space. SOD1 is located in the mitochondrial intermembrane space and cytosol, SOD2 is located in the mitochondrial matrix, and SOD3 is tethered to the extracellular matrix (95). We hypothesized that chemical manipulation of these enzymes would alter \( \text{O}_2^- \) levels and subsequently alter levels of \( rpl13a \) snoRNAs in the cytosol. To increase levels of \( \text{O}_2^- \), we co-treated cells with doxorubicin and diethyldithiocarbamate (DETC), an SOD inhibitor (Figure 4.4A). We also co-treated cells with doxorubicin and MnTBAP, an SOD mimic, to increase \( \text{O}_2^- \) scavenging and subsequently decrease \( \text{O}_2^- \) levels (Figure 4.4A). \( rpl13a \) cytosolic snoRNA expression correlated with these changes in \( \text{O}_2^- \) levels (Figure 4.4B). Relative to doxorubicin treatment alone, U32a, U33, and U34 all showed increased cytosolic accumulation following co-treatment with doxorubicin and DETC and decreased cytosolic accumulation following co-treatment with doxorubicin and MnTBAP. U35a was unaltered by doxorubicin treatment but was up-regulated following co-treatment with doxorubicin and DETC. Although doxorubicin co-treatment with MnTBAP lowered cytosolic snoRNA levels relative to doxorubicin treatment alone, snoRNA levels remained higher in the presence of doxorubicin and MnTBAP than in \( \text{H}_2\text{O}_2 \)-treated control cells, despite lower \( \text{O}_2^- \) levels in the MnTBAP treated cells. These data suggest \( \text{O}_2^- \) contributes to cytosolic snoRNA localization, but other factors likely contribute and may be important for localization of U35a in particular.
CONCLUSIONS

The *rpL13a* snoRNAs are critical for ROS propagation in response to metabolic stress. Cytosolic localization of these snoRNAs is thought to be an important feature of the function of these non-coding RNAs during lipid-induced oxidative stress. Here, we demonstrate a link between ROS induction and cytosolic snoRNA accumulation. Specifically, $\text{O}_2^-$ induction precedes cytosolic snoRNA accumulation under lipotoxic conditions. Doxorubicin induced $\text{O}_2^-$ also causes *rpL13a* snoRNAs to localize to the cytosol independent of metabolic stress. Manipulation of $\text{O}_2^-$ demonstrates that $\text{O}_2^-$ levels directly correlate with cytosolic snoRNA expression. Our data are consistent with a model in which palmitate and direct $\text{O}_2^-$ inducers stimulate higher levels of $\text{O}_2^-$ in the cells. This $\text{O}_2^-$ provides a signal to nuclear snoRNAs, through a pathway yet to be elucidated, causing translocation of snoRNAs to the cytosol (Figure 4.5).

Additional experimentation will be required to determine the source of $\text{O}_2^-$ under lipotoxic conditions. We hypothesize that NADPH oxidase is that source of $\text{O}_2^-$. Inhibition of NADPH oxidase or Nox enzyme knockdown will enable determination of the role of NADPH oxidase in cytosolic snoRNA accumulation. Apocynin is an NADPH oxidase inhibitor that prevents the assembly of NADPH oxidase subunits. Diphenylene iodium (DPI) is a non-specific inhibitor of flavoenzymes including NADPH oxidase, quinone oxidoreductase, cytochrome P450 reductase, and nitric oxide synthase (59). Inhibition with either of apocynin or DPI could be used to test whether NADPH oxidase is generally involved in cytosolic snoRNA localization. Additionally, directed knockdown of Nox2, p22phox, or p47phox would help elucidate whether specific NADPH oxidase components are related to snoRNA localization. Nox2 knockdown would directly target
a predominant source of $O_2^-$. Knockdown of $p22^{phox}$ would test whether any of the Nox enzymes 1-4 are involved, and knockdown of $p47^{phox}$ would test whether a key cytosolic component required for Nox1-3 activity is related to snoRNA expression.

Additionally, we propose that palmitate-induced oxidative damage is amplified by cytosolic snoRNA accumulation (72). Following $O_2^-$ reduction by MnTBAP or NADPH oxidase inhibition/knockdown, we hypothesize that oxidative damage will be reduced during lipotoxicity. To test this hypothesis, protein carbonylation and palmitate-induction of cell death will be quantified in future studies in cells expressing various levels of $O_2^-$. Finally, it will be of great interest to determine the specificity of relationship between specific ROS species and the broader genomic program of snoRNAs. We hypothesize that specific ROS regulate different snoRNAs. Preliminary data from our lab suggests a number of additional snoRNAs are found in the cytosol during lipotoxicity (not shown). Even though U32a, U33, U34, and U35a are produced from the same pre-mRNA, our data suggest that the kinetics or the specificity of ROS-stimulated relocation to the cytosol differs among these snoRNAs. Since oxidative stress is the result of the propagation of various ROS induced at different times, we propose that individual snoRNAs respond specifically to different ROS. To test this hypothesis, we will use a custom Agilent microarray developed in our lab that enables quantitative detection of approximately 300 known and predicted snoRNAs to determine the program of snoRNAs that accumulate in the cytosol following specific induction of $O_2^-$ vs. $H_2O_2$ vs. other ROS species. Changes identified in snoRNA expression will be confirmed by qPCR and in situ hybridization.
Together, our data suggest an intricate connection between ROS and snoRNA localization. Future studies will be needed to elucidate the mechanisms through which $O_2^-$ and other ROS signal to snoRNAs and will broaden our understanding of the contributions of these non-coding RNAs to metabolic stress.
Figure 4.1 Palmitate induces superoxide. (A) H9c2 cardiomyoblast cells were treated with BSA or treated with 500 µM palmitate for 3, 6, 12, or 24 h. Cytosolic fractions were isolated from cells by sequential detergent solubilization. Total RNA was prepared from the cytosolic fraction and analyzed for rpl13a snoRNA and mRNA abundance relative to 36B4 by qRT-PCR. Graph shows mean ± SE (n = 3) from a representative experiment. (B) H9c2 cells were untreated (UT) or treated with palmitate (palm) in 2-methyl-6-(4-methoxyphenyl)-3,7-dihydroimidazo[1,2-A]pyrazin-3-one, hydrochloride
(MCLA) buffer for 60 min. Luminescence was measured every 30 sec and normalized to a superoxide dismutase-treated control.
Figure 4.2

