The RNase Rny1p cleaves tRNAs and promotes cell death during oxidative stress in *Saccharomyces cerevisiae*

Debrah M. Thompson and Roy Parker

Department of Molecular and Cellular Biology, and Howard Hughes Medical Institute, University of Arizona, Tucson, AZ 85721

The cellular response to stress conditions involves a decision between survival or cell death when damage is severe. A conserved stress response in eukaryotes involves endonucleolytic cleavage of transfer RNAs (tRNAs). The mechanism and significance of such tRNA cleavage is unknown. We show that in yeast, tRNAs are cleaved by the RNase T2 family member Rny1p, which is released from the vacuole into the cytosol during oxidative stress. Rny1p modulates yeast cell survival during oxidative stress independently of its catalytic ability. This suggests that upon release to the cytosol, Rny1p promotes cell death by direct interactions with downstream components. Thus, detection of Rny1p, and possibly its orthologues, in the cytosol may be a conserved mechanism for assessing cellular damage and determining cell survival, analogous to the role of cytochrome *c* as a marker for mitochondrial damage.

Introduction

Stress conditions stimulate a variety of responses in eukaryotic cells, ranging from stress neutralization to cell death. Recent studies have described a novel aspect of stress responses in eukaryotic cells wherein cytosolic tRNAs are separated into half molecules by cleavage in the anticodon loop. Such tRNA cleavage has been observed during amino acid starvation in *Tetrahymena thermophila* and *Aspergillus fumigatus* (Lee and Collins, 2005; Jochl et al., 2008), during development in the bacterium *Streptomyces coelicolor* (Haiser et al., 2008), and in human, plant, and yeast systems during oxidative stress (Thompson et al., 2008; see Yamasaki et al. on p. 35 of this issue). Moreover, large-scale small RNA sequencing studies have identified cleaved tRNAs in human cells (Kawaji et al., 2008). Interestingly, tRNA fragments are also observed in the urine and sera of cancer patients, with levels correlated with the tumor burden (Waalkes et al., 1975; Borek et al., 1977; Speer et al., 1979; Tamura et al., 1987). An unresolved issue is the mechanism and significance of stress-induced tRNA cleavage.

We demonstrate that cytosolic RNAs in yeast are cleaved during oxidative stress by release of the endonuclease RNase in Yeast 1 (Rny1p) from the vacuole into the cytosol. Rny1p also modulates cell survival during oxidative stress, but this effect is independent of its catalytic ability. These observations argue that release of Rny1p from the vacuole both promotes tRNA cleavage and separately activates a downstream pathway that promotes cell death. This is analogous to the classical example of cytochrome *c* release from mitochondria promoting apoptosis, and may be a general principle of how cells sense damage to membrane-bound compartments. Strikingly, the human orthologue of Rny1p, RNASET2, is a tumor suppressor that functions in a manner independent of its catalytic activity (Acquati et al., 2005; Smirnoff et al., 2006), which suggests that a similar control circuit occurs in mammalian cells and impacts on tumor progression.

Results and discussion

The endonuclease Rny1p is responsible for tRNA cleavage

To identify the endonuclease responsible for tRNA cleavage, we screened several mutant yeast strains lacking predicted endonucleases for their effect on tRNA cleavage during H_20_2 exposure or entry into stationary phase, when tRNA fragments are easily detected (Thompson et al., 2008). Only strains lacking *RNY1*, which is a member of the RNaseT2 family (MacIntosh et al., 2001),

Correspondence to R. Parker: rrparker@email.arizona.edu

Abbreviations used in this paper: Rny1p, RNase in Yeast 1; SCM, synthetic complete medium; WT, wild type.

The Rockefeller University Press $30.00
J. Cell Biol. Vol. 185 No. 1 43–50
www.jcb.org/cgi/doi/10.1083/jcb.200811119

