LSM1 AND RNY1: CLUES IN THE SEARCH FOR HOW RNA METABOLIC PATHWAYS CONTROL CANCER

by

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ABSTRACT

Carcinogenesis requires numerous alterations to gene expression to evade normal controls on cellular growth, invasion, and immortality. Traditionally, these changes have been examined in the context of deregulated transcriptional control of oncogenes and tumor suppressors. However, in recent years, research has revealed that processes outside of transcription such as RNA splicing, translation, and decay are also deregulated in cancers, sustaining tumorigenic potential. This dissertation details our investigation into the cellular functions of two RNA metabolic proteins whose human orthologs are deregulated in tumors: a putative oncogene, Lsm1, and a putative tumor suppressor, Rny1. Herein, we reveal interesting functions for these proteins that might provide insight into their roles in carcinogenesis. First, we demonstrate a role for Lsm1 over-expression in altered splicing through depletion of U6 snRNA levels. Second, we clarify the mechanism for Rny1’s activity against RNA substrates and identify cis regions required for its non-catalytic role in growth inhibition. Overall, this knowledge expands our understanding of how RNA metabolism might be deregulated in cancer and could provide novel pathways to target for synthetic lethal responses in cancers with altered expression of these proteins.
DEFINITION OF DISSERTATION FORMAT

To comply with the guidelines for the *Manual of Theses and Dissertations* of the University of Arizona Graduate College, I present my work here in two chapters and five appendices. The first chapter provides an introduction to the two problems addressed in my studies and introduces a published review of the literature, appended as Appendix A. The second chapter presents and summarizes results of the two studies. These studies are addressed in complete detail as an appended, published paper on Lsm1 (Appendix B) and a manuscript in preparation for studies of Rny1 (Appendix C). I have included an additional appendix (Appendix D) to address remaining experimental questions to ask in future studies of Rny1. Permissions for copyrighted materials are included in Appendix E.

My individual contributions to the individual publications and manuscripts are delineated as follows:

Appendix A is my own work in its entirety.

Appendix B is my own work in its entirety.

Appendix C is my own work in its entirety.

All of these studies were guided and shaped by Roy Parker, my mentor, and were influenced by discussions and recommendations from past and present members of the lab.
CHAPTER I: INTRODUCTION

Rationale for studies of Lsm1 and Rny1

In order to understand the contribution of RNA metabolism to cancer, we selected the yeast proteins Rny1 and Lsm1, two RNA metabolic factors whose human orthologs are altered in expression in certain tumors. Initially, we investigated the role of Lsm1 whose over-expression has been demonstrated in multiple tumor types, including over 80% of pancreatic cancers (Schweinfest, Graber et al. 1997). Prior publications revealed that increased Lsm1 expression affects proliferation, anchorage-independent growth, and growth factor requirements (Kelley, Brown et al. 2000; Fraser, Watson et al. 2005; Streicher, Yang et al. 2007). Consistent with a model for Lsm1-induced carcinogenesis, in vivo targeting of Lsm1 in pancreatic tumor models decreased tumor establishment, tumor volumes and metastases, and improved survival in a hepatic metastasis model (Kelley, Brown et al. 2000; Yan, Rubinchik et al. 2005; Yan, Rubinchik et al. 2006). While studies in yeast and humans have investigated the effects of Lsm1 on mRNA stability, a model for how increased levels of Lsm1 predispose the cell to transformation was lacking.

We hypothesized that two functions could be affected by Lsm1 over-expression (depicted in Figure 1.2). First, Lsm1 over-expression might interfere with the stochiometry of the cytoplasmic decay machinery. Evidence for this idea originates from Lsm1’s role in regulating degradation of mRNAs in both human and yeast cells through associations with Lsm2-7 in the cytoplasm (Boeck, Lapeyre et al. 1998; Bouveret, Rigaut et al. 2000; Tharun, He et al. 2000; Ingelfinger, Arndt-Jovin et al. 2002; Sheth and
Parker 2003)). In this manner (see Figure 1.1), a polyadenylated mRNA is shortened 3’-to-5’ in a rate-limiting process involving deadenylases until it reaches an oligoadenylated length (7-16 nucleotides) (Decker and Parker 1993) at which time the Lsm1-7 complex can then bind to the 3’end of mRNA transcripts, interacting with decapping factors at the 5’ end to ensure efficient mRNA decay. Thus, over-expression might somehow alter the constitution or number of cytoplasmic Lsm1-7 complexes, altering mRNA decay.

Alternatively, Lsm1 might also alter the balance between cytoplasmic Lsm1-7 complexes and nuclear Lsm2-8 complexes. This idea is supported in that Lsm2-7 also associate with Lsm8 in a similar complex that does not contain Lsm1 (Mayes, Verdone et al. 1999). This complex binds and regulates U6 snRNA to coordinates splicing (Mayes, Verdone et al. 1999; Pannone, Kim et al. 2001). Thus, over-expressing Lsm1 might squelch Lsm2-7 factors from their association with Lsm8 in the nucleus, inhibiting U6 snRNA protection by these factors and hence splicing.

Given Lsm1’s possible effects on splicing and mRNA decay, we proposed to investigate whether mutations to these pathways might display synthetic lethal responses with Lsm1 over-expression in yeast. Hence, our studies might reveal novel factors that could be manipulated to elicit a synthetic lethal response under these conditions in human tumors over-expressing human Lsm1.

The second RNA regulatory protein examined, RNASET2, the human ortholog of yeast Rny1, is a T2 ribonuclease whose expression is decreased in certain tumors and has been proposed to act as a tumor antagonizing malignancy suppressor gene (Acquati, Bertilaccio et al.; Acquati, Possati et al. 2005). T2 enzymes are ubiquitously found in
organisms and regulate interesting aspects of cellular biology (for a review of these enzymes, see Appendix A). The role of RNASET2 in cancer is not well understood, but it appears to function in a catalytic-independent manner to prevent metastasis in certain cancers, presumably through its ability to stimulate competent immune cells (Acquati, Bertilaccio et al.). Since Rny1, its ortholog in yeast, also possesses catalytic and catalytic-independent functions (Thompson and Parker 2009), we proposed to study this protein as a model for catalytic and catalytic-independent roles of RNASET2. In this manner, we could rapidly assay genetic interactions and cis mutations to elucidate the mechanism for Rny1’s function, perhaps revealing how the human ortholog RNASET2 acts to maintain normal tissue growth and architecture through nuclease and nuclease-independent activities.
Figure 1.1: Lsm1 functions in mRNA decay

This figure depicts a major pathway for Lsm1-mediated decay in yeast and human cells. Lsm1 binds to the 3’ end of mRNAs in the cytoplasm once poly(A) shortening to an oligoadenylated length (10-12 residues) is achieved. Through interactions with factors at the 5’ end, Lsm1 enables the decapping of mRNA which is followed by exonucleolytic decay in the 5’ to 3’ direction.
Figure 1.2: Two possible models

This figure illustrates the possible models investigated in seeking the effects of Lsm1 over-expression on cellular growth. The left panel shows possible effects of Lsm1 on stochiometry of cytoplasmic Lsm1-7 complexes, and the right panel presents a model for squelching of Lsm2-7 from the nuclear Lsm complex.
CHAPTER II: PRESENT STUDY

Summary of studies clarifying the effects of Lsm1 over-expression

In order to understand Lsm1 over-expression in human tumors, we examined the effects of yeast Lsm1 over-expression, screening for synthetic interactions with yeast gene deletion strains disrupted for RNA metabolic pathways. These screens revealed that Lsm1 over-expression achieved the strongest inhibition of growth when U6 snRNA function is compromised. Our experiments linked growth inhibition by Lsm1 over-expression to depletion of U6 snRNA, likely through squelching of Lsm2-7 factors of the nuclear Lsm complex whose function affects U6 snRNA and RNA splicing. In addition, Lsm1 over-expressing cells were sensitive to inhibition of deadenylase function, and these effects could not be wholly attributed to U6 snRNA depletion, suggesting a possible effect of Lsm1 over-expression on mRNA decay. Taken together, this study identified two possible pathways, consistent with our initial hypotheses, which might be targeted to elicit synthetic lethal responses in human tumors over-expressing Lsm1. Additional details concerning the methods, results, and a conclusion for this project have been published and are appended to this document as Appendix B.
Summary of studies to elucidate T2 ribonuclease functions

RNASET2, the only identified T2 ribonuclease in humans, inhibits tumor establishment and possesses nuclease and nuclease-independent functions. In order to understand this protein’s roles, we analyzed the nuclease and nuclease-independent properties of yeast Rny1, also a T2 ribonuclease. To elucidate Rny1’s functions, we sought to identify cis regions conferring non-catalytic cytotoxicity and trans factors required for catalytic function. First, we asked whether specific cis regions of Rny1 might be involved in interactions with other factors (e.g. RNA, lipids, or proteins) and thus be necessary for cytotoxic effects observed with Rny1 over-expression. To probe for regions required for non-catalytic cytotoxicity, we made cis mutations in Rny1 and over-expressed these in a yeast strain whose growth was sensitive to these effects, looking for regions of Rny1 required for the growth inhibition. These studies revealed that the N-terminal peptide sequence, the conserved T2 core, and the unique C-terminal domain of Rny1 all contribute to the non-catalytic cytotoxicity.

Second, we predicted that trans factors required for Rny1’s catalytic function might regulate vesicular trafficking and/or RNA quality control pathways. To examine these possibilities, we analyzed tRNA and rRNA fragments generated by Rny1 in strains mutant for aspects of autophagy, ribophagy, RNA quality control, multi-vesicular body (MVB) formation, and vacuole fusion. We identified factors affecting vacuolar fusion, vacuolar acidification, multi-vesicular body formation, and microautophagy that influence accumulation of tRNA and rRNA fragments during nutrient-limited growth. We propose that a microautophagy/vacuole fusion-like process and an MVB-dependent
process both function in parallel to control translation during stress in an autophagy-independent manner. Additional details on methods, results, and a conclusion for this project are appended as Appendix C.
APPENDIX A: T2 FAMILY RIBONUCLEASES: ANCIENT ENZYMES WITH DIVERSE ROLES

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T2 Family ribonucleases: ancient enzymes with diverse roles

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Ribonucleases of the T2 family are found in the genomes of protozoans, plants, bacteria, animals and viruses. A broad range of biological roles for these ribonucleases have been suggested, including scavenging of nucleic acids, degradation of self-RNA, serving as extra- or intracellular cytopathins, and modulating host immune responses. Recently, RNaseT2-family members have been implicated in human pathologies such as cancer and parasitic diseases. Interestingly, certain functions of RNaseT2-family members are independent of their nuclease activity, suggesting that these proteins have additional functions. Moreover, human homologs of RNaseT2 manifest a defect in neurodevelopment, perhaps due to aberrant control of the immune system. We review the basic structure and function of RNaseT2-family members and their biological roles.

The T2 family, an interesting subclass of ribonucleases

Ribonucleases catalyze the cleavage of RNA, acting on the single-stranded (ss), double-stranded (ds), and DNA–RNA hybrid substrates that are ubiquitous components of cells. A great deal of research has focused on specific ribonucleases that function in a variety of cellular processes, including DNA synthesis, RNA processing, cytoplasmic or nuclear RNA degradation, RNA, and antiviral defense. However, cells also produce a set of general RNases that are typically secreted or targeted to membrane-bound compartments, such as the lysosome or vacuole. Such enzymes include members of the RNaseA, RNaseT1, and RNaseT2 families (Table 1). Interestingly, recent work suggests that RNaseA (reviewed in Ref. 1) and RNaseT2-family members often play important roles in a variety of biological settings. Here, we focus on ribonucleases of the T2 family, a specific subclass of endoribonucleases that cleave ssRNA and exhibit diverse functions important to the biology of prokaryotes, eukaryotes, and human diseases.

Properties of T2 family ribonucleases

Ribonucleases of the T2 family are transferase-type RNases and are classified by their similarity to the RNase T2 protein from Aspergillus oryzae [2,3]. The other major families of transferase-type RNases are the RNaseA and T1 protease families. All of these enzymes catalyze the cleavage of ssRNA through a 2',3'-cyclic phosphate intermediate, producing monoo- or oligonucleotides with a terminal 3' phosphate group. Three features distinguish T2 ribonucleases from the RNaseA and RNaseT1 protease families. First, T2 ribonucleases are distributed more widely and are found in protozoans, plants, bacteria, animals and viruses [3]. By contrast, RNaseT1-family enzymes exist only in bacteria and fungi, and RNaseA-family enzymes are highly represented in animals. Second, the optimal pH of activity of many T2 ribonucleases is 4.5–6, contrasting with the 5.5–7 pH of RNaseA or the more acidic (pH 4.5–6) activity of RNaseT1-family enzymes [2,3]. This acidic activity of T2 enzymes is consistent with their localization to the vacuole or lysosome, and suggests that cleavage of self-RNAs within an acidic compartment could be one function of these ribonucleases. Third, the T2-family ribonucleases generally cleave at all four bases, whereas the A-family tends to be specific for pyrimidine RRs and the T1-family members tend to be specific for guanine RRs [2,3].

The RNaseT2-family members are typically secreted from the cell or localized to internal compartments such as the lysosome or vacuole [2,3]. As these proteins enter the secretory pathway, they are generally glycosylated in eukaryotic cells [2,3]. However, there are cases where RNaseT2 proteins enter the cytoplasm. For example, in budding yeast, the RNaseT2 protein Ryl1 is released from the vacuole into the cytosol during oxidative stress [4,5]. Moreover, in some cases, secreted T2 ribonucleases can be internalized by other cells in trans, a process which is often cytotoxic to the target cell. Furthermore, release of T2 ribonucleases from internal membrane-bound compartments correlates with cleavage of some RNA substrates [4,5]. Thus, compartmentalization of T2 ribonucleases and their subsequent release into the cytosol might play an important role in modulating their activity.

Structure and catalysis by T2 ribonucleases

The structure and mechanism of RNA cleavage by T2 ribonucleases is well understood. A variety of crystal structures of T2-family members from bacteria, plants, and fungi have revealed a conserved α/β core structure, as observed in the structure from the fungal ribonuclease Rh from Rhizopus rouxii (Figure 1a) [2,19]. Substrate binding regions have been identified by X-ray crystallography in RNaseT2-family members with mono- or dinucleotides; two conserved regions, named B1 for sites 5' and B2 for sites 3' of the scissile bond, can be occupied by nucleotides and presumably function to position the phosphate bond to be cleaved in the active site of the enzyme [2,3,12,16,19].

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Catalysis is promoted by one or three histidine residues that are found in two blocks of conserved amino acids (designated CAS1 and CAS2) located in the active site of the enzyme [20,21]. In several proteins, altering these histidines through DNA mutagenesis or chemical modification can inactivate the RNase activity both in vitro [22-28] and in vivo [4]. The ability to inactivate the catalytic ability of these proteins specifically provides a genetic mechanism to distinguish if functions of RNaseT2 proteins are dependent on their catalytic ability. Similar to other transferase ribonucleases, RNA degradation by T2 enzymes occurs in two steps: transphosphorylation and hydrolysis (Figure 1b) [15]. Two histidines are required to perform acid-base catalysis at each of these steps, with these histidines reversing roles as acid and base at each step [5].

C-terminal extensions in certain T2 ribonucleases
Some RNaseT2 family members contain C-terminal extensions downstream of the conserved T2 region. For example, one of the envelope glycoproteins produced by several positiveviruses is a glycoprotein termed Env, with an RNaseT2 domain and ribonuclease activity, as well as a 97KDa C-terminal extension [29,30]. This EnvC-terminal extension functions to allow receptor-independent translocation across cellular membranes due to its high positive charge, which is similar to the manner in which some other cytotoxic RNases enter cells [31,32]. Thus, the C-terminal region of positiveviruses T2 ribonucleases could be a means of delivering the RNA to immune cells and altering their response to infection.

In another case, a C-terminal extension with regions of conservation exists in three T2 ribonucleases of Basidiomycetes fungi and in RoV1 of budding yeast [33,34]. In the Basidiomycetes fungi, this region contains a conserved serine-threonine-rich region, which resembles that of fungal glycine-rich proteins, followed by a 10KDa C-terminus [33]. As fungal glycine-rich proteins contain a substrate-binding domain downstream of their serine-threonine-rich region, it has been hypothesized that the 10KDa C-terminal region might contain a substrate-binding site [33].

Biological roles for T2 ribonucleases
T2 ribonucleases are present in organisms across kingdoms and exhibit a conserved C-terminal extension. T2 ribonucleases have been suggested to perform a variety of functions in different organisms, including scavenging of nucleic acids, degradation of self-RNA, modulating host immune response, and serving as extracellular cytokotixin. Interestingly, some of these roles appear to be independent of the ability to hydrolyze RNA.

Scavenging phosphates/nucleic acids
Scavenging of extracellular nucleic acids for nutrients is one possible function for T2 ribonucleases (Figure 2a). In plants, severe T2 ribonucleases are induced during phosphate starvation and have been shown to provide phosphates from nucleic acids [35-37]. Specifically, in tomato (Solanum lycopersicum) cells, ribonuclease LE is one of several phosphatases that are induced upon phosphate starvation [37], and the extracellular activity of this T2 ribonuclease is increased during these conditions [36]. A phosphodiesterase with increased synthesis and secretion during phosphate starvation was identified, supporting a model for scavenging of extracellular RNA substrates during phosphate starvation [39]. Moreover, plants can scavenge phosphates from nucleic acids, as the inhibition of tomato cell growth due to phosphate starvation can be eliminated by providing yeast RNA in place of inorganic phosphate (KH₂PO₄) [39]; however, there is no direct evidence that this effect is due to the action of RNase LE. In another example, an RNaseT2 protein secreted by the pathogenic oomycete Peronospora parasitica cleaves phosphatases from this amoeba's host [40].

The presence of RNaseT2 proteins in the lysosome/vacuole seems likely to allow them to function also in the recycling of cytoplasmic RNAs that are delivered to the lysosome/vacuole during autophagy. One possible source of RNA substrates for autophagic degradation within an acidic compartment might be found in RNA granules. Aggregated proteins, for instance, can be removed by a selective form of autophagy involving specific receptors or bridging molecules that recognize ubiquitin and ubiquitin-like modifications on these cytoplasmic proteins [41]. Moreover, some cytoplasmic aggregates of proteins also contain RNA, suggesting that these might also be targeted by autophagy. One such example is provided by P-granules, aggregates of proteins and RNA found in germinal, but not somatic, cells during metasenescence development [42]. Specifically, autophagy, selectively mediated by two P-granule components, SFP/1 (suppressor of ectopic P granule in autophagy mutants-1) and PGL-3 (P-granule abnormality-3), interacting with the autophagy protein LGG4 is required for the degradation of certain P-granule components [43]. Hence, it is plausible to propose that mRNAs interacting with these proteins might also be targeted by autophagic processes and degraded by T2 ribonucleases within acidic compartments. Taken together, one likely role of RNaseT2 proteins is to degrade extracellular or intracellular RNAs to obtain nutrients.

A role in neural development: possible clearance of RNAs to modulate the immune response
Recent results have indicated that the human RNASET2 protein plays an important role in neurodevelopment,
which might be related to effects on the immune system. Specifically, mutations in RNASET2 were identified as the causative lesion in an autosomal recessive disorder leading to cystic leukoencephalopathy [64]. How the loss of RNASET2 causes this phenotype is not known, but the specific neurological abnormalities are indistinguishable from those seen in infants infected intratermally with congenital cytomegalovirus (CMV) [64]. This raises the possibility that CMV infection triggers a change in physiology similar to that induced by the loss of RNASET2. For example, CMV infection inactivates RNaseL, as a host evasion mechanism [65] and, as suggested by Henneke et al. [64], either RNaseL inactivation or loss of RNASET2 could lead to a failure to degrade either extracellular and/or intracellular sRNAs, which triggers the innate immune response with downstream consequences on development. An important area of research here will be the study of mice lacking RNASET2 to determine the contribution of this enzyme to mammalian immune function and neural development.

An RNASET2 in schistosome infection: a role in modulating host immune response

A more direct connection of T2 ribonuclease activity to the immune response is illustrated by the manner in which a secreted RNASET2 from the parasitic blood fluke Schistosoma mansoni affects host immune cells. During a human infection this worm lays its eggs in its definitive mammalian host, and these eggs secrete a factor termed omega-1, which is a known RNASET2 family member. Omega-1 provokes a host immune response that aids excretion of the egg and transmission to a molluscan intermediate host [46-48]. Recently, two groups showed that omega-1 is the major component in priming dendritic cells for Th2 polarization of CD4+ T cells during infection [49,50]. This function appears to depend on the RNase activity of omega-1, as inactivation of the ribonuclease activity of soluble egg extracts or omega-1 inhibited their Th2-polarizing function [50]. This finding suggests that omega-1 might be taken up by dendritic cells and affect their function; this idea also is supported by the observation that omega-1 can alter the cytoskeletal structure of dendritic cells [50].

RNASET2 family members as cytotoxic agents

In several cases, RNASET2 family members act as cytotoxic agents, both in trans on other cells or in cis, acting as an intracellular rather than a secreted protein. Interestingly, these cytotoxic roles can be both catalytic-dependent and catalytic-independent.

One example of RNASET2 proteins inducing cytotoxicity, and modulating immune cell function, comes from analysis of certain poxviruses, including classical swine fever virus (CSFV) and bovine viral diarrhea virus (BVDV) (Figure 2b). In these viruses, one of the envelope glycoproteins, termed E2, has an RNASET2 domain and ribonuclease activity, and is secreted by infected cells [29,30,51]. In cell culture experiments, this enzyme produced cytotoxic effects against the lymphocytes, but not epithelial cells, of various host species [29], suggesting that viable infected cells secrete E2 to dampen the host immune response. Consistent with this possibility, mutations inactivating the RNase activity of E2 do not affect viral replication in cell culture [63,64], but do reduce viral infection in animals [65,66]. Moreover, certain amino acid substitutions, but not all catalytically dead versions of the E2 protein, prevent the reduction of host immune cell levels typically seen during CSFV infection [67]. Taken together, these observations argue that E2 proteins in both catalytic and catalytic-independent manners as a trans-acting cytotoxin that dampens the immune response during viral infection.

Another example of RNASET2 family members as cytotoxic agents comes from the well-characterized sRNases (Figure 2b). S-RNases are found in plants of the Solanaceae, Rosaceae and Scrophulariaceae families, and function to maintain self-incompatibility in pollination [25,57] and other pollen rejection mechanisms [58].
pistil proteins that are secreted into the extracellular matrix [59,60] and enter both incompatible and compatible pollen tubes [61]. Within incompatibly pollinated styles, pollen RNA is degraded, and self-fermentation is disrupted [62]. By contrast, compatible pollen RNA is protected from degradation by S-RNase [63].

The nuclease activity of S-RNase is required to exert cytotoxic effects on incompatible pollen tube development [25,57,63]. However, initial studies of heat-inactivated S-RNase demonstrated greater inhibition than the intact protein against pollen tubes [60], perhaps implicating S-RNases in a catalytically independent function within other pollen rejection pathways.

The mechanism by which compatible pollen is protected from cytotoxicity is the subject of ongoing research. The pollen protein S-Locus F-box (SLF) is required for maintaining self-incompatibility [64,65] and S-RNase interacts with SLF, a putative ubiquitin epigene that has been proposed to target the T2 enzyme for proteasomal degradation [66]. However, other pistil factors and the regulated compartmentalization of S-RNase within an acidic compartment might also play a role in controlling the cytotoxicity of S-RNase [6]. Further research is necessary to ascertain how S-RNase activity is modulated to differentiate compatible and incompatible pollen.

Another T2 ribonuclease that exhibits a catalytic-independent function in cytotoxic responses is Rny1 (Figure 2d). During oxidative stress, Rny1 is released from the vacuole and cleaves cytoplasmic tRNA and rRNA [4,67]. Rny1 plays some role in modulating cell viability, as strains lacking Rny1 are sensitive to some triggers of cell death [34], and over-expression of Rny1 decreases cell growth and leads to hypersensitivity to oxidative stress [4]. Strikingly, the Rny1-dependent effects on cell viability are independent of its catalytic activity, as mutations that inactivate the nuclease function have an effect on cell
viability similar to that of the wild type protein [5]. Ryn1 is also secreted [64] but does not appear to be taken up by other yeast cells, suggesting its effects in this case occur only within the cell where it is produced [4]. Hence, increased levels of Ryn1 are cytotoxic to the cell during oxidative stress, independent of its catalytic activity.

Human RNASET2: possible roles in tumor progression and growth control

Evidence for a conserved, catalytic-independent function for T2 ribonucleases is provided by studies of RNASET2, the only T2 ribonuclease identified in humans [58,60]. RNASET2 is expressed as a tumor suppressor, as its expression is reduced in various ovarian tumors and cancer cell lines [59]; however, some groups have argued that this effect is not significant [70]. Moreover, introduction of RNASET2 into cells inhibits the clonogenicity of ovarian cancer cells in culture [60] and reduces metastatic potential [71]. Tumorigenesis [69], and tumor volume [72] in mouse xenograft models. Inactivation of the T2 enzyme through mutation or denaturation failed to block anti-carcinogenic effects [71,72], consistent with a cleavage-independent role for RNASET2 in tumor suppression. To date, mutations within the RNASET2 coding region have not been identified in cancers [59,70], and Hennecke et al. [64] noted that human subjects harboring mutations at the RNASET2 locus did not exhibit an increased susceptibility to cancer; however, the age (<20 years) of these subjects might preclude this phenotype. Thus, although it is clear that RNASET2 can affect cell transformation in a cleavage-independent manner, additional work will be required to determine how and if this activity contributes to tumor formation in mammals.

Possible cleavage-independent functions of RNASET2 and ACTIBIND

An unresolved issue is the mechanism by which RNASET2 proteins can affect cell function independent of their catalytic ability. One possibility is that these proteins can bind to and modulate the cytoskeleton, which is suggested by the analysis of ACTIBIND, a T2 ribonuclease from the mold Aspergillus niger, that, as well as some other RNaseT2 family members, can bind actin in vitro [72,73]. Interestingly, ACTIBIND inhibits the clonogenicity and savagiveness of various tumors in culture and in mouse models [73,74]. Both ACTIBIND and the catalytically inactive form of the ribonuclease reduced aberrant crypt formation (an early marker of colon cancer development) when they were administered locally to mouse colon along with dimethylhydrazine, which promotes the development of colon cancer [73]. Consistent with this being an effect on the cytoskeleton, ACTIBIND disrupts the actin network and the migration of cancer cells [75]. The interaction of RNaseT2 proteins with the actin cytoskeleton provides a possible mechanism for RNaseT-independent cytotoxicity. However, until a specific mutation disrupting the T2 ribonuclease-actin interaction can be identified and shown to affect the cytotoxicity, this remains an intriguing hypothesis.

Concluding remarks

RNaseT2 family members function in a surprising diversity of biological roles. Some of these functions rely on their activity as nucleases, and it will be important to determine if there is any specificity in the RNAs cleaved by T2 enzymes in cells. The use of cross-linking and immunoprecipitation (CLIP) in conjunction with high-throughput sequencing might enable the identification of novel substrates of T2 ribonucleases. By contrast, other functions appear to be independent of the nuclease activity of these enzymes, and understanding these nuclease-independent functions might reveal new, specific roles for RNaseT2 family members. The identification and characterization of novel proteins whose interactions might indicate how these enzymes function in a catalytic-independent manner. Moreover, mutational analysis of T2 enzymes might reveal the regions necessary for nuclease-independent functions. Finally, it will be of interest to understand how RNaseT2 proteins enter cells, either from extracellular pools, or from membrane-bound compartments within the cytoplasm; such studies might reveal novel mechanisms by which proteins cross membranes.

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APPENDIX B: LSM1 OVER-EXPRESSION IN *S. CEREVISIAE* DEPLETES U6 SNRNA LEVELS

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LSM1 over-expression in *Saccharomyces cerevisiae* depletes U6 snRNA levels

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**ABSTRACT**

Lsm1 is a component of the Lsm1-7 complex involved in cytoplasmic mRNA degradation. Lsm1 is over-expressed in multiple tumor types, including over 80% of pancreatic tumors, and increased levels of Lsm1 protein have been shown to induce carcinogenic effects. Therefore, understanding the perturbations in cell process due to increased Lsm1 protein may help to identify possible therapeutic targets for tumors over-expressing Lsm1. Herein, we show that LSM1 over-expression in the yeast *Saccharomyces cerevisiae* inhibits growth primarily due to U6 snRNA depletion, thereby altering pre-mRNA splicing. The decrease in U6 snRNA levels causes yeast strains over-expressing Lsm1 to be hypersensitive to loss of other proteins required for production or function of the U6 snRNA, supporting a model wherein excess Lsm1 reduces the availability of the Lsm1-7 proteins, which also assemble with Lsm8 to form a complex that binds and stabilizes the U6 snRNA. Yeast strains over-expressing Lsm1 also display minor alterations in mRNA decay and demonstrate increased susceptibility to mutations inhibiting cytoplasmic deadenylation, a process required for both 5′-to-3′ and 3′-to-5′ pathways of exonucleolytic decay. These results suggest that inhibition of splicing and/or deadenylation may be effective therapies for Lsm1-over-expressing tumors.

**INTRODUCTION**

Tumors of the pancreas pose a critical problem in eliminating mortality due to cancer in that incidence and morbidity rates for this disease are nearly equal (NCI SEER database, http://seer.cancer.gov/statfacts/html/pancreas.html). Even with recent technological advances in genomic analysis, the overall relative 5-year survival rate of pancreatic tumors from 1996 to 2004 was 5.1%, and trend analysis of the period from 2003 to 2005 revealed no significant changes in mortality rate (NCI SEER database, http://seer.cancer.gov/statfacts/html/pancreas.html). Given that the current prognosis for patients with these tumors is dismal, it is vital that we search for novel therapeutics targeting this disease.

In 1997, Lsm1 was identified through subtractive hybridization cloning in pancreatic cancer cells (1) and was shown to be over-expressed in 87% of pancreatic cancers. Subsequently, its over-expression has been described in 40% of prostate cancers (2), a subset (15–20%) of breast cancers that are amplified at the 8p11–12 region (3,4) and most recently in lung cancers and mesotheliomas (5). The direct involvement of Lsm1 in carcinogenesis in these tissues has been demonstrated through analyses of Lsm1’s effects on growth and anchorage dependence (2,6,7), contact inhibition (2), autocrine activity (7) and tumor establishment and metastases (2,6,8,9). The increase in Lsm1 levels in these tumors is moderate (about 2- to 5-fold) (7), suggesting that subtle changes in the levels of Lsm1 can affect the growth properties of mammalian cells. Thus, it is important to elucidate the processes affected by LSM1 over-expression in order to provide new targets for therapeutic development against pancreatic and other Lsm1-over-expressing cancers.

Lsm1 over-expression could affect cellular metabolism in several manners. For example, Lsm1 over-expression has been suggested to destabilize certain tumor suppressor transcripts, allowing for carcinogenesis (3). This model is based on the fact that Lsm1 in yeast and humans assembles with the Lsm2/Lsm7 proteins to form a heteroheptameric Lsm1-7 complex that binds mRNAs, components of the decapping machinery, and promotes mRNA decapping and degradation (10–14). Alternatively, Lsm1 over-expression might inhibit the function of the related Lsm2-8 complex, wherein the Lsm1 protein is replaced by the Lsm8 protein. The Lsm2-8 complex binds the 3′-end of the U6 snRNA protecting it from degradation and thereby allowing normal rates of pre-mRNA splicing (15–17). Consistent with Lsm1 over-expression affecting the nuclear Lsm2-8 complex, over-expression of Lsm1...
in budding yeast increased the cytoplasmic localization of Lam1p (18). Hence, over-expression of LSM1 may actually reduce U6 levels and selectively influence splicing, allowing for carcinogenesis.