(A) Superoxide (O$_2^-$) is rapidly dismutated by the antioxidant enzyme superoxide dismutase (SOD), leading to the formation of hydrogen peroxide (H$_2$O$_2$) and molecular oxygen. 

B

C

Figure 4.2 Doxorubicin is not a potent H$_2$O$_2$ inducer. (A) Superoxide (O$_2^-$) is rapidly dismutated by the antioxidant enzyme superoxide dismutase (SOD), leading to the formation of hydrogen peroxide (H$_2$O$_2$) and molecular oxygen. (B,C) H9c2 cells pre-loaded with CM-H$_2$DCFDA (DCF) dye were untreated (UT) or treated with menadione (mena) or doxorubicin (dox) for 60 min. H$_2$O$_2$ induction was assessed by DCF fluorescence every 2 min using a Tecan microplate reader.
Figure 4.3

H9c2 cells were untreated (UT) or treated with doxorubicin (dox) for 60 min. Superoxide induction was assessed by MCLA. Luminescence was measured every 30 sec and integrated luminescence over 3 min is reported normalized to an SOD treated control.

(B) H9c2 cells were untreated (UT) or treated with 20 µM doxorubicin for 20 min, 60 min, 12 h, or 24 h. Cytosolic fractions were isolated from cells by sequential detergent solubilization. Total RNA was prepared from the cytosolic fraction and analyzed for
*rpL13a* snoRNA and mRNA abundance relative to 36B4 by qRT-PCR. Graph shows mean ± SE (n = 3) from a representative experiment.
Figure 4.4 Manipulation of superoxide alters cytosolic *rpL13a* snoRNA accumulation. (A,B) H9c2 cells were untreated (UT) or treated with 5 µM doxorubicin (dox) for 60 min. Cells were co-treated with H$_2$O, 400 µM diethyldithiocarbamic acid (DETC), or 400 µM manganese(III) tetrakis(4-benzoic acid)porphyrin chloride (MnTBAP). (A) Superoxide induction was assessed by MCLA. Luminescence was measured every 30 sec and integrated luminescence over 3 min is reported normalized to an SOD treated control. (B) Cytosolic fractions were isolated from cells by sequential detergent solubilization. Total RNA was prepared from the cytosolic fraction and
analyzed for *rpL13a* snoRNA and mRNA abundance relative to 36B4 by qRT-PCR. 

Graph shows mean ± SE from a representative experiment.
Figure 4.5 Superoxide is linked to cytoplasmic snoRNA accumulation. A model for the role of superoxide in cytoplasmic snoRNA accumulation is shown. Treatment with palmitate or doxorubicin causes increased levels of superoxide radicals. Through an unknown mechanism, superoxide signals nuclear snoRNAs to accumulate in the cytoplasm. SnoRNAs are shown in green, purple, yellow, and blue.
CHAPTER 5

Discussion

Genetic screens provide powerful approaches to dissect cell biological phenomena. Inherent in this approach is the advantage that genes are identified on the basis of their functional contributions to the pathway of study. Since multiple mutations may affect different components in the pathway, genetic approaches also have the ability to elucidate interacting elements. Our screen for mutations that render cells resistant to lipotoxicity identified \textit{rpL13a} snoRNAs as critical mediators of metabolic stress. Identification of a second, independent mutation that disrupts the expression of these snoRNAs highlights the importance of ncRNAs in lipotoxicity. With this second mutant, we identified SmD3 as an upstream regulatory element necessary for the expression of \textit{rpL13a} snoRNAs. Thus, in an unbiased genetic screen, we identified unexpected elements of the lipotoxic response.

Phenotypic characterization of the 6F2 and 6H2 mutants revealed that \textit{rpL13a} snoRNAs are also involved in the general response to oxidative stress. Previous biochemical studies in diverse cell types have correlated lipotoxic stress with the generation of ROS (7, 45). The \textit{rpL13a} snoRNAs provide evidence of a functional link between the progress of lipotoxic cell death and the deleterious cellular response to oxidative stress. While transcriptional, posttranslational, and signaling mechanisms are known to contribute to the cellular response to oxidative stress (33, 41), our studies are the first to implicate snoRNAs in the response to environmental perturbations.

