PNAS SPECIAL FEATURE: INTRODUCTION OPEN ACCESS

RNA dynamics in oxidative stress: From obscurity to mechanistic understanding in health and disease

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Stress is a fact of life for *all* creatures great and small, and it places enormous burdens on cells, often threatening their very survival. This feature is about bringing RNA to the rescue to allow these organisms to cope with the stress, from microbes to humans. A mere decade ago, discovering RNAs decorated with m^5C, m^6A, and 8-oxo-rG was viewed as an alphabet-soup of curious findings. Today these modifications are known to be associated with cellular adaptation to stress. The stressors include environmental conditions, like oxidative stress and heat shock, and diseases ranging from drug-resistant chronic infections, through cancers, to neurodegenerative diseases. Indeed, transfer RNA (tRNA) modification-dependent redox homeostasis has been shown to regulate bacterial cell survival on the one hand and synapse formation and memory in insect cells, on the other. These are exciting times, and these topics as well as the role of small-RNAs (sRNAs) in regulation during stressful conditions lie at the heart of this Special Feature "RNAs and Their Intracellular Processing During Oxidative Stress."

Molecular oxidation in response to reactive oxygen species (ROS) occurs both spontaneously in cellular systems and as a consequence of environmental exposures to various toxins. Given that RNAs are more susceptible to modifications than DNA, there is vast interest in understanding how cells process oxidized RNAs, how different proteins, like RNA modifying enzymes (readers, erasers, and writers) manage cellular oxidative stress, and how ultimately this affects various cellular pathways. In this special issue on RNA oxidation, we explore this topic in the context of original and review articles. Consistent themes that emerge across different cell types, ranging from bacteria to human cells, underscore the importance of posttranscriptional modifications on tRNAs as the result of oxidative stress. They also point to the functional relevance of sRNA regulation of key oxidative stress-resistance gene clusters, recognition and processing of oxidized RNAs in cells, and the role of oxidized RNAs in several of the above-mentioned diseases. Likewise, the continual challenges in the types of methods that enable studies of RNA oxidation are also highlighted in several articles, as well as many of the gaps that remain understudied as far as the interplay of oxidative stress, RNA oxidation, and the broader epitranscriptome.

The first three articles, describing bacterial systems, yield deep mechanistic insights (1–3). The article, *"Stress induced modification of E. coli tRNA generates 5-methylcytidine in the variable loop"* by Valesyan et al. focuses on tRNA modification in the context of stress. While the field of RNA modifications has emerged to the point of identifying hundreds of naturally occurring modifications in a wide range of RNAs, there is still a gap in knowledge on how and under what cellular conditions specific modifications arise; of key interest to this issue is which ones arise under oxidative stress. The authors detail the identification and biochemical characterization of a novel ROS-induced posttranscriptional modification of tRNA containing the modified noncanonical nucleoside queuosine (tyr-QUA-II) (1). Functional m^5C modifications are widespread throughout archaea, bacteria, and eukaryotes; however, this specific tRNA stress-induced modification has not been previously reported in *Escherichia coli* tRNA. Through liquid chromatography/mass spectroscopy (LC–MS/MS), several modifications were found to be increased either via the Fenton reaction or H_2O_2 exposure, most notably m^5C accumulation in the UCA[m^5C]AGp motif of Tyr-QUA-II. Mutants of known bacterial cytidine methyltransferases were evaluated and RsmF, the ribosomal RNA methyltransferase, was the only enzyme identified in m^5C generation and was found to be increased under oxidative stress conditions. Although heat-shock stress also leads to the accumulation of this modification, ultraviolet (UV) light does not. In addition, the authors identify a functional role of the RsmF methyltransferase in promoting survival during increased ROS conditions. In an exciting way, this article opens up questions about the structural and functional role of the m^5C modification on tRNA, which the authors speculate could promote RNA structural alteration and could possibly contribute to survival in environmentally stressful conditions beyond oxidative stress. An important question that this article raises is whether or not other methylation targets are linked to the oxidative stress response.