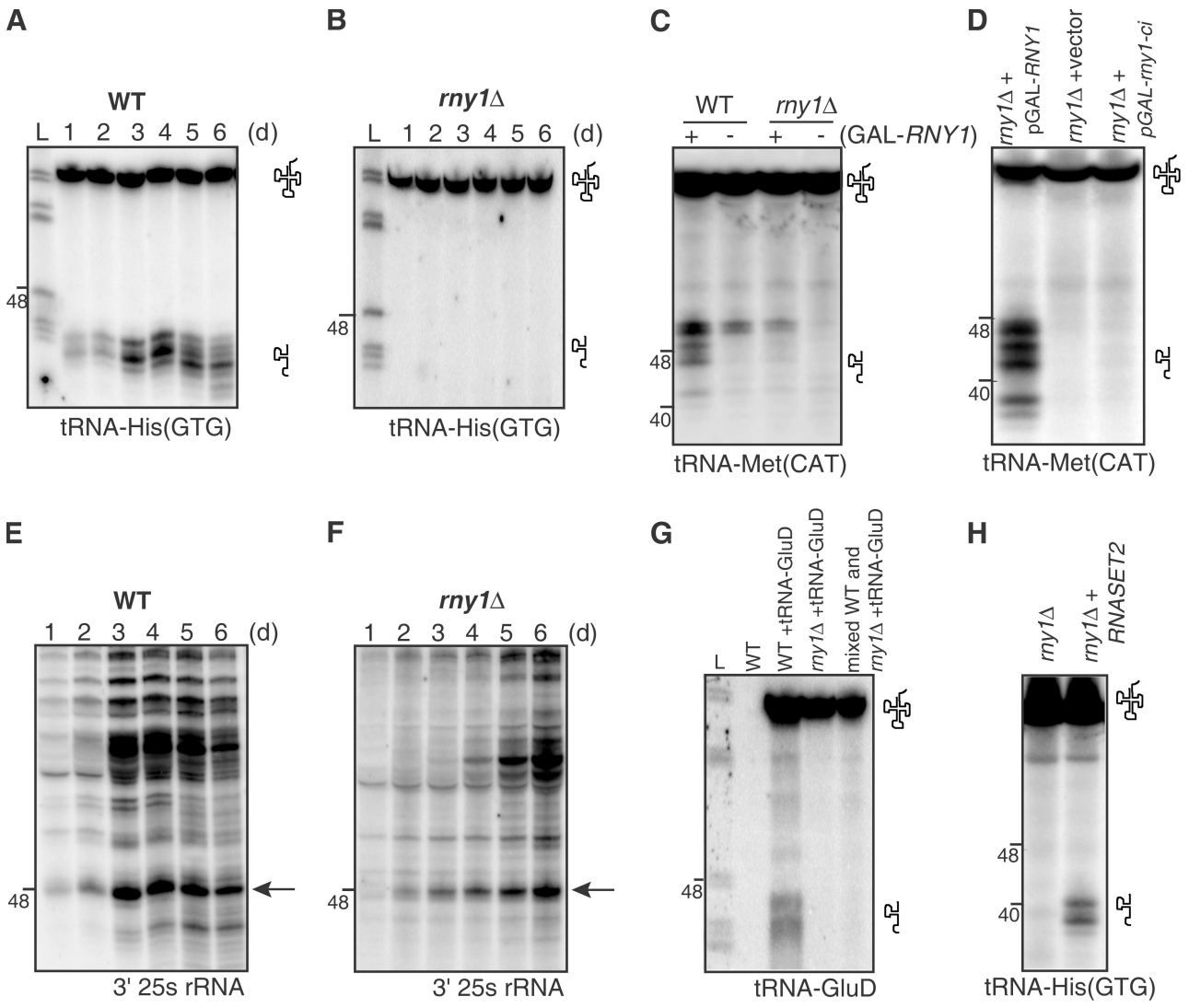

Figure 1. **Rny1p is the endonuclease responsible for yeast tRNA cleavage.** (A) 5′ tRNA-His(GTG) probe. tRNA cleavage in WT yeast during stationary phase entry. WT yeast were grown for 6 d. (B) 5′ tRNA-His(GTG) probe. tRNA cleavage does not occur in yeast lacking *RNY1*. *rny1Δ* yeast were grown as in A. The contrast in panels A and B were adjusted to similar levels to facilitate comparison. (C) 3′ tRNA-Met(CAT) probe. Expression of *RNY1* from a plasmid restores tRNA cleavage. The pGAL-*RNY1* vector (+) or the empty vector pRS426 (−) were transformed into WT and *rny1Δ* cells. Yeast were grown to mid-log phase. (D) 3′ tRNA-Met(CAT) probe. Overexpression of *rny1-ci* in *rny1Δ* cells does not rescue tRNA cleavage; compare this with the overexpression of *RNY1*. Yeast were grown as in C. (E) 3′ 25s rRNA probe (arrow). rRNA cleavage in WT yeast during stationary phase entry. This is a reprobing of the blot shown in A. (F) 3′ 25s rRNA probe (arrow). rRNA cleavage in *rny1Δ* yeast during stationary phase entry. This is a reprobing of the blot shown in B. (G) 3′ tRNA-GluD probe. tRNA cleavage does not occur during postharvest cell lysis. WT (yRP840) and *rny1Δ* strains carrying a *D. discoideum* tRNA (tRNA-GluD) were cocultured or grown separately for 3 d in selective medium. (H) 5′ tRNA-His(GTG) probe. Overexpression of human *RNASET2* in an *rny1Δ* strain rescues tRNA cleavage. Yeast were grown as in C. tRNA illustrations indicate full-length and fragment species. L = φX174/*Hinf 1* ladder (sizes are indicated in nucleotides). Experiments were repeated at least three times; representative blots are shown.

failed to produce tRNA fragments during oxidative stress, entry into stationary phase, or other stress conditions (Fig. 1, A and B; and not depicted). tRNA fragment production in the *rny1Δ* strain can be rescued by providing *RNY1* on a plasmid, and is increased when *RNY1* is overexpressed from the *GAL* promoter (Fig. 1 C). Rny1p is likely to directly cleave tRNAs, as expression of a catalytically inactive version (*rny1-ci*), with two key histidine residues in the active site replaced by phenylalanines (H87F and H160F; Deshpande and Shankar, 2002; Acquati et al., 2005), fails to restore tRNA fragment production in an *rny1Δ* strain (Fig. 1 D). These observations argue that Rny1p is the nuclease cleaving tRNAs in yeast.

Rny1p can also cleave ribosomal RNAs (rRNAs)

rRNA fragments are also detected in yeast, and increased rRNA fragment levels are observed during oxidative stress conditions (Thompson et al., 2008). Therefore, we asked whether loss of Rny1p also affects rRNA fragment levels during oxidative stress, using a probe against the 3′ end of the 25S rRNA that corresponds to a common rRNA cleavage product (Fig. 1, E and F, arrows; Thompson et al., 2008). We observed that the *rny1Δ* strain did have fewer rRNA fragments overall, but rRNA fragments were still observed (Fig. 1, E and F). This demonstrates that Rny1p can affect rRNA fragment production, but that there

are likely to be additional nucleases that can act on rRNA during oxidative stress. That Rny1p is able to cleave other RNAs in addition to tRNAs is consistent with work showing that T2 RNases have broad specificity (Deshpande and Shankar, 2002).

Rny1p does not act in trans

Rny1p contains a putative N-terminal signal peptide for entry into the secretory pathway and is thought to be a secreted protein (MacIntosh et al., 2001), but it also accumulates in yeast vacuoles (see the following paragraph). This partitioning of Rny1p away from the cytosol raised the possibilities that tRNA cleavage was either an artifact of cell lysis, with extracellular or vacuolar Rny1p interacting with tRNAs released from the cytoplasm during lysis, or that Rny1p could be transferred between cells. To test these possibilities, we cocultured two yeast strains: an *rny1Δ* strain expressing a *Dictyostelium discoideum* tRNA (referred to as tRNA-GluD; Shaheen and Hopper, 2005), and a wild-type (WT) strain lacking tRNA-GluD. We observed that tRNA-GluD is not cleaved by Rny1p provided in trans, although when tRNA-GluD is expressed in a WT strain, tRNA-GluD fragments are clearly visible (Fig. 1 G). This demonstrates that Rny1p-mediated tRNA cleavage only occurs within cells expressing Rny1p and is not an artifact of cell lysis.

Vacuolar release of Rny1p correlates with tRNA cleavage

An unresolved issue was how cytoplasmic tRNAs and secreted or vacuolar localized Rny1p might interact. One possibility was that tRNAs enter the vacuole during stress conditions, possibly via autophagy (Scherz-Shouval and Elazar, 2007). However, tRNA fragment production is identical in autophagy-deficient and WT yeast strains (Fig. S1). A second possibility was that oxidative stress induces release of Rny1p from the vacuole into the cytoplasm. To address this issue, we localized a GFP-tagged version of Rny1p before and during oxidative stress.

