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Stressing Out over tRNA Cleavage

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A conserved response to stress involves endonucleolytic cleavage of cytoplasmic transfer RNAs (tRNAs) by ribonucleases that are normally secreted or sequestered. Ribonuclease activation or release is an intriguing new aspect of cellular stress responses, with a potential impact on translation, apoptosis, cancer, and disease progression.

Eukaryotic cells contain robust mechanisms to respond to and mitigate environmental stress. These responses include neutralization of stress stimuli, repair of cellular damage, and induction of cell death pathways. One important component of stress responses is the regulation of RNA metabolism, which often involves a decrease in general translation and an increase in preferential translation of stress-response genes (reviewed in Holcik and Sonenberg, 2005).

Transfer RNAs (tRNAs) are a fundamental component of the translation machinery but also play roles in modulating cell proliferation and stress responses. For example, overexpression of initiator tRNAs contributes to cellular transformation (Marshall et al., 2008). Additionally, during amino acid starvation, uncharged tRNAs act as signaling molecules to activate the Gcn2 kinase, which modulates the starvation response (Wek et al., 1995). Moreover, during stress responses, tRNA transcription is downregulated, and there is retrograde transport of tRNAs into the nucleus (Hopper and Shaheen, 2008).

Several observations now indicate that tRNAs can be endonucleolytically cleaved, primarily in the anticodon loop, under a variety of stress conditions. The nucleases responsible for stress-induced tRNA cleavage in yeast (Rny1) and mammalian cells (angiogenin) are secreted or sequestered proteins that gain access to cytosolic tRNAs during stress. New work suggests that cleaved tRNAs may inhibit translation or may target specific mRNAs for degradation. Moreover, Rny1 and its human ortholog RNASET2 promote cell death and limit tumor formation in a manner that is independent of their nuclease activity, suggesting that they may also serve as sensors of cellular damage.

tRNAs Are Cleaved during Stress

The earliest reports of tRNA cleavage come from work in the bacterium *Escherichia coli* on the nuclease PrrC. This nuclease cleaves tRNAs in their anticodon loop, destroying full-length tRNAs in response to bacteriophage infection (Levitz et al., 1990). Anticodon-loop cleavage of specific tRNAs also occurs in cells targeted in *trans* by colicin D or E5, resulting in cell death from translational arrest. Cytotoxic colicins are normally used as a defense mechanism against competing organisms (Masaki and Ogawa, 2002).

Cleavage of tRNAs within the anticodon loop has now been reported during a variety of stress responses in eukaryotic cells (Table S1 available online). For example, tRNA cleavage occurs during amino acid starvation in the unicellular organism *Tetrahymena* (Lee and Collins, 2005) and in human, plant, and *Saccharomyces cerevisiae* (budding yeast) systems during oxidative stress (Thompson et al., 2008; Yamasaki et al., 2009). Starvation-induced hyphae formation in the bacterium *Streptomyces coelicolor* correlates with the production of tRNA fragments (Haiser et al., 2008), and cleaved tRNAs are also observed in the primitive eukaryote *Giardia* during starvation-induced encystation (Li et al., 2008). Several large-scale small RNA sequencing projects have demonstrated the presence of half tRNAs in various organisms, including the fruit fly *Drosophila* (Aravin et al., 2003) and the fungus *Aspergillus* (Jochl et al., 2008), and in human cell lines (Kawaji et al., 2008). tRNA halves are also found in pumpkin phloem sap (Zhang et al., 2009). Taken together, these observations demonstrate that tRNA fragments are present in a wide variety of organisms and often increase during stress conditions. Notably, stress-induced cleavage of tRNAs is not a mechanism to degrade misprocessed or hypomodified tRNAs. Such tRNAs are primarily degraded 5′ to 3′ in the cytosol or adenylated and degraded 3′ to 5′ in the nucleus (Chernyakov et al., 2008; Copela et al., 2008); yeast strains with aberrant tRNAs do not show an increase in tRNA fragments (Thompson et al., 2008).

Although these observations come from numerous organisms, they have several common features. First, cleavage is not limited to specific tRNAs, as sequencing and northern blot analyses have shown that many tRNAs undergo endonucleolytic cleavage, although the relative amount of cleavage can differ (Fu et al., 2009; Haiser et al., 2008; Jochl et al., 2008; Kawaji et al., 2008; Lee and Collins, 2005; Li et al., 2008; Thompson et al., 2008; Yamasaki et al., 2009). In contrast, colicin-mediated cleavage tends to target specific tRNAs (Masaki and Ogawa, 2002). Second, cleavage occurs primarily in the anticodon loop, though cleavage in other loops has been observed (Kawaji et al., 2008; Lee and Collins, 2005; Li et al., 2008; Thompson et al., 2008; Zhang et al., 2009) (Table S1). Given that the tRNA cleavage enzymes have broad specificity (see below), the anticodon loop is likely targeted because it is the most accessible region of the tRNA. Third, in most cases, full-length tRNA levels do not decline significantly, and tRNA fragment levels are consistently lower than those of full-length tRNAs (Lee and Collins, 2005; Thompson et

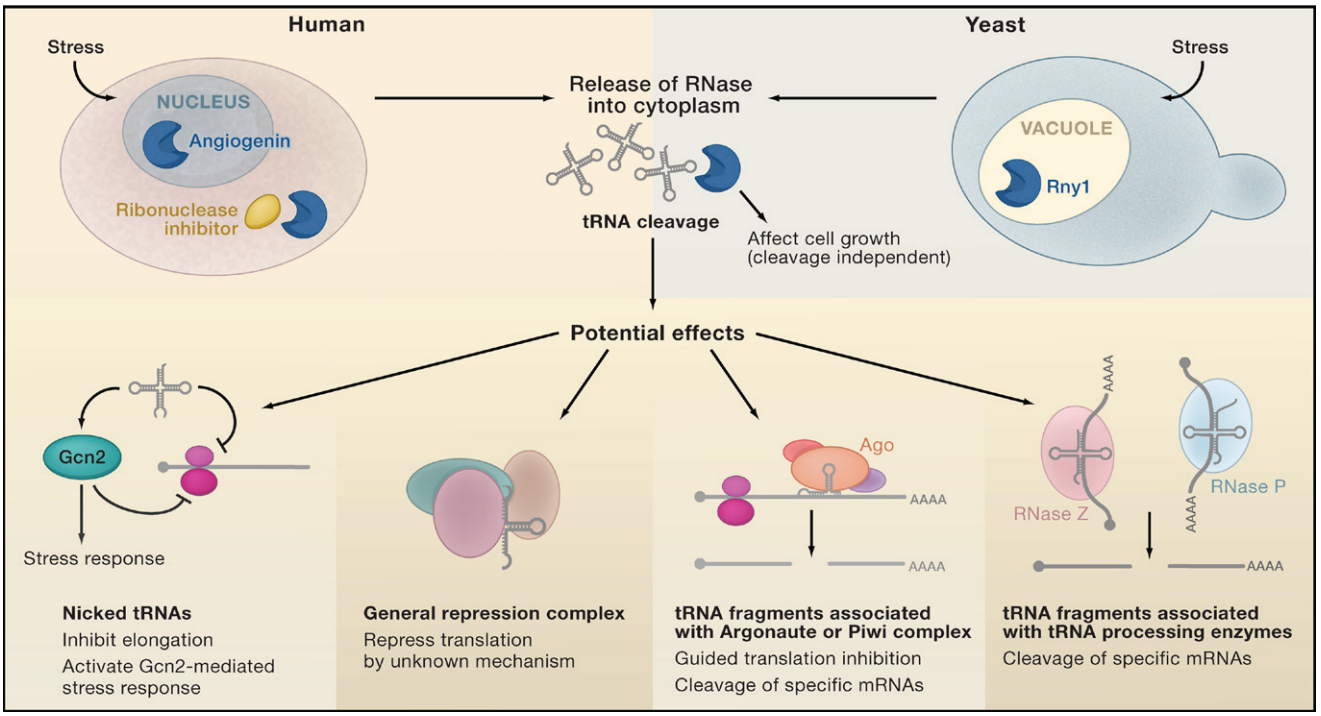

Figure 1. Stress-Induced tRNA Cleavage

Stress conditions induce the release of sequestered ribonucleases (RNases): angiogenin in mammalian cells (left) and Rny1 in yeast (right). The RNases move into the cytoplasm where they cleave tRNAs into fragments. There are several potential mechanisms by which cleaved or nicked tRNAs might inhibit mRNA function. For example, nicked tRNAs might activate a stress response or stall elongation by interacting with the translation machinery. tRNA fragments could interact either with an unknown general repression complex or with known complexes such as Argonaute or Piwi to inhibit translation. tRNA fragments may also direct cleavage of specific mRNAs in conjunction with tRNA processing or Argonaute complexes.

al., 2008; Yamasaki et al., 2009), suggesting that only a small proportion of tRNA is targeted. This is in contrast to the complete depletion of tRNAs targeted by colicins (Masaki and Ogawa, 2002). Fourth, fragments are likely to derive from fully mature tRNAs, as sequencing indicates that tRNA fragments do not contain introns, have mature 5′ ends, and often include 3′ CCA or partial CCA sequences (Haiser et al., 2008; Kawaji et al., 2008; Lee and Collins, 2005; Li et al., 2008; Thompson et al., 2008; Zhang et al., 2009).

Cleavage of tRNAs is not induced by all stress conditions. Cleavage was not induced in γ-irradiated (Fu et al., 2009), etoposide-treated (Yamasaki et al., 2009), or caffeine-treated (Thompson et al., 2008; Yamasaki et al., 2009) human cells, or in yeast cells undergoing amino acid or glucose starvation or UV irradiation (Thompson et al., 2008). The failure of these stress stimuli to increase tRNA fragment levels indicates that cleavage is neither a general consequence of all stresses nor a general effect of a decrease in translation rates. However, a low level of cleavage may occur even in the absence of stress, as some tRNA cleavage is observed in unstressed yeast cells (Thompson et al., 2008).

Stress-induced cleavage is not limited to tRNAs. In yeast, specific ribosomal RNA (rRNA) fragments also increase during stress (Thompson et al., 2008), and sequence analysis indicates that fragments of other RNAs are present in small RNA populations (Table S1). Given that rRNA and tRNA species can be affected by this cleavage mechanism, one anticipates that some mRNAs will also be subjected to stress-induced endonucleolytic cleavage.

Stress-Activated Nucleases

The nucleases responsible for tRNA cleavage during stress have been identified in budding yeast and mammalian cells (Figure 1). In yeast, tRNAs are cleaved by Rny1, a member of the RNase T2 family (Thompson and Parker, 2009). Although mammalian cells do contain an ortholog of Rny1 called RNASET2, this nuclease does not affect tRNA cleavage during oxidative stress in mammalian cells (Thompson and Parker, 2009; Yamasaki et al., 2009). Instead, stress-induced tRNA cleavage in mammalian cells depends on angiogenin, a member of the RNase A family (Fu et al., 2009; Yamasaki et al., 2009). Ribonucleases from the RNase T2 and RNase A families typically have little substrate specificity, suggesting that angiogenin and Rny1 might cleave additional RNAs during stress. Consistent with this view, Rny1 affects rRNA cleavage during stress in budding yeast (Thompson and Parker, 2009), and angiogenin is known to cleave both tRNAs and rRNAs (Saxena et al., 1992; St Clair et al., 1988).

A surprising property of both Rny1 and angiogenin is that these nucleases are either secreted or targeted to membrane-bound organelles and thus are normally segregated from cytoplasmic RNAs. Specifically, Rny1 is both secreted (MacIntosh et al., 2001) and localized to the vacuole (the

yeast equivalent of the lysosome). During oxidative stress, Rny1 and other vacuolar proteins appear to exit the vacuole and move into the cytoplasm via an unknown mechanism, allowing access to cytosolic RNAs (Thompson and Parker, 2009). Angiogenin is also a secreted protein but is known to enter cells by endocytosis. It is concentrated in the nucleus/nucleolus (Moroianu and Riordan, 1994) and is also found in the cytoplasm, where it is bound by the ribonuclease inhibitor RNH1 (Shapiro and Vallee, 1987; Tsuji et al., 2005). Consistent with cytoplasmic angiogenin being responsible for tRNA cleavage, RNH1 depletion increases tRNA fragment production (Yamasaki et al., 2009). This suggests that stress conditions may activate angiogenin by promoting either its dissociation from RNH1 or its release from the nuclear compartment. Therefore, a common theme is that these nucleases are normally secreted or sequestered and then enter the cytosol during stress. This resembles a process in stressed mammalian cells in which several lysosomal proteases exit the lysosome as initiators or executors of the apoptotic response (Guicciardi et al., 2004). The release of ribonucleases from sequestration may be an important aspect of the stress response, not only because these nucleases can cleave cytosolic RNAs but also because their release may be a marker of cellular damage.

Biological Roles of Cleaved RNAs

Cleavage of RNAs during stress has several potential consequences. For instance, tRNA cleavage could inhibit translation by depletion of the tRNA pool. However, full-length tRNA is not significantly depleted in any of the studies published to date, regardless of tRNA fragment levels (Fu et al., 2009; Haiser et al., 2008; Lee and Collins, 2005; Li et al., 2008; Thompson et al., 2008; Yamasaki et al., 2009; Zhang et al., 2009). Even in *Aspergillus*, where full-length tRNA levels fluctuate over time, tRNAs do not disappear, and their decline does not correspond with higher levels of tRNA fragments (Jochl et al., 2008). Thus, stress-induced tRNA cleavage is unlikely to be a mechanism for depletion of cellular tRNA pools.

Some evidence suggests that cleaved tRNAs themselves may function to inhibit translation. For example, Zhang et al. (2009) demonstrated that small RNAs in phloem sap—which include many tRNA halves—inhibit translation of several mRNAs in vitro. To determine whether this was an effect of tRNA fragments or other species in the phloem small RNA pool, they treated yeast tRNA with RNase A (to achieve partial cleavage) and showed that these cleaved tRNAs also repressed translation in vitro. Strikingly, denaturation of the RNase A-treated tRNAs prevented the inhibition of translation (Zhang et al., 2009). This suggests that translation was inhibited by fully folded tRNA with a nick in the anticodon loop, not by separated half tRNAs. Such nicked tRNAs would be more likely than tRNA fragments to associate with the translation machinery, form effective blocks to elongation or initiation, activate the GCN2 stress response, or impair the function of proteins that normally bind to tRNAs (Figure 1).

Additional experiments suggest that tRNA fragments might also have a biological function. For example, Yamasaki et al. (2009) demonstrated that 5′ tRNA half molecules can inhibit protein synthesis, both in vitro and when transfected into mouse or human cells. Although the decrease in overall protein synthesis was small, transfection of 3′ tRNA halves did not show a similar effect, suggesting that this was a specific effect and that 5′ halves may preferentially affect translational repression. These exciting data are tempered with the caveat that the 5′ tRNA halves used for transfection were a pool of gel-purified small RNAs from stressed mammalian cells in culture and may contain small RNAs that do not originate from tRNAs (Yamasaki et al., 2009).

There are several possible mechanisms by which tRNA fragments might inhibit translation (Figure 1). One possibility is that they interact with unknown proteins to form a repression complex. Alternatively, they may serve as unique substrates for known pathways. tRNA fragments have been identified in pools of small RNAs copurifying with Argonaute and Piwi complexes (Kawamura et al., 2008; Lau et al., 2006), suggesting that such tRNA fragments could function as siRNAs or miRNAs. Another intriguing possibility is that tRNA fragments guide cleavage and destruction of specific mRNAs by recruiting tRNA processing enzymes to mRNAs. Both tRNase Z, a 3′ pre-tRNA processing enzyme, and RNase P, which processes the 5′ ends of pre-tRNAs, can cleave RNAs containing artificial pre-tRNA-like structures composed of engineered partial tRNA fragments base-paired to mRNAs (Nashimoto, 2000; Yuan et al., 1992) (Figure 1). Strikingly, new work suggests that endogenous half tRNAs can guide tRNase Z-mediated cleavage of engineered target sequences and potentially of endogenous targets in vivo (Elbarbary et al., 2009). Taken together, these observations build a view that either nicked tRNA molecules or tRNA fragments may inhibit global translation or affect the degradation or repression of specific mRNAs. An important goal of future work will be to determine the mechanisms by which nicked tRNAs or tRNA fragments affect mRNA function.

RNA Cleavage and the Stress Response

Stress-induced release of RNases into the cytosol may modulate the stress response in several ways. First, cleavage of tRNAs, and potentially rRNAs, may inhibit specific steps in translation, leading to a global decrease in translation. Importantly, general inhibition of a specific aspect of translation can lead to preferential translation of specific mRNAs, as individual mRNAs have different rate-limiting steps during translation. For example, cleavage of tRNAs by angiogenin might decrease general translation but upregulate translation of IRES-containing mRNAs such as that encoding vascular endothelial growth factor (VEGF), which promotes new blood vessel formation (angiogenesis) (Holcik and Sonenberg, 2005). Alternatively, cytosolic Rny1 or angiogenin might directly cleave specific mRNAs or produce tRNA fragments that target specific mRNAs in conjunction with tRNA processing enzymes or Argonaute proteins. Finally, the activation of these nucleases during stress may

provide a new paradigm for intercellular communication during stress. For example, treatment of cultured mammalian cells with angiogenin stimulates tRNA cleavage (Yamasaki et al., 2009) and with exogenous RNASET2 affects growth (Smirnoff et al., 2006).

Rny1 and RNASET2 also have a role in the stress response that is independent of their nucleolytic activity. Specifically, overexpression of the *RNY1* gene reduces cell viability, especially during oxidative stress. Also, *RNY1* has genetic interactions with genes involved in the oxidative stress response and apoptosis in yeast (Thompson and Parker, 2009). Intriguingly, this effect of Rny1 on cell survival is independent of its catalytic activity, arguing that Rny1 has a second function involving promotion of cell death during stress that is separate from its ability to cleave RNA (Thompson and Parker, 2009). A possible explanation is that upon release to the cytosol, Rny1 promotes cell death by direct interactions with components of an unknown pathway. Strikingly, expression of the Rny1 homolog RNASET2, or treatment with recombinant RNASET2 protein, inhibits colony formation and metastasis of tumor cell lines in a manner that is independent of the catalytic activity of RNASET2 (Acquati et al., 2005; Smirnoff et al., 2006). Thus, detection of Rny1, and possibly its orthologs, in the cytosol may be a conserved mechanism for assessing damage and modulating cell survival following stress, analogous to the role of mitochondrial cytochrome c in promoting apoptosis.

Several observations suggest that stress-induced RNA cleavage and release of RNases into the cytosol may be involved in cancer and other diseases. First, as discussed above, the Rny1 homolog RNASET2 reduces the metastatic potential of tumor cell lines both in vitro and in vivo (Acquati et al., 2005; Smirnoff et al., 2006). Oxidative stress in mammalian cells causes release of lysosomal proteins into the cytosol, which is thought to promote apoptosis (reviewed in Guicciardi et al., 2004). Proton-pump inhibitors have proven effective for 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). Second, antagonists of angiogenin can delay or prevent tumor development in vivo (e.g., Olson et al., 1995), and angiogenin mutations (many leading to reduced RNase activity) have recently been found in patients with amyotrophic lateral sclerosis (Crabtree et al., 2007). Third, tRNA fragments—identified by detection of modified ribonucleosides—have been reported in the serum and urine of humans and mice with certain tumors (Borek et al., 1977; Speer et al., 1979), suggesting that tRNA cleavage occurs in tumors in situ. Finally, Onconase, an RNase A family member in clinical trials, is toxic to human cancer cells in culture at least in part due to its ability to cleave tRNAs (Ardelt et al., 1991; Iordanov et al., 2000). These observations suggest that understanding the mechanisms and roles of RNA cleavage during stress may lead to the development of new therapies for treating cancer and possibly other diseases.

Supplemental Data

Supplemental Data include one table and can be found with this article online at http://www.cell.com/supplemental/S0092-8674(09)00795-8.

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