To understand how Lam1 over-expression influences cell processes, we took advantage of the conservation of Lam1 function in both budding yeast and humans to determine how Lam1 over-expression affects RNA metabolism in yeast. We found that over-expression of LSM1 in the yeast Saccharomyces cerevisiae leads to defects in pre-mRNA splicing, which is caused by decreased levels of the U6 snRNA. The splicing defect causes yeast strains over-expressing Lam1 to be hypersensitive to loss of other components required for maintaining levels of U6 snRNA. Moreover, yeast strains over-expressing Lam1 are more susceptible to mutations inhibiting cytoplasmic deadenylatation, which is normally a prerequisite for mRNA decay. These results suggest that inhibition of splicing and/or deadenylation may be effective therapies for LSM1-over-expressing tumors.

MATERIALS AND METHODS

Yeast strains, growth conditions and plasmids

The genotypes of the strains used are listed in Supplementary Table S3. Cells were cultured in either yeast extract-peptone medium or synthetic medium supplemented with appropriate amino acids and 2% sugar (sucrose or galactose) and were grown at 30°C. Yeast strains were transformed as previously described (19) and maintained in the appropriate selective media. Over-expression studies were performed by culturing strains continuously in galactose. Plasmids utilized in this study are found in Supplementary Table S4.

Plasmid construction

The GAL LSM1 2µ plasmid was constructed by amplifying the LSM1 coding region 64 nt prior to its start through 240 nt following the stop, A BamHI restriction site immediately upstream of the 5'-end and a SalI restriction site at the 3'-end of this product facilitated its ligation to a GAL 2µ vector (pRP861) and placed the LSM1 gene under transcriptional control of a GAL promoter. The GAL LSM1 CEN plasmid was constructed by amplifying the LSM1 coding region 59 nt prior to its start through 240 nt following the stop. A SacI restriction site immediately upstream of the 5'-end and a SalI restriction site at the 3'-end of this product facilitated its ligation to a GAL CEN vector (pRP23) and placed the LSM1 gene under transcriptional control of a GAL promoter.

RNA analysis

RNA analyses were performed as previously described (20). Total RNA was isolated (21) from midlog cultures grown in appropriate media, and 20µg of each sample were electrophoresed on 6% acrylamide, 8 M Urea gels. Northern blots were performed using the indicated oligonucleotides radiolabelled with 32p at the 5'-end.

Oligonucleotides used in this study are detailed in Supplementary Table S5.

For decay time course experiments, transcriptional shutoff was achieved by resuspending galactose-induced cultures in media containing 4% dextrose (22) and then collecting samples over a brief time course.

Protein analysis

Midlog cultures were collected and harvested for protein analysis. Samples were lysed using 5 M urea, boiled, then vortexed in glass beads for 5 min. A solution of 125 mM Tris-Cl pH 6.8, 2% SDS was added to 2.5x the volume of 5 M urea used, and this was vortexed into the mixture, then samples were boiled a second time. The lysate was clarified by spinning at 16,000 RCF, and the supernatant was resuspended in protein loading buffer (0.05 M Tris pH 6.8, 1% SDS, 0.01% bromophenol blue, 10% glycerol), boiled, and run on a 12% Tris-SDS acrylamide gel. Gels were transferred to nitrocellulose and probed using standard Western blotting protocols using an antibody to Lam1 (a generous gift of Allen Sachs and Karsten Wei), and an anti-rabbit secondary coupled to HRP (Pierce). Lam1 signal was revealed using Pierce SuperSignal West Dura and exposing the blots to film and developing in a film processor (Konica).

Films were scanned into .tif format using an HP Scanjet Pro flatbed scanner, and images were analyzed and quantified in Adobe Photoshop following the method outlined at http://www.lukemiller.org/journal/2007/08/quantifying-western-blots-without.html. References from Supplementary Tables are Table S3 (23–25) and Table S4 (26–28).

RESULTS

LSM1 over-expression in yeast can affect cell growth

In order to understand the effects of LSM1 over-expression in budding yeast, we first expressed LSM1 from the GAL promoter on a CEN plasmid in a wild-type yeast strain. To determine the degree of over-expression, we utilized antisera against Lam1 to determine the increase in the Lam1 protein levels as compared to a vector only control strain. We observed that strains transformed with a GAL-LSM1 centromere plasmid showed ~3x the levels of wild-type yeast strains (Figure 1A and B), an increase in levels similar to what is seen in various human tumor cell lines. We observed that cells carrying this plasmid exhibited a slight decrease in growth as compared to the same strain carrying the vector backbone, although the strains were still able to grow at some rate (Figure 1C, uppermost panel). Thus, over-expression of LSM1 inhibits, but does not completely prevent, the growth of wild-type yeast strains.

LSM1 over-expression shows genetic interactions with proteins affecting the U6 snNP and mRNP deadenylases

There are two likely possibilities for how LSM1 over-expression might affect cell function. First, since the Lam1L complex functions in the control of mRNA
Figures 1 and 2. Over-expression of the LSM1 gene.

**Figure 1.** Moderate levels of Lsm1 affect cell growth. (A) LSM1 over-expression on a GAL CEN plasmid yields 2- to 4-fold protein expression. (B) Quantitative analysis of Lsm1 over-expression. (C) LSM1 over-expression on a GAL CEN plasmid affects growth and yields synthetic growth defects with strains mutant for U6 snRNP function, (A, B, C) OE CEN = Lsm1 expressed on GAL CEN plasmid (pRP1831), V = CEN vector (pRP33). (A) Western blot. Strains were transformed as indicated. Lsm1 protein levels were normalized to a control strain. Error bars represent standard deviation. (C) BY4741(WT) and isogenic deletion strains transformed as indicated and were grown on YPD medium. (D) Over-expression on a GAL CEN plasmid in 293T cells transfected with both plasmids. (E) Over-expression on an integrated plasmid in 9L cells.

**Figure 2.** LSM1 over-expression affects cell growth. (A) LSM1 over-expression on a GAL 2 μ plasmid affects growth of wild-type yeast. (B) LSM1 over-expression on a GAL 2 μ plasmid yields synthetic growth defects with strains mutant for U6 snRNP or (C) deoxyribonuclease functions, BY4741(WT) and isogenic deletion strains transformed with either the GAL-LSM1 2 μ plasmid construct (pRP1830, OE) or GAL 2 μ plasmid control (pRP8661, V) were grown on YPD medium. (D) Over-expression on a GAL CEN plasmid in 293T cells transfected with both plasmids. (E) Over-expression on an integrated plasmid in 9L cells.

In order to extend this genetic analysis, we decided to over-express Lsm1 from the GAL promoter on a multi-copy 2 μ plasmid, thereby increasing the probability of us detecting additional genetic interactions. We observed that over-expression from the GAL 2 μ plasmid gave a stronger inhibition of growth than the GAL centromere plasmid (compare Figure 1C uppermost panel and Figure 2A), although the cells do still grow at a reduced rate (Figure 2A). Using this system of expressing LSM1 from the GAL 2 μ plasmid, we then examined Lsm1 over-expression in a variety of yeast strains lacking non-essential proteins that function in pre-mRNA splicing or mRNA degradation. A complete list of these mutants and their phenotypic effects observed with LSM1 over-expression is provided in Supplementary Tables S1 and S2.

Our broader genetic analysis revealed that factors interacting with U6 snRNA were indeed important for growth when Lsm1 was over-expressed. Expressing LSM1 from the GAL 2 μ plasmid still demonstrated strong synthetic growth defects with strains deleted for LSM7 or LHP1 (Figure 2B). Furthermore, snu6Δ, which removes a
component of the U4/U6/U5 tri-snRNP (33,34), also showed a strong exacerbation of the growth defect observed when LSM1 was over-expressed from the GAL 2 μ plasmid (Figure 2B). Taken together, these results suggest that LSM1 over-expression may interfere with growth by inhibiting U6 snRNA biogenesis or function.

We also observed that over-expressing LSM1 displayed strong synthetic growth defects with deletions of the CCR4 and POP2 genes, which are key components of the predominant mRNA deadenylase (35). As seen in Figure 2C, expression of GAL-LSM1 on a 2 μ plasmid in strains deleted for CCR4 or POP2 inhibited growth more strongly than over-expression in a wild-type strain (Figure 2A). Significant synthetic growth defects were not demonstrated with deletions in many other decay factors (Supplementary Table S1), including strains deleted for F451, DHH1, and EDC3 (Figure 2C), which encode factors that enhance decapping and/or translational repression (15,22,36,37). The enhanced toxicity of Lsm1 over-expression in the ccr4Δ and pop2Δ strains suggests that mRNA decay might be altered when Lsm1 is over-expressed, increasing the relative requirement for deadenylation.

**LSM1 over-expression induces defects in splicing**

Since strains lacking LSM7, LHP1, and SNU66 are defective for U6 or U4/U6/U5 snRNP function (29,32,34), we hypothesized that LSM1 over-expression might further interfere with splicing in these deletion strains. To assess the effects of Lsm1 over-expression on splicing we examined the accumulation of the intron-containing precursor to the U3 snoRNA, which is a sensitive measure of splicing in budding yeast (29). In this analysis, we also included the cer4Δ and pop2Δ strains, in case these mutations had some previously unobserved effect on splicing. For this experiment, cells containing the GAL-LSM1 2 μ plasmid, or a vector control, were continuously grown in galactose, cells were harvested at mid-log, and the RNA was analyzed on northern blots for the U3 snoRNA.

We observed that over-expression of Lsm1 in a wild-type strain did not lead to accumulation of the intron-containing precursor to the U3 snoRNA (pre-U3 snoRNA). However, over-expression of Lsm1 in an lsm7Δ strain, increased the accumulation of the pre-U3 snoRNA seen in this strain (Figure 3A, compare lanes 3 and 4). Similarly, a nud6Δ strain accumulated small amounts of the pre-U3 snoRNA, and these levels increased with LSM1 over-expression (Figure 3A, compare lanes 7 and 8). In an lhp1Δ strain, no pre-U3 snoRNA was evident, and small amounts accumulated with Lsm1 over-expression, which were visible on longer exposures (Figure 3A, compare lanes 5 and 6). Finally, cer4Δ and pop2Δ strains failed to accumulate pre-U3 snoRNA with or without LSM1 over-expression (Figure 3A, examine lanes 9 through 12). Moreover, in a similar analysis, over-expressing LSM1 on the GAL CEN plasmid demonstrated similar synthetic splicing defects in strains deleted for LSM7 or LHP1 as compared to vector only controls (Figure 3B, compare lane 3-4 and lane 5-6). These results indicate that Lsm1 over-expression can lead to defects in splicing, even at moderate levels of over-expression as observed in tumors (7), which are most easily revealed in strains lacking proteins affecting U6 snoRNA biogenesis or function.
**LSM1** over-expression leads to a decrease in U6 snRNA levels

A simple hypothesis for how Lsm1 over-expression leads to defects in splicing is that Lsm1 over-expression reduces the levels of the Lam2-8 complex, which binds to and stabilizes the U6 snRNA (17,29). This hypothesis predicts that the levels of U6 snRNA should be reduced by Lsm1 over-expression. To test this prediction, we examined the levels of U1, U2, U4, U5 and U6 snRNAs in both wild-type and various mutant strains over-expressing Lsm1.

We observed that over-expressing LSM1 on a GAL 2 μ plasmid had little effect on U1, U2, U4 and U5 snRNA levels (Figure 3C and data not shown), but U6 levels were decreased in all cells over-expressing Lsm1. Specifically, in wild-type cells over-expressing LSM1 on a GAL 2 μ plasmid, U6 snRNA levels decreased by an average of 2.5-fold (Figure 3C, compare lane 2 to 1 and histogram), and this level of U6 depletion was also observed when moderately over-expressing LSM1 on a GAL CEN plasmid (data not shown). Moreover, and consistent with the genetic interactions, U6 levels were most depleted (4.5-fold less on average, Figure 3C histogram) in an Lsm7 deletion background over-expressing LSM1 on a GAL 2 μ plasmid when compared to wild-type expressing a vector control (Figure 3C, examine lanes 3 and 2 and histogram).

In these two instances, the results imply that LSM1 over-expression exerts its inhibition of growth through a reduction of U6 levels, yielding an inhibition of splicing. However, the levels of U6 snRNA in lhp1Δ, smu66Δ, ccr4Δ, and pop2Δ strains over-expressing LSM1 on a GAL 2 μ plasmid were not consistently lower than that of a wild-type strain over-expressing LSM1. These results suggest that the consequences of Lsm1 over-expression on growth can be made more significant without further reductions in the U6 level. In the case of the smu66Δ, this effect may be due to the assembly of a defective U4/U6/U5 tri-snRNP, whereas the growth inhibition in the ccr4Δ and pop2Δ strains may be due to defects in mRNP decay (see ‘Discussion’ section).

Depletion of U6 is responsible for synthetic growth and splicing defects in wild-type, lam7Δ and lhp1Δ strains over-expressing LSM1

The decrease in U6 snRNA levels with Lsm1 over-expression suggests that the inhibition of growth in wild-type and possibly various mutant strains could be due to the decreased U6 snRNA levels. This hypothesis predicts that the growth and splicing defects due to Lsm1 over-expression should be reversed by increasing levels of U6. To test this possibility, we introduced a high-copy plasmid expressing U6 snRNA into each strain and examined its effects on growth and splicing in the presence and absence of Lsm1 over-expression from a GAL 2 μ plasmid.

Our results revealed that at least some of the growth defects due to LSM1 over-expression are attributable to low levels of U6 snRNA. First, over-expression of U6 snRNA suppressed the growth defect seen in wild-type cells due to Lsm1 over-expression (Figure 4, uppermost panel). Second, consistent with its severe growth defects and U6 snRNA reduction, lam7Δ over-expressing LSM1 was nearly completely rescued by the increased U6 expression (Figure 4) and U6 snRNA over-expression suppressed the splicing defect seen with Lsm1 over-expression in this strain (Figure 5, compare lanes 5, 6 and 7). Third, a similar complementation of synthetic growth defects was observed in lhp1Δ over-expressing LSM1 when U6 was over-expressed (Figure 4). These observations indicate that at least some of the growth and splicing defects in Lsm1 over-expressing strains arise from depletion in the levels of U6.

However, the growth defects in the smu66Δ strain were not rescued by over-expression of U6 snRNA (Figure 4). We speculate that the synthetic growth defects in the smu66Δ strain may be due to the assembly of a U6 snRNA that is compromised in function due to loss of both the Smu66p and the Lam2-8 complex. Consistent with that possibility, over-expression of U6 snRNA did not suppress the splicing defect seen in the smu66Δ strain (Figure 5, compare lanes 9, 10 and 11). This suggests that
in the absence of the Snu66 protein, the Lam1p complex may contribute directly to U6 snRNP function (see 'Discussion' section).

We observed that the cer4Δ and pop2Δ strains were partially rescued by over-expression of U6 snRNA (Figure 4). This suggests that the growth defects in these strains is in part due to reduced U6 snRNA levels and in part due to alterations in deadenylation, which might affect some aspect of mRNA decay.

**Lam1 over-expression does not globally alter mRNA decay**

The strong synthetic growth defects seen in the pop2Δ and cer4Δ strain with over-expression of Lam1 on a GAL 2 μ plasmid led us to hypothesize that Lam1 over-expression could also alter mRNA decay in some manner. This was also supported by the fact that the strong synthetic growth defects in cer4Δ and pop2Δ over-expressing LSm1 was only partially rescued by increasing U6 levels. To test if Lam1 over-expression affects decapping, we examined the effects of LSm1 over-expression on mRNA decay in a wild-type strain using the MFA2 pG mRNP, which is a commonly used reporter for mRNA decapping in yeast (36). In order to analyze the decay of MFA2 pG mRNA, we expressed it as a low-copy, galactose-inducible promoter fusion along with our GAL-LSm1 2 μ plasmid. Cells were grown in galactose and transcription was repressed by the addition of 4% dextrose (22), with samples taken for RNA analysis over a brief time course to avoid impacting the pool of Lam1 protein whose half-life has been reported to be 76 min (39). We observed no significant change in the decay rate of the MFA2 pG mRNA in a wild-type strain over-expressing Lam1 as compared to a vector control (t1/2 less than 5 for both). Similarly, over-expressing Lam1 at moderate levels on a GAL-LSm1 CEN plasmid did not affect the decay profile of the MFA2 pG mRNA (data not shown). This indicates that Lam1 over-expression does not have a strong effect on the decay of all mRNAs although it remains possible that Lam1 over-expression affects the decay of a subset of mRNAs.

**DISCUSSION**

Lam1 over-expression alters pre-mRNA splicing

In this work, we provide several lines of evidence that Lam1 over-expression can inhibit cell growth in budding yeast by affecting the biogenesis and/or function of the U6 snRNP. First, over-expression of Lam1 inhibited growth and decreased the levels of the U6 snRNA (Figures 1, 2 and 3). Second, the toxicity of the Lam1 over-expression, and in certain strains its impact on splicing, was increased in the lsm7A, lhp1A and snu66A strains, all of which impact in some manner on the function/biogenesis of the U6 snRNP (Figures 1, 2 and 3). Third, over-expression of the U6 snRNA rescued growth and the splicing defect in some strains mutant for U6 snRNP function (Figures 4 and 5). The simplest interpretation of these observations is that Lam1 over-expression impacts on U6 snRNA by depleting the levels of the Lam2-8 complex due to competition between Lam1 and Lam8 for the Lam2-7 complex members. This interpretation is also consistent with synthetic growth defects, as well as depletion of U6 snRNA levels, seen when LSm1 was over-expressed in an lsm6-1 mutant and with the observation that LSm1 over-expression increases the concentration of Lam7p in cytoplasmic foci, presumably reflecting an increased formation of the Lam1-7p complex (18).

Our results suggest that Lam1 over-expression can impact on the U6 snRNP in two manners depending on other alterations in the strain. In the lsm7Δ strain, the primary effect is on the levels of the U6 snRNA, and over-expression of the U6 snRNA can suppress the growth defect seen in these cases. This is consistent with other observations that in an otherwise wild-type strain the predominant role of the Lam2-8 complex is to enhance the stability of the U6 snRNA (29,40). However, in the snu66Δ, over-expression of the U6 snRNA fails to suppress the Lam1 growth and splicing phenotypes resulting from Lam1 over-expression (Figures 4 and 5). The simplest interpretation here is that when Snu66p, a component of the U4/U6/U5 tri-snRNP, is missing, the Lam2-8 complex now plays a more important role in the tri-snRNP’s function in splicing. Thus, even when U6 snRNA is over-expressed any resulting U4/U6/U5 complex lacking both the Snu66p and Lam2-8 proteins would be defective for function.

A clear implication of these observations is that changes in Lam1 levels might impact on splicing in pancreatic tumors. Our studies reveal that when Lam1 is over-expressed in yeast at moderate levels comparable to that observed in cancer cells (7), splicing is altered due to a depletion in U6 snRNA levels. In this light, it is notable that alterations in the splicing machinery have been previously described in human pancreatic tumors and transgenic mouse models. For instance, a serine/arginine
protein kinase involved in splicing, SRPK1, has been shown to be upregulated in pancreatic tumors and its downregulation correlated to decreased proliferation and increased apoptosis in these tumors (41). In addition, an analysis of genomic changes in an E1a-e- myc transgenic mouse model for pancreatic cancer revealed that splicing factors and spliceosome-related genes were part of a major class of genes upregulated in primary tumors and liver metastatic regions as compared to normal pancreas (42). Hence, it is possible that LSM1 over-expression also contributes to changes in splicing patterns by altering the stoichiometry of the spliceosomal machinery, allowing for carcinogenesis to occur in pancreatic cells. An implication of this analysis, and the increased toxicity of Lsm1 over-expression in yeast strains compromised for U6 snRNA function, is that therapies directed at reducing U6 snRNA biogenesis and/or function might be effective therapies for any tumor over-expressing Lsm1.

LSM1 over-expression decreases cell viability when deadenylation is inhibited

We also provide evidence that Lsm1 over-expression leads to a change in the cell’s requirement for different mRNA decay factors. Specifically, we observed that the toxicity of the Lsm1 over-expression was increased in ccr4Δ and pop2Δ strains (Figure 1C), which are compromised for the predominant cytoplasmic deadenylase (35). Moreover, the synthetic growth defects in these deadenylase mutants could only be partially rescued by increasing U6 snRNA levels (Figure 4). Overall, these results indicate that some process of mRNA decay is altered by Lsm1 over-expression, perhaps due to the assembly of mRNA decay complexes that are defective in function. However, since we did not observe an alteration in the MFA2/Gm mRNA, any alterations in degradation must be limited to subsets of mRNAs.

Prior studies in human cells do suggest that alterations in the decay of subsets of mRNAs may exist in Lsm1 over-expressing cancer cells. Array analyses of how Lsm1 over-expression affects the transcriptome (2,7) have demonstrated that increased Lsm1 expression alters levels of certain transcripts, some of which encode factors important in carcinogenesis. However, only one example is published providing an mRNA decay analysis. The p21/Cip1 mRNA, encoding a cyclin-dependent kinase inhibitor (43), was shown to be stabilized by targeted reduction of Lsm1 in a cancer cell line, yet this did not correspond to an increase in its protein expression (2).

Thus, it is still unclear whether therapeutic effects achieved with Lsm1 targeting in cancer cells are due to altering the decay of specific transcripts. Nevertheless, the synthetic lethality of Lsm1 over-expression in strains defective in mRNA deadenylation implies that therapeutic agents targeting deadenylase activity could be possible mechanisms for the treatment of tumors with over-expression of Lsm1.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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REFERENCES

APPENDIX C: ANALYSIS OF CIS AND TRANS FACTORS REQUIRED FOR RNY1’S FUNCTIONS IN S. CEREVISIAE

Natalie Luhtala and Roy Parker

Manuscript in process
ABSTRACT

T2 ribonucleases perform interesting roles in cell biology from regulation of self-incompatibility by S-RNases in plants to modulation of host immune cell responses by viral and schistosome T2 enzymes, (reviewed in (Luhtala and Parker 2010)). In humans, the only identified T2 ribonuclease, RNASET2, protects cells from ovarian tumor establishment in a catalytic-independent manner, and loss-of-function of this ribonuclease has been linked to a neurologic condition proposed to arise from accumulation of rRNA within the lysosome. We probed for cis regions and trans factors regulating the functions of Rny1, the sole T2 ribonuclease in budding yeast, to better understand the general mechanisms deregulated by loss of RNASET2 in human disease. Herein, we reveal interesting aspects of Rny1’s catalytic and non-catalytic functions.

First, we show that non-catalytic cytotoxicity and catalytic functions require Rny1’s signal peptide, and the unique C-terminal domain is sufficient to direct a non-catalytic function. Second, we reveal that Rny1’s cleavage of RNA substrates is independent of autophagy and ribophagy pathways. Third, we identify factors involved in vacuole-vacuole fusion, microautophagy, vacuole acidification, and multivesicular body (MVB) formation that alter normal levels of cleavage of RNA substrates by Rny1. We propose an autophagy- and MVB-independent mechanism by which microautophagy, vacuolar acidification and vacuolar fusion regulators deliver RNA substrates within the cytoplasm to Rny1 within vacuoles. Our evidence suggests that MVBs might also participate in a pathway for regulation of RNA, and loss of this pathway’s function could increase Rny1-mediated cleavage of RNA substrates.
INTRODUCTION

T2 ribonucleases affect cell growth and survival in multiple organisms (reviewed in (Luhtala and Parker 2010)), and understanding these roles could elucidate pathways for host-pathogen interactions and human disease. While evidence for how certain T2 enzymes affect immune cell functions and rRNA half-life has been presented (Acquati, Bertilaccio et al.; Haud, Kara et al. 2011; Hillwig, Contento et al. 2011), the basic mechanisms for how these proteins exert their effects are unknown. We investigated Rny1, the sole T2 ribonuclease in budding yeast, in order to determine how T2 ribonuclease functions are regulated.

Rny1 shares many characteristics with RNASET2 and other ribonucleases of the T2 family. First, Rny1 cleaves tRNA and rRNA during various stresses (Thompson and Parker 2009). This activity appears to be required for growth in certain strain backgrounds (MacIntosh, Bariola et al. 2001), but how gene expression is affected by tRNA and rRNA cleavage is unknown. Second, Rny1 is a glycosylated, vacuolar protein (Acquati, Bertilaccio et al.; MacIntosh, Bariola et al. 2001; Thompson and Parker 2009). Glycosylation and acidic nuclease activity are typical of T2 ribonucleases, but the mechanisms governing access of these enzymes to their substrates are unknown. Third, Rny1 affects cellular growth and sensitivity to stress independently of its nuclease activity (Thompson and Parker 2009). Although other T2 ribonucleases have been shown to influence cellular growth independent of catalytic activity, evidence clearly supporting a mechanism for this function is lacking.
Rny1’s nuclease activity was recently identified to be responsible for tRNA and rRNA cleavage during oxidative stress and stationary phase growth (Thompson and Parker 2009), a time in which yeast cells undergo nutrient depletion. Cleavage of tRNA is not unique to yeast and is conserved in prokaryotes and eukaryotes as a response to specific stresses (reviewed in (Thompson and Parker 2009) (Thompson, Lu et al. 2008; Yamasaki, Ivanov et al. 2009; Zhang, Sun et al. 2009)), producing tRNA cleavage products mapping primarily to the anticodon loop. Coupled with the fact that rRNA fragments accumulate during similar conditions that induce tRNA cleavage (Thompson, Lu et al. 2008; Thompson and Parker 2009), these data suggest the possible regulation of translation complexes in a stress-specific manner by ribonucleases such as Rny1, and loss-of-function of these enzymes might impinge on cellular survival during stresses.

Loss-of-function of Rny1 can inhibit cellular growth and survival during stress, and these defects can be rescued by nuclease activity. In the initial characterization of Rny1, MacIntosh et al. identified a specific strain background with a temperature sensitive phenotype with deletion to RNY1, and they were able to rescue this defect by expressing RNase A in a catalytic-dependent manner (MacIntosh, Bariola et al. 2001). Moreover, cells lacking Rny1 in this background exhibited an increase in cell and vacuole size (MacIntosh, Bariola et al. 2001). Hence, Rny1’s nuclease function is important for growth during heat stress, and this activity might regulate cell and vacuole size and morphology in an as-of-yet uncharacterized manner.

The question of how T2 ribonucleases act on their RNA substrates has been addressed in the literature. In that T2 ribonucleases are compartmentalized and their
RNA substrates are cytoplasmic, some type of regulation must occur to allow these factors to meet within the cell. Two models exist and have been supported by previous data.

First, T2 ribonucleases might exit their compartmentalized state and enter the cytoplasm to act on RNA substrates. This event would resemble the leaking of vacuolar cathepsins observed during lysosomal, caspase-independent, cell death (reviewed in Johansson, Appelqvist et al. 2010). In support of this model, Rny1 localization to the vacuole, in addition to that of other vacuolar enzymes, was shown to decrease in response to oxidative stress, corresponding to the onset of tRNA and rRNA cleavage (Thompson and Parker 2009). However, levels of Rny1-GFP during these conditions were difficult to detect over those of autofluorescent particles resulting from hydrogen peroxide treatment, and the specific localization of Rny1-GFP was unclear following its disappearance from vacuoles. Thus, it is difficult to ascertain whether decompartmentalization of Rny1 actually occurs.

Second, cytoplasmic RNA might enter the vacuole, where the T2 enzyme resides, via known or unknown vesicular trafficking pathways during stress. Recently, Haud et al. revealed the accumulation of rRNA within lysosomes with loss of RNASET2 in zebrafish (Haud, Kara et al. 2011). The authors of this and another recent publication demonstrating effects of a plant T2 ribonuclease on rRNA half-life (Hillwig, Contento et al. 2011) have proposed the possible involvement of pathways for targeting of cytoplasmic RNA substrates to the vacuole: ribophagy or nonfunctional ribosomal RNA decay.
Both of these pathways might be predicted to enable degradation of tRNA or rRNA substrates at the vacuole. The cleavage of tRNA and rRNA occurs during nutrient-limiting conditions that would induce autophagy (Thompson, Lu et al. 2008; Thompson and Parker 2009), and ribophagy entails the selective autophagy-dependent degradation of ribosomes through ubiquitin targeting of ribosomal proteins to vacuoles (Beau, Esclatine et al. 2008; Kraft, Deplazes et al. 2008; Ossareh-Nazari, Bonizec et al. 2010). Similarly, nonfunctional rRNA decay (NRD) involves ubiquitin and activates the degradation of RNA mutated at sites of codon-anticodon recognition and large ribosomal subunit binding (LaRiviere, Cole et al. 2006; Cole, LaRiviere et al. 2009; Fujii, Kitabatake et al. 2009). Nevertheless, previous data fails to reveal a requirement for autophagy in tRNA and rRNA cleavage precluding a model for ribophagy, and a test of the hypothesis that NRD targeting is required for tRNA and rRNA cleavage is lacking.

Another unknown aspect of Rny1’s function relates to its non-catalytic effects on growth, survival and stress response. Over-expression of Rny1 sensitizes cells to oxidative stress, a stress which induces tRNA and rRNA cleavage (Thompson and Parker 2009). Over-expression of Rny1 also increases cleavage of tRNA and rRNA substrates in the absence of stress (Thompson and Parker 2009). However, inactivating Rny1’s catalytic activity against RNA substrates does not alleviate its effects on growth and survival (Thompson and Parker 2009). Furthermore, loss of Yap1, an oxidative stress response factor or Bir1, an apoptotic inhibitor exacerbates non-catalytic growth defects, implicating a non-catalytic function for Rny1 that affects cellular survival and response to stress (Thompson and Parker 2009). Nevertheless, suppressors of Rny1’s over-
expression induced cytotoxicity have yet to be identified, leaving the mechanism for
Rny1’s non-catalytic function a mystery.

We studied the cis regions of Rny1 and screened for its regulatory factors in
trans, and our studies revealed interesting aspects of Rny1’s catalytic and non-catalytic
roles. First, we found that signal peptide targeting directs Rny1’s catalytic function and
its non-catalytic effects on growth, yet abrogating loops within the T2 core or eliminating
the T2 conserved region inhibits nuclease function without affecting non-catalytic
cytotoxicity. Second, we revealed that pathways for autophagy, ribophagy, and
nonfunctional ribosomal RNA decay do not play a major role in regulating Rny1’s
nuclease activity. Third, we identify factors regulating vacuole-vacuole fusion,
microautophagy, vacuole acidification, and multivesicular body (MVB) formation that
affect the accumulation of Rny1-dependent tRNA and rRNA fragments. We present
evidence supporting a possible mechanism by which microautophagy, vacuolar
acidification and vacuolar fusion proteins deliver RNA substrates within the cytoplasm
to Rny1 within vacuoles. Although this pathway does not require MVB or autophagy
function, MVBs could function within a parallel pathway that also regulates RNA that is
subject to downregulation by Rny1 with loss of MVB function.
MATERIALS AND METHODS

Yeast strains and growth conditions

Yeast strains used in this study are described in Table S1. Cells were grown at 30°C in all experiments. For experiments over-expressing Rny1, pRP1584 and pRP1587 were used with pRP861 as a vector control. Cells were grown in selective synthetic media containing 2% sucrose to saturation, and these cultures were pelleted, aspirated, and diluted (OD600=0.1) in selective synthetic media with 2% galactose as the sole carbon source and grown to early midlog (OD600=0.3-0.5). For experiments to analyze tRNA and rRNA fragment cleavage, cells were grown to saturation in media specified in figure legends (either selective synthetic media or yeast extract/peptone media both containing 2% dextrose), then diluted and grown to early midlog. Early midlog times were recorded, and cells were grown three days from this time to represent stationary phase growth. For frog ponding, yeast strains were patched to selective media plates, diluted to OD600=0.1 in selective media containing 2% sucrose in the first column of a 96-well plate, then diluted by 10-fold across into four additional columns. These columns were directly plated without additional growth.

RNA analyses

Total RNA was prepared from liquid nitrogen flash frozen pellets. All steps prior to acid phenol addition were performed at 4°C. Samples were suspended in TNE buffer (50mM Tris-Cl pH7.4, 100mM NaCl, 10mM EDTA), lysed with beads (two one-minute high speed vortexes interrupted by a one-minute incubation on ice to prevent overheating),
then vortexed with SDS added to 1% and an equivalent volume of acid phenol chloroform. Vortexing was repeated, then samples were heated to 65°C, followed by additional vortexing. After acid phenol chloroform extraction, an additional acid phenol chloroform extraction, and one chloroform extraction, RNA was precipitated, washed, dried, and resuspended in deionized formamide. Equal amounts of RNA (20 μg) as determined by A260, were resolved on 10% acrylamide, 47% urea, 1XTBE gels, and transfer to Ambion Bright White membranes in 0.5XTBE buffer. Blots were UV crosslinked twice, prewashed once at 65°C, using 0.1% SDS 0.1XSSC, then prehybridized in 6XSSC 0.1% SDS 10X Denhardt’s at 42°C. Hybridization with $\gamma^{32}$P-5’ end-labelled probes (see Table S3) was performed in the prehybridization buffer. Blots were washed with 6XSSC 0.1% SDS and placed against phosphor screens to expose, and screens were scanned into a Typhoon scanner and quantitated using ImageQuant software.

**Protein analyses**

Samples were lysed using 5M urea, boiled, then vortexed in glass beads for 5 minutes. A solution of 125mM Tris-Cl pH6.8, 2% SDS was added at 2.5x the volume of 5M urea used, and this was vortexed into the mixture, then samples were boiled a second time. Collected lysate was clarified by spinning at 16000 RCF, and the supernatant was resuspended in protein loading buffer (0.05M Tris pH6.5, 1% SDS, 0.01% bromophenol blue, 10% glycerol), boiled, and run on a 10% Tris-SDS acrylamide gel. Gels were transferred to nitrocellulose and probed using standard Western blotting protocols using an antibody to the His tag (Novagen, 70796-3, used at 1:1000) and an anti-mouse secondary coupled to HRP (Sigma A4416, used at 1:2500). Rny1 signal was revealed
using Pierce SuperSignal West Dura and exposing the blots to film and developing in a film processor (Konica). Films were scanned into .tif format using an HP Scanjet Pro flatbed scanner, and images were analyzed using a cross-reactive band as a loading control.

**Plasmids and plasmid construction**

Plasmids used in this study are listed in Table S2. The \( GAL\ RNY1\ 2\mu \) plasmid (pRP1584) and its counterpart plasmid containing mutations to produce catalytically inactive Rny1 (pRP1587) were the templates utilized to generate \( cis \) mutants by QuikChange (http://www.genomics.agilent.com/CollectionSubpage.aspx?PageType=Product&SubPageType=ProductDetail&PageID=383). Primers were designed using the software at this website and are listed in Table S3.
RESULTS

Alanine mutagenesis of \textit{cis} regions encoding predicted loop structures of Rny1 disrupts catalytic but not non-catalytic functions

Since Rny1’s non-catalytic effects on growth are exhibited in other T2 ribonucleases (reviewed in (Luhtala and Parker 2010)), we expected that Rny1’s effects on cytotoxicity might involve interactions with other factors (e.g. proteins, RNA, lipids) within the T2 conserved region. These interactions might involve conserved or non-conserved loops within the T2 region.

In order to understand if specific interactive \textit{cis} loop regions might confer cytotoxicity, we utilized known T2 ribonuclease structure data to predict important loops for mutagenesis. Using Swiss Model (http://swissmodel.expasy.org/) and the known three-dimensional structure for the fungal T2 ribonuclease, ACTIBIND (de Leeuw, Roiz et al. 2007), we generated a predicted structure for Rny1 based on its 39% homology to this enzyme (Figure 1B). In addition, using COBALT (http://www.ncbi.nlm.nih.gov/tools/cobalt/cobalt.cgi), we aligned Rny1 to ACTIBIND and another fungal T2 ribonuclease, Rh, of known structure (Kurihara, Nonaka et al. 1996) (Figure 1A). This information revealed the position of eleven loops available for surface interactions, two of which (L4 and L7) could involve RNA binding based on previously published alignments of Rh to T2 ribonucleases with known nucleotide-bound structures (Rodriguez, Panjikar et al. 2008). We identified 11 loops for mutagenesis (labeled on Figure 1A and 1B), and we were successful in mutating 10 of these loops.
(L2-L11) through alanine mutagenesis, probing effects on non-catalytic growth-inhibition in wild-type and/or catalytically inactive backgrounds.

In order to exacerbate the growth inhibition arising from Rny1 over-expression to produce conditions suitable for mapping cis loops important for this function of Rny1, we examined several mutant strains shown previously in large-scale synthetic genetic arrays to exhibit interactions with Rny1 (Wilmes, Bergkessel et al. 2008). These screens revealed a significant synthetic catalytic-independent growth defect by combining over-expression of Rny1 in a background deleted for HIR2, encoding a component of the HIR nucleosome assembly complex (Sherwood, Tsang et al. 1993; Spector, Raff et al. 1997).

Using the hir2Δ strain, we over-expressed RNY1 with loops individually mutated by switching all residues to alanine, expressing in either the wild-type or catalytic inactive RNY1 background, each made on a GAL-promoted 2μ plasmid. Comparisons were performed of the cis mutant constructs to over-expression of a full-length, wild-type or catalytic inactive form of Rny1 or a GAL 2μ vector control. Growth was analyzed by first streaking for single colonies to identify possible suppressing plasmids. Subsequently, frog ponds on selective media plates containing galactose to induce over-expression or dextrose to assay for growth independent of over-expression were performed to clarify the effects. Our results failed to reveal any consistent effects of loop mutagenesis on abrogating the non-catalytic effects of Rny1 on growth inhibition (data summarized in Table 1).

In order to understand if we had successfully abrogated RNA binding and hence catalytic function, we grew the rny1Δ strain expressing the cis loop mutant constructs
that correctly sequenced in the catalytically active background of \textit{GAL-RNY1}. These strains were harvested after inducing over-expression in galactose in parallel with strains expressing vector, unmutated \textit{GAL-RNY1} or its catalytically inactive counterpart. Total RNA was prepared and equivalent amounts of RNA were loaded and resolved by urea-acrylamide gel electrophoresis. We transferred these gels to membranes and Northern blotted using a probe to tRNA Met (CAT), shown in a previous publication to yield tRNA fragments with over-expression of Rny1 (Thompson and Parker 2009).

We found that RNA cleavage was disrupted with most \textit{cis} loop mutations that we tested. Of our \textit{cis} loop mutants, only the alanine-mutated L10 construct, which mutates two non-conserved residues, was capable of duplicating the cleavage of an unmutated \textit{GAL-RNY1} (Figure 1C, compare lane 9 to lane 1). Interestingly, one of the mutations to a loop region predicted to be involved in RNA binding appeared to shift the cleavage of the RNA to a larger size, suggesting that positioning of the RNA within the binding site of Rny1 is altered (Figure 1C, compare lane 7 to lanes 1 and 3). All other mutants failed to produce cleavage that differed significantly from the vector control (Figure 1C, compare lanes 4, 5, 6, 8, and 10 to lane 3).

\textbf{Catalytic-independent growth inhibition requires signal peptide targeting but not the T2 core}

Since single loop mutations within the T2 conserved region in \textit{cis} failed to disrupt inhibition of growth by Rny1, we wondered if the cytotoxic effects of Rny1 in \textit{cis} might be redundant or involve larger regions than initially mutated. We identified three larger
cis regions of RNY1 to screen for their influence on the non-catalytic function (diagrammed in Figure 2A). First, the N-terminal signal peptide of Rny1 presumably targets its translation to the ER and directs its localization to the vacuole which could be required for the growth inhibition (MacIntosh, Bariola et al. 2001; Thompson and Parker 2009). Second, the conserved T2 core contains putative nucleotide binding and catalytic residues and might be expected to participate in a non-catalytic role given the conservation of this function in other organisms. Third, the unique C-terminus of Rny1 is conserved in closely related fungal species and might contribute in an as-of-yet uncharacterized manner (Kobayashi, Itagaki et al. 2003).

Using the hir2Δ strain to exacerbate the growth defects, we over-expressed RNY1 with mutations inactivating catalytic activity as 3 separate cis deletion constructs, each made on a GAL-promoted 2μ plasmid. These 3 cis deletions individually tested the requirement of the N-terminal signal peptide, the conserved T2 core, or the unique C-terminal domain for the catalytic-independent growth inhibition. Growth was analyzed by frog ponds on selective media plates containing galactose to induce over-expression or dextrose to assay for growth independent of over-expression Comparisons were made of the cis deletion constructs to over-expression of a full-length, catalytic inactive form of Rny1 or a GAL 2μ vector control.

Our results revealed that the signal peptide targeting sequence is required for catalytic-independent growth inhibition. The strongest alleviation of growth defects was observed with loss of the signal peptide sequence (Figure 2B). Since this sequence would be expected to target Rny1’s translation to the ER and allow its glycosylation and
delivery to the vacuole, partial rescue of cytotoxicity with loss of Rny1’s signal peptide suggests that its non-catalytic role requires its normal trafficking within the cell.

Intriguingly, over-expressing the unique C-terminal domain or the conserved T2 region under the targeting of the signal peptide inhibited growth to similar extents (Figure 2B). In that the C-terminal domain is only conserved in close fungal homologs, this evidence implies the existence of conserved and divergent interactions involved in regulation of non-catalytic cytotoxicity.

Since these results could arise from loss of expression of the mutant proteins, we examined levels of these mutant proteins as compared to controls. We grew cells in the same hir2Δ strain over-expressing the cis mutants or full-length, wild-type Rny1, the catalytic inactive mutant or vector controls to midlog in selective media containing galactose to induce over-expression. Total protein lysates were prepared and analyzed by SDS-PAGE and Western blot for the His affinity tag on the C-terminus of Rny1.

This analysis revealed that cis mutant proteins were over-expressed at similar levels to that of full-length Rny1 (Figure 2C). Thus, these data suggest that Rny1 exerts its function within its signal peptide-targeted cellular regions, possibly through interactions at both the T2 core and within its unique C-terminal domain.

**Rny1’s nuclease activity requires signal peptide targeting and the conserved T2 region**

In order to understand if these regions affect Rny1’s nuclease function, we examined their ability to cleave the tRNA Met(CAT) when over-expressed, previously shown to occur with over-expression of Rny1 (Thompson and Parker 2009). We cultured
the rnyΔ strain expressing the cis deletion constructs in the catalytically active background of GAL-RNY1. These strains were harvested after inducing over-expression in galactose in parallel with strains expressing vector, unmutated GAL-RNY1 or its catalytically inactive counterpart. After total RNA preparation, equivalent amounts of RNA were loaded and resolved by urea-acrylamide gel electrophoresis. After transfer to membranes, Northern blotting was utilized to reveal tRNA fragments using our probe to tRNA Met (CAT).

We observed that both the T2 conserved region and signal peptide targeting were required to induce normal levels of tRNA cleavage (Figure 2D, compare lanes 4 and 6 to lanes 1 and 3). Loss of the T2 conserved regions produced a similar profile to vector, and lack of signal peptide targeting produced only very faint levels of cleavage. In contrast, loss of the unique C-terminal domain did not inhibit cleavage of tRNA (Figure 2D, compare lane 5 to lanes 1 and 3).

**Multiple factors could regulate Rny1’s nuclease activity in trans**

It is possible that both catalytic and non-catalytic roles might involve interactions with similar trans regulatory factors. Consequently, understanding the pathways regulating Rny1’s nuclease activity might elucidate factors acting in trans to regulate catalytic and non-catalytic roles of Rny1..

Several pathways might be expected to be involved in regulating Rny1’s nuclease function. We expected that due to Rny1’s compartmentalized localization within the cell, known or novel vesicular trafficking pathways and targeting processes might be required to enable the joining of RNA substrates and Rny1. We explored the following pathways
in our analysis: RNA quality control, autophagy, vacuole-vacuole fusion, and multivesicular bodies.

**Rny1’s nuclease activity against substrates does not require known RNA quality control pathways**

Rny1’s RNA substrates might be targeted for degradation as part of an RNA quality control process (diagrammed in Figure 3A). Since one substrate of Rny1 is rRNA, we considered known pathways for quality control of rRNA. Nonfunctional ribosomal RNA decay (NRD) is a process by which 18s rRNAs with decoding site mutations and 25s rRNAs with peptidyl transferase mutations are degraded (LaRiviere, Cole et al. 2006; Cole, LaRiviere et al. 2009; Fujii, Kitabatake et al. 2009). If Rny1’s nuclease activity were employed by cells to remove damaged, nonfunctional RNAs during stresses, we might expect this pathway’s involvement.

We analyzed mutants to genes encoding proteins regulating 18s NRD and 25s NRD. Degradation of nonfunctional 25s rRNA has been shown to require Rtt101, an E3 ubiquitin ligase, and its interactor, Mms1 (Fujii, Kitabatake et al. 2009). In contrast, loss of Dom34, a translation termination paralog with proposed involvement in stalled ribosome release from mRNAs, stabilizes nonfunctional 18s rRNA (Cole, LaRiviere et al. 2009), linking NRD to another RNA quality control process for eliminating mRNAs with translation stalls, no-go decay (NGD) (Doma and Parker 2006). We predicted that any of these factors involved in NRD and NGD might target RNA substrates to Rny1.

We grew strains deleted for *RTT101, MMS1*, or *RNY1* and used the isogenic wild-type for comparison. Strains were isolated at 3 days from midlog to allow for analysis of
tRNA and rRNA fragment accumulation. Total RNA was prepared and resolved by urea-acrylamide gels and Northern blot for previously used tRNA His(GTG) and 25s rRNA probes. Data was normalized to U6 snRNA levels.

Strains lacking Rtt101, Mms1, or Dom34 did not influence levels of tRNA or rRNA fragment accumulation (Figure 3B and 3C). Thus, Rny1-mediated cleavage processes do not require intact NRD or NGD function.

**RNA substrate delivery to Rny1 is autophagy- and ribophagy-independent**

One pathway which might be expected to compartmentalize cytoplasmic RNA and deliver these to vacuoles is autophagy (diagrammed in Figure 4A). Consistent with this model, Rny1-dependent fragments accumulate during nutrient-limiting conditions that induce autophagy (Thompson and Parker 2009). Moreover, two groups recently linked T2 ribonuclease deficiency to increased rRNA half-life and accumulation of undigested rRNA within lysosomes, and targeting of these RNA to lysosomes was proposed to function via a selective autophagy process targeting ribosomes and rRNA (Haud, Kara et al. 2011; Hillwig, Contento et al. 2011). Nevertheless, previous studies from our lab failed to reveal a dependence of Rny1-mediated tRNA and rRNA cleavage on autophagy (Thompson, Lu et al. 2008). However, we supposed that autophagy factors not initially investigated might participate in substrate targeting to Rny1.

We predicted that if cleavage of tRNA and rRNA substrates by Rny1 occurred as part of ribophagy or another autophagic process, Atg7, an autophagy factor previously unexamined, would be required for generating Rny1-dependent tRNA and rRNA fragments. Atg7 is an E3-like enzyme involved in two ubiquitin-like conjugations
necessary for autophagy (reviewed in (Geng and Klionsky 2008)), and loss of this protein blocks the sorting of ribosomal proteins to the vacuole during ribophagy (Kraft, Deplazes et al. 2008). Hence, Rny1’s RNA substrates might associate with ribosomal proteins targeted by ribophagy to vacuoles, and this process would require Atg7’s function.

We also supposed that Atg15 might play a role in enabling Rny1’s activity against its RNA substrates. Atg15 is required for efficient lysis of autophagic bodies and cytoplasm-to-vacuole trafficking (CVT) vesicles and appears to contribute to intravacuolar multivesicular body (MVB) vesicle lysis, all compartmentalized structures that could contain cytoplasmic components such as Rny1’s RNA substrates (Epple, Suriapranata et al. 2001; Teter, Eggerton et al. 2001; Epple, Eskelinen et al. 2003). Therefore, we expected that this lipase might be important for lysing intravacuolar vesicles containing RNA substrates to allow their cleavage by Rny1 within vacuoles.

To ascertain whether these factors affect Rny1’s nuclease activity in *trans*, we investigated strains deleted for *ATG7*, *ATG15*, or *RNY1* and used the isogenic wild-type for comparison. Strains were grown for 3 days from midlog to allow for detection of tRNA and rRNA fragment accumulation. Total RNA was prepared and examined by urea-acrylamide gels and Northern blot for previously used tRNA His(GTG) and 25s rRNA probes. A probe for U6 snRNA, a nuclear snRNA, was utilized as a normalization control.

Our analysis of tRNA fragment (Figure 4B) and rRNA fragment (Figure 4C) accumulation showed that neither Atg7 nor Atg15 affected Rny1’s nuclease activity. These results reveal that Rny1’s cleavage of RNA substrates is independent of autophagy
and ribophagy, and it does not require intravacuolar lysis by Atg15. However, we wondered whether Rny1’s nuclease activity might be enabled independently of Atg7 and Atg15 yet require additional factors involved in substrate targeting for selective autophagic processes.

**Rny1’s nuclease activity requires Vac8 but not other proteins regulating selective autophagy**

We surmised that Rny1’s nuclease activity might require proteins involved in selective autophagy but not require an autophagosome structure. Accordingly, ubiquitin pathways utilized for selective degradation processes during starvation might route RNA substrates to the vacuole independently of an autophagosome. Moreover, receptors for organellar degradation might deliver localized translation complexes containing RNA substrates to vacuoles for scavenging during starvation in an autophagy-independent process. Thus, we studied several mutants to selective autophagic receptors and ubiquitin modifying pathways to probe for effects on Rny1’s nuclease activity in *trans*.

We analyzed how loss-of-function of proteins with known or possible influences on selective forms of autophagy (mitophagy, pexophagy, piecemeal micronucleophagy, ribophagy) affected Rny1’s cleavage of tRNA and rRNA substrates (diagrammed in Figure 5A). Mutant strains lacking either receptors for autophagic substrate delivery to the vacuole (Uth1, Vac8, Nvj1) (Pan, Roberts et al. 2000; Kissova, Deffieu et al. 2004) or ubiquitin binding and modifying enzymes (Pex10, Bre5, Ubp3, Ufd3) (Kraft, Deplazes et al. 2008; Williams, van den Berg et al. 2008; Ossareh-Nazari, Bonizec et al. 2010) were grown to 3 days from midlog and harvested. We prepared total RNA from these strains...
and resolved samples on urea-acrylamide gels, transferred to membranes, and Northern blotted for tRNA and rRNA. Results are shown in Figure 5B and 5C.

Our analyses revealed that tRNA and rRNA accumulation were significantly reduced in a Vac8 mutant, but not an Nvj1 mutant (Figure 5B and 5C, compare lanes 8 and 9 to lanes 1 and 2). Vac8 and Nvj1 physically interact, and both factors are required for degradation of piecemeal micronucleophagy substrates at the vacuole (Pan, Roberts et al. 2000; Kvam and Goldfarb 2004). However, Vac8 also plays roles in autophagy-independent vacuole fusion and cytoplasm-to-vacuole trafficking (CVT) (Scott, Nice et al. 2000; Veit, Laage et al. 2001). Since lack of significant effects on tRNA and rRNA fragment accumulation were observed with loss of Nvj1, Atg7, Atg15, or other selective autophagy proteins affecting organellar autophagy, we suspected that RNA substrates might be targeted to the vacuole in a vacuole fusion-dependent but autophagy-independent process involving Vac8.

**Bem1, a Vac8 interactor, increases tRNA and rRNA fragment accumulation**

We supposed that other factors interacting with Vac8 might be important for regulating Rny1’s nuclease activity in *trans*. One factor identified, Bem1, was previously demonstrated in synthetic genetic array analysis to be sensitive to loss of Rny1 (Costanzo, Baryshnikova et al. 2010). Furthermore, Bem1, a regulator of cell polarity establishment and budding, positively affects vacuole-vacuole fusion putatively via lipid mixing, and in these studies, it cosedimented with Vac8 and other vacuole fusion factors (Chenevert, Corrado et al. 1992; Xu and Wickner 2006) (roles diagrammed in Figure...
6A). Consequently, we reasoned that Bem1 might participate with Vac8 in regulating Rny1’s nuclease activity.

We compared total RNA from strains deleted for \( \text{BEM1}, \text{VAC8}, \text{or RNY1} \) to a wild-type control strain. As performed for previous analyses, we resolved equivalent amounts of total RNA on urea-acrylamide gels, transferred to membranes, and Northern blotted for tRNA and rRNA fragments and U6 snRNA as a normalization control.

Interestingly, we found that Rny1-dependent tRNA and rRNA markedly increased with loss of Bem1 (Figure 6B and 6C). Given the reported sensitivity of a \( \text{bem1} \Delta \) strain to loss of Rny1 in synthetic genetic array analysis, this suggests that loss of Bem1 increases the requirement and level of Rny1’s nuclease activity. This effect contrasts with the decrease in Rny1-dependent RNA fragment accumulation displayed by a strain mutant for Vac8. Taken together, these data suggest that Bem1-mediated fusion is not required for Rny1’s activity. However, loss of Bem1-mediated fusion might increase the requirement for Rny1’s cleavage of RNA substrates in a Vac8-dependent manner.

**Factors that regulate vacuole docking/fusion, vacuole acidification and microautophagy affect RNA fragment accumulation**

To understand whether other Vac8 and/or Bem1 interactors at the vacuole might also influence levels of RNA cleavage by Rny1, we examined additional strains mutant for factors interacting with Vac8 or Bem1 (Pan, Roberts et al. 2000; Veit, Laage et al. 2001; Tang, Kauffman et al. 2003; Cheong, Yorimitsu et al. 2005; Hou, Subramanian et al. 2005; Xu and Wickner 2006; Collins, Kemmeren et al. 2007; Tarassov, Messier et al. 2008). Strains examined were deleted for genes encoding factors involved in multiple
pathways (functions diagramed in Figure 7A): membrane-associated transport (Hxt1, Cot1, Fth1, Pmc1, Vma7) (Lewis and Bisson 1991; Cunningham and Fink 1994; Graham, Hill et al. 1994; Nelson, Mandiyan et al. 1994; Marchi, Sorin et al. 1999; Urbanowski and Piper 1999; MacDiarmid, Gaither et al. 2000), nucleus-vacuole junctions (Sw1) (Levine and Munro 2001), cytoplasm-to-vacuole- trafficking (Atg13) (Scott, Nice et al. 2000), endocytosis (Nce102, Ubi4) (Shih, Sloper-Mould et al. 2000; Grossmann, Malinsky et al. 2008), vacuole inheritance (Vac17) (Ishikawa, Catlett et al. 2003), microautophagy (Gtr1, Meh1) (Dubouloz, Deloche et al. 2005; Wang, Kurihara et al. 2009), vacuole docking/fusion (Vam3, Vam7, Vps33, Yck3) (Sato, Rehling et al. 2000; LaGrassa and Ungermann 2005), and an MVB cargo protein (Sna3) (Reggiori and Pelham 2001).

We probed for altered tRNA and rRNA cleavage by isolating total RNA from deletion strains to strains deleted for RNY1 and a wild-type control strain. As in previous experiments, we compared equivalent amounts of total RNA on urea-acrylamide gels, transferred to membranes, and Northern blotted for tRNA and rRNA fragments and U6 snRNA as a control.

Our results (Figure 7B and 7C) demonstrated that tRNA and rRNA accumulation were decreased with loss of factors affecting either vacuole acidification (Vma7, Meh1, lanes 10 and 16, left panel)(Graham, Hill et al. 1994; Nelson, Mandiyan et al. 1994; Gao, Wang et al. 2005), microautophagy (Meh1, lane 16, left panel), or vacuole docking/fusion (Vam3, lane 6, right panel; Vps33, Vam7, lanes 1-2, right panel). These data suggest that RNA substrates might be delivered to vacuoles utilizing vacuole docking/fusion machinery. In addition, vacuolar acidification might participate in
substrate delivery to vacuoles, and/or it could be required for enabling nuclease activity of Rny1 through optimizing substrate/enzyme interactions.

We wondered whether microautophagy, the direct invagination of vacuoles to form lumenal compartments, could also be involved in targeting RNA substrates to vacuoles. Meh1, a factor required this process (Duboulouz, Deloche et al. 2005), was necessary for normal accumulation of tRNA and rRNA fragments. While Gtr1, a factor colocalizing with other microautophagic proteins (Wang, Kurihara et al. 2009), did not affect tRNA and rRNA fragment accumulation, its requirement for this process has not been clearly shown. Nevertheless, effects of loss of Meh1 might relate to lack of optimal substrate-enzyme interactions vacuolar acidification is inhibited in a strain deleted for MEHI, and. Therefore, we pursued additional possibilities for compartmentalization of cytoplasmic RNA.

**Loss of core factors involved in MVB formation increases RNA cleavage**

We opted to examine multivesicular body (MVB) mutants as another possible mechanism for compartmentalization of cytoplasmic substrates. Endosomal microautophagy, a process described in mammalian cells, is a selective degradative process that utilizes endocytic and autophagic pathways to take up cytoplasmic components (Sahu, Kaushik et al. 2011). We predicted that a similar process in yeast, using the MVB machinery and/or microautophagy factors, might also be utilized to take up cytoplasmic RNA substrates into lumenal compartments that fuse with vacuoles where Rny1-mediated degradation can occur.
Several deletion strains were analyzed for their accumulation of tRNA and rRNA fragments. Deletions encoding factors affecting multiple steps of the MVB pathway were examined (see Figure 8A)(functions reviewed in (Hurley and Hanson 2010)): substrate targeting (Ear1, Ssh4) (Leon, Erpapazoglou et al. 2008; Nikko and Pelham 2009), ESCRT-I (Mvb12 Stp22), ESCRT-II (Vps25, Vps36), ESCRT-III (Vps2, Vps20, Vps24, Snf7), and late-acting processes affecting ESCRT factor recycling and vacuolar delivery of substrates (Vps4, Ist1, Vta1). In addition, we examined mutants for factors involved in alkaline pH response since Rim20 interacts with Snf7 (Xu, Smith et al. 2004), and Rim8 associates with components of ESCRT-I (Herrador, Herranz et al. 2010).

Cleavage of tRNA and rRNA was analyzed by isolating total RNA from deletion strains to strains deleted for RNY1 and a wild-type control strain. As in previous experiments, we compared equivalent amounts of total RNA on urea-acrylamide gels, transferred to membranes, and Northern blotted for tRNA and rRNA fragments and U6 snRNA as a control.

Our results (Figure 8B and 8C) demonstrated that tRNA accumulation was increased in all mutant strains except those involving the alkaline pH response and early and late functions of MVB sorting of cargoes and their internalization into lumenal vesicles: substrate targeting (ear1Δ, ssh4Δ; lanes 1-2), alkaline pH response factors (rim8Δ, rim20Δ; lanes 4-5) a non-core component of ESCRT-I (mvb12Δ, lane 3), and two late-acting factors (vta1Δ, ist1Δ; lane 15 and lane 2). These results were also observed with the 25s rRNA probed fragments, albeit to a lesser degree.
Interestingly, additional cleavage fragments were observed using this probe for the *ssh4Δ* (8C, lane 2) strain and the *vta1Δ* (8C, lane 15) strain (bracketed region on the figure). In that another rRNA can be cleaved independently of Rny1’s function, this data suggests that these mutations might route substrates to another ribonuclease.
DISCUSSION

Rny1’s non-catalytic effects do not require the T2 conserved region or RNA binding

Several of our experiments support a model for a non-catalytic function of Rny1 that is exerted through its unique C-terminal region mediating interactions with trans factors other than RNA. First, our studies of the effects of mutations to multiple cis loop regions within the T2 conserved region failed to rescue growth even though most mutants completely abrogated cleavage of RNA (Table 1 and Figure 1C). Second, over-expressing the unique C-terminal domain under signal peptide targeting was sufficient to inhibit growth, even expressed at slightly lower than wild-type levels of protein (Figure 2B). Third, the unique C-terminal domain failed to rescue tRNA cleavage in a strain deleted for RNY1 (Figure 2D) which suggests its involvement in a nuclease-independent process.

Divergent C-terminal extensions could regulate non-catalytic functions of T2 enzymes. Unique roles for divergent C-terminal extensions of T2 ribonucleases have been discussed in prior publications. The viral E<sup>ns</sup> protein, which is capable of host immune cell depletion in certain catalytic mutants, possesses a unique C-terminal domain that enables its receptor-independent translocation across host’s cellular membranes (Langedijk 2002). In another type of fungi, Basidiomycetes, C-terminal extensions of T2 enzymes contain serine+threonine rich regions similar to those in fungal glycanhydrolases, and it has been postulated that these regulate binding to substrates downstream of this site (Kobayashi, Itagaki et al. 2003). While the serine+threonine rich regions are not conserved within Rny1’s C-terminal domain, some sequence similarities
amongst these fungal proteins’ C-terminal regions does exist (Kobayashi, Itagaki et al. 2003). Hence, Rny1’s non-catalytic function might involve binding to non-RNA substrates, perhaps regulating its association with membranes or proteins.

**Localization of Rny1 is important for its non-catalytic and catalytic functions**

Our analysis of cis deletion constructs supports the idea that signal peptide-directed translation of Rny1 is important for its non-catalytic activity, most likely due to its ability to direct Rny1’s localization to vacuoles. Loss of the sequence encoding the signal peptide exhibited the strongest relief of the non-catalytic growth inhibition (Figure 2B), and this construct was expressed at similar levels to other cis deletion constructs that were less capable of rescuing the growth defects (Figure 2C).

Similarly, over-expression of Rny1 lacking its signal peptide sequence produced only trace amounts of tRNA cleavage in a strain deleted for RNY1 (Figure 2D). Coupled with the signal peptide’s requirement for complete non-catalytic cytotoxicity, these data support a model in which Rny1 exerts catalytic and non-catalytic functions at vacuoles.

The idea that Rny1 would cleave RNA within vacuoles is supported by recent evidence. Haud et al. examined the neurons of zebrafish mutant for RNASET2 and found undigested ribosomal RNA accumulating within their lysosomes (Haud, Kara et al. 2011). Thus, Rny1 might cleave tRNA and rRNA in vacuolar-localization-dependent manner, and loss of the signal peptide disrupts this activity.

