Localized control of oxidized RNA

Yu Zhan¹,⁴, James Dhaliwal¹,⁴, Pauline Adjibade², James Uniacke³, Rachid Mazroui², and William Zerges¹,⁵

1) Biology Department & Centre for Structural and Functional Genomics, Concordia University, 7141 Sherbrooke W, Montreal, Quebec, Canada, H4B 1R6
2) Department of Molecular Biology, Medical Biochemistry, and Pathology, Laval University, Centre de Recherche le CHU de Quebec, Quebec, Canada
3) Current address: Department of Molecular and Cellular Biology, Science Complex University of Guelph, Guelph, Ontario, Canada, N1G 2W1
4) These authors contributed equally to this work.
5) Corresponding author: william.zerges@concordia.ca Tel: (514) 848-2424 extension 3416

Key words: oxidized RNA, Rubisco, stress granule, RNA, chloroplast.
ABSTRACT

The oxidation of biological molecules by reactive oxygen species can render them inactive or toxic. This includes the oxidation of RNA, which appears to underlie detrimental effects of oxidative stress, aging, and certain neurodegenerative diseases. Here we investigate the management of oxidized RNA in the chloroplast of the green alga *Chlamydomonas reinhardtii*. Our results of immunofluorescence microscopy reveal oxidized RNA (with 8-hydroxyguanine) localized in the pyrenoid, a chloroplast microcompartment where CO₂ is assimilated by the Calvin cycle enzyme Rubisco. Results of genetic analyses support a requirement for the Rubisco large subunit, but not Rubisco, in the management of oxidized RNA. An RBCL pool that could carry out such a “moonlighting” function is revealed by results of biochemical fractionation experiments. We also show that human (HeLa) cells localize oxidized RNA to cytoplasmic foci which are distinct from stress granules, processing bodies, and mitochondria. Our results suggest that the compartmentalization of oxidized RNA management is a general phenomenon and therefore has some fundamental significance.
INTRODUCTION

The oxidation of biological molecules by reactive oxygen species (ROS) can render them inactive or toxic (Holmstrom and Finkel, 2014). While DNA, lipids and proteins have long been considered as critical targets of oxidation, recent evidence suggests roles of RNA oxidation in oxidative stress, aging, and certain neurodegenerative diseases (Wurtmann and Wolin, 2009). For example, the translation of oxidized mRNAs generates aberrant proteins (Nunomura et al., 2009; Tanaka et al., 2007) and elevated RNA oxidation in neurons is associated with Alzheimer disease, Parkinson disease, and amyotrophic lateral sclerosis (Nunomura et al., 2009).

Molecular quality control systems specifically recognize oxidized molecules and subject them to repair, sequestration, or degradation (Stoecklin and Bukau, 2013). Quality control systems have been characterized for oxidized DNA, proteins, and lipids, but not for oxidized RNA (Wurtmann and Wolin, 2009). However, oxidized RNA quality control may involve YB-1, Auf1 and PNPase because these proteins bind oxidized RNA and control its accumulation (Li et al., 2014). Moreover, the intracellular location(s) of oxidized RNA quality control in eukaryotic cells have been hypothesized to include stress granules (SGs) and processing bodies (PBs) (Thomas et al., 2011; Walters and Parker, 2014; Wurtmann and Wolin, 2009). For SGs, this is supported by their recruitment of YB-1 and Auf1, and their formation under oxidative stress conditions when excess ROS causes oxidative damage (Bravard et al., 2010; Onishi et al., 2008; Tanaka et al., 2014). Similarly, PBs increase in size and number during oxidative stress and they contain mRNA degradation machinery, a component of RNA quality control (Thomas et al., 2011; Walters and Parker, 2014). While our understanding of the quality control of oxidized RNA is advancing, the precise intracelluar locations are still unknown.

In the chloroplasts of plants and green algae, antioxidant systems and molecular quality control are particularly important because photosynthesis produces ROS as hydrogen
peroxide (H$_2$O$_2$), singlet oxygen, superoxide and the hydroxyl radical (Foyer and Shigeoka, 2011). Moreover, chloroplasts have a genome and a gene expression system which are targets of oxidation and mutagenesis by ROS (Wurtmann and Wolin, 2009; Zheng et al., 2014). While chloroplasts have known quality control systems for oxidized DNA, proteins, and lipids (Apel and Hirt, 2004), nothing is known about how they manage oxidized RNAs.

An avenue to study RNA quality control and its localization in chloroplasts arose with our discovery of SG-like bodies that form during oxidative stress in the chloroplast of the unicellular green alga *Chlamydomonas reinhardtii* (hereafter “Chlamydomonas”) (Uniacke and Zerges, 2008). These “chloroplast stress granules” (cpSGs) were seen by confocal fluorescence microscopy as stress-induced foci containing mRNAs encoded by the chloroplast genome and SG marker proteins. cpSGs form at the inner perimeter of the pyrenoid, a micro-compartment in the chloroplasts of most algae where CO$_2$ fixation is catalyzed by the Calvin cycle enzyme ribulose bisphosphate carboxylase/oxygenase (Rubisco) (McKay and Gibbs, 1991). cpSGs are enriched in the large subunit of Rubisco, RBCL, but not the Rubisco holoenzyme; they are not similarly enriched in the small subunit of this complex, RBCS (Uniacke and Zerges, 2008). The RBCL in cpSGs might function in RNA metabolism because, under oxidizing conditions, the protein has been shown to acquire an RNA-binding activity and form aggregates which might represent a biochemical form of cpSGs (Knopf and Shapira, 2005; Yosef et al., 2004). Together, these results suggest that cpSGs, RBCL, and the pyrenoid have some undefined role(s) in chloroplast RNA metabolism during stress. This hypothesis and the previously reported role of RBCL in autoregulatory feedback translational repression are mutually compatible (Cohen et al., 2006).

Here we show that oxidized RNA localizes to the pyrenoid with results of immunofluorescence (IF) microscopy using an antibody against a major oxidized base in RNA and DNA, 8-hydroxyguanine (8-oxoG) (Nunomura et al., 1999; Wurtmann and Wolin, 2009). Results of genetic analyses support a requirement for the Rubisco large subunit, but
not Rubisco, in the management of oxidized RNA. An RBