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## RNA under attack: Cellular handling of RNA damage

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### Abstract

Damage to RNA from ultraviolet light, oxidation, chlorination, nitration, and alkylation can include chemical modifications to nucleobases as well as RNA-RNA and RNA-protein crosslinking. In vitro studies have described a range of possible damage products, some of which are supported as physiologically relevant by in vivo observations in normal growth, stress conditions, or disease states. Damage to both messenger RNA and noncoding RNA may have functional consequences, and work has begun to elucidate the role of RNA turnover pathways and specific damage recognition pathways in clearing cells of these damaged RNAs.

### Keywords

oxidative stress; UV; alkylation; RNA photoproducts; RNA crosslinking; RNA decay

### Introduction

Both exogenous and endogenous agents can damage cellular DNA, RNA, proteins and lipids. Cellular components come into contact with ultraviolet light, reactive oxygen species and other oxidants, hypohalous acids, nitric oxide, and alkylating agents, all of which are known from in vitro experiments to cause chemical modification or crosslinking of nucleic acids. For RNA, modification can in theory impact the function of both messenger and noncoding RNAs in numerous ways, such as interfering with base-pairing interactions in the transcription and translation of mRNAs or by altering the chemical properties of ribosomal RNA nucleotides necessary for RNA-driven catalysis in the ribosome. As studies have begun to demonstrate that many types of RNA damage that were first described in vitro also occur in vivo under physiologic conditions, so too have observations begun to indicate that the damage interferes with RNA function. It is also becoming clear that the cell may respond to RNA damage with a number of different defense mechanisms, including repair mechanisms specific for certain forms of damage as well as normal turnover pathways. This review focuses on the emerging understanding of the scope of RNA damage within the cell and its consequences for cellular function. In addition, this review considers both described and candidate response mechanisms for RNA damage.

## Ultraviolet damage

Ultraviolet (UV) irradiation can cause several types of damage to RNA: photochemical modification, crosslinking, and oxidative damage. Much of the work describing UV damage to RNA has been carried out *in vitro*, with a few studies suggesting damage may also occur *in vivo* under physiologic conditions. Additionally, the extensive study of UV damage to DNA is useful in suggesting the effects that UV damage could have on RNA, as the photochemistry of nucleic acids occurs primarily at the nucleobase (DNA photodamage is reviewed in Cadet *et al.*, 2005; Ravanat *et al.*, 2001).

### In vitro evidence for UV-induced chemical modification of RNA

In early experiments to detect RNA photoproducts, isolated RNA was irradiated, hydrolyzed enzymatically, and then separated by chromatography to resolve photoproducts such as modified nucleotides and dimers (Small *et al.*, 1968). With this technique, *in vitro* irradiation of synthetic poly(U) and poly(C) or isolated tobacco mosaic virus (TMV) RNA was observed to induce cyclobutane pyrimidine dimers, uridine hydrate, and cytidine hydrate (Singer, 1971; Miller and Cerutti, 1968). These studies identified commonly formed photoproducts (Figure 1) as well as general principles about the photoreactivity of RNA (Gordon *et al.*, 1976). In particular, single-stranded RNA is more likely to form photoproducts than double-stranded RNA, as seen in the comparison of poly(U) to a complex of poly(U) and poly(A) (Pearson and Johns, 1966). The presence of  $Mg^{2+}$ , which can promote the folding of RNAs, also leads to decreased susceptibility of RNA to photodamage (Singer, 1971). Additionally, studies on purified RNAs and viral particles have shown that protein binding can alter photoreactivity (Remsen *et al.*, 1970; Gordon *et al.*, 1976).

### Evidence for UV-induced chemical modification of RNA under physiologic conditions

It is not clear whether these RNA photoproducts observed *in vitro* are formed *in vivo* under physiologic conditions. One important consideration is the wavelength of UV. The early *in vitro* studies of RNA photodamage primarily used UVC (200–290 nm), which is the highest energy form of UV irradiation but is not environmentally relevant as it is absorbed by the ozone layer in Earth's atmosphere. However, the lower energy UVB (290–320 nm) wavelengths do reach the Earth's surface. Additionally, UVA (320–400 nm) wavelengths are of even lower energy, but reach the Earth's surface at levels ~10–100 times that of UVB and can travel farther into tissue (Kaminer, 1995). Direct absorption of UVB by nucleic acids only very inefficiently creates photoproducts, and UVA absorption by nucleic acids is even weaker. Thus, the formation of photoproducts at these wavelengths, particularly for UVA, may also involve the action of photosensitizer molecules within the cell. Although this is an area of ongoing investigation, it is speculated that molecules such as NADPH, flavins, or porphyrins could absorb UV and then in turn react with nucleic acids (Cadet *et al.*, 1992; Cadet *et al.*, 2005; Mitchell, 2006; Mouret *et al.*, 2006).

Further, the doses of UV used in experiments must be evaluated. A useful reference point for UV doses is the minimal erythema dose (MED), or the dose where sunburn is first apparent. The UVB MED has a mean of  $704 \pm 188 \text{ J/m}^2$ , and the MED for UVA has a median of 207,000  $\text{J/m}^2$  as measured on Caucasian subjects (Gambichler *et al.*, 2006); these doses can be reached within an hour during the summer in Europe (Ambach and Blumthaler, 1993).

Considering these criteria, some observations have been made that are consistent with RNA photoproduct formation as a result of physiologically relevant irradiation. Early studies in insect eggs used chromatography to detect pyrimidine dimers in RNA following irradiation at a UVB wavelength of 295 nm (Jäckle and Kalthoff, 1978). Conversion of ~0.15% of

pyrimidines to dimers occurred at a dose of 110 J/m<sup>2</sup>, which although strong enough to cause inactivation of the eggs, is within a relevant exposure range for humans.

Additionally, a report on rRNA damage caused by UV in cultured mammalian cells is consistent with formation of RNA photoproducts (Jordanov *et al.*, 1998). At either a UVB dose of 600 J/m<sup>2</sup> or high doses of UVC, lesions in the 28S rRNA were detected by a primer extension assay. The nature of the lesions was not determined, but the lesions occur primarily at adjacent pyrimidine nucleotides, which is consistent with cyclobutane pyrimidine dimer formation. Interestingly, the UV-induced lesions occurred in a site-specific, biased pattern within the 28S rRNA, particularly affecting the active site of the ribosome, and a decrease in translation activity was observed (Jordanov *et al.*, 1998). Further, this damage to actively translating ribosomes is associated with the activation of a kinase-mediated stress response.

Although the relative susceptibility of RNA and DNA to UV-induced lesion formation in vivo is unknown, there is evidence that environmentally relevant doses of UVB and UVA lead to photoproduct formation in DNA at levels that could cause cellular consequences. High performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS) quantification of pyrimidine dimer formation in human skin after low doses of UVB demonstrated a dimer formation rate of ~0.0520 lesions per 10<sup>6</sup> normal bases per J/m<sup>2</sup>. Additionally, exposure to low doses of UVA results in a rate of pyrimidine dimer formation of ~0.000008 lesions per 10<sup>6</sup> normal bases per J/m<sup>2</sup> (Mouret *et al.*, 2006). Using MED values to evaluate these rates of lesion formation in DNA, it appears that relevant levels of UVA and UVB exposure could cause damage on the order of 10<sup>5</sup> lesions per cell. It is thus possible that significant RNA damage also occurs, as average prokaryotic and eukaryotic cells contain ~4–6 times more RNA than DNA, most of which is noncoding RNA with a half-life on the order of days.

### **In vitro evidence for UV-induced RNA crosslinking**

In addition to modification of nucleobases, UV can induce long-range covalent crosslinks involving RNA. This was first suggested from the experimental use of UV irradiation to probe ribosome structure, where both RNA-RNA and RNA-protein crosslinks were observed. RNA-RNA crosslinks can be induced both by UVC and UVB irradiation (Zwieb *et al.*, 1978; Wilms *et al.*, 1997). The frequency of crosslinking between nucleotides is correlated with the distance and angle between nucleotides as well as the ability of the nucleotides to undergo transient conformational change to a conformation that would allow crosslinking within the timescale of photoexcitation (reviewed in Huggins *et al.*, 2005).

For RNA-protein crosslinks, the likelihood of crosslinking is also determined by the geometry and photoreactivity of the nucleotide and amino acid (Smith, 1976). As with RNA-RNA crosslinks, there is a long history of using RNA-protein crosslinking experimentally to study ribonucleoprotein (RNP) complexes including the ribosome (Möller *et al.*, 1978). These studies provide insight into the types of crosslinks that are possible. For example, it has been noted that crosslinking does not normally occur in double-stranded regions (Noah *et al.*, 2000). Thus, certain cellular RNPs may be more or less pre-disposed by RNA structure to UV crosslinking damage. Above all, the experimental use of UV to generate RNA-protein crosslinks reflects the inherent photoreactivity of RNPs.

### **Evidence for UV-induced RNA crosslinking under physiologic conditions**

RNA-RNA crosslinking occurs within tRNAs in *Escherichia coli* treated with relevant doses of broad spectrum UVA light (320–405 nm). The observed crosslinks occur between cytidine and the naturally modified pseudouridine nucleotide (Ramabhadran *et al.*, 1976). In vitro experiments showed that tRNAs containing this crosslink had lower rates of aminoacylation

and caused inefficient amino acid incorporation in translation assays (Ramabhadran *et al.*, 1976; Chaffin *et al.*, 1971).

RNA-protein crosslinks have been described in UVB-irradiated insect eggs, although the RNAs involved were not identified (Jäckle and Kalthoff, 1979), and in maize (Casati and Walbot, 2004). In the maize study, environmentally relevant doses of UVB caused the formation of covalent crosslinks between protein and RNA within the ribosome. The observed crosslinks occurred at locations where there is close proximity between RNA and protein in the 3D structure of the ribosome. Further, this crosslinking damage may have functional consequences for the cell. As the duration of UVB exposure increased, the accumulation of crosslinked products increased, and the total cellular level of protein synthesis decreased. This correlation suggests that the protein-RNA crosslinks may have contributed to the loss of ribosome function, although other UV-induced mechanisms for inhibition of translation are also possible.

## Oxidative damage

Oxidative damage to RNA has been reported in animal models of aging, human neurodegeneration and UV-irradiated cells. There is also growing evidence that oxidative RNA damage can lead to defects in protein synthesis, including decreased rates of protein synthesis and the production of aggregated and truncated peptides.

Under most conditions, the bulk of cellular oxidative damage is a result of reactive oxygen species (ROS) formation due to metabolic reactions. Multiple steps in the electron transport chain involve a free radical semiquinone anion ( $\cdot Q^-$ ) that can react with oxygen to form the superoxide radical ( $O_2^-$ ). The cell eliminates this superoxide radical through the action of superoxide dismutase to form hydrogen peroxide ( $H_2O_2$ ), followed by enzymatically catalyzed reduction of  $H_2O_2$  to  $H_2O$  and  $O_2$  (reviewed in Finkel and Holbrook, 2000). However, the reaction of  $H_2O_2$  with intracellular iron in the form of  $Fe^{2+}$  and  $Fe^{3+}$  can lead to a cascade of radical chemistry through the Fenton and Haber-Weiss reactions and the production of ROS, including the highly reactive hydroxyl radical ( $\cdot OH$ ) (reviewed in Halliwell and Gutteridge, 1984).

The reaction of ROS with free nucleobases, nucleosides, nucleotides or oligonucleotides can create numerous distinct modifications in RNA (Figure 2; Barciszewski *et al.*, 1999). In studies of DNA, guanine bases have been shown to be particularly reactive, and a major product observed in vitro and in vivo is 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxodG) (Cadet *et al.*, 2007; Ravanat *et al.*, 2001). In vivo studies of RNA oxidation (discussed below) have also detected 8-oxo-7,8-dihydroguanosine (8-oxoG) in RNA through the use of HPLC-MS/MS and immunocytochemistry, indicating that this common oxidative modification to DNA occurs in RNA as well. The formation of 8-oxoG occurs by reaction of guanine with the  $\cdot OH$  radical followed by oxidation, or by reaction of guanine with singlet oxygen  $^1O_2$  followed by reduction (Cadet *et al.*, 2007). Oxidation of other nucleobases is also possible (Cadet *et al.*, 2007), and at least two other modifications have been identified in yeast RNA (Yanagawa *et al.*, 1992).

## In vivo measurement of oxidative damage to RNA

Oxidized RNA has been measured in vivo in a growing number of contexts, both as a result of experimental treatment of cells with oxidative agents and in diseased tissue. Treatment of *E. coli* cultures with  $H_2O_2$  has been shown to cause 16S rRNA damage that can be detected by a reverse transcription polymerase chain reaction (RT-PCR) assay and is correlated with an increase in the 8-oxoG content in the cell (Gong *et al.*, 2006). Experimental treatment has also been shown to result in RNA damage in animals. Exposure of rats to ethanol is associated with increased 8-oxoG RNA immunostaining in the pituitary gland (Ren *et al.*, 2005), while

ammonia exposure triggers RNA oxidation in the rat brain (Görg *et al.*, 2008). Additionally, in a study of the carcinogen 2-nitropropane (2-NP), intraperitoneal injection of rats with 2-NP led to a significant increase in the levels of 8-oxoG in liver RNA (Fiala *et al.*, 1989). In addition to increased 8-oxoG levels, HPLC-electrochemical detection (HPLC-EC) also detected other forms of unidentified modified ribonucleotides, indicating that oxidative damage to RNA *in vivo* may lead to the formation of numerous chemical modifications.

### Measurement of oxidative damage to RNA under physiologic conditions

Oxidative damage to RNA has also been measured from natural biological processes. Oxidative RNA damage to cells under normal physiologic conditions has been suggested by detection of the oxidized ribonucleoside 8-oxoG in the urine of rats and humans as well as in human blood plasma (Park *et al.*, 1992; Weimann *et al.*, 2002). Further, because metabolic reactions are major sources of ROS, metabolic rate as well as mitochondrial dysfunction can be significant factors in oxidative damage. One model system is aging rats, which display mitochondrial dysfunction. In the brains of such rats, levels of 8-oxoG in RNA are significantly higher than levels in young rats (Liu *et al.*, 2002). Indeed, treatment of old rats with metabolites that boost mitochondrial function resulted in oxidized RNA levels comparable to those of younger rats, demonstrating a link between mitochondrial function and intracellular RNA damage. Additionally, studies of aging muscle displaying deregulation of iron homeostasis are consistent with the role of intracellular metals in generating ROS through Fenton chemistry, and *in turn* oxidative damage (Hofer *et al.*, 2008). Muscle from 32-month old rats was shown to have higher levels of 8-oxoG levels in total cellular RNA as well as higher free, non-heme iron levels compared to 6-month old rats. Additionally, imposing disuse atrophy on the older rats further elevated both the levels of free, non-heme iron and 8-oxoG. Thus, both the misregulation of metabolism and the loss of metal homeostasis that occur with age are correlated with increased oxidative damage to RNA.