The nature of the non-catalytic role and its reliance on signal peptide-targeting are still a mystery. Our evidence implies that the interactions required for non-catalytic cytotoxicity are with non-RNA factors, and these interactions are optimized by Rny1’s
localization to vacuoles. We would predict that the C-terminal domain mediates at least some of these effects, perhaps in concert with the T2 conserved region, through interactions with *trans* factors at vacuoles. Nevertheless, the clarification of this process and the *trans* factors conferring cytotoxic effects is a topic for further investigation.

**Known pathways for autophagy or quality control of RNA do not significantly impact Rny1’s nuclease activity**

Our results reveal that Rny1’s nuclease activity does not rely on RNA quality control, ribophagy or other autophagic processes (Figures 3-5). First, loss of factors required for the efficient degradation of no-go RNA substrates and nonfunctional rRNA failed to affect accumulation of Rny1-dependent RNA fragments during nutrient-limited growth (Figure 3). Second, Atg7, required for autophagy and specifically demonstrated to be necessary for large ribosomal protein trafficking to the vacuole during ribophagy, did not impact normal levels of tRNA or rRNA fragment accumulation (Figure 4). Third, none of the factors tested for selective autophagy, except Vac8, demonstrated significant effects on Rny1’s nuclease activity (Figure 5). Taken together, these data support a model for the autophagy-independent delivery of RNA substrates to Rny1.

Our data reveal the involvement of autophagy-independent processes in targeting tRNA and rRNA to vacuoles. Previous data from our lab had indicated that autophagy was not required for accumulation of tRNA and rRNA fragments. Since the autophagy mutants originally tested by our lab (Thompson, Lu et al. 2008) (Atg3, Atg4) did not match the mutant used in characterizing ribophagy (Kraft, Deplazes et al. 2008) (Atg7), we assayed this strain to rule out the possibility that ribophagy is the sole mediator of
delivery of Rny1’s RNA substrates to vacuoles. Our studies of known pathways for RNA quality control, ribophagy and other selective autophagic processes demonstrate that these functions are not necessary to enable Rny1’s cleavage of RNA substrates.

**Vacuole-vacuole fusion, microautophagy and vacuolar acidification enable Rny1’s nuclease activity through a novel mechanism**

We identified several factors, involved in autophagy-independent vacuolar processes, that affected Rny1’s nuclease activity. Initially, we identified, Vac8, involved in piecemeal micronucleophagy (PMN) (Pan, Roberts et al. 2000), vacuole-vacuole-fusion (Veit, Laage et al. 2001), and cytoplasm-to-vacuole trafficking (Scott, Nice et al. 2000), as a factor necessary for wild-type levels of tRNA and rRNA fragment accumulation during nutrient-limited growth (Figure 5). This effect was not demonstrated in a strain deleted for *NVJ1* (Figure 5), encoding the nuclear receptor for PMN, or with loss of another factor at nucleus-vacuole junctions (Swh1, Figure 7). These observations suggest that functions outside of PMN contribute to Vac8’s effects on Rny1’s nuclease activity.

By examining the effects of loss of physical interactors of Vac8 on tRNA and rRNA fragment accumulation, we discovered that microautophagy (Meh1), vacuolar acidification (Vma7 and Meh1), and vacuole-vacuole fusion (Vam3, Vam7, and Vps33) were important for Rny1’s nuclease activity (Figure 5). To our knowledge, these factors have not been described to act within a pathway that impinges on RNA cleavage. We supposed that these factors might orchestrate a microautophagy-like process by which vacuoles directly engulf cytoplasmic RNA substrates for digestion.
**Loss of MVB formation increases Rny1’s cleavage of RNA**

Multivesicular body (MVB) formation resembles microautophagy of vacuoles, so our studies led us to investigate the role of the MVB machinery in Rny1’s nuclease activity. A recent publication presented a microautophagy-like pathway in dendritic cells which utilizes portions of the MVB machinery in conjunction with selective autophagy targeting to direct cytosolic proteins to lysosomes in an Atg7-independent manner (Sahu, Kaushik et al. 2011). This finding proposed the possibility that factors from multiple pathways might coordinate novel pathways direct vacuolar delivery of substrates.

Our studies, however, found that loss of core components of ESCRTs, required for MVB formation, increased accumulation of Rny1-dependent RNA fragments (reviewed in (Hurley and Hanson 2010)). Loss of Stp22, a core ESCRT-I component, or loss of any of the ESCRT-II or ESCRT-III components tested, all vital for coordinating membrane budding and scission steps of MVB biogenesis, increased levels of tRNA and rRNA cleavage (Figure 8). Likewise, Vps4, required for disassembly of ESCRT-III, augmented levels of Rny1-dependent tRNA and rRNA fragment accumulation (Figure 8).

In contrast, loss of factors required for targeting of certain substrates to MVBs failed to increase accumulation of tRNA and rRNA fragments (Ear1, Ssh4, Figure 8). Furthermore, Mvb12, an ESCRT-I component, and Ist1 and Vta1, both with published roles in ESCRT-III disassembly, failed to affect levels of tRNA and rRNA cleavage (Figure 8).

One possible interpretation of this data is that loss of certain aspects of MVB function produce RNA that requires cleavage by Rny1 at vacuoles. Links between
multivesicular bodies, RNA binding proteins and RNA have been described in previous publications. In mammalian cells, multivesicular exosomes, formed by the MVB machinery, have been shown to contain RNA binding proteins and RNA (Pegtel, Cosmopoulos et al.; Gibbings, Ciaudo et al. 2009), and extracellular Snf7-dependent vesicles, containing ribosomal proteins and other protein biosynthetic factors, have been described in yeast (Oliveira, Nakayasu et al. 2010). It is possible that the MVB machinery regulates aspects of translation, and failure of this process increases the requirement for Rny1-dependent cleavage of RNA.

This does not rule out the possibility that MVB’s might normally deliver RNA substrates to vacuoles for degradation by Rny1. Nevertheless, the MVB machinery is not the sole mediator of RNA substrate delivery to vacuoles since loss of core ESCRT components increases and does not block accumulation of RNA fragments.

**Loss of Bem1 increases the requirement for Rny1-mediated cleavage of RNA**

Loss of Bem1, a regulator of cell polarity establishment and vacuole-vacuole fusion, also increased accumulation of tRNA and rRNA fragments, much like MVB mutants (Figure 6). While Bem1 and Vac8 interact and are purported to act together in vacuole-vacuole fusion (Xu and Wickner 2006), they have opposing effects on tRNA and rRNA cleavage. Moreover, loss of Bem1 has been shown in synthetic genetic arrays to be sensitive to loss of Rny1 (Costanzo, Baryshnikova et al. 2010). Taken together, these evidence suggest that loss of Bem1 might increase the requirement for Rny1-mediated cleavage of RNA substrates.
It is tempting to speculate that loss of Bem1 increases RNA substrates originating from localized translation associated with cell polarity establishment. Bem1 acts as a scaffold for cell polarity establishment, and loss of its normal function decreases competition between cell polarization complexes (Chenevert, Corrado et al. 1992; Howell, Savage et al. 2009). If these processes involve localized translation, and Bem1’s function at vacuoles involves downregulation of ineffective polarization complexes, Rny1 might be necessary during nutrient-limited times to perform this process in the absence of Bem1.

**Model for RNA substrate delivery to Rny1**

We propose an autophagy-independent and MVB-independent mechanism for delivering RNA substrates to Rny1 (Figure 9). Loss of MVB formation and/or Bem1 might provide RNA and translation for this process which utilizes microautophagy and the vacuole docking and fusion machinery to mediate the cleavage of RNA by Rny1 within vacuoles, independent from MVBs.

In this manner, invagination of vacuoles might be driven by Meh1, a factor already demonstrated to drive this process during recovery from rapamycin-induced growth arrest (Dubouloz, Deloche et al. 2005). While we did not observe effects on RNA cleavage fragment accumulation with loss of Gtr1, another factor colocalizing with other factors regulating microautophagy (Dubouloz, Deloche et al. 2005; Wang, Kurihara et al. 2009), a direct role for this factor in microautophagy has not been demonstrated. Other factors involved in microautophagy might participate in this process, and future experiments might reveal their requirement for Rny1’s nuclease activity.
Vac8 might serve to engulf cytoplasmic RNA fragments after invagination occurs. Two lines of evidence support this model. First, Vac8 has been shown to affect cytoplasm-to-vacuole trafficking (CVT) of substrates, but it does not affect autophagy as does Atg13, another CVT protein (Scott, Nice et al. 2000). Second, in *Pichia pastoris*, loss of Vac8 during glucose-induced micropexophagy leads to accumulation of an invaginated vacuole with arm-like extensions surrounding peroxisomes, and this group’s analysis of Vac8-GFP localization led them to hypothesize a function for Vac8 in fusing arm-like extensions (Fry, Thomson et al. 2006; Oku, Nishimura et al. 2006). Thus, Vac8 might mediate the autophagy-independent trafficking of RNA substrates to the vacuolar lumen.

The requirement for additional vacuole fusion factors (Vam3, Vam7, Vps33) for RNA cleavage by Rny1 suggests that these factors might also participate in delivering RNA substrates to vacuoles. In this manner, vacuole docking and fusion regulators would participate with Vac8 in fusion between the arms of the invaginated vacuole, similarly to other processes of vacuole-vacuole fusion (review of SNARE and Sm interactions mediating this process in Sudhof and Rothman 2009). Furthermore, delivery of the lumenal compartment containing RNA substrates to vacuoles would require fusion pore opening. A recent publication has revealed that an interaction between Vps33 and Vam3 is necessary for fusion pore opening and content delivery within fused vacuoles (Pieren, Schmidt et al. 2010), so the requirement for these factors in Rny1’s cleavage activity is consistent with our model. Thus, the vacuole docking and
fusion machinery could mediate the delivery of a lumenal compartment to vacuoles containing Rny1.

**Exit of RNA substrates from vacuoles might involve additional lipases or vacuolar acidification**

How RNA substrates exit this intralumenal vesicle is unclear. While this might require lipase activity, Atg15 is not necessary for RNA cleavage fragment accumulation (Figure 4). Vacuolar acidification could play a role, since both Vma7 (a H⁺-ATPase subunit) and Meh1 affect these processes and are required for optimal cleavage. Additional lipases other than Atg15 exist, and any of these could contribute to release of RNA substrates into vacuoles. Alternatively, these effects might involve the hypothesized intermediates in the vacuole fusion process which could account for leakiness observed in vacuoles (idea discussed in (Engel and Walter 2008)). In this manner, asymmetric opening of the fusion pore and lipid mixing might allow RNA substrates to leak into the vacuole, perhaps allowing their access to Rny1. These are all interesting possibilities, but future research is necessary to isolate the veritable mechanism for this process.
FIGURE LEGENDS

Figure 1: Loops within Rny1’s structure contribute to catalytic but not non-catalytic function. (A) COBALT alignment (http://www.ncbi.nlm.nih.gov/tools/cobalt/cobalt.cgi) of Rny1 of S. cerevisiae (top, in blue) to other ribonucleases of known structure (Rh, R. niveus, middle, in red; ACTIBIND, A. niger, bottom, in khaki) (Kurihara, Nonaka et al. 1996; de Leeuw, Roiz et al. 2007). T2 ribonuclease conserved amino acid sequences (CAS) are underlined and shown in red (I) and light red (II). Predicted nucleotide binding residues are shown in blue (B1 site) and yellow (B2 site) and are based on an alignment of Rh to ribonucleases whose structures are known in complex with nucleotides (Rodriguez, Panjikar et al. 2008). Residues that overlap involvement in B1 and CAS are shown in purple while those that overlap B2 and CAS are designated by orange (conserved sequence elements of T2 ribonucleases are reviewed in (Luhtala and Parker 2010)). Putative N-linked glycosylation sites are depicted by underlined pink N residues which were identified by analysis with predictive glycosylation software (http://comp.chem.nottingham.ac.uk/cgi-bin/glyco/bin/getparams.cgi (Caragea, Sinapov et al. 2007)). Possible acetylation of lysine residues was predicted using a software that recognizes human motifs for acetylation (http://www.phosida.com/ (Gnad, Ren et al. 2007)), and these lysines are highlighted in gray. Loops targeted for mutation are boxed in pink with an identifier above that corresponds to the structure in (B). (B) Swiss Model predicted structure (Swiss Model (http://swissmodel.expasy.org/) was generated by 39% homology to ACTIBIND (de Leeuw, Roiz et al. 2007), and the image was illustrated in cyan using
PyMol (www.pymol.org) with color coding and loop designations referring to those used in (A). Catalytic histidine residues are shown as protrusions within the T2 core in orange and purple. Loops L4 and L7 are predicted to participate in nucleotide binding based on our alignment to Rh which was previously aligned to ribonucleases with known regions of nucleotide binding (Rodriguez, 2008 #476). (C) Cleavage of tRNA Met(CAT) by over-expressed full-length (WT), full-length catalytically inactive (ci), vector control (v), or RNY1 containing catalytic histidines but mutated in cis at indicated loops (L#).

Strains deleted for RNY1 were grown as described to induce plasmid over-expression of GAL-RNY1 in the plasmid mutant or control indicated, and equivalent amounts of RNA were resolved and transferred to gels for Northern blots using oRP1401, all performed as indicated in the Materials and Methods. Top bracket indicates the presence of a shifted band that might result from the L4 mutation altering the RNA binding site. Bottom bracket reveals where expected bands accumulate with over-expression of the full-length (WT) RNY1.

Figure 2: Larger cis regions all contribute to non-catalytic cytotoxicity but have varying effects on nuclease function.

(A) Diagram indicating the positions within the amino acid sequence of larger cis regions targeting for deletion. (B) Frog ponds (performed as described in Materials and Methods section) on synthetic selective media plates containing galactose to induce Rny1’s over-expression in catalytic mutant background in a hir2Δ strain as a GAL-RNY1 plasmid either full-length (WT), deleted for either the signal peptide sequence (ΔSP), the
T2 conserved region (ΔT2) or the unique C-terminal region (ΔCTD) or a vector control (v).  (C) Western blot (performed as indicated in Materials and Methods) of strains expressing constructs as shown in (B) except that the first lane shows a non-catalytic, full-length mutant GAL-RNY1’s expression in the same strain (WT) (D) Northern blot performed, blotting for tRNA Met(CAT), as detailed in Materials and Methods. Strains deleted for RNY1 expressing GAL-RNY1 constructs as in (B) and (C) except that all constructs (except ci which is full-length catalytically inactive) were expressed in the catalytically active background.

Figure 3: Rny1’s cleavage of RNA substrates does not involve known RNA quality control processes.  (A) Diagram of nonfunctional ribosomal RNA decay (NRD) and no-go decay (NGD) pathways detailed and referenced in the text. (B) Northern blot using oRP1398 probe of equivalent amounts of total RNA from yeast strains grown in yeast extract peptone dextrose media for 3 days from early midlog.Samples were all harvested, resolved, and transferred as part of the same blot. See additional details in the Materials and Methods. (C) Same as (B), but blot was stripped and reprobed for 25s rRNA (oRP1423). Arrow indicates the position of an Rny1-dependent band.

Figure 4: Rny1’s nuclease activity is not regulated by ribophagy or autophagy.  (A) Diagram of processes coordinated by Atg7 and Atg15 function. See text for additional details and references. Northern blots in (B) and (C) were performed as in Figures 3B and 3C, respectively.
**Figure 5:** Factors regulating selective autophagy do not affect Rny1’s cleavage of RNA. (A) Diagram of multiple factors and processes analyzed and referenced in the text. Northern blots in (B) and (C) were performed as in (B) and (C) of Figures 3 and 4.