In the absence of oxidative stress, Rny1p was primarily concentrated in the vacuole, as assessed by colocalization with the vacuolar luminal dye 7-amino-4-chloromethylcoumarin (CMAC; Fig. 2 A). Smaller puncta of Rny1p are also observed, which may be vesicles involved in Rny1p biogenesis, as expected for a protein with a secretion peptide. After H_2O_2 exposure, the level of Rny1p in the vacuole is reduced, which suggests that Rny1p is delocalized from the vacuole during stress. Vacuolar exit does not appear to be the result of stress-induced lysis or breakdown of the vacuole, as the luminal CMAC dye is retained in vacuoles during oxidative stress (Fig. 2 A). Moreover, by Western blot analysis, the amount of Rny1p does not appreciably decline after exposure to oxidative stress, which argues that loss of the vacuolar signal is not caused by degradation of Rny1 protein (Fig. 2 B). The strongly fluorescent puncta observed after H_2O_2 treatment do not represent a novel cytosolic location for Rny1p, as they colocalize with mitochondria, and are present even in control strains without a GFP-tagged protein (Fig. S2 A), which suggests mitochondrial autofluorescence, possibly from oxidized flavoproteins (see Foster et al., 2006). Therefore, after oxidative stress, our results suggest that Rny1p is distributed in the cytosol, which provides a mechanism by which Rny1p could access tRNAs and rRNAs for cleavage.

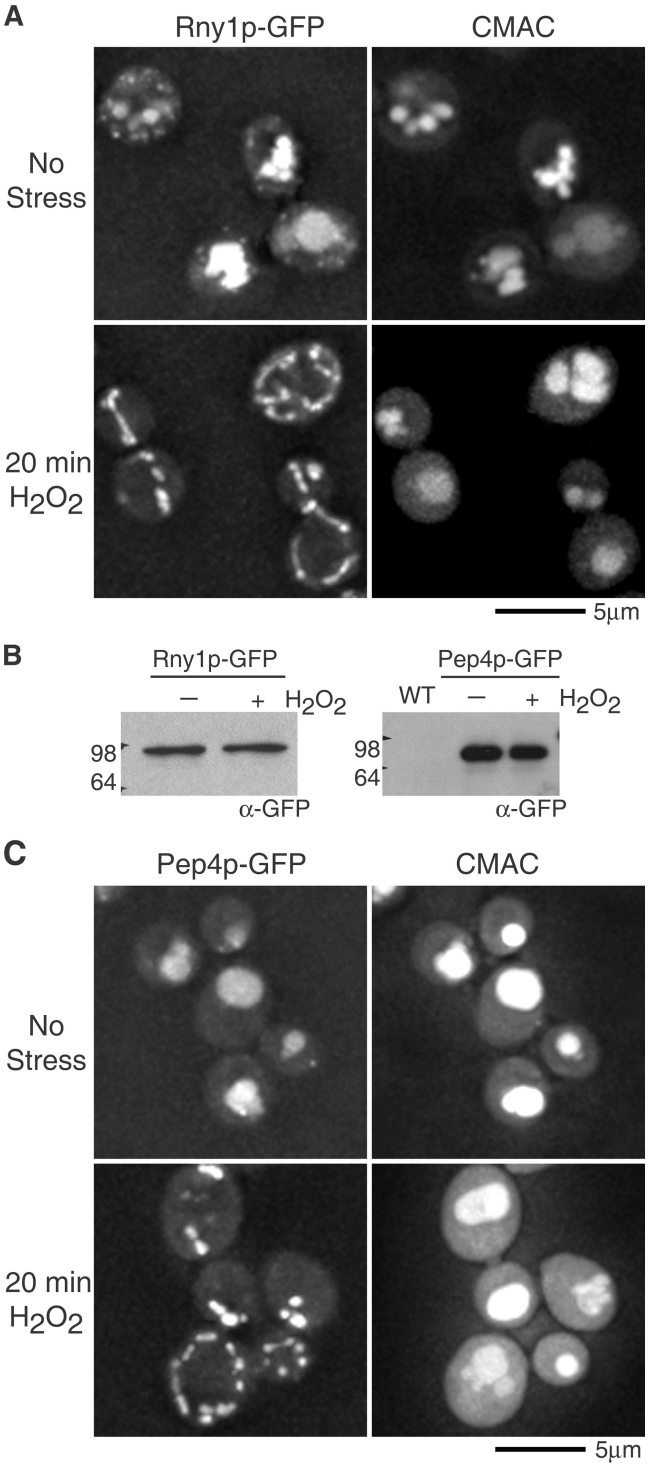

Figure 2. **Rny1p localization before and during oxidative stress.** (A) Localization of Rny1p-GFP during log phase and after a 20-min exposure to 3 mM H_2O_2. (B) Western blot analysis of Rny1p-GFP or Pep4p-GFP levels in cells before and after 1 h exposure to 3 mM H_2O_2. Approximate molecular masses are indicated in kD. (C) Localization of Pep4p-GFP during log phase and after a 20-min exposure to 3 mM H_2O_2. The vacuolar luminal dye CMAC was used to mark vacuoles. Experiments were repeated at least three times; representative examples are shown.

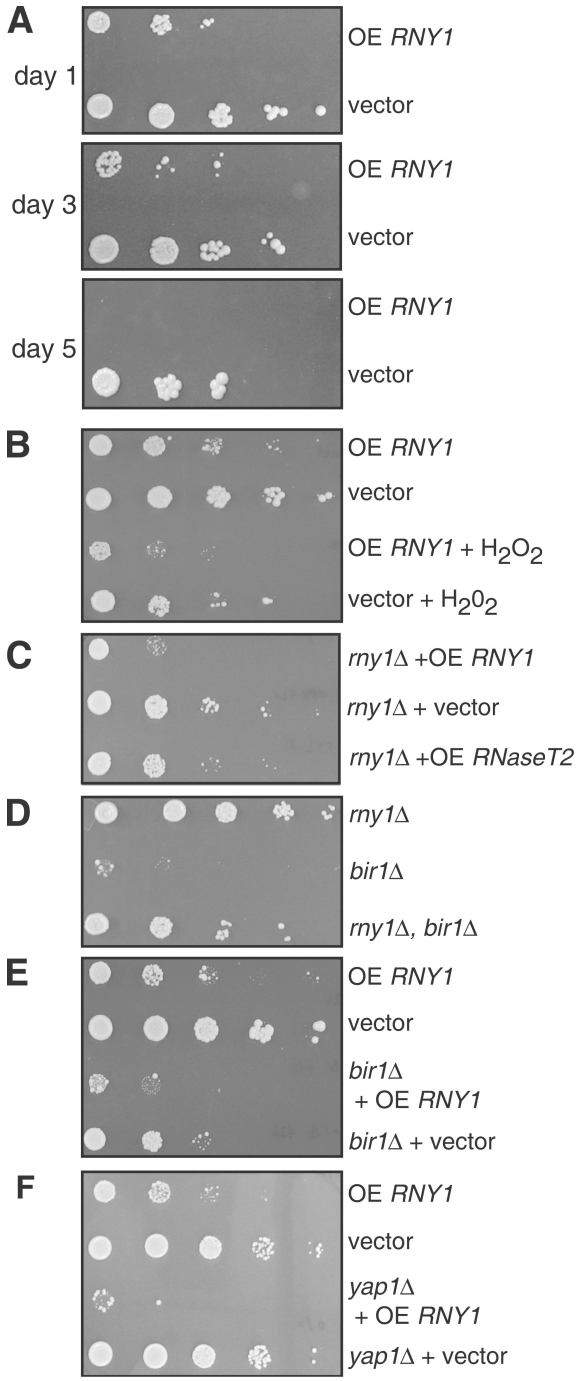

Figure 3. ***RNY1* promotes cell death during stationary phase and oxidative stress.** (A) Overexpression of *RNY1* affects viability. Yeast carrying the pGAL-*RNY1* (OE *RNY1*) or an empty vector were grown for the indicated number of days. 10-fold serial dilutions of cells were plated. (B) Overexpression of *RNY1* sensitizes cells to oxidative stress. Indicated strains were grown to mid-log phase, then exposed to 0 or 3 mM H_2O_2 for 1 h. 10-fold serial dilutions were plated. (C) Overexpression of human *RNASET2* in an *rny1Δ* strain results in a growth defect. Yeast carrying the pGAL-*RNY1* (OE *RNY1*), the pGAL-*RNASET2* (OE *RNASET2*), or vector were grown as in A. 10-fold serial dilutions were plated. (D) Deletion of *RNY1* rescues the *bir1Δ* growth defect. Indicated strains were grown for 3 d, then 10-fold serial dilutions were plated. (E) Overexpression of *RNY1* exacerbates the *bir1Δ* growth defect. *bir1Δ* cells carrying either the pGAL-*RNY1* plasmid (OE *RNY1*) or vector were grown for 3 d, then 10-fold serial dilutions were plated. (F) Overexpression of *RNY1* exacerbates the *yap1Δ* growth defect in response to oxidative stress. *yap1Δ* cells carrying either the pGAL-*RNY1* plasmid (OE *RNY1*) or vector were grown as in E. Experiments were repeated at least three times; representative experiments are shown. pRS426 was the empty vector control.

To determine whether Rny1p is unique in being released from vacuoles during oxidative stress, we examined whether GFP-tagged versions of the vacuolar proteases Pep4 and Prc1, or the luminal protein Npc2 were also released into the cytosol after oxidative stress. We observed that all three of these vacuolar proteins are concentrated in vacuoles during log-phase growth (Fig. 2 C and Fig. S3, A and B). During oxidative stress conditions, these proteins, like Rny1p, decline in concentration in the vacuole and increase in the cytosol (as assessed away from the mitochondrial background), although their overall levels do not significantly decline during oxidative stress (Fig. 2 B and Fig. S3 C). These observations indicate that during oxidative stress, multiple proteins are released from the vacuole, presumably because of damage to, or increased permeability of, the vacuolar membrane. Moreover, this release of proteins from the vacuole is likely to be conserved, as previous studies have indicated that human lysosomal proteins are released during oxidative stress (for review see Guicciardi et al., 2004).

Rny1p promotes cell death

Because oxidative stress can promote apoptosis and also resulted in high levels of tRNA cleavage, we hypothesized that Rny1p and/or tRNA cleavage might contribute to cell death. To examine this possibility, we first determined the effects of overexpression or deletion of *RNY1* on yeast undergoing oxidative stress, either from entry into stationary phase or from exogenously added H_2O_2. We observed that strains overexpressing *RNY1* show reduced viability as cells enter stationary phase (Fig. 3 A). Additionally, yeast overexpressing *RNY1* are hypersensitive to oxidative stress from H_2O_2 exposure (Fig. 3 B). This suggests that overexpression of *RNY1* hypersensitizes cells to oxidative stress, thus promoting cell death during peroxide exposure and stationary phase onset.

Expression of human *RNASET2* in yeast results in tRNA cleavage and a growth defect

Because tRNA cleavage during oxidative stress is conserved among eukaryotes (Thompson et al., 2008; Yamasaki et al., 2009), we hypothesized that orthologues of Rny1p might be responsible for tRNA cleavage in other organisms. To begin to address this issue, we examined the effects of expressing the human orthologue of *RNY1*, *RNASET2*, in an *rny1Δ* yeast strain. We observed that overexpression of *RNASET2* restores tRNA fragment production in an *rny1Δ* yeast strain (Fig. 1 H) and confers a similar loss of viability in response to oxidative stress, as does overexpression of *RNY1* (Fig. 3 C). siRNA knockdowns of *RNASET2* levels in human cells do not reduce tRNA cleavage during stress conditions (unpublished data; Yamasaki et al., 2009), which is consistent with the angiogenin nuclease playing a predominant role in stress-induced tRNA cleavage in mammalian cells (Fu et al., 2009; Yamasaki et al., 2009). However, the ability of RNASET2 to complement an *rny1Δ* strain suggests that RNASET2 may have some role in tRNA cleavage in mammalian cells.

Rny1p and yeast apoptosis

WT and *rny1Δ* cells showed similar viability during entry into stationary phase and after H_2O_2 treatment (unpublished data), which suggests that the role of Rny1p in cell death is not limiting under these conditions. However, if Rny1p plays a significant, but nonlimiting, role in promoting cell death, then the loss of Rny1p might be expected to suppress mutations that predispose yeast cells to premature apoptosis. The yeast protein Bir1 is an apoptotic inhibitor, and the *bir1Δ* strain shows both increased cell death in response to stress and a growth defect due to premature cell death (Walter et al., 2006). Strikingly, we observed that deletion of *RNY1* suppressed the growth defect of a *bir1Δ* mutant strain, resulting in increased viability (Fig. 3 D). Moreover, overexpression of *RNY1* exacerbates the sensitivity of *bir1Δ* yeast to oxidative stress (Fig. 3 E). In similar fashion, overexpression of *RNY1* also increases the sensitivity of *yap1Δ* yeast to oxidative stress (Fig. 3 F), Yap1p being a key factor in the response to oxidative stress in yeast (Ikner and Shiozaki, 2005). The fact that deletion of *RNY1* suppresses the premature death of the *bir1Δ* strain, and that overexpression of *RNY1* hypersensitizes *yap1Δ* and *bir1Δ* cells to oxidative stress, indicates that Rny1p contributes to the modulation of cell survival during oxidative stress.

The role of Rny1p in cell death is independent of its catalytic activity

The similarity of tRNA cleavage in yeast and mammalian cells, and the ability of the human orthologue RNASET2 to complement an *rny1Δ* yeast strain, suggested that the biological response to oxidative stress involving these nucleases is a conserved process. Previous work has shown that additional expression of *RNASET2*, or addition of recombinant RNASET2 protein, inhibits colony formation and metastasis of tumor cell lines, though in a manner independent of the catalytic activity of RNASET2 (Acquati et al., 2001, 2005; Smirnoff et al., 2006). This suggested two possible overlapping mechanisms by which Rny1p could influence cell fate. In one, release of Rny1p from the vacuole increases tRNA cleavage, resulting in an inhibition of cellular function, either by the reduction of critical RNA levels or by the tRNA fragments themselves having an inhibitory function. Alternatively, in a manner similar to release of cytochrome *c* from the mitochondria triggering apoptosis (for review see Eisenberg et al., 2007), movement of Rny1p out of the vacuole could inhibit cellular function in a manner independent of its nuclease activity, possibly as part of a mechanism to sense vacuolar damage.

To distinguish between these possibilities, we generated a catalytically inactive form of Rny1p (rny1p-ci) by replacing two highly conserved residues in the catalytic core. Similar mutations in other RNase T2 family members inactivate the enzyme (Deshpande and Shankar, 2002; Acquati et al., 2005). Overexpression of *rny1-ci* does not rescue tRNA fragment production in an *rny1Δ* mutant strain (Fig. 1 D), which demonstrates that these mutations inhibit the nuclease activity of Rny1p. However, rny1-ci protein is expressed at levels similar to WT Rny1p (both expressed from the *GAL* promoter), localizes to the vacuole during mid-log phase growth, and is released during oxidative stress (Fig. S2, B and C). Thus, rny1p-ci behaves similarly to WT Rny1p but is unable to cleave RNAs.

Examination of the phenotype of cells expressing *rny1-ci* led to two important observations. First, like WT *RNY1*, overexpression of *rny1-ci* reduces cell viability in response to stationary phase entry or peroxide stress, or in a *yap1Δ* or *bir1Δ* strain (Fig. 4 and unpublished data). Second, expression of *RNY1* or *rny1-ci* at endogenous levels in the *bir1Δ rny1Δ* strain suppressed the enhanced viability conferred by deletion of *RNY1* (compare Fig. 4 D to Fig. 3 E). These results demonstrate that the effects of Rny1p on growth are independent of its nuclease activity, similar to the role of RNASET2 in tumor suppression in mammalian cells.

A conserved control circuit controlling cytosolic RNA cleavage and cell fate

Our results describe a control circuit that modulates the response to oxidative stress, affecting both cytosolic RNA cleavage and, separately, cell fate. This circuit has the following key features (Fig. 5). First, oxidative stress prompts relocalization of Rny1p, as well as other vacuolar proteins (Mason et al., 2005), from the vacuole to the cytosol (Fig. 2). Second, the presence of Rny1p in the cytosol allows tRNA cleavage, as well as

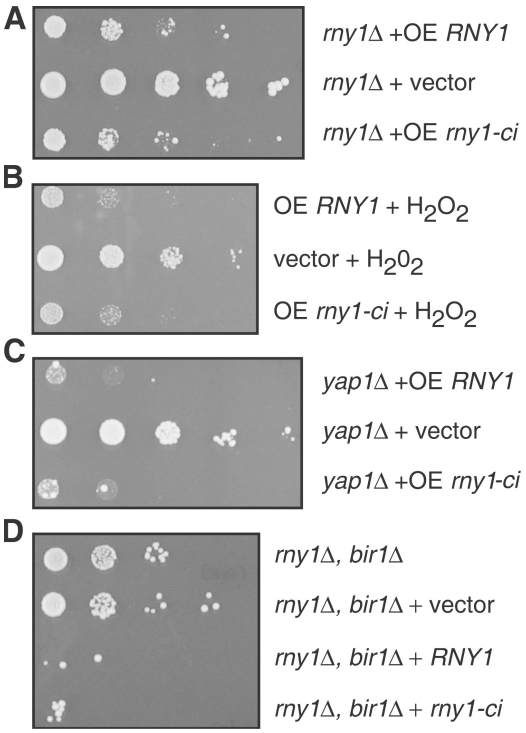

Figure 4. **Catalytically inactive and WT *RNY1* both promote cell death during oxidative stress.** (A) Overexpression of *RNY1* or *rny1-ci* results in a growth defect. Indicated strains were grown for 3 d, then 10-fold serial dilutions of cells were plated to assess viability. (B) Overexpression of *RNY1* or *rny1-ci* sensitizes cells to oxidative stress. Indicated strains were grown to mid-log phase and exposed to 3 mM H_2O_2 for 1 h, then 10-fold serial dilutions were plated. (C) Overexpression of *RNY1* or *rny1-ci* exacerbates the *yap1Δ* growth defect in response to oxidative stress. Strains were grown as in A. (D) Expression of *RNY1* or *rny1-ci* at endogenous levels in a *bir1Δ rny1Δ* strain results in reversion to the *bir1Δ* phenotype. Cells were grown as in A. Experiments were repeated at least three times; representative experiments are shown.

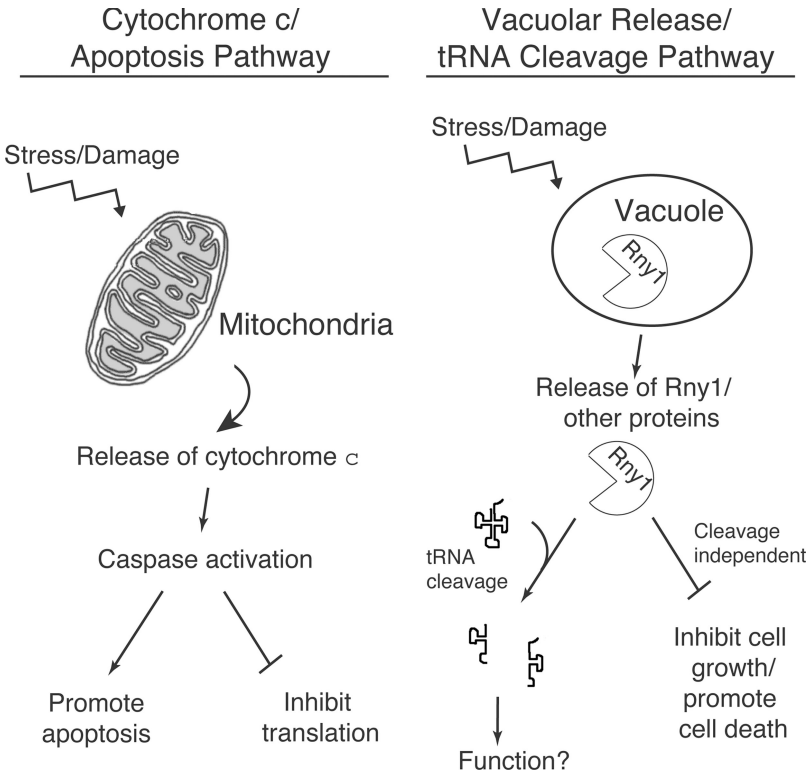

Figure 5. **Organellar release of proteins involved in stress responses.** This model compares the cytochrome *c*–apoptosis pathway to how release of Rny1p from the vacuole might affect cell death after stress or damage.

cleavage of rRNA and potentially some mRNAs (Fig. 1). Third, Rny1p reduces cell growth independently of its nuclease activity (Fig. 4), presumably by affecting signaling pathways. Note that the accumulation of an organellar component in the cytosol inhibiting growth is analogous to cytochrome *c* release from the mitochondria promoting apoptosis (Eisenberg et al., 2007). Thus, the cellular response to stress involves parallel pathways for sensing cellular damage to membrane-bound compartments, the summation of which determines whether cells grow or commit to cell death.

Evidence suggests that a related pathway is conserved in mammalian cells. First, endonucleolytic cleavage of tRNAs in response to oxidative stress is observed in mammalian cells (Thompson et al., 2008; Yamasaki et al., 2009). Second, overexpression of the human *RNY1* orthologue *RNASET2* both complements an *rny1Δ* strain with regard to tRNA cleavage and also reduces yeast viability after stress (Figs. 1 H and 3 C). Third, expression of *RNASET2* has been shown to affect cell growth in mammalian cells and to reduce the metastatic potential of tumor cell lines both in vivo and in vitro, independent of its nuclease activity (Acquati et al., 2001, 2005; Smirnoff et al., 2006). Finally, release of lysosomal proteins into the cytosol during oxidative stress has been observed in mammalian cells and is thought to promote entry of cells into apoptosis (for review see Guicciardi et al., 2004). However, the observations that angiogenin cleaves tRNAs during oxidative stress (Fu et al., 2009) and that tRNA fragments may have an additional role in inhibiting translation (Yamasaki et al., 2009) suggest that the mammalian process may be more complex. One possibility is that mammalian cells have evolved separate mechanisms to inhibit cell growth, using RNASET2, and to cleave tRNAs and alter translation, performed by angiogenin.

Vacuole/lysosome homeostasis appears to affect cells and tumor progression in a manner consistent with our observations. For example, we have observed that in a yeast strain defective in vacuolar pH maintenance, Rny1p-GFP is not concentrated in vacuoles, and tRNA cleavage is increased (unpublished data). Moreover, lysosomal proton pumps are up-regulated in several human cancers, which contributes to the tendency of tumor microenvironments to acidity (see Fais et al., 2007). Finally, proton pump inhibitors have proven effective in inducing apoptosis in human tumor cells both in vitro and ex vivo by a mechanism involving increased permeability of the lysosomal membrane (De Milito et al., 2007). These observations, and the effect of RNASET2 on tumor progression, suggest that release of Rny1p or its orthologues in response to oxidative stress is not only a conserved process, but one that may modulate tumor progression in humans.

Materials and methods

Yeast strains and growth conditions

Strains used in this study are listed in Table S1. BY4741 (Thermo Fisher Scientific) was the WT strain used unless indicated. The *bir1Δ* strain was obtained from F. Madeo (Karl-Franzens University, Graz, Austria; Walter et al., 2006). Yeast were cultured at 30°C in yeast extract-peptone (YEP) medium with 2% glucose (YEPD) or in synthetic complete medium (SCM) supplemented with 2% glucose. As all plasmids used in this study carried a *URA3* yeast auxotrophic marker, "selective SCM" indicates SC medium lacking uracil. For galactose induction of gene expression, strains were grown overnight in selective SCM containing 2% sucrose, then diluted to OD_{600} = 0.1 in fresh medium containing 2% galactose. "Mid-log phase" indicates growth to OD_{600} = 0.3–0.5. Coculturing of strains (Fig. 1 G) involved mixing equal amounts of cells (based on OD_{600}) from overnight cultures for a total starting OD_{600} of 0.1. Cultures were grown for 2 d, then harvested for RNA.

To delete *rny1Δ* in yRP840 (yRP2449), the *rny1Δ::kanMX* deletion cassette was amplified from strain Y02129 using oRP1381 and oRP1382, and transformed into yRP840. To replace *RNY1* with a *URA3* cassette in the *bir1Δ* strain (which already has a *kanMX* marker), we generated a *rny1Δ::URA3* cassette by amplifying the *URA3* gene from pRS426 (Christianson et al., 1992) using oRP1383 and oRP1384, and transformed the PCR product into *bir1Δ* yeast. Strain yRP2496 was generated by plating yRP2446 yeast on 5-fluoroorotic acid–containing plates to select for loss of the *URA3* marker.

Apoptosis and stationary phase

To assess the viability of cells after oxidative stress, or to collect protein extracts from stressed yeast, strains were grown in selective SCM with 2% galactose to mid-log phase, and treated with 0 or 3 mM H_2O_2 for 1 h; then, either serial dilutions (10-fold, starting at OD_{600} = 0.3) were plated on the same media, or cells were collected and frozen for protein extraction.

For stationary phase growth experiments, cultures were diluted into fresh medium and grown for 3–6 d at 30°C. WT and *rny1Δ* strains were grown in SCM with 2% glucose; yeast carrying GAL overexpression vectors or the empty vector pRS426 were grown in selective SCM with 2% galactose, and yeast carrying the single-copy *RNY1* vectors were grown in selective SCM with 2% glucose. Aliquots were taken from the culture each day for analysis, and serial dilutions (10-fold, starting at OD_{600} = 2) were plated on the same media to assess viability.

Plasmids

All plasmids used in this study carried a *URA3* yeast auxotrophic marker. The GAL-*RNY1* plasmid (pRP1584) was obtained from Thermo Fisher Scientific, and pRS426 (Christianson et al., 1992) was used as an empty vector. GAL-*rny1-ci* (pRP1587) was generated using QuikChange (Agilent Technologies) and pRP1584, changing both residue 87 and 160 from histidine to phenylalanine.

pRP1618 was generated by PCR-amplifying *RNY1* coding, promoter, and 3′ end sequences using oRP1381 and oRP1382. The PCR product was TA cloned (pGEM T-Easy; Invitrogen), and the cassette was digested with NotI and cloned into the single-copy *cen* plasmid pRS416 (Christianson et al., 1992). pRP1618 was QuikChange mutated as with pRP1587 to generate pRP1619.

pRP1621 expresses human *RNASET2* using the *GAL* promoter, the *RNY1* 3′ end, and the *RNY1* secretion sequence instead of the *RNASET2* secretion sequence (amino acids 1–24). It was constructed by yeast in vivo recombination using RsrII-linearized pRP1620 and a recombination cassette made by PCR-amplifying human *RNASET2* with primers oRP1387 and oRP1388. For pRP1620, *RNY1* coding and 3′ end sequences were amplified with oRP1404 and oRP1382, and the PCR product was TA cloned, then digested with NotI and cloned downstream of the *GAL* promoter in pRP1623 (pRS426 with the *GAL* promoter cloned between the XhoI and BamHI sites). An in-frame *Rsr* II site was added immediately after the signal sequence using QuikChange and oRP1385 and oRP1386.

Two GFP-*RNY1* vectors with GAL promoters were constructed, with GFP in-frame either at the C terminus (shown in Fig. 2) or immediately downstream of the secretion signal. Localization of both proteins was identical. pRP1622 (internal GFP) was constructed by in vivo recombination using *Rsr*II-linearized pRP1620 and the *GFP* coding sequence amplified from pRP1391. pRP1547 was generated from pRP1622 using QuikChange as with pRP1587. pRP1729 (C-terminal GFP) was constructed by yeast in vivo recombination using *Kpn1*-linearized pRP1584 and a GFP cassette amplified using oRP1428 and oRP1429.

The *D. discoideum* tRNA-Glu plasmid (ptRNA-GluD) was a gift from A. Hopper (Ohio State University, Columbus, OH; Shaheen and Hopper, 2005). It was transformed into WT and *rny1Δ* strains (yRP840 background).

RNA analysis

RNA extraction and blotting were performed essentially as described previously (Caponigro et al., 1993). Probe sequences are listed in Table S2.

Protein analysis

For Western blot analysis, 5–20 µg of total protein was separated on 10% (wt/vol) SDS-PAGE gels and blotted onto Protran membrane (GE Healthcare). GFP-tagged proteins were detected using an anti-GFP antibody (1:1,000; Covance), followed by HRP-conjugated goat anti–mouse secondary antibody (1:5,000; Sigma-Aldrich). Proteins were detected using SuperSignal DuraWest Extended Duration Substrate (Thermo Fisher Scientific), followed by exposure to film. Films were digitized on a scanner Scanjet 4890 (Hewlett-Packard).

Microscopy

MitoTracker Red chloromethyl-X-rosamine (CMXRos) and Cell Tracker blue CMAC were obtained from Invitrogen. Yeast were routinely grown to mid-log phase in selective SCM with 2% galactose; WT yeast were grown in SCM with 2% galactose; and those carrying GFP-tagged Npc2p, Pep4p, and Prc1p were grown in SCM with 2% glucose. CMAC was added at a final concentration of 10 nM, 30 min before the initial viewing. MitoTracker was added at a final concentration of 50 µM, 15 min before the initial viewing. H_2O_2 was added to a final concentration of 3 mM for 20 min to induce oxidative stress.

Cells were harvested and spotted onto slides for immediate microscopic examination. All images were acquired using a Deltavision RT microscope system running softWoRx 3.5.1 software (Applied Precision, LLC), using a 100×, oil immersion, 1.4 NA objective lens (Olympus). Images were acquired as z-series stacks of 10–15 images, and collected as 512 × 512 pixel files with a CoolSnapHQ camera (Photometrics) using 2 × 2 binning. All images (except those in Fig. S3 B, which were not deconvolved but otherwise treated identically) were deconvolved using standard softWoRx deconvolution algorithms (enhanced ratio, low noise filtering). ImageJ (National Institutes of Health) was used to collapse stacks to a single image and to adjust images to equal contrast ranges for each fluorochrome within individual experiments.

Online supplemental material

Fig. S1 shows that autophagy defects do not appear to affect tRNA fragment production. Fig. S2 documents mitochondrial autofluorescence during oxidative stress and shows that rny1p-ci also localizes to vacuoles. Fig. S3 provides additional examples of vacuolar protein release during stress. Table S1 contains yeast strain information. Table S2 lists the sequences of oligos used in this study. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200811119/DC1.

We thank Anita Hopper and Frank Madeo for strains and plasmids, Carolyn Decker for critical review of the manuscript, and the Parker laboratory for support and discussions.

This work was supported by the Howard Hughes Medical Institute.

Submitted: 24 November 2008
Accepted: 26 January 2009

References

Acquati, F., C. Nucci, M.G. Bianchi, T. Gorletta, and R. Taramelli. 2001. Molecular cloning, tissue distribution, and chromosomal localization of the human homolog of the R2/Th/Stylar ribonuclease gene family. *Methods Mol. Biol.* 160:87–101.

Acquati, F., L. Possati, L. Ferrante, P. Campomenosi, S. Talevi, S. Bardelli, C. Margiotta, A. Russo, E. Bortoletto, R. Rocchetti, et al. 2005. Tumor and metastasis suppression by the human RNASET2 gene. *Int. J. Oncol.* 26:1159–1168.

Borek, E., B.S. Baliga, C.W. Gehrke, C.W. Kuo, S. Belman, W. Troll, and T.P. Waalkes. 1977. High turnover rate of transfer RNA in tumor tissue. *Cancer Res.* 37:3362–3366.

Caponigro, G., D. Muhlrad, and R. Parker. 1993. A small segment of the MAT alpha 1 transcript promotes mRNA decay in *Saccharomyces cerevisiae*: a stimulatory role for rare codons. *Mol. Cell. Biol.* 13:5141–5148.

Christianson, T.W., R.S. Sikorski, M. Dante, J.H. Shero, and P. Hieter. 1992. Multifunctional yeast high-copy-number shuttle vectors. *Gene.* 110:119–122.

De Milito, A., E. Iessi, M. Logozzi, F. Lozupone, M. Spada, M.L. Marino, C. Federici, M. Perdicchio, P. Matarrese, L. Lugini, et al. 2007. Proton pump inhibitors induce apoptosis of human B-cell tumors through a caspase-independent mechanism involving reactive oxygen species. *Cancer Res.* 67:5408–5417.

Deshpande, R.A., and V. Shankar. 2002. Ribonucleases from T2 family. *Crit. Rev. Microbiol.* 28:79–122.

Eisenberg, T., S. Buttner, G. Kroemer, and F. Madeo. 2007. The mitochondrial pathway in yeast apoptosis. *Apoptosis.* 12:1011–1023.

Fais, S., A. De Milito, H. You, and W. Qin. 2007. Targeting vacuolar H+-ATPases as a new strategy against cancer. *Cancer Res.* 67:10627–10630.

Foster, K.A., F. Galeffi, F.J. Gerich, D.A. Turner, and M. Muller. 2006. Optical and pharmacological tools to investigate the role of mitochondria during oxidative stress and neurodegeneration. *Prog. Neurobiol.* 79:136–171.

Fu, H., J. Feng, Q. Liu, F. Sun, Y. Tie, J. Zhu, R. Xing, Z. Sun, and X. Zheng. 2009. Stress induces tRNA cleavage by angiogenin in mammalian cells. *FEBS Lett.* 583:437–442.

Guicciardi, M.E., M. Leist, and G.J. Gores. 2004. Lysosomes in cell death. *Oncogene.* 23:2881–2890.

Haiser, H.J., F.V. Karginov, G.J. Hannon, and M.A. Elliot. 2008. Developmentally regulated cleavage of tRNAs in the bacterium *Streptomyces coelicolor. Nucleic Acids Res.* 36:732–741.

Ikner, A., and K. Shiozaki. 2005. Yeast signaling pathways in the oxidative stress response. *Mutat. Res.* 569:13–27.

Jochl, C., M. Rederstorff, J. Hertel, P.F. Stadler, I.L. Hofacker, M. Schrettl, H. Haas, and A. Huttenhofer. 2008. Small ncRNA transcriptome analysis from *Aspergillus fumigatus* suggests a novel mechanism for regulation of protein synthesis. *Nucleic Acids Res.* 36:2677–2689.

Kawaji, H., M. Nakamura, Y. Takahashi, A. Sandelin, S. Katayama, S. Fukuda, C.O. Daub, C. Kai, J. Kawai, J. Yasuda, et al. 2008. Hidden layers of human small RNAs. *BMC Genomics.* 9:157.

Lee, S.R., and K. Collins. 2005. Starvation-induced cleavage of the tRNA anticodon loop in *Tetrahymena thermophila. J. Biol. Chem.* 280:42744–42749.

MacIntosh, G.C., P.A. Bariola, E. Newbigin, and P.J. Green. 2001. Characterization of Rny1, the *Saccharomyces cerevisiae* member of the T2 RNase family of RNases: unexpected functions for ancient enzymes? *Proc. Natl. Acad. Sci. USA.* 98:1018–1023.

Mason, D.A., N. Shulga, S. Undavai, E. Ferrando-May, M.F. Rexach, and D.S. Goldfarb. 2005. Increased nuclear envelope permeability and Pep4p-dependent degradation of nucleoporins during hydrogen peroxide-induced cell death. *FEMS Yeast Res.* 5:1237–1251.

Scherz-Shouval, R., and Z. Elazar. 2007. ROS, mitochondria and the regulation of autophagy. *Trends Cell Biol.* 17:422–427.

Shaheen, H.H., and A.K. Hopper. 2005. Retrograde movement of tRNAs from the cytoplasm to the nucleus in *Saccharomyces cerevisiae. Proc. Natl. Acad. Sci. USA.* 102:11290–11295.

Smirnoff, P., L. Roiz, B. Angelkovitch, B. Schwartz, and O. Shoseyov. 2006. A recombinant human RNASET2 glycoprotein with antitumorigenic and antiangiogenic characteristics: expression, purification, and characterization. *Cancer.* 107:2760–2769.

Speer, J., C.W. Gehrke, K.C. Kuo, T.P. Waalkes, and E. Borek. 1979. tRNA breakdown products as markers for cancer. *Cancer.* 44:2120–2123.

Tamura, S., H. Fujioka, T. Nakano, T. Hada, and K. Higashino. 1987. Serum pseudouridine as a biochemical marker in small cell lung cancer. *Cancer Res.* 47:6138–6141.

Thompson, D.M., C. Lu, P.J. Green, and R. Parker. 2008. tRNA cleavage is a conserved response to oxidative stress in eukaryotes. *RNA.* 14:2095–2103.

Waalkes, T.P., C.W. Gehrke, R.W. Zumwalt, S.Y. Chang, D.B. Lakings, D.C. Tormey, D.L. Ahmann, and C.G. Moertel. 1975. The urinary excretion of nucleosides of ribonucleic acid by patients with advanced cancer. *Cancer.* 36:390–398.

Walter, D., S. Wissing, F. Madeo, and B. Fahrenkrog. 2006. The inhibitor-of-apoptosis protein Bir1p protects against apoptosis in *S. cerevisiae* and is a substrate for the yeast homologue of Omi/HtrA2. *J. Cell Sci.* 119:1843–1851.

Yamasaki, S., P. Ivanov, G.-f. Hu, and P. Anderson. 2009. Angiogenin cleaves tRNA and promotes stress-induced translational repression. *J. Cell Biol.* 185:35–42.