Other biological processes may also generate reactive oxygen species that can contribute to RNA damage. For example, as a part of the immune response to pathogens, neutrophils and eosinophils use NADH oxidase to produce  $O_2^-$  and heme proteins to generate hypohalous acids, both of which can then lead to  $\cdot OH$  formation. In a study designed to approximate conditions generated at sites of inflammation by the activation of neutrophils and eosinophils, fibroblasts were treated with the hypohalous acid HOCl in combination with hyperoxia (Shen *et al.*, 2000). Such treatment resulted in increased 8-oxoG to levels of 7–8 per  $10^5$  G in poly (A) RNA and in the free nucleotide pool, as compared to 1 per  $10^5$  G in control cells. Yet, most treated cells remained viable, indicating that cells are able to survive this level of damage. Notably, other tissues also express NADH oxidases. The catalytic subunit of the phagocytic NADH oxidase has been shown to be expressed in a variety of tissues, including colon, reproductive tissues, and muscle (Suh *et al.*, 1999), while the kidney expresses an NADH oxidase called Renox that is proposed to be an oxygen-sensing protein (Geiszt *et al.*, 2000). Thus, many tissues may have a heightened requirement for cellular systems that respond to oxidative RNA damage.

### UV-induced oxidation of RNA

UV is also a source of oxidative damage to RNA. Numerous pathways of excitation and oxidation are possible for nucleobases, as has been studied extensively in research on DNA damage (reviewed in Ravanat *et al.*, 2001). Radical anion species can be formed from nucleobases due to the direct absorption of UVB light by a nucleobase; the radical anion species can then undergo further reaction to generate oxidized nucleotides. Oxidative damage can also occur from UVA, mediated either by photosensitizer molecules or by (ROS) formation (Cadet *et al.*, 2007). For DNA, both UVB- and UVA-induced oxidation most commonly result in the formation of (8-oxodG), although photooxidation of adenine, cytosine, and thymine bases is

also detected in DNA from irradiated cells (Cadet *et al.*, 2005). Importantly, much of the chemistry described for photooxidation of DNA is also applicable for RNA, as is borne out by measurement of UV-induced oxidative damage to RNA in cells.

UV-induced oxidative damage to RNA has been demonstrated in experiments with human skin fibroblasts. Exposure of fibroblasts to UVA at sublethal doses led to significantly increased levels of (8-oxoG) in RNA as measured by (HPLC-EC) analysis of total RNA (Wamer and Wei, 1997). Increasing intensity of UVA irradiation caused a dose-dependent increase in 8-oxoG. Interestingly, as seen in other examples of oxidative stress (discussed below), the RNA damage was greater than the DNA damage observed. Importantly, this study used UVA at doses comparable to the MED, supporting the physiologic relevance of UVA-induced oxidative damage to RNA.

### Oxidative damage to RNA observed in disease

There is a growing body of evidence correlating oxidative damage to disease states, especially for neurodegenerative disorders (Moreira *et al.*, 2008; Nunomura *et al.*, 2006). In Alzheimer's disease (AD), high levels of 8-oxoG RNA levels in the cytoplasm and nucleoli of neurons have been seen by immunostaining on DNase-treated postmortem brain sections from AD patients, as compared to the levels seen for control patients (Nunomura *et al.*, 1999). Indeed, quantification of oxidized poly(A) RNA from frontal cortices by immunoprecipitation of 8-oxoG-containing RNAs indicated that  $52.3 \pm 6.15\%$  of mRNAs contained 8-oxoG in AD patients compared to  $1.78 \pm 0.56\%$  in age-matched controls (Shan and Lin, 2006). Elevated levels of oxidatively damaged RNA also have been observed in neurons from the degenerating substantia nigra of patients with Parkinson's disease, as compared to unaffected regions of the same patient brains (Zhang *et al.*, 1999). Similar findings are reported for patients with dementia with Lewy bodies, where high levels of 8-oxoG RNA were seen by immunostaining in neuronal populations susceptible to degeneration (Nunomura *et al.*, 2002). Oxidation of poly (A) RNA has also been observed in neurons from affected regions of the brain and spinal cord of patients with amyotrophic lateral sclerosis (ALS) (Chang *et al.*, 2008).

It has even been suggested that RNA oxidation may be an early event in the progression of neurodegenerative diseases, as in one study of familial AD, elevated 8-oxoG RNA staining was seen in cells that had not developed  $\alpha$ -synuclein aggregation, a hallmark of degeneration (Nunomura *et al.*, 2004). Further, in a mouse model of familial ALS, mRNA oxidation was observed to have preceded neurodegeneration and then to have subsided by the onset of neurodegenerative symptoms (Chang *et al.*, 2008). The role of mRNA oxidation in disease progression appears complex, as vitamin E treatment blocked mRNA oxidation and delayed disease onset but did not alter mean life-span.

In addition to neurodegenerative diseases, RNA damage has also been noted in atherosclerosis. In atherosclerotic artery samples from carotid endarterectomies, the 8-oxoG RNA immunostaining levels were found to be elevated compared to non-diseased mammary arteries (Martinet *et al.*, 2004).

### Underlying causes of the susceptibility of RNA to oxidative damage

In many studies of oxidative damage, a common finding is that damage levels are higher in total RNA than in total DNA. This observation has been made by comparing the level of 8-oxoG immunostaining after RNase or DNase treatment, such as in carcinogen treatment of rats (Fiala *et al.*, 1989) and studies of neurodegeneration (Nunomura *et al.*, 1999). Several experiments have also sought to quantify nucleic acid damage. In one study on livers of rats treated with doxorubicin to induce oxidative stress, RNA and DNA was isolated simultaneously and quantified by HPLC-EC (Hofer *et al.*, 2006). This approach found that in

untreated animals, the ratio of 8-oxoG:guanosine in RNA was 1.4-fold higher than the ratio of 8-oxodG:2'-deoxyguanosine in DNA. Further, RNA damage increased significantly upon doxorubicin treatment while DNA damage did not. Quantification of 8-oxoG content in RNA versus 8-oxodG content in DNA has also been performed by *in vitro*  $^{18}\text{O}$ -labeled  $\text{H}_2\text{O}_2$  treatment of human lung epithelial cells followed by HPLC-MS/MS (Hofer *et al.*, 2005). In this context, the level of RNA damage per nucleoside was measured to be 14–25 times greater than that in DNA.

One explanation for the higher levels of oxidized nucleosides in RNA compared to DNA under normal cellular conditions is that there is a higher incidence of damage to molecules in closer proximity to mitochondria. This is supported by observations that levels of oxidized nucleosides are higher in mitochondrial DNA than in nuclear DNA (Shen *et al.*, 2000). Indeed, in experimental treatment of cells with oxidizing reagents, this effect may be reproduced because exogenous oxidizing agents reach the cytosol first. Differential susceptibility of RNA and DNA to oxidation could also result from differences in protein association and in the degree of single-strandedness. The higher levels of oxidization observed for RNA could also be a consequence of different rates of removal of RNA and DNA damage.

RNA susceptibility to oxidative damage may also vary between noncoding and coding RNAs, among different classes of noncoding RNAs, and among different mRNA species. One suggested factor is the degree of protein association, which may serve to protect RNAs from damage. Indeed, different RNAs have varying degrees and temporal patterns of association with proteins, from the stable coating of rRNAs with ribosomal proteins to the progressive association of mRNAs with transcriptional machinery, splicing and export factors, and eventually polyribosomes. While the basis behind the differences in damage is not yet fully understood, it has been reported that different mRNA transcripts show differing levels of oxidative damage. For example, in ammonia-treated rat astrocytes, rRNAs and some mRNAs are oxidized while oxidation of other mRNAs is not detected (Görg *et al.*, 2008). This issue has also been addressed by a study of oxidized RNAs in the brains of Alzheimer's patients (Shan *et al.*, 2003). Starting from purified poly(A) RNA, oxidized mRNAs were isolated by immunoprecipitation with an antibody against 8-oxoG. RT-PCR for mRNAs of interest found that not all mRNAs were present in the pool of oxidized RNA, independent of transcript abundance. Further, even for mRNAs identified as oxidized, the prevalence of damage differed for particular mRNAs, in the range of ~54–75% damage-containing transcripts (Shan and Lin, 2006). Therefore, different cellular RNAs may be unequally susceptible to oxidative damage or certain damaged transcripts may be preferentially degraded or repaired. The consequences of such patterns of damage on disease states remain to be further elucidated.

Another factor that may render some RNAs particularly susceptible to oxidative damage is association with iron, as iron catalyzes Haber-Weiss and Fenton reactions that produce ROS (Halliwell and Gutteridge, 1984). This hypothesis has been raised particularly in regard to ribosomal RNAs. In studies of neurons from Alzheimer's patients, rRNA was found to be oxidatively damaged at levels higher than for control patients (Ding *et al.*, 2005; Honda *et al.*, 2005). Intriguingly, purified ribosomes from Alzheimer's patients showed elevated levels of associated redox-active iron (Honda *et al.*, 2005). *In vitro* experiments correlated binding of redox-active iron with oxidative damage to rRNAs: rRNA was found to have higher *in vitro* iron-binding than tRNA or mRNA, and this iron-rich rRNA had a 13-fold greater formation of 8-oxoG in Fenton reaction oxidation experiments as compared to the iron-poor tRNA. Thus, iron-binding properties of certain classes of RNAs may correlate with vulnerability to oxidative damage under basal conditions and especially in disease states involving perturbation of intracellular metal homeostasis.

## Consequences of oxidative RNA damage

The consequences of oxidative RNA damage include impairment of translation due to damage to mRNAs and due to decreased ribosome function. For example, oxidation of some mRNAs in affected regions of AD patient brains is correlated with low protein expression for those genes (Shan *et al.*, 2007). The effect of oxidatively damaged mRNA on translation has been tested by introducing in vitro oxidized luciferase mRNA into cultured human HEK293 cells (Shan *et al.*, 2003). The transfected oxidized mRNA was not degraded over the time course of the experiment, but compared to undamaged controls, luciferase activity and protein level from the damaged mRNA were strongly reduced. Additionally, proteins translated from the oxidized mRNA were shown to form aggregates suggestive of misfolding. In a similar study, transfected oxidized luciferase mRNA was shown to have normal polysome association, yet the expression of full-length protein decreased with a dose-dependent relationship to level of mRNA oxidation (Tanaka *et al.*, 2007). In that study, use of protease inhibitors revealed that short polypeptides result from the translation of the oxidized mRNAs, perhaps due to both premature termination and protease degradation of aberrant full-length protein. Similar effects were seen when HEK293 cells were treated with paraquat to induce oxidative damage, demonstrating that following cellular oxidative stress, mRNAs are translated yet often generate truncated proteins that must be degraded by the proteasome (Tanaka *et al.*, 2007). Another consequence of mRNA oxidation may be ribosome stalling, as the size of polyribosome complexes formed on in vitro oxidized mRNAs increases in rabbit reticulocyte translation systems, consistent with a slowed rate of elongation (Shan *et al.*, 2007).

Considerable evidence exists that translation defects after oxidative stress may also result from damage to the ribosome. In vitro, oxidative damage to purified ribosomes has been shown to result in decreased translation of an undamaged poly(U) mRNA in a translation assay (Honda *et al.*, 2005). Similarly, in vivo defects in ribosome function are suggested by the finding that for AD patients, polyribosomes isolated from regions of the brain known to incur oxidative damage show a reduced rate of protein synthesis in an in vitro assay compared to polyribosomes from nondiseased regions within AD patient brains or from control patients (Ding *et al.*, 2005). At least two types of damage have been observed in ribosomes under oxidizing conditions. First, studies of 8-oxoG levels in rRNAs from affected regions of AD brains have revealed that rRNA is oxidized under conditions of oxidative stress (Ding *et al.*, 2006). A second form of oxidative damage to ribosomes is the crosslinking of rRNA to ribosomal proteins. This has been observed in H<sub>2</sub>O<sub>2</sub>-treated yeast cells, where 85% of ribosomal proteins were seen to be oxidized and HPLC-MS/MS identified numerous sites of ribosomal protein-nucleotide crosslinking (Mirzaei and Regnier, 2006).

## Chlorinating and nitrating damage

Radical chlorine and nitrogen species arise from multiple intracellular sources and can also cause damage to RNA. Phagocytic cells produce a range of radical species including hypochlorous acid (HOCl), nitric oxide (NO<sup>\*</sup>), and peroxynitrate (ONOO<sup>-</sup>) to fight infections, and many cell types use nitric oxide as a signaling molecule. However, these radical species can lead to the chlorination, nitration, and oxidation of biological molecules including RNA (Hawkins *et al.*, 2007).

### In vitro evidence for chlorinating and nitrating damage to RNA

Reaction of HOCl with DNA nucleotides in stop-flow experiments shows that guanine and thymine are especially reactive, most likely due to chlorination at the heterocyclic nitrogen (Prütz, 1996). In cellular RNA from a human monocytic cell line treated with HOCl, HPLC-MS/MS has been used to detect 5-chlorocytidine, 8-choloroguanosine and to a lesser extent, 8-chloroadenosine and to show that chlorination of RNA occurs at higher levels than for DNA



(Figure 2; Badouard *et al.*, 2005). Interestingly, denaturation of double-stranded DNA is also seen *in vitro* upon HOCl treatment, suggesting that RNA base pairing and folding could also be disrupted by chlorination (Prütz, 1996).

The types of damage described for DNA from nitration include base deamination leading to depurination and strand breakage, DNA-DNA and DNA-protein crosslinking, and numerous base modifications including 8-oxodG and 8-nitro-2'-deoxyguanosine; these lesions and modifications are chemically possible for RNA as well (Reiter, 2006; Ohshima *et al.*, 2006). Indeed, *in vitro* deamination of purified adenine, guanine, yeast tRNA, and bovine liver tRNA by nitric oxide has been measured (Nguyen *et al.*, 1992). Additionally, *in vitro* exposure of purified total RNA to nitrogen radical species including ONOO<sup>-</sup> leads to stable 8-nitroguanosine and 8-oxoG formation (Masuda *et al.*, 2002). Treatment of cultured human lung cancer cells with ONOO<sup>-</sup> also is associated with the formation of 8-nitroguanosine (Masuda *et al.*, 2002).

### Measurement of nitrating damage to RNA under physiologic conditions

*In vivo*, nitric oxide is generated as a part of the inflammatory response, and this has been observed to cause a concomitant increase in 8-nitroguanosine levels in the RNA from lungs of mice infected with virus (Akaike *et al.*, 2003). Cytoplasmic immunostaining for 8-nitroguanosine is seen both in the infection of hamsters with parasites (Pinlaor *et al.*, 2003) and in human patients with *Helicobacter pylori* infection (Ma *et al.*, 2004), although controls were not performed in these experiments to conclusively identify RNA rather than DNA as the source of the cytoplasmic staining. These results suggest a pattern of damage to RNA from nitration in cases of infection, and further studies may extend the current understanding of the scope of chlorination and nitration under physiologic conditions.

### Alkylation damage

RNA, like DNA, can undergo alkylation by S<sub>N</sub>1 or S<sub>N</sub>2 nucleophilic reactions with methylating agents (reviewed in Sedgwick, 2004). Endogenous sources of methylation are still under investigation but include methyl halides, compounds arising from the nitrosation of amines, and *S*-adenosylmethionine. Methylation can occur at nucleophilic N and O atoms on all of the ribonucleobases as well as at the O atoms of the phosphate backbone. As single-stranded nucleic acids are more vulnerable to methylation, many RNAs may be more susceptible than DNA to this damage.

While methylation is a naturally occurring rRNA modification, only specific, largely conserved positions are methylated in a process requiring a large class of small nucleolar ribonucleoproteins (snoRNPs) to guide methylation. Indeed, experimental re-engineering of snoRNPs to direct methylation of normally unmethylated sites in yeast rRNA can cause growth defects and inhibition of protein synthesis (Liu and Fournier, 2004). When alkylation occurs at random, one source of deleterious effects is the inhibition of base-pairing interactions. For example, methylation can interfere with tRNA-rRNA interactions in translation (Yoshizawa *et al.*, 1999) and mRNA-tRNA interactions in translation (Ougland *et al.*, 2004) and can theoretically interfere with any other RNA function that relies on base pairing, such as siRNA and miRNA function. Additionally, loss of base pairing interactions can cause structured RNAs to misfold. Protein recognition of RNA bases, such as in the aminoacylation of tRNAs, can also be inhibited by methylation (Ougland *et al.*, 2004).

### Cellular handling of specific forms of RNA damage

The current understanding of turnover or repair mechanisms for damaged RNA varies for different forms of damage. For some, such as alkylation, a clear mechanism has been identified.

For most other types of damage, the roles of normal RNA turnover mechanisms and alternate pathways are still under investigation.

### Mechanisms for handling of RNA with alkylative damage

Alkylated nucleic acids can be repaired by enzyme-catalyzed oxidative demethylation. The *E. coli* AlkB enzyme and one of the human oxidative demethylases, hABH3, demethylate 1-methyladenine and 3-methylcytosine in RNA in addition to having activity on DNA (Aas *et al.*, 2003). In vivo, the action of AlkB and hABH3 can lead to the restoration of RNA function, as exemplified by reactivation of alkylated RNA bacteriophages (Aas *et al.*, 2003), and tRNA (Ougland *et al.*, 2004).

Interestingly, because these oxidative demethylases only function to remove methyl groups from 1-methyladenine and 3-methylcytosine, certain classes of RNAs may be more likely to accumulate alkylation damage that cannot be repaired. Alternatively, demethylases with other specificities may remain to be identified. The efficacy of AlkB in restoring tRNA function as measured by in vitro assays is less than that for mRNA, consistent with the more double-stranded nature of tRNA, which would lead to alkylation damage predominately at positions not repaired by AlkB (Ougland *et al.*, 2004). This suggests that in vivo certain classes of RNAs may show higher capacity for repair, while others may be targeted by other mechanisms for decay.

Finally, it has been noted that AlkB has only weak affinity for DNA and may interact with other DNA-binding proteins in order to increase its affinity in vivo (Yang *et al.*, 2008). This may be true for RNA as well, and it will be of interest to identify proteins that function with these demethylases.

### Mechanisms for handling UV-induced RNA photoproducts and crosslinks

Cyclobutane pyrimidine dimers are actively repaired in some organisms by photolyase enzymes that catalyze light-dependent dimer splitting. While many prokaryotes and eukaryotes have DNA photolyases, the physiological relevance of DNA photolyases in RNA repair is most likely minimal, as exemplified by the poor RNA binding of the *E. coli* photolyase (Kim and Sancar, 1991). To date, RNA photolyase activity has only been observed in plants (Gordon *et al.*, 1976) and insects (Jäckle and Kalthoff, 1980). Therefore, it is unclear to what extent most organisms actively repair cyclobutane pyrimidine dimers in RNA.

For the rRNA-protein crosslinking observed in maize subjected to UVB irradiation (Casati and Walbot, 2004), a cellular handling mechanism is implied by the disappearance of the crosslinked products in a recovery period following UVB exposure. However, the mechanism that recognizes and degrades the crosslinked RNAs and proteins is not yet known.

### Mechanisms for handling oxidized nucleotides

A mechanism has been described for the turnover of oxidized free nucleotides. Clearing oxidatively damaged free nucleotides can prevent incorporation of these nucleotides into newly synthesized RNAs. Besides triggering problems caused by the presence of oxidized nucleotides in a particular RNA, 8-oxoG can be incorporated opposite adenosine by RNA polymerase and thus cause mutations at the level of transcription (Taddei *et al.*, 1997). Such mutations can cause errors in protein synthesis if they occur in mRNAs or affect the folding or function of noncoding RNAs. In *E. coli*, the MutT protein hydrolyzes 8-oxoguanosine triphosphate (8-oxoGTP) to 8-oxoguanosine monophosphate (8-oxoGMP) in addition to catalyzing the same hydrolysis reaction on 8-oxo-2'-deoxyguanosine triphosphate (Taddei *et al.*, 1997). In human cells, the MutT-related protein MTH1 has hydrolytic activity on 8-oxoGTP, and the resulting 8-oxoGMP is prevented from re-entering the nucleoside triphosphate pool because guanylate

kinase lacks activity on 8-oxoGMP (Hayakawa *et al.*, 1999). A second human MutT-related protein, NUDT5, hydrolyzes 8-oxoGTP to 8-oxoGDP, which can be further hydrolyzed by MTH1 (Ishibashi *et al.*, 2005). Such enzymatic activity is important in ensuring transcriptional fidelity despite oxidative damage to nucleotides both by ongoing metabolic generation of ROS and by oxidative stress conditions. In kainate-induced oxidative stress within the rat hippocampus, MTH1 expression is upregulated (Kajitani *et al.*, 2006). Further, MTH1-null mutant rats show significantly higher levels of 8-oxoG immunostaining in RNA and DNA in kainate-sensitive regions of the hippocampus and return to basal 8-oxoG levels with a slower time course after induced oxidative stress. Thus, hydrolysis of 8-oxoG by MutT-related proteins functions to protect the nucleotide pool both under normal growth and in stress conditions.

### Evidence that oxidatively damaged RNAs are degraded

There is also evidence for the turnover of damaged mRNAs and noncoding RNAs. The quantification of 8-oxoG in RNA after oxidative treatment of cultured human lung epithelial cells shows that approximately a third of the 8-oxoG in RNA is cleared by 3 hours after removal of oxidative stress (Hofer *et al.*, 2005). As 95% of cellular RNA is noncoding RNA with a normal half-life much greater than 3 hours, mechanisms must exist to rapidly degrade some damaged RNAs. Interestingly, after the 3 hour time point, the turnover of 8-oxoG slows, leading to an overall half-life of ~12.5h for 8-oxoG in RNA. Similar observations have been made for HeLa cells treated with sub-lethal doses of H<sub>2</sub>O<sub>2</sub> (Wu and Li, 2008). In that study, 8-oxoG levels in RNA rapidly peaked following a pulse of oxidative stress, were reduced by half within 30 minutes of recovery, and then slowly reached basal levels by 24h. Additionally, turnover or degradation has been reported in total RNA isolated from rat astrocytes subjected to ammonia stress (Görg *et al.*, 2008). However, elevated levels of oxidized RNA measured in studies of aging and neurodegeneration (Liu *et al.*, 2002; Nunomura *et al.*, 1999) imply that at least some fraction of damaged RNA accumulates in those contexts.

For rRNA in particular, there are reports of degradation following oxidative damage. In human atherosclerotic plaques, reduction of 18S and 28S rRNA levels coincides with high 8-oxoG RNA levels (Martinet *et al.*, 2004). Similarly, isolation of total RNA and ribosomes from affected regions of AD patient brains has shown decreased levels of all four individual rRNAs (5S, 5.8S, 18S, and 28S) and in turn decreased levels of the 40S and 60S ribosomal subunits and mature 80S ribosomes (Ding *et al.*, 2005; Ding *et al.*, 2006). Degradation of the 25S and 5.8S rRNAs is also seen in yeast under oxidative stress (Mroczek and Kufel, 2008; Thompson *et al.*, 2008). However, for these observations, it is unclear to what extent the rRNA degradation was an effect of ongoing apoptosis, rather than a specific mechanism to degrade damaged RNAs.

Finally, oxidative stress has also been noted to induce the accumulation of cleaved tRNA halves in yeast, *Arabidopsis thaliana*, and human cells (Thompson *et al.*, 2008). As mature tRNA levels are not reduced, it is unclear whether these cleavages affect cell function.

### Cytoplasmic domains implicated in the handling of UV- and oxidatively damaged RNAs

Conditions that cause RNA damage can also lead to translational arrest and induction of two sites of mRNA accumulation within the cytoplasm, stress granules and processing bodies (P-bodies) (reviewed in Anderson and Kedersha, 2008). The role of these foci in the turnover of damaged RNA is just beginning to be understood. The first step in the formation of stress granules occurs through pathways that converge on eukaryotic initiation factor 2 $\alpha$  (eIF2 $\alpha$ ) to stall translation. For example, arsenite-induced oxidative stress leads to heme-regulated initiation factor 2 $\alpha$  kinase (HRI) inactivation of eIF2 $\alpha$ , the inhibition of protein synthesis, and the formation of stress granules (McEwen *et al.*, 2005). UV irradiation also leads to stress

granule formation, possibly because UV irradiation activates protein kinase R, which is a regulator of eIF2 $\alpha$  (Kedersha *et al.*, 1999; Anderson and Kedersha, 2008). Ribosomes blocked at initiation accumulate on mRNA as 48S structures, which then begin to nucleate stress granules through the binding of a variety of aggregate-forming RNA-binding proteins (Kedersha *et al.*, 1999). Translational arrest of mRNAs that had been undergoing translation during normal growth conditions may allow cells to degrade damaged RNAs and alter the profile of mRNA translation in response to stress.

mRNAs are thought to exit stress granules by reinitiation of translation or transfer to P-bodies (Kedersha *et al.*, 2005). P-bodies are sites of mRNA decapping and decay and are found in cells under normal growth conditions but are further induced by stress (reviewed in Eulalio *et al.*, 2007). Decay of a deadenylated mRNA via the 5'-3' pathway is initiated by removal of the 5' cap structure by the decapping complex Dcp1-Dcp2. Numerous activators of decapping also act at this step, including the Sm-like (Lsm) 1–7 complex, Pat1, and the helicase Dhh1. After decapping, the mRNA is degraded by the 5'-3' exoribonuclease Xrn1. The proteins involved in the 5'-3' decay pathway are all localized to P-bodies (Sheth and Parker, 2003). Indeed, the decay of mRNA has been demonstrated to occur within these foci, as yeast strains that lack Xrn1 have enlarged P-bodies that contain accumulating mRNA. Interestingly, at least one type of mRNA quality control, nonsense-mediated decay (NMD), is thought to use P-bodies as a site of degradation of defective mRNAs. Several factors essential for NMD are localized to P-bodies (Fukuhara *et al.*, 2005; Unterholzner and Izaurralde, 2004) and mRNAs containing premature stop codons are recruited to P-bodies by the NMD protein Upf1 (Sheth and Parker, 2006). The finding that NMD may occur in P-bodies raises the possibility that P-bodies may be sites of turnover for other types of aberrant mRNAs.

The function of P-bodies in the handling of damaged RNA is supported by the increased sensitivity of cells lacking certain P-body proteins to conditions that cause RNA damage. Notably, a mutant *xrn1* strain displays UV sensitivity (Tishkoff *et al.*, 1991). This is consistent with the UV-sensitive phenotypes for strains lacking Pat1 and strains lacking Lsm1 (Birrell *et al.*, 2001; Wang *et al.*, 1999). Interestingly, in addition to the UVC sensitivity of the *lsm1 $\Delta$*  deletion strain, both the *lsm1 $\Delta$*  and *lsm6 $\Delta$*  deletion strains are sensitive to the alkylating agent methyl methanesulfonate (MMS) (Chang *et al.*, 2002). These phenotypes suggest that P-bodies may be important for the decay of several different types of damaged mRNAs and that failure to degrade damaged RNAs can impact cell growth.

Moreover, in addition to these stress sensitive phenotypes of P-body proteins, the role of P-bodies in conditions that cause RNA damage is indicated by findings that while P-bodies exist under normal cellular conditions, they enlarge or change subcellular localization in response to certain stress conditions. Yeast cells exposed to UV light show intensified localization of the RNA helicase Dhh1 and the decapping protein Dcp2 to P-bodies during recovery following treatment (Teixeira *et al.*, 2005). Following arsenite-induced oxidative stress of mammalian cells, stress granules form and P-bodies localize adjacent to stress granules, possibly to expedite the degradation of damaged mRNAs accumulating in stress granules (Yu *et al.*, 2005; Kedersha *et al.*, 2005).

Additional, distinct cytoplasmic bodies named UV-induced mRNA granules (UVG) have also been proposed (Gaillard and Aguilera, 2008). Following UV treatment of yeast cells, overexpressed reporter mRNAs were observed to be stabilized and to accumulate in UVGs, which do not colocalize with P-body or stress granule components. It is possible that such localization serves to sequester mRNAs from translation, but it is not yet known how sorting to UVGs occurs or whether these RNAs contain UV damage.

## Additional proteins implicated in the handling of oxidatively damaged RNAs

Another candidate for involvement in turnover of oxidatively damaged RNA that is also localized to P-bodies and stress granules (Yang and Bloch, 2007) is the Y-box-binding protein (YB-1). A member of the cold-shock domain superfamily, YB-1 participates in a wide range of processes, including transcription regulation, translation regulation, and DNA repair (reviewed in Kohno *et al.*, 2003). In vitro experiments suggest that YB-1 may have specific binding for 8-oxoG-containing oligonucleotides (Hayakawa *et al.*, 2002). A role for YB-1 in removal of oxidatively damaged RNAs is also consistent with the observation that expression of human YB-1 in *E. coli* increases resistance to paraquat-induced oxidative stress (Hayakawa *et al.*, 2002) while YB-1 knockdown induces UV sensitivity in human epidermoid cells (Ohga *et al.*, 1996). Further, YB1<sup>-/-</sup> mouse embryonic fibroblasts have increased sensitivity to oxidative stress and senesce prematurely in hyperoxic conditions (Lu *et al.*, 2005). Interpretation of these phenotypes is complicated by the numerous roles of YB-1, as stress resistance could also be a result of YB-1 regulation of transcription. One additional indication of a role for YB-1 in handling damaged nucleic acids is that YB-1 has the capacity to stimulate the base excision repair activity of the NEIL2 protein, which removes oxidized bases from DNA (Das *et al.*, 2007). While oxidatively damaged RNAs are not thought to be repaired, this finding suggests a model wherein YB-1 binding to 8-oxoG-containing RNAs may assist turnover of these RNAs by other proteins.

Much research has focused on the human polynucleotide phosphorylase protein (hPNPase), a homolog of the well-characterized bacterial 3'-5' exoribonuclease. In bacterial cells, PNPase participates in mRNA turnover as well as the degradation of some noncoding RNAs, including aberrant precursor tRNAs and rRNAs (Deutscher, 2006). hPNPase emerged as a candidate for turnover of oxidatively damaged RNAs when in vitro binding experiments suggested hPNPase has some specificity for oxidatively damaged RNA (Hayakawa *et al.*, 2001). Additionally, overexpression of hPNPase lowers levels of 8-oxoG RNA while siRNA knockdown of hPNPase raises 8-oxoG levels (Wu and Li, 2008). However, a possible role for hPNPase in degrading oxidized RNAs must be reconciled with the localization of hPNPase to the mitochondrial intermembrane space, which is not known to contain RNA (Chen *et al.*, 2006). While hPNPase mobilization into the cytosol is seen in conditions that induce apoptosis, it is not clear whether hPNPase is also released into the cytosol under other stress conditions.

## Proteins and pathways that may have a general function in handling damaged RNAs

### Exoribonucleases

In eukaryotes, an important candidate for degradation of damaged RNA is the exosome complex of 3'-5' exonucleases. As this complex acts in the nuclear degradation of aberrant precursor mRNAs, tRNAs, and rRNAs as well as in the cytoplasmic degradation of mRNAs that contain premature stop codons (reviewed in Houseley *et al.*, 2006), the exosome may also be recruited to damaged RNAs. Interestingly, yeast strains mutant for exosomal proteins show increased sensitivity to 5-fluorouracil (5FU) treatment (Fang *et al.*, 2004), which may be consistent with a role for the exosome in turning over RNAs containing that unnatural base.

In bacteria, several exoribonucleases have been implicated in the decay of damaged RNAs. In *Deinococcus radiodurans*, PNPase is important for viability following UV irradiation and for growth on H<sub>2</sub>O<sub>2</sub>-containing media, indicating a potential role for that nuclease in degradation of damaged RNAs (Chen *et al.*, 2007). Another processive 3'-5' exoribonuclease, RNase R, which is remarkable for its ability to processively degrade through regions of significant secondary structure, shows increased activity in *E. coli* in a number of stresses such as growth in minimal media, starvation, and cold shock (Chen and Deutscher, 2005). Additional evidence

that PNPase and RNase R function in the turnover of aberrant RNAs has been provided by experiments in *E. coli*. At 42°C, the PNP<sup>tsR</sup> strain accumulates fragments of the 16S and 23S rRNAs and is deficient in 70S ribosome assembly (Cheng and Deutscher, 2003). This suggests that these two nucleases function to degrade defective rRNAs that cannot be incorporated into ribosomes. Further, PNPase has been shown to degrade mutant precursor tRNAs (Li *et al.*, 2002). These findings suggest that these nucleases may also act to clear the cell of defective noncoding RNAs after RNA damage.

While different nucleases are known for having varying tolerance for RNA secondary structure (Deutscher, 2006), it is unclear whether different nucleases may show particular tolerance or sensitivity toward unusual structures formed by RNA damage such as RNA-RNA or RNA-protein crosslinks or modified nucleotides. This point has not been addressed directly, but interestingly, the RNase R homolog from *Mycoplasma genitalium* stalls at sites of 2'-O-methylation, while the *E. coli* RNase R does not show this sensitivity (Lalonde *et al.*, 2007). Thus, the activity of different nucleases in degrading through crosslinks or other nucleotide modifications resulting from RNA damage may determine what nucleases are most active in the turnover of damaged RNAs.

Finally, it is notable that both prokaryotes and eukaryotes have mechanisms for large-scale turnover of rRNA. In *E. coli*, starvation induces degradation of most of the rRNA in the cell, presumably in order to scavenge nucleotides, although the mechanism is not well understood (Jacobson and Gillespie, 1968). In *S. cerevisiae*, starvation causes ribosomes to be targeted to the lysosome for degradation in a process termed 'ribophagy' (Kraft *et al.*, 2008). While the extensive rRNA degradation seen during starvation has not been reported for conditions known to damage RNA, it is possible that cells use the same underlying mechanisms to target damaged RNAs for degradation.

### Poly(A) polymerases and helicases

The activities of some prokaryotic nucleases and the eukaryotic exosome are enhanced by poly(A) polymerases and helicases. In prokaryotes, the degradation of mRNAs and noncoding RNAs is stimulated by the addition of poly(A) tails by poly(A) polymerase (Deutscher, 2006). In eukaryotes, polyadenylation of RNAs by the TRAMP complex promotes degradation by the nuclear exosome (LaCava *et al.*, 2005; Vanáčová *et al.*, 2005). The TRAMP complex consists of the poly(A) polymerase Trf4 as well as an RNA helicase Mtr4 and one of two closely related RNA-binding proteins, Air1 and Air2. Polyadenylation may play a role in targeting certain RNAs for degradation, as the TRAMP proteins have been reported to preferentially polyadenylate incorrectly folded tRNAs *in vitro* (Vanáčová *et al.*, 2005). It is speculated that this specificity arises from greater accessibility of the 3' end (Reinisch and Wolin, 2007), but it is unclear how this preference may extend to many types of damaged RNAs. Interestingly, polyadenylated rRNA precursors accumulate in yeast treated with 5FU, especially in strains mutant for the exosomal subunit Rrp6 (Fang *et al.*, 2004). As 5FU-containing RNAs can form stable RNA-protein adducts with the modifying enzyme pseudouridylase, the accumulating defective rRNA precursors are hypothesized to require TRAMP/exosome-mediated degradation (Hoskins and Butler, 2008).

Helicases are thought to aid degradation by facilitating translocation of the nucleases over the RNA, removal of RNA secondary structure, and removal of proteins from the RNA. The helicase activity of the TRAMP complex is required for the degradation of substrates including aberrant tRNAs (Wang *et al.*, 2008), while the cytoplasmic exosome interfaces with the Ski complex, of which Ski2 has helicase activity (Houseley *et al.*, 2006). In bacteria, PNPase can function with at least two different helicases, one of which may be a cold adaptation (Prud'homme-Généreux *et al.*, 2004), suggesting that different accessory proteins may function to adapt nuclease activity to different environmental stresses.

## mRNA quality control pathways

Cells have multiple mRNA quality control pathways that eliminate faulty transcripts, including nonsense-mediated decay of mRNAs containing premature stop codons, non-stop decay of mRNAs lacking stop codons, and no-go decay of mRNAs stalled in translation (Doma and Parker, 2007; Isken and Maquat, 2007). These mechanisms may also participate in the handling of some forms of RNA damage. For example, the production of truncated protein products by the translation of oxidized mRNAs (Tanaka *et al.*, 2007) could potentially trigger NMD, and likewise, stalling of elongation in the translation of oxidatively damaged mRNAs (Shan *et al.*, 2007) could lead to mRNA degradation by no-go decay.

There is evidence that certain types of stress may activate NMD activity. One regulator of NMD activity is hSMG-1 kinase, which phosphorylates hUpf1 to promote NMD activity. Interestingly, activity of the hSMG-1 kinase increases with UV or ionizing radiation (IR), and higher NMD activity was observed after IR (Brumbaugh *et al.*, 2004). However, it has not yet been demonstrated that the increased NMD activity is required for the degradation of damaged RNA, rather than another function.

## Other RNA binding proteins

The Ro autoantigen, a ring-shaped RNA binding protein, also has roles both in RNA quality control and cell stress response. In vertebrate cells, the Ro protein associates with both misfolded pre-5S rRNAs (O'Brien and Wolin, 1994) and aberrant U2 snRNAs (Chen *et al.*, 2003), and is thought to act in quality control for these RNAs, most likely by a scavenger mechanism of binding to RNAs that fail to properly associate with processing factors or incorporate into mature RNPs (Fuchs *et al.*, 2006). Further, in the bacterial species *D. radiodurans*, the Ro homolog Rsr immunoprecipitates with PNPase and acts with the exoribonucleases RNase PH and RNase II in 23S rRNA maturation (Chen *et al.*, 2007). This suggests that Ro proteins could act with nucleases in other processes as well, such as the turnover of damaged RNAs. Consistent with this hypothesis, Ro contributes to survival after UV in *D. radiodurans* (Chen *et al.*, 2000) and in mammalian cells, where an accumulation of Ro is seen in the nucleus during recovery from UVC irradiation (Chen *et al.*, 2003). Ro also exhibits genetic interactions with PNPase in both UV and oxidative stress in *D. radiodurans* (Chen *et al.*, 2007). It will be of interest to determine what RNAs are bound by Ro during recovery from UV and other stresses, how Ro binding may lead to RNA degradation, and how Ro activity and subcellular localization are regulated in stress conditions.

The host factor I (Hfq) protein is another bacterial ring-shaped RNA binding protein and, like components of the Lsm1–7 ring, is a member of the Sm-like family of proteins. Hfq has a variety of functions, including modulation of several steps in RNA decay. Hfq protects some RNAs from RNase E cleavage, which often functions as an initiating event in RNA degradation (Moll *et al.*, 2003). Hfq also promotes polyadenylation by poly(A) polymerase I (PAPI), which in turn increases mRNA turnover by making mRNAs better substrates for PNPase-mediated degradation (Mohanty, Maples & Kushner 2004). Such activities could be critical to promoting or regulating the turnover of damaged RNAs. Indeed, hfq insertion mutants show sensitivity to UV (Tsui *et al.*, 1994) and H<sub>2</sub>O<sub>2</sub> (Muffler *et al.*, 1997), among other growth phenotypes. However, H<sub>2</sub>O<sub>2</sub> sensitivity was correlated with another Hfq function, the translation of the RNA polymerase  $\sigma^s$  subunit responsible for transcription of stress-response genes (Muffler *et al.*, 1997), and could also result from the action of Hfq in the post-transcriptional regulation of some mRNAs (Zhang *et al.*, 2002). Therefore, it is not currently possible to conclude whether Hfq modulation of RNA decay is important for survival following stress.