The molecular mechanisms used by proteins that have been thought to uniquely recognize and degrade oxidized RNAs to confer cellular protection under oxidative stress remain poorly understood. In the article *"Selective 8-oxo-rG stalling occurs in the catalytic core of polynucleotide phosphorylase (PNPase) during degradation,"* Miller et al. study the degradation and binding activity of *E. coli* PNPase, an enzyme conserved across different domains of life, to 8-oxoG-containing RNA oligomers (2). Using different conditions that affect enzymatic activity as well as mutant versions of PNPase, the authors use biochemical experiments, cryo-electron microscopy (cryo-EM) structural analysis, and phenotypic characterization of cells lacking the enzyme to elucidate how this enzyme might confer protective cellular effects under oxidative stress. Binding of PNPase to 8-oxoG results in catalytic stalling of PNPase RNA degradation

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L.M.C. and M.B. are organizers of this special feature.

Author contributions: L.M.C. and M.B. wrote the paper.

The authors declare no competing interest.

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Published November 4, 2024.

activity. Major conclusions from this study are that PNPase stalls upon encountering an 8-oxoG within an RNA, in a process mediated by hydrogen bonding. Moreover, the authors show that 8-oxoG stalling occurs within the catalytic site of the enzyme, is specific to 8-oxoG, and is affected by the number of 8-oxoG modifications. Importantly, the catalytic activity of the enzyme is shown to be important for survival of *E. coli*, as mutations within the catalytic site compromise cell survival under stress. An open question is if and how the enzyme "unstalls" to be able to degrade oxidized RNAs in cells.

The article "*Small RNA OxyS induces resistance to aminoglycosides during oxidative stress by controlling Fe-S cluster biogenesis in Escherichia coli*" by Baussier et al. proposes a model of how two regulatory sRNAs, OxyS and FnrS, regulate iron–sulfur cluster regulator (IscR)-mediated Fe–S cluster biogenesis as a response to oxidative stress (3). Interesingly. FnrS and OxyS act as mechanistic opposites, wherein FnrS represses the expression of the Fe–S-containing transcription factor, IscR and, conversely, OxyS activates *iscR* expression under oxidative stress conditions. The primary challenge being addressed in the manuscript is elucidating the regulatory mechanisms that govern Fe–S clusters in bacteria—as these represent networks that are not well understood as they relate to bacterial metabolic control and stress response. Molecular methods are used in this study, e.g., an *iscR-lacZ* translational fusion to screen a 26-sRNA library for potential posttranscriptional activation, along with gel electrophoresis to confirm direct binding of the sRNAs to the proposed IscR target. Also, OxyS's impact on survivability under oxidative stress, induced by H_2O_2, was determined using a strain exhibiting catalase/peroxidase deficiency. An interesting feature of this mechanism is the oxidation of OxyR itself, a master regulator and activator of OxyS, resulting in the production of OxyS and, consequently, in the posttranscriptional activation of IscR. This oxidative-stress regulatory network is also relevant to a higher-order regulation of bacterial virulence factors, given the finding that this alternative route of Fe–S biogenesis reduces aminoglycoside sensitivity, increasing cellular resistance to aminoglycoside antibiotics. It is noted by the authors that the genes encoding this network are present in the important bacterial pathogens *Salmonella, Klebsiella, Yersinia,* and *Pseudomonas.*