A key observation that facilitated our understanding of the contributions of the \textit{rpL13a} locus to lipotoxicity was the extent of sequence conservation of this gene
beyond the protein-coding exons. Four box C/D snoRNAs, U32a, U33, U34, and U35a located in \textit{rpL13a} introns 2, 4, 5, and 6, respectively, are highly conserved across mammalian species both in terms of their primary sequence and their position within the locus. While box C/D snoRNAs function as guides for 2'-O-methylation in yeast (Lowe and Eddy, 1999), the sequences of the U32a, U33, U34, and U35a snoRNAs and their genomic organization diverge substantially from yeast to mammals. In mammals, a role in the modification of ribosomal RNAs has not been demonstrated for these snoRNAs. We show here in a stable mutant cell line that decreased basal expression of U32a, U33, and U35a snoRNAs and loss of palmitate induction of these snoRNAs is not associated with changes in 2'-O-methylation of predicted rRNA targets, yet is associated with resistance to lipotoxicity. While it is possible that these snoRNAs contain more than one functional guide sequence to target multiple substrates including rRNAs (13, 110), their accumulation in the cytosol during lipotoxicity suggests a non-nucleolar function for these snoRNAs.

Movement of snoRNAs to the cytosol is not without precedent, since the independently transcribed box C/D snoRNA, U8, has been shown to be exported from the nucleus (121). The present study provides the first direct evidence for intronic snoRNAs in the cytosol. Our data are most consistent with a model in which the \textit{rpL13a} snoRNAs function in lipotoxicity and oxidative stress response pathways as non-canonical box C/D snoRNAs. Our results demonstrate that U32a, U33, and U35a function coordinately in propagation of oxidative stress \textit{in vivo}. Thus, our study provides evidence for snoRNA regulation of metabolic stress response pathways.
Cytosolic functions of snoRNAs

The cytosolic function of snoRNAs has yet to be elucidated. We hypothesize that snoRNAs serve to guide the modification of cytosolic target RNAs during metabolic stress. Cytosolic ncRNAs, particularly tRNAs, are known targets for modification. tRNAs are subject to over 75 different modifications throughout their sequence. Modifications to the anticodon loop can influence the recognition of codons and maintenance of the translational reading frame (32). Although pre-mRNA are known to undergo 5' m7G capping, 3' polyadenylation, and RNA splicing, modification of individual nucleotides within cytosolic mRNAs is not well described. The abundance of modifications in tRNA, rRNA, and snRNA raises the possibility that mRNAs might also be modified.

SnoRNAs could function in the regulation of mRNA translation, possibly through targeted modification or sequestration of specific mRNAs. Recently, it was demonstrated that targeted pseudouridylation of termination codons suppresses translation termination in vitro (48). Given the manifold of changes in gene expression during the lipotoxic and oxidative stress responses (10), the coordinate function of the rpL13a snoRNAs (72), lack of a well-defined long stretch of perfect antisense homology to targets in snoRNAs (53), and the possibility that snoRNA-directed modifications of RNA targets may not be reflected by changes in abundance of those RNAs, identification of specific targets for each of these snoRNAs will be a complex task and the subject of future investigations. To identify individual targets of rpL13a snoRNAs, transfection of cells with synthetic biotin-labeled snoRNA molecules could facilitate pull-down of RNA species that interact specifically with rpL13a snoRNAs. While initial approaches may utilize biochemical isolation of specific snoRNA-targetRNA complexes,
it will also be of great interest to examine 2′-O-methylation and pseudouridylation on a genome-wide level.

Identification of precise snoRNA localization within the cytosol will also be useful for understanding the function of snoRNAs during metabolic stress. Moreover, our initial studies suggest that snoRNAs are subject to cellular export. Treatment of cultured cells with palmitate leads to detectable snoRNA expression in culture media. Additionally, snoRNAs are stably expressed in human and mouse serum, and treatment of 12-week-old mice with LPS results in increased serum snoRNA expression (not shown). Thus, snoRNAs may be readily exported from cells. A number of recent studies have shown that miRNAs are released from cells after cell death, in apoptotic bodies, through microvesicles including exosomes, and as part of protein complexes giving precedence for extracellular ncRNAs (106, 113, 114). If future studies confirm a role for snoRNA secretion in response to lipotoxicity and oxidative stress, circulating snoRNAs could ultimately serve as a biomarkers for diseases characterized by oxidative stress.

**Regulation of snoRNA processing**

Identification of SmD3 through our genetic screen unmasks a previously unsuspected role for this protein in production of intronic snoRNAs. The precise mechanisms through which SmD3 regulates intronic snoRNA expression remain to be determined. One possibility is that SmD3 has a direct role in snoRNA biogenesis unrelated to its function in snRNPs. Unlike SmD3 haploinsufficient cells, SmB knockdown cells remain sensitive to palmitate-induced death suggesting that SmD3 has a function distinct from other Sm protein family members. Additionally, SmD3 has been
observed to function independently from the Sm core in the cytoplasm (30). SmD3 has also been previously shown to bind small Cajal body RNAs (scaRNAs), a class of snoRNAs specifically localizing to Cajal bodies, and human telomerase (hTR) via a CAB box site (28). However, H/ACA snoRNAs U17b and U64, which are affected by haploinsufficiency of SmD3, both lack CAB box sequences. Additional studies will be required to decipher whether SmD3 can function independently from the Sm core during snoRNA biogenesis.

Our data are most consistent with a model in which SmD3 does not directly interact with the maturing snoRNA during processing, but rather controls processing indirectly through a role in snRNP assembly and/or stabilization (Figure 3.10). Knockdown of SmD3 results in decreased expression of U4 and U5 snRNPs that are required for precatalytic spliceosome formation and initiation of an active spliceosome capable of intron lariat formation (68). Furthermore, reduced expression of snoRNA lariat precursors, resulting from decreased lariat production or stability, leads to decreased abundance of the mature intronic snoRNAs and failure to support their induction during lipotoxicity in 6H2 cells. These differences in intron lariat abundance and snRNP expression suggest that the phenotype observed in 6H2 cells is related to spliceosomal machinery, even though haploinsufficiency of SmD3 is sufficient to maintain production of mRNAs.

We also observed that wild type levels of SmD3 are critical for the ability to express a number of intronic non-coding RNA elements including other box C/D snoRNAs, H/ACA snoRNAs, and mirtrons. These ncRNAs do not share significant sequence similarities and each assembles with a unique set of proteins during
processing, making it unlikely that SmD3 directly recognizes and associates with the nascent RNP. It has been shown that the C-terminal tail of SmD3 interacts with the central Tudor domain of splicing factor SPF30 (62), indicating that SmD3 may be important for the assembly of splicing factors specific to individual snRNPs. Individual Sm proteins may be critical for the assembly of specific factors within different snRNPs consistent with the different snRNA expression profiles observed following SmD3 knockdown versus SmB knockdown. In follow up, it will be of interest to determine whether Sm protein-splicing factor interactions are critical for SmD3’s role in snRNP assembly and/or stabilization and to better define the distinct but overlapping roles of SmD3 and SmB in assembly and stabilization of snRNPs.

Regulation of snoRNA localization

SnoRNAs are processed in the nucleus and canonically reside within the nucleolus. Our studies indicate that nuclear snoRNA levels are multiple orders of magnitude higher in the nucleus than the cytoplasm even during metabolic stress. Unlike snRNAs, snoRNAs are not thought to undergo a cytosolic phase during maturation (69), suggesting that accumulation of snoRNAs in the cytosol requires an active mechanism. We demonstrate that O$_2^-$ induction precedes cytosolic snoRNA accumulation consistent with the hypothesis that O$_2^-$ functions as an upstream trigger of snoRNA translocation from the nucleus to the cytosol. O$_2^-$ participates in multiple signaling pathways (34). ROS driven signal transduction includes induction of gene expression, protein phosphorylation, and alteration of redox status (73). Examination of
signaling pathways, particularly in the nucleus, will be of great interest in future studies of ROS regulated snoRNA localization.

O$_2^-$ diffuses only short distances before reacting with a target, so it is likely that the source of O$_2^-$ related to snoRNAs is generated in close proximity to its intended target. Although NADPH oxidases are implicated to be involved in lipotoxicity, the source of O$_2^-$ leading to cytosolic snoRNAs remains to be elucidated. Nox enzymes each have distinct subcellular localizations. Nox2 is found predominantly on the plasma membrane, whereas Nox4 is located in intracellular membranes. O$_2^-$ can also be generated from several additional sources, including xanthine oxidases, peroxidases, lipoxygenases, cyclooxygenases, and mitochondrial respiratory chain complexes (129). Determination of the source of O$_2^-$ will help elucidate how O$_2^-$ is linked to cytosolic snoRNA accumulation.

**Summary**

Together, our data demonstrate that snoRNAs are highly regulated in response to metabolic stress and contribute significantly to the pathogenesis of oxidative stress. To carry out this role, snoRNAs must be efficiently processed and properly localized. Elucidation of the regulatory steps in snoRNA production and localization and identification of targets for non-canonical snoRNAs will advance our understanding of the pathogenesis of common human diseases. Moreover, regulation of snoRNAs provides potential therapeutic targets for oxidative stress-related diseases including type 2 diabetes.
CHAPTER 6
Materials and Methods

Materials. Palmitate was from Nu-Chek Prep. \(^{14}\text{C}\)-palmitate and \(\alpha^{-32}\text{P}\)-UTP were from PerkinElmer Life Sciences. Camptothecin, actinomycin D, hygromycin, and MnTBAP were from Calbiochem. Staurosporine, \(\text{H}_2\text{O}_2\), menadione, phloretin, clotrimazole, doxorubicin, and DETC were from Sigma-Aldrich. Fatty acid-free bovine serum albumin (BSA) was from SeraCare. Propidium iodide, CM-H\(_2\)DCFDA, DHE, and MCLA were from Invitrogen. All synthetic oligonucleotides were from IDT (see Table 1 for primer sequences). Restriction enzymes were from New England BioLabs.

Cell Culture. CHO-K1 cells (American Type Culture Collection) 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)) media with 5% non-inactivated fetal bovine serum, 2 mM L-glutamine, 50 units/ml penicillin G sodium, 50 units/ml streptomycin sulfate, and 1 mM sodium pyruvate. C2C12 myoblasts (American Type Culture Collection) were maintained in high glucose (4.5 mg/ml Dulbecco’s modified Eagle’s medium with 10% heat-inactivated fetal bovine serum, 50 units/ml penicillin G sodium, and 50 units/ml streptomycin sulfate. NIH 3T3 cells (American Type Culture Collection) were maintained in high glucose (4.5 mg/ml Dulbecco’s modified Eagle’s medium) media with 10% bovine calf serum, 2 mM L-glutamine, 50 units/ml penicillin G sodium, and 50 units/ml streptomycin sulfate. H9c2 cardiomyoblast cells (American Type Culture Collection) were maintained in high glucose (4.5 mg/ml Dulbecco’s modified Eagle’s
medium) media with 10% fetal bovine serum, 2 mM L-glutamine, 50 units/ml penicillin G sodium, and 50 units/ml streptomycin sulfate. For lipotoxicity experiments, cell culture media was supplemented with 500 µM (WT CHO and 6H2) or 250 µM (shRNA transfected cells) palmitate complexed to BSA at a 2:1 M ratio, as described previously (61). For ROS induction, media was supplemented with the indicated concentrations of H$_2$O$_2$, menadione, or doxorubicin.

**Generation of CHO Cell Mutants.** Vesicular stomatitis virus G protein pseudotyped murine retrovirus encoding the ROSAβgeo retroviral promoter trap was generated as described previously (26, 81). CHO cells were transduced with retrovirus at a low multiplicity of infection (1 integration per 10 genomes on average) and mutants were isolated as described previously (7). Number of retroviral insertions within the mutant cell genome was assessed by Southern blotting. Genomic DNA was digested with restriction enzymes, separated by agarose gel electrophoresis, transferred to nylon membranes, and probed with a $^{32}$P-labeled probe corresponding to the ROSAβgeo proviral sequence.

**Cell Death Assays.** Cell death was assessed by membrane permeability to propidium iodide (PI) staining and flow cytometry (61). Following treatments, cells were harvested by trypsinization and stained with 1 µM PI. Analyses were performed on $10^4$ cells/sample.
**14C Palmitate Uptake Assay.** 2 × 10^6 cells were resuspended in 1 ml phosphate-buffered saline (PBS) containing 500 µM 14C-palmitate complexed to 250 µM BSA and incubated for one minute at 37°C. Cells were washed with 10 ml PBS containing 0.1% BSA and 500 µM phloretin, filtered, and cell-associated 14C was quantified by scintillation counting. A parallel aliquot of cells was used for quantification of protein by bicinchoninic acid assay (Pierce).

**Identification of Trapped Gene.** The endogenous 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; Clontech). The 5' RACE product was TA cloned and sequenced, and tested for sequence similarity by NCBI BLAST. PCR was used to verify retroviral integration within the snrpD3 gene.

**Quantitative Real Time PCR (qPCR).** RNA was isolated using TRIzol or TRIzol LS reagent (Invitrogen) and reverse transcribed to cDNA using the SuperScript III First-strand Synthesis System for RT-PCR (Invitrogen) following the manufacturer's instructions. Reverse transcription was performed with oligo(dT) to detect mRNA or random hexamers to detect pre-mRNA and intron lariats. For quantification of snoRNAs and pre-miRNAs, RNA was isolated using Trizol LS (Invitrogen). cDNA synthesis was primed with hairpin stem-loop oligos as previously described (72), with overhang complementarity to the 3’ end of the processed snoRNA or pre-miRNA. cDNA was amplified for 40 PCR cycles using SYBR Green PCR master mixture (Applied Biosystems) and 100 nM template specific primers in a ABI Prism 7500 Fast Real-Time
PCR System. Relative quantification of gene expression was performed using the comparative threshold method as described by the manufacturer.

**Flow Cytometry Detection of Reactive Oxygen Species.** Cells \((2 \times 10^5)\) were plated in 12-well plates 24 h prior to various treatments. Cells were rinsed with PBS and incubated with PBS containing 0.5 mM MgCl\(_2\), 0.92 mM CaCl\(_2\), and 1 µM (C2C12 cells) or 3 µM (CHO cells) 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate, acetyl ester (CM-H\(_2\)DCFDA, Invitrogen) in the dark at 37°C for 1 h. Cells were then rinsed with PBS, harvested by trypsinization, and quenched with culture media. Mean fluorescence was determined by flow cytometry on \(10^4\) cells/sample.

**Generation of SmD3 antibody.** For immunoblot detection of SmD3, polyclonal rabbit anti-peptide antibody was generated from ProSci Incorporated. Animals were immunized with the unique peptide NH\(_2\)-CTGEVYRGKLEAED-OH (murine sequence) conjugated to KLH. Animals received six rounds of immunization followed by affinity purification of serum.

**Immunoblot Analyses.** Whole cell protein lysates were prepared using RIPA buffer (50 mM Tris-Cl, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, and 5 mM EDTA) containing 1 mM phenylmethylsulfonyl fluoride and 1 × Protease Complete inhibitor mixture (Roche). Subcellular fractions were isolated by sequential detergent solubilization as described previously (40). Proteins (40 µg) were resolved by 15% (for rpL13a, SmD3, and SmB) or 12% (for hsp90, fibrillarin, lamin B1, CHOP-10,
and β-actin) SDS-PAGE gel electrophoresis and transferred to nitrocellulose membrane (Whatman). Membranes were probed with antibodies to rpL13a (1:500), SmD3 (1:500), β-actin (A 2066, Sigma, 1:5000), hsp90 (SPA-846, Stressgen, 1:2000), fibrillarin (MMS-581S, Covance, 1:500), lamin B1 (ab16048, Abcam, 1:1000), CHOP-10 (F-168, Santa Cruz, 1:500), and SmB (S0698, Sigma, 1:1000). Proteins were visualized using appropriate horseradish peroxidase-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories, 1:10,000) and chemiluminescence reagents (PerkinElmer Life Sciences). Band intensities were quantified by densitometry (Bio-Rad Image Lab Software).

**snoRNA Probe Synthesis and RNase Digestion.** Hamster- and mouse-specific snoRNA probes were generated with Megashortscript kit (Ambion). dsRNA templates were generated for probe for each rpL13a snoRNA by PCR amplification of cloned hamster or mouse rpL13a genomic sequence templates using primers containing the T7 RNA polymerase promoter and used for in vitro RNA transcription of 32P-labeled snoRNA probes. miR-16 probes were synthesized using templates from mirVana miRNA Detection kit (Ambion). RNA was isolated from cells using mirVana miRNA isolation kit (Ambion) and hybridized to 32P-labeled RNA probes (mirVana miRNA Detection kit) overnight at 42-52°C, followed by RNase digestion and ethanol precipitation. RNA was separated by 10% or 15% polyacrylamide electrophoresis and visualized by autoradiography.
snoRNA Knockdown in vitro. Anti-sense oligos (ASOs) were designed to specifically target murine U32a, U33, U35a, U50, U57, and U60 snoRNA sequences according to Ideue et al. (44). For snoRNA “knock-down” experiments, $10^6$ C2C12 myoblasts were nucleofected using Nucleofector Kit V (Amaxa) and a total of 600 pmol of ASO.

Mapping of 2'-O-methyl modification by primer extension. Protocol was based on methods from Lowe and Eddy (64). Total RNA from Trizol extraction was annealed with $^{32}\text{P}$ end-labeled primers at 55°C for 4 min. Primer extension reactions were carried out in the presence of 50 mM Tris-Cl (pH 8.6), 60 mM NaCl, 9 mM MgCl$_2$, 10 mM DTT, 1 mM dNTP, and using avian myeloblastosis virus reverse transcriptase for 30 min at 37°C. For rRNA sequencing, ddNTPs were used in individual reactions. For 2'-O’methyl mapping, reactions were carried in “High” (4mM), or “Low” (0.004 mM) dNTP concentrations, and 5 mM MgCl$_2$. Reaction products were separated by 6% polyacrylamide electrophoresis (PAGE) and visualized by autoradiography.

In situ hybridization of snoRNA probes. Synthesis and labeling of sense and antisense RNA probes were adapted from Darzacq et al. (16). C2C12 myoblasts were fixed in PBS containing 3% paraformaldehyde for 10 min, rinsed with PBS and permeabilized with 0.1% Triton X-100 for 10 min at room temperature. Cells were dehydrated serially in 70% ethanol, 95% ethanol, 100% ethanol and then air-dried. Sequence-specific oligonucleotide probes containing aminoallyl UTP were generated using the FISH Tag RNA Kit (Invitrogen) and labeled with an amine-reactive Alexa Fluor 594 dye. Fluorescent probe (0.5 ng/µL) was denatured in hybridization buffer (2x SSC,
50% formamide, 10% dextran sulfate, 20 mM vanadyl ribonucleoside complexes) for 10 min at 80°C. Cells were incubated with probe/hybridization buffer at 37°C for 10 hr, followed by sequential washes with 2x SSC, 1x SSC, 0.1x SSC all containing 1% SDS for 15 min each at 37°C. Nuclei were counter-stained using SYTOX Green (Invitrogen). Slides were mounted with SlowFade antifade reagent (Invitrogen). Images were captured on a ZEISS LSM 510 META confocal laser scanning microscope using constant pinhole size, detector gain, and offset for each probe.

**Mouse model of LPS-mediated oxidative stress and in vivo snoRNA knockdown.**

Female FVB mice were obtained from Charles River Laboratories and housed in Washington University Division of Comparative Medicine facilities. Diet was standard 6% fat breeding chow supplied ad libitum, and food was withheld at the time of LPS injection. Between 10 and 16 weeks of age, LPS was administered at 8 mg/kg intraperitoneally (IP), and animals were euthanized 12-24 h later. For in vivo knockdown experiments, LNA-modified ASOs were purchased from Exiqon and used to specifically target snoRNAs U32a, U33, and U35a. An ASO targeting GFP was used as a control. Mice were injected IP with a total of 2.5 mg/kg of LNA every other day for a total of three injections, and then dosed with LPS as above 48 h after the last LNA injection. Individual snoRNA ASO concentrations were 1.25, 0.5, and 0.75 mg/kg per dose, targeting U32a, U33, and U35a respectively (empirically determined based on knockdown). Liver tissue was divided and either snap-frozen in liquid nitrogen (for RNA), fixed in 10% neutral buffered formalin, or frozen in O.C.T. Compound (Tissue-Tek). Experimental procedures were approved by the Washington University Animal
Studies Committee and were conducted in accordance with USDA Animal Welfare Act and the Public Health Service Policy for the Humane Care and Use of Laboratory Animals.

**In vivo detection of ROS.** Formalin-fixed, paraffin-embedded samples or liver frozen in O.C.T. were mounted on slides in serial sections by the Washington University Anatomic and Molecular Pathology Core Lab. Detection of superoxide was performed on frozen sections using dihydroethidium (DHE; Invitrogen, Cat# D11347). Sections were incubated with 2 µM DHE for 30 min at 37°C or pre-treated with 200 Units/ml PEG-SOD (Sigma), followed by co-incubation of 2 µM DHE and 200 Units/ml PEG-SOD for 30 min at 37°C to verify the specificity of staining as indicated. For each animal (n = 4 for GFP ASO; n = 5 for SNO ASO), intensity of staining was quantified in three independent fields from each of six sections using ImageJ software. Protein carbonyls were detected by immunoblot using the OxyBlot Protein Oxidation Detection Kit (Chemicon) according to the manufacturer’s instructions. Blots were quantified by densitometry using actin as a loading control. Tissue oxysterols (7-ketocholesterol, 7-keto; 3β,5α,6β-cholestantriol, triol) per mg liver protein were quantified using LC/MS/MS as described (87).