**Figure 6:** Bem1, a regulator of cell budding and vacuole fusion, affects Rny1’s cleavage of RNA. (A) Diagram of Bem1’s roles in cell polarity establishment and budding and its role in complex with Vac8 in vacuole-vacuole fusion. References and details are in the text. (B) Strains indicated were grown 3 days from early midlog in selective synthetic media containing dextrose and were harvested and equal amounts of total RNA were electrophoresed and transferred to membranes, all as a single blot. Northern blot was performed using oRP1398 probe. See additional details in the Materials and Methods section. (C) Same blot as in (B), probed here for oRP1423. Arrow pointing to the lower band is the same Rny1-dependent band indicated in Figures 3C and 4C. Arrow pointing to the upper band is an additional Rny1-dependent band that is visible utilizing synthetic media.

**Figure 7:** Specific Vac8 and Bem1 interactors affect Rny1-dependent cleavage of RNA. (A) Diagram of the multiple functions of Vac8 and Bem1 interactors examined in this analysis. Details and references are within the text. Northern blots in (B) and (C) were conducted as described in (B) and (C) of Figure 6.
Figure 8: Loss of MVB formation increases levels of Rny1-dependent RNA cleavage. (A) Diagram of the processes involved in vacuolar delivery by MVB sorting of cargoes originating from either the plasma membrane or the Golgi. Additional details and references for these processes are within the text. Factors investigated are grouped by function at the bottom of the diagram. Northern blots in (B) and (C) were conducted as described in (B) and (C) of Figures 6 and 7. (C) also uses brackets here to indicate the presence of possible intermediates or products of cleavage by a ribonuclease other than Rny1 in lanes 7 and 15.

Figure 9: Possible mechanism for Rny1’s control of translation during stress. (A) The model depicts a mechanism for delivery of cytoplasmic RNA substrates to Rny1 within vacuoles involving microautophagy, vacuole fusion and non-autophagic CVT function, and vacuolar acidification and possibly unknown lipases (or leaky vacuolar fusion processes as detailed in the text). Loss of MVB or Bem1 function might increase RNA substrates, explaining how these mutants increase RNA fragment accumulation, if these processes do indeed regulate translation in an as-of-yet uncharacterized manner. Additional details and references for this model are included in the text.
FIGURES AND TABLES

Table 1: Effects of over-expressed RNY1 cis mutants on growth and tRNA cleavage

<table>
<thead>
<tr>
<th>Mutation</th>
<th>Background</th>
<th>Growth in a hir2Δ strain</th>
<th>tRNA cleavage in an rny1Δ strain</th>
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<tbody>
<tr>
<td>L2</td>
<td>active</td>
<td>Equal to wild-type</td>
<td>No cleavage - equal to vector</td>
</tr>
<tr>
<td>L2</td>
<td>inactive</td>
<td>Equal to wild-type</td>
<td>Not tested</td>
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<tr>
<td>L3</td>
<td>active</td>
<td>Equal to wild-type</td>
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<td>L3</td>
<td>inactive</td>
<td>Equal to wild-type</td>
<td>Not tested</td>
</tr>
<tr>
<td>L4</td>
<td>active</td>
<td>Equal to wild-type</td>
<td>Size of cleavage products shifted</td>
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<td>inactive</td>
<td>Equal to wild-type</td>
<td>Not tested</td>
</tr>
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<td>Not tested</td>
</tr>
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<td>No cleavage - equal to vector</td>
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<td>Equal to wild-type</td>
<td>Not tested</td>
</tr>
<tr>
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<td>No cleavage - equal to vector</td>
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<td>Not tested</td>
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Table 1: Plasmids (listed under mutation column and detailed in Table S2) were based on pRP1584 (active) or pRP1587 (inactive) backbones containing GAL-RNY1 or GAL-RNY1 containing mutations to catalytic histidines, respectively. Growth indicates results of their over-expression in the hir2Δ strain by frog ponds on plates containing galactose. All strains were tested and grew equivalently on dextrose. The tRNA cleavage was tested by harvesting rny1Δ cells over-expressing the indicated constructs, resolving equivalent amounts of their total RNA by gel electrophoresis, and probing by Northern
blot for tRNA Met(CAT). Additional details can be found within Materials and Methods.

NA=not applicable.
FIGURE 1

A.

B.
FIGURE 1

C. \(my1^{\Delta} + GAL-RNY1\) loop mutants

tRNA Met (CAT) probe
FIGURE 2

A.

<table>
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<tr>
<th>signal peptide</th>
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<th>unique C-terminal domain</th>
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<td>294</td>
</tr>
<tr>
<td></td>
<td></td>
<td>433</td>
</tr>
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</table>

B.

C.
FIGURE 2

D. $my1\Delta + GAL-RNY1$ cis deletions

tRNA Met (CAT) probe
FIGURE 3

A. 25s rRNA with mutations at peptidyl transferase → 25s NRD → Mms1, Rtt101 → Ribosomal protein ubiquitination 25s rRNA decay

STOP → mRNA with stalled translation → NGD 18s NRD → Dom34 → Possible release of translation complex to allow 18s rRNA and mRNA decay by NGD

18s rRNA mutant at decoding center

B. WT  myrΔ  dom34Δ  mms1Δ  rtt101Δ

tRNA 5' His (GTG) probe

C. WT  myrΔ  dom34Δ  mms1Δ  rtt101Δ

25s rRNA probe
FIGURE 4

A.

Processes require Atg7 E3-like functions

Large ribosomal subunit proteins

Other cytoplasmic factors

Pre-autophagosomal structure (PAS)

PAS expansion and closure to form autophagosome

Vacuolar fusion and delivery of single membrane-bound vesicle

Release of lumenal vesicle contents

B.

WT

myr1Δ

atg7Δ

atg15Δ

C.

25s rRNA probe

tRNA 5' His (GTG) probe
FIGURE 5

A. RIBOPHAGY

Deubiquitination of large ribosomal subunit proteins

pre-autophagosomal structure

MITOPHAGY

Receptor-mediated organelar autophagic process

Vacuolar delivery

PEXOPHAGY

Pex10, E3 ubiquitin ligase, enables peroxisome matrix protein import

Pex10 routing of substrates to vacuole?

PIECEMEAL MICRO-NUCLEOPHAGY (PMN)

Nvj1 and Vac8, receptors for exchange at nucleo-vacuolar junctions

B. tRNA 5' His (GTG) probe

C. 25s rRNA probe
FIGURE 6

A. Bem1 serves as a scaffold for cell polarity establishment and budding

B. tRNA 5' His (GTG) probe

C. 25s rRNA probe

Bem1 interacts with Vac8 and positively affects vacuole-vacuole fusion
FIGURE 7

A. VACUOLE INHERITANCE

Vac8

Sna3

MVB CARGO

Atg13

Vac8

Cot1

Pmc1

Zn++

Ca++

Fe

H+

Ftr1

Vma7

Vac8

Vam3

Vam7

Bem1

Vps33

Yck3

VACUOLE-VACUOLE FUSION

B. tRNA 5' His (GTG) probe

vac8Δ

sna3Δ

ubl4Δ

cot1Δ

hxt1Δ

frl1Δ

pmc1Δ

meh1Δ

atg13Δ

yck3Δ

gtr1Δ

swr1Δ

bem1Δ

vps33Δ

vma7Δ

myo2Δ

nec102Δ

vam7Δ

vma7Δ

frl1Δ
FIGURE 7

C.

25s rRNA probe
**FIGURE 8**

**A.**

PLASMA MEMBRANE CARGOES

- Alkaline pH response: Affects filamentation in *C. albicans*

GOLGI CARGOES

- Targeting of Cargoes

Ubiquitin

- Endosomal compartments

MVB

- MVB sorting through ESCRT function

Pre-vacuolar compartments

Vacuole

- Vacular delivery of cargoes and recycling of ESCRT proteins requiring late-acting proteins

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<tr>
<th>Ear1</th>
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<th>Vps36</th>
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<th>Vps4</th>
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<td>Vpa20</td>
<td>Vpa24</td>
<td>Snf7</td>
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**Targeting**

- ESCRT-I

- ESCRT-II

- ESCRT-III

- Late-acting

**B.**

tRNA 5' His (GTG) probe
FIGURE 8

C.

![Image of gel electrophoresis with labeled lanes and arrows indicating bands.](image)

25s rRNA probe
FIGURE 9

A. CONTROL OF TRANSLATION BY RNY1 DURING STRESS

- Translation failing to sort into MVBs or deregulated by loss of Bem1
- Substrates originating from other pathways?
- Microautophagic invagination
- Activation of Rny1’s access to RNA
- Fusion pore opening to release RNA substrates
- Compartmentalization by vacole docking/fusion and CVT (Vac8)
- Vma7? Lipase?
**SUPPLEMENTAL DATA**

**Table S1: Strains used in this study**

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<td>BY4741</td>
<td>Mat a his3D1 leu2D0 met15D0 ura3D0</td>
<td>(Brachmann, Davies et al. 1998)</td>
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<tr>
<td>All yeast gene</td>
<td>EUROSCARF deletion library isogenic to BY4741</td>
<td>(Winzeler, Shoemaker et al. 1999)</td>
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**Table S2: Plasmids used in this study**

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<td>pRP1584</td>
<td>RNY1 under a GAL promoter on a 2μ URA3 plasmid, obtained from Thermo Scientific</td>
<td>(Thompson and Parker 2009)</td>
</tr>
<tr>
<td>pRP1587</td>
<td>pRP1584 mutated at two DNA sites encoding catalytic histidines to make catalytically inactive protein</td>
<td>(Thompson and Parker 2009)</td>
</tr>
<tr>
<td>pRP861</td>
<td>Used as vector in experiments using GAL-RNY1. pPS293: GAL1 promoter (EcoRI/XbaI fragment from pPS231), 2μ,URA3</td>
<td>(Lee, Henry et al. 1996)</td>
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<td>3a</td>
<td>L7 active</td>
<td>Quikchange mutated pRP1584 (active GAL-RNY1) or pRP1587 (inactive GAL-RNY1) to replace all residues within loops (see Figure 1) with alanines. Primer sequences used to make these constructs are listed in Table S3. This study</td>
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<td>9b</td>
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<td>ΔSP</td>
<td>Quikchange mutated using pRP1584 template to delete the DNA sequence</td>
<td>This study</td>
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encoding the putative signal peptide (see Figure 1)  

ΔSPci Quikchange mutated using pRP1587 template to delete the DNA sequence encoding the putative signal peptide (see Figure 1)  

ΔCTD Quikchange mutated using pRP1584 template to delete the DNA sequence encoding the unique C-terminal domain (see Figure 1)  

ΔCTDci Quikchange mutated using pRP1587 template to delete the DNA sequence encoding the unique C-terminal domain (see Figure 1)  

ΔT2 Quikchange mutated using pRP1587 template to delete the conserved T2 region (see Figure 1)  

ΔT2ci Quikchange mutated using pRP1587 template to delete the conserved T2 region (see Figure 1)  

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<td>GTACTAACACTATACTAAG</td>
<td>Probe for 5’ tRNA His (GTG) region</td>
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<td>oRP1401</td>
<td>GCGCCGCTCGGTTTCGATCC</td>
<td>Probe to tRNA Met (CAT)</td>
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<td>oRP1423</td>
<td>CAAAGGCTTAATCTCAGCAGATCG</td>
<td>Probe for 25s rRNA</td>
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<td>oRP1437</td>
<td>ATCTCTGTATTTCTCAAATTTGACCAA</td>
<td>Probe for U6 snRNA</td>
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<td>oNLm97</td>
<td>CCT ATA AAC ATT CCA GCA GCA GCA GCA GCA GCA GCA GCA GCA GCA GCA GCA GCA GCA</td>
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We thank Dr. Pamela A. Silver for providing the vector control plasmid used in our studies (pRP861). We are also grateful to Dr. Greg Odorizzi for valuable advice and feedback in the analyses and interpretations of the multivesicular body mutants. We especially appreciate the guidance of Anne Webb in designing the illustrations for several of the figures. Finally, we thank all members of the Parker lab for their valuable feedback and useful discussions in the preparation of this manuscript.
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REFERENCES


APPENDIX D: FUTURE STUDIES
We are currently pursuing answers to the following questions:

**Do factors influencing tRNA cleavage affect Rny1’s expression or localization?**

Our data indicating effects of Vac8 and Bem1 on levels of tRNA and rRNA fragment accumulation could arise from alterations to Rny1’s expression and/or localization. We need to analyze expression of Flag-Rny1 in the deletion strain exhibiting effects on tRNA and rRNA cleavage to rule out any possible effects on Rny1’s expression. We will also examine Rny1’s localization (either in live cells using GFP or in fixed cells using Flag-Rny1) in strains deleted for *VAC8* or *BEM1* to see whether this is altered.

**Does Rny1’s nuclease activity affect specific mRNAs’ expression?**

We are considering possible ways in which Rny1’s catalytic and non-catalytic functions against tRNA and rRNA affect gene expression. Rny1’s nuclease effects could be global, perhaps entailing downregulation and/or quality control of translation complex RNA (tRNA, rRNA, and possibly mRNA) during damaging or nutrient-limiting stresses, or there might be a subset of mRNAs whose entire translation complex is targeted for degradation by Rny1. In addition, non-catalytic functions of Rny1, perhaps through interactions with other factors, could affect gene expression independent of catalytic activity.

To address these questions, we utilized microarrays to probe for effects on gene expression, comparing cells deleted for *RNY1* to those expressing Rny1 or its catalytically inactive form, to assay for the role of nuclease activity in gene expression.
Comparisons were performed under unstressed (exponential growth) and stressed, nutrient-limited (stationary phase growth) conditions.

Two possible results are currently under investigation. First, a subset of RNAs might be catalytically downregulated by Rny1 during stress. If this is true, we would expect to see specific mRNAs decreased with expression of wild-type Rny1, but not the catalytic inactive mutant. Second, Rny1 might influence gene expression independent of its catalytic activity. In this scenario, we would expect to see a subset of genes increased or decreased with expression with either wild-type Rny1 or the catalytically inactive mutant’s expression.

**How are RNA (cytoplasmic) and Rny1 (compartmentalized) brought together within the cell to enable cleavage to occur?**

The simplest model, supported by our evidence, is that Vac8 mediates delivery of RNA substrates to the vacuole where acidic-dependent nuclease activity by Rny1 occurs. Consistent with this idea, our analysis of Vac8 and Bem1 interactors identified several factors required for tRNA and rRNA cleavage whose functions impinge on possible modes for RNA substrate delivery to vacuoles: vacuole docking and fusion (Vam3, Vam7, Vps33) and a protein regulating a process by which cytoplasmic material can be enveloped and released within the vacuole, microautophagy, (Meh1). Also in accordance with this model, Rny1 has been visualized within the vacuole, an acidic compartment, and as a T2 ribonuclease would be expected to cleave RNA optimally at acidic pH. Moreover, vacuolar acidification appears to be required for tRNA and rRNA cleavage by Rny1, suggesting that RNA substrates and Rny1 coexist within vacuoles to enable RNA
cleavage. Nevertheless, it is possible that Rny1 could exit the vacuole through a novel mechanism. Thus, direct analysis of localization of Rny1 and RNA substrates in strains wild-type or mutant for trans-regulatory proteins is necessary.

If Rny1 cleaves tRNA or rRNA within vacuoles, we would expect that uncleaved tRNA and/or rRNA might accumulate within vacuoles in an Rny1 mutant or a vacuole fusion mutant (such as \textit{VAC8}). We are currently attempting to isolate vacuoles in wild-type and Rny1 mutant strains to probe for tRNA and rRNA substrates, as well as Flag-Rny1 localization.

Rny1-dependent tRNA and/or rRNA fragments might also be visible within wild-type vacuoles, and the mutants showing increased RNA fragment accumulation. These mutant strains might be useful in probing for the location of tRNA or rRNA fragments within vacuoles. Alternatively, these fragments might be routed to other areas of the cell and exert roles in other aspects of gene expression regulation, for instance, transcription at the nucleus. In this case, it could be useful to do crude sedimentation analyses to determine whether other organelles (such as the nucleus) or the cytoplasm are the site of localization of these fragments.
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