As well as the connection of bacterial regulatory RNAs to oxidative stress responses, the regulation of micro-RNAs (miRNAs) in human cells during oxidative stress has also been of interest. In the article "*Apurinic/apyrimidinic endodeoxyribonuclease 1 [APE] controls miR-92b expression in cancer cells by regulating RNA G-quadruplex folding*", Bellina et al. explore the role of the human APE, a DNA repair enzyme, in the miRNA biogenesis pathways (4). Specifically, the authors focus on the interplay between G-rich miRNA precursors that can form G-quadruplex structures, APE1 regulation of these precursors and impact of APE1 depletion on the maturation process, as well as the effect on the corresponding target genes of these miRNAs. Using a number of assays, they show that the APE1 protein can bind these G-rich miRNAs precursors and that its N-terminal lysines can regulate the folding of their G-quadruplex structures. The study uses miR-92b as an example, given the known presence of a quadruplex structure in this miRNA, and its role in several cancer subtypes. Importantly, since the protein has also been connected with decay pathways of oxidized miRNAs during oxidative stress, this work presents a potential mechanism by which a protein like APE1, traditionally linked to DNA repair, can affect regulatory activity of RNAs during oxidative stress. These studies raise questions about the basal frequency of the oxidized base 8-oxoG in miRNAs, the rates of 8-oxoG accumulation in miRNAs postoxidative stress and the potential mechanism of oxidized base repair in miRNAs. The authors speculate on the potential importance of the regulation of guanine oxidation for miRNA processing and function. As such the potential role of proteins like APE1 in recognizing and processing oxidized miRNAs along or in complex with other oxidized-RNA binding proteins represents an interesting avenue for future studies given the regulatory functions of G-quadruplex in several miRNAs. Another interesting question is how the presence of 8-oxoG might impact G-quadruplex folding and structure, and finally whether such miRNAs that are implicated in cancer-related pathways have prognostic value in common tumors.

Continuing on the theme of oxidative stress during tumor progression is the review article "*Stress Response Regulation of mRNA translation: Implications for antioxidant enzyme expression in cancer*", by Soo Kim et al. The review focuses on various mechanisms to achieve regulation of translation rates to globally downregulate protein synthesis to conserve ATP, while upregulating translation of proteins that are needed for stress survival (5). Some of the mechanisms that are reviewed include 1) downregulation of cap-dependent translation through the integrated stress response and mammalian target of rapamycin (mTOR) signaling pathways, 2) activation of selective translation via 5'untranslated regions (5'UTR) structures like upstream open reading frames and internal ribosome entry sites, 3) RNA binding proteins to alter messenger RNA (mRNA) turnover, decay, and direct translation control depending on the binding site and competitive/cooperative interactions, and 4) RNA modifications and tRNA fragmentation. For the last topic, oxidative stress has been shown to induce tRNA fragmentation in which different fragments have been associated with supporting tumor suppression or progression. Likewise, m^6A in mRNAs and a variety of modifications in tRNAs are correlated with oxidative stress and alter translation rates. The similarities in cancer cells' response to oxidative stress to other cell types, including radiation-resistant bacteria, are also highlighted. For instance, the article points out how enzymes like glutathione peroxidases and thioredoxin reductases, among others, help to reduce ROS stress in cancer cells. While this is an exciting area for future therapeutic development, the review begs additional studies to better understand how oxidative stress influences the likelihood of tRNA mischarging as well as the potential roles that oxidative stress-induced tRNA modifications have on translation in cancer cells.

In contrast to the articles that focus on RNA oxidation and the oxidative stress responses from the angle of RNA biology and regulation, the review by Jaafar and Aguiar "*Dynamic multilayered control of m6A RNA demethylase activity*" contributes a systems perspective to the topic of oxidative stress, highlighting a hypothesized broad interplay between oxidative stress and methylation (6). They offer a fresh perspective on the dynamic m^6A RNA modification, focusing predominantly on the myriad controls that dictate m^6A RNA demethylase function, through enzymes FTO and ALKBH5, and how these regulatory mechanisms should be considered when investigating the role of m^6A in cancer biology. m^6A is a dynamically

regulated RNA modification present in coding and non-coding RNA species. Many additional levers of dynamic control of m^6A exist within the context of RNA demethylases—transcription factor activation/suppression, subcellular localization, post-translational modification, abundance of intermediary metabolites, to name a few. Of particular interest to the lens of oxidative stress, the authors make intriguing connections between FTO, a well-known m^6A demethylase of relevance to several diseases, oxidative stress, ROS generation, and mitochondrial biogenesis. The interconnectedness of other epitranscriptomic modifications and of how these affect RNA oxidation remains unknown.

RNA oxidation has not only been implicated in various cancers, but also in a number of neurodegenerative diseases. The article "*Mapping the future of oxidative RNA damage in neurodegeneration: rethinking the status quo with new tools"* by Wheeler et al. reviews studies that have linked RNA oxidation as an early, possibly causative sign of amyotrophic lateral schlerosis (ALS) Alzheimer disease, and Parkinson disease (7). Indeed, 8-oxoG accumulation might be part of the mechanism by which these diseases progress. The authors highlight the ways in which new technologies can fill the gaps in understanding neurodegenerative diseases. One suggestion is improving disease 2-dimensional (2D) and 3-dimensional (3D) model tissues (e.g., induced pluripotent stem cells (iPSC) derived tissues and organoids) to help us understand better the interplay between RNA-binding proteins, 8-oxoG-modified RNA, and neurodegenerative diseases. Another suggestion is extending the focus beyond 8-oxoG to characterize other relevant oxidative lesions that are thought to exist in neurodegenerative diseases but that remain understudied (e.g., 8-oxoA, fapyrG, Gh, Sp). The article also points out a general challenge in characterizing oxidized RNA: the limitation of reliable and accurate methods. To date, most methods for characterizing 8-oxoGs (LC–MS, antibody, and sequencing technologies) are not necessarily base-specific. Nevertheless, antibody-based sequencing approaches are the predominant method used for transcriptome-wide studies of RNA oxidation. It is also important to identify RNA-binding proteins that differentially interact with 8-oxoG RNA in neurodegenerative diseases (and in diseases in general) and to determine whether these differences ultimately contribute to the disease progression.

Finally, again on the topic of the nervous system, a family of enzymes that is important to oxidative stress and is thought to transfer methyl groups to and from RNA is the AlkB homolog (ALKBH) family. In the article "*tRNA modification enzyme-dependent redox homeostasis regulates synapse formation and memory*" by Madhwani et al. the authors explore the role of ALKBH8 in methylation of wobble uridines in tRNA (8). ALKBH8 is relevant to neurological disorders given that mutations in the last codon of ALKBH8 cause autosomal-recessive intellectual disability. The main findings from this article were that ALKBH8 also plays a key role in the synthesis of selenoproteins by methylating tRNA-selenocysteine, and if this modification is abolished, there are significant downstream effects since selenoproteins regulate synapse overgrowth and oxidative stress. The authors make these conclusions using genetic methods in *Drosophila*, by producing null alleles of ALKBH8 and comparing these mutants' ability to form synapses, produce selenoprotein, survive under oxidative stress, and form associative memory, as compared with wildtype ALKBH8 flies. Overall, the authors highlight the significant downstream effects that can occur if this tRNA modification is abolished. Perhaps the most notable demonstration of this is that *Drosophila* lacking ALKBH8 exhibited 53% greater mortality than control animals post exposure to paraquat, and a severe deficit in associative memory as compared to control animals. By exploring the use of catalase and superoxide dismutase as a treatment to suppress synaptic overgrowth in animals with mutant ALKBH8, the authors make the interesting suggestion of antioxidants as therapeutic interventions for diseases that originate from mutations causing weakened responses to oxidative stress.

There are three major take-home messages in this compilation of articles. First is the importance of the "RNA-modification code," which lay buried until recently and that is only now being deciphered. This code can account for myriad cellular adaptations to stress in organisms as simple as anucleate bacteria and those as complex as humans. Second is the versatility of a single modification in a broad spectrum of organisms. Take for example 8-oxoG, which in two of the articles functions variously to protect a bacterial cell from oxidative stress, while regulating the quadruplex structure and activity of an miRNA associated with human cancer. In another article, this very same 8-oxoG modification is associated with neurodegeneration in ALS, Alzheimer, and Parkinson diseases. Third is the impressive role of sRNAs in bacteria and miRNAs in mammals, in regulatory functions as different as iron trafficking and cell division, respectively. However, despite these breathtaking discoveries, the cellular function of many dozens of RNA modifications, regulatory sRNAs and miRNAs remain unknown, as do their precise relationship to disease. There is little doubt that the field of RNA regulation in oxidative stress is evolving rapidly, and we need to stay tuned for the next spate of insights, because despite good progress in recent years, we have discovered merely the tip of an iceberg. The challenges and opportunities presented by this field have been explored as a topic of the recently published National Academy Report "Toward Sequencing and Mapping of RNA modifications."

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