Additionally, multiple ribosomal proteins have secondary activities that suggest roles in response to RNA damage. In *Arabidopsis*, one part of the UV-induced stress response is the

rapid degradation of bulk mRNA, but not noncoding RNA (Revenkova *et al.*, 1999). This extreme turnover of mRNA may allow more responsive upregulation of stress response genes as well as function to degrade damaged mRNAs. Disruption of the promoter of one isoform of ribosomal protein S27 inhibits this mRNA degradation and causes UVC sensitivity. Additionally, in *Drosophila melanogaster*, the small ribosomal subunit protein S3 and the ribosomal stalk protein P0 have been reported to have activities that are a part of DNA base excision repair (Yacoub *et al.*, 1996; Yacoub *et al.*, 1996). S3 was shown to have endonucleolytic activity on 8-oxodG-containing DNA, and both S3 and P0 were reported to have cleavage activity on abasic sites. The activity of P0 on single-stranded as well as double-stranded DNA raises the possibility that these proteins might act on RNA lesions as well.

## Conclusions and Perspectives

A growing collection of findings suggests that RNA damage occurs in cells during normal growth and also during stress. In some cases, the functional consequences of RNA damage have been observed, including decreased protein synthesis as a result of ribosomal crosslinking and/or mRNA oxidation. As RNA damage is increased in models of aging and is in some instances correlated with disease, such as neurodegeneration and atherosclerosis, it will be important to elucidate in more detail how RNA damage inhibits cell function. In particular, a better understanding is needed of the *in vivo* susceptibility of particular RNAs to damage and how the varying levels of damage seen for different mRNAs and noncoding RNAs impact cellular function and disease progression. In addition, as most of the experimental work on the functional consequences of RNA damage has focused on mRNAs and rRNAs, future efforts will likely focus on the consequences of damage to more recently described classes of small noncoding RNAs including miRNAs, siRNAs, and piRNAs.

As knowledge of the turnover of damaged RNA has expanded, it has become clear that RNA turnover has critical significance to cellular stress responses. For example, mutant phenotypes for proteins that act in RNA turnover, such as Xrn1, Lsm1, Lsm6, and Pat1, are seen in stress conditions that cause RNA damage. Such phenotypes indicate that failure to clear damaged RNAs may negatively affect cell viability. These phenotypes, combined with the identification of other mechanisms to handle specific types of damage, such as MutT hydrolysis of oxidized free RNA nucleoside triphosphates and AlkB-catalyzed repair of alkylation, demonstrate the existence of cellular machinery for clearing RNA damage. Nonetheless, important questions remain regarding the extent to which turnover of damaged RNAs is a result of mechanisms that specifically recognize certain damage products or whether damaged RNAs are handled by general turnover pathways. Additionally, for the accumulation of damaged RNAs seen in aging and disease, it is unclear whether RNA degradation mechanisms become impaired or simply overwhelmed. Future studies will likely identify roles for known and novel factors in the handling of damaged RNAs and address the function of RNA turnover mechanisms in disease states.

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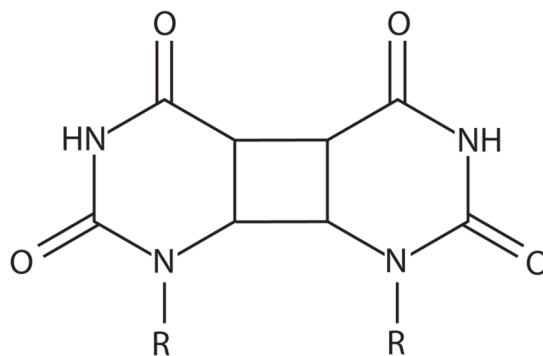
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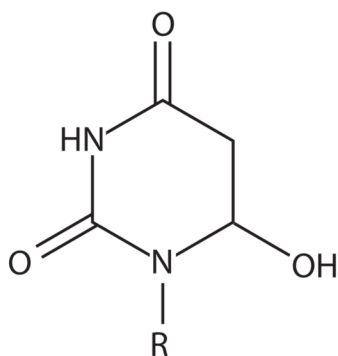
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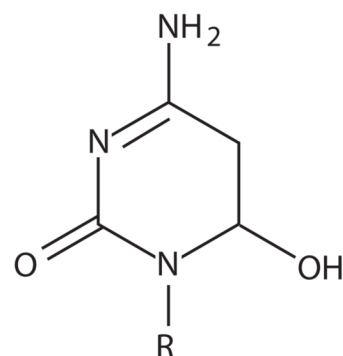
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**A**

Uracil cyclobutane dimer

**B**

Uracil hydrate

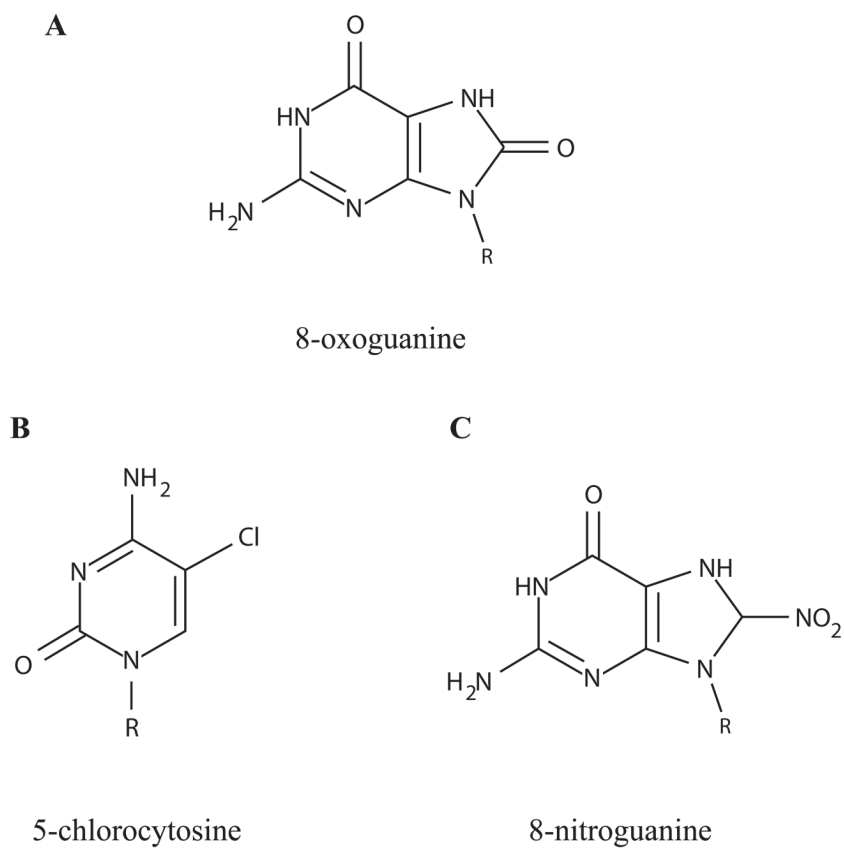
**C**

Cytosine hydrate

**Fig. 1.**

UV photoproducts. UV modification of bases can occur to free nucleobases (R = H) or in ribonucleosides, ribonucleotides, or in the context of an oligoribonucleotide (R = ribose). Figures produced with MarvinSketch (ChemAxon).





**Fig. 2.**  
Chemically modified bases. R = H for free nucleobases or R = ribose for ribonucleosides.