**Generation of SmD3 shRNA Clones.** Hamster *snrD3* cDNA sequence was used to design siRNA oligonucleotides using Ambion’s siRNA Target Finder Program (ambion.com/techlib/misc/siRNA_finder.html). shRNA oligonucleotides were designed from siRNA sequences that conferred effective knockdown in transient transfection
assays, and each was cloned into a pSilencer 4.1-CMV hygro vector (Ambion) containing a hygromycin resistance cassette. shRNA vectors were transfected into CHO cells with Lipofectamine 2000 reagent (Invitrogen). Cells were plated at limiting dilution and treated with 80 µg/ml hygromycin. Clonal lines were isolated, and SmD3 knockdown assessed by immunoblot.

**Luciferase Plasmids and Transient Transfection.** The split luciferase vector containing a β-globin intron was as described previously (128). All constructs generated by PCR or Quick-Change (Stratagene) mutagenesis were confirmed by sequencing. The β-globin intron in the split luciferase reporter was replaced with *rpL13a* intron 2 containing snoRNA U32a or the *rpL13a* intron 2 lacking the 83 nucleotide U32a snoRNA sequence. Cells were transfected with Lipofectamine 2000 (Invitrogen) as per the manufacturer’s protocol and assayed 20 h post-transfection.

**Luciferase Detection.** Cells (3 × 10^4) were plated in triplicate in 96-well plates. Following transfection of luciferase reporters as described above, cells were lysed with Dual-Glo Luciferase Reagent (Promega) according to the manufacturer’s protocol. Luciferase was detected using a Tecan Infinite M200 microplate reader and Magellan software.

**Microarray Sample Preparation and Data Analysis.** NIH 3T3 cells (2 × 10^5) were plated in 6-well plates 24 h pre-transfection. Cells were transfected with 40 pmol LNA/DNA oligonucleotides specifically targeting GFP or SmD3 (Exiqon) using
lipofectamine 2000 (Invitrogen) as per the manufacturer’s protocol, and total RNA was harvested 23 h post-transfection using TRIzol reagent (Invitrogen). Resulting RNA was quantified by A260 and A280 readings using a Nanodrop spectrophotometer (Nanodrop Technologies) and qualitatively assessed using a BioAnalyzer 2100 (Agilent Technologies). cDNA was prepared using the NuGen Ovation System and microarray data was generated with Affymetrix GeneChip Mouse Exon 1.0 ST arrays containing 266,200 probesets in the Siteman Cancer Center Molecular and Genomic Analysis Core at Washington University. Partek Genomics Suite 6.5 was used to calculate probeset intensities from .CEL files using the RMA algorithm with default settings at both the gene level and probeset level. Probesets with RMA intensity below 3 across all samples were excluded to eliminate probesets with low expression levels. Alternative splicing multiway ANOVA was applied using Partek defaults to identify alternative splicing events with a false discovery rate (FDR) < 0.1. Core exon level analysis was also applied at the exon level to determine differential expression of exons not grouped by transcript.

**Transient Knockdown.** For SmD3 knockdown, NIH 3T3 cells (2 × 10^5) were plated in 6-well plates 24 h pre-transfection. Cells were transfected with 40 pmol LNA/DNA oligonucleotides specifically targeting GFP or SmD3 (Exiqon) using lipofectamine 2000 (Invitrogen) as per the manufacturer’s protocol, and RNA was harvested 23 h post-transfection. For SmB knockdown, NIH 3T3 cells (1 × 10^5) were plated in 6-well plates 24 h pre-transfection. Cells were transfected with 50 pmol control (Ambion) or snrpb
Silencer Select siRNA using lipofectamine RNAiMAX (Invitrogen) as per the manufacturer’s protocol, and RNA was harvested 24 h post-transfection.

**Immunoprecipitation.** For snRNA immunoprecipitation, NIH 3T3 cells were plated and transfected as performed for SmD3 transient knockdown. 23 h post-transfection, cells were harvested in lysis buffer (50 mM Tris-Cl pH 8, 150 mM NaCl, 0.5% Nonidet P-40) containing 1 mM phenylmethylsulfonyl fluoride and 1 × Protease Complete inhibitor mixture (Roche) and incubated on ice for 30 min. Cells were sonicated with five 5-second pulses using a Branson Sonifier 250, incubating on ice for 20 sec between each pulse. Cell lysates were centrifuged at 15,000 × g for 30 min at 4°C to remove insoluble material. Cell lysates were immunoprecipitated using α-Sm Y12 antibody (ab3138, Abcam, 1:100) or IgG control. RNA was isolated from immunoprecipitated samples using TRIzol (Invitrogen) and reverse transcribed to cDNA using the SuperScript III First-strand Synthesis System for RT-PCR (Invitrogen) following the manufacturer’s instructions. Quantification of α-Sm immunoprecipitated snRNA expression relative to control IgG precipitated snRNA was performed by qRT-PCR.

**Superoxide Detection in vitro.** Cells (8 × 10^4) were plated in 96-well opaque white microplates 24 h prior to treatment. Cells were incubated for 1 hr in reaction buffer containing 125 mM KCl, 10 mM HEPES, 5 mM MgCl_2_, 2 mM K_2_HPO_4_, and 5 μM 2-methyl-6-(4-methoxyphenyl)-3,7-dihydroimidazol[1,2-A]pyrazin-3-one, hydrochloride (MCLA) during continuous treatment (palmitate) or rinsed with PBS and incubated in reaction buffer immediately following treatment (doxorubicin). MCLA light emission was
quantified using a Tecan Infinite M200 microplate reader and iControl software. The photomultiplier was set with an integration time of 1000 ms. The MCLA signal was quantified in real-time or as an integral of the signal measured every 30 sec over 3 min.

**Real-time Reactive Oxygen Species Detection in vitro.** Cells \((5 \times 10^4)\) were plated in 12-well plates 24 h prior to CM-H\(_2\)DCFDA loading. Cells were rinsed with PBS and incubated with PBS containing 0.5 mM MgCl\(_2\), 0.92 mM CaCl\(_2\), and 10µM CM-H\(_2\)DCFDA in the dark at 37°C for 10 min. Cells were then rinsed with PBS prior to various treatments in PBS containing 0.5 mM MgCl\(_2\) and 0.92 mM CaCl\(_2\). Mean fluorescence was quantified every 3 min with excitation/emission wavelengths of 492/517 nm using a Tecan Infinite M200 microplate reader and iControl software.
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<td>ACA28 SLRT</td>
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<td>U12 forward</td>
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<td>U4atac forward</td>
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<td>U4atac reverse</td>
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<td>5'RACE ROSAβgeo reverse</td>
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<td>siRNA snrpD3 sense</td>
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<td>snrpD3 antisense</td>
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<td>scrambled control sense</td>
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<tr>
<td>scrambled control antisense</td>
<td>5'-AACAUCUAUGCUCUA-3'</td>
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<td>LNA/DNA oligos GFP</td>
<td>5' +T*+C*+A*+<em>C</em>T<em>C</em>A<em>C</em>C<em>T</em>C<em>T</em>+C*+A*+<em>C</em>T<em>C</em>+T-3'</td>
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<tr>
<td>U32a</td>
<td>5' +G*+C*+G*+G*+T*+G<em>C</em>A<em>T</em>G*+G*+T*+G*+T*+C*+T*+C 3'</td>
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<tr>
<td>U33</td>
<td>5' +T*+G*+G*+T*+A*+G<em>T+C+C</em>A*+T*+G*+A*+G*+T*+C*+T*+C 3'</td>
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<td>U35a</td>
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<td>snrpD3</td>
<td>5' -T*+C*+G*+G*+T*+A*+G<em>T</em>C+C<em>A</em>T<em>G</em>+G*+T*+A*+G*+T*+C*+G*+G 3'</td>
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<td>RNase protection &amp; FISH CHO U32 forward</td>
<td>5'-TAC TGG GTA AGT TTC ATT CAG-3'</td>
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<td>5'-GTA ATA CGA CTC ACT ATA GGG AGG AAG GAG TCC AGG AGG G-3'</td>
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<td>CHO U33 forward</td>
<td>5'-GGG TGC CAT GGA GAA TGG G-3'</td>
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<td>5'-GTA ATA CGA CTC ACT ATA GGG AAG</td>
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<td>CHO U34 forward</td>
<td>5’-GCA AGC CTA GCT TTC CAC AG-3’</td>
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<td>CHO U35 forward</td>
<td>5’-TTG CAG AGT GGT CTA GGT GG-3’</td>
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<tr>
<td>FISH mouse U3 forward</td>
<td>5’-TGT AGA GCA CCC GAA ACC AC-3’</td>
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<tr>
<td>mouse U3 reverse</td>
<td>5’-TCC ACT CAG ACT GCG TTC C-3’</td>
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<td>mouse rpL13a intron 1 forward</td>
<td>5’-AAT TAA CCC TCA CTA AAAG GGA GCA ATA AAC AGG GTG GCT GT-3’</td>
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<tr>
<td>mouse rpL13a intron 1 reverse</td>
<td>5’-GTA ATA CGA CTC ACT ATA GGG TCC TCA GAT GCT CAA GCA GA-3’</td>
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<td>Primer extension</td>
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<td>U32a, U33: 18S target</td>
<td>5’- GTAACTAGTTAGCATGCCAGAGTCTCG -3’</td>
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<td>U32a: 28S target</td>
<td>5’- GCTACGGACCTCCACCAGAG-3’</td>
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<td>U35a: 28S target</td>
<td>5’-TCGTACTGAGCAGATTACCATGGC-3’</td>
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<td>Z17a, Z17b, U45a, U45b: 18S target</td>
<td>5’- CCCGTCGGCATGTATTAGCTCTAG-3’</td>
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*, phosphorothioate linkage; +, LNA residue
REFERENCES


Sm core domain that binds a transport receptor independently of the m3G cap. EMBO J 12:573-583.


65. **Lyman SK, Gerace L, Baserga SJ.** 1999. Human Nop5/Nop58 is a component common to the box C/D small nucleolar ribonucleoproteins. RNA **5**:1597-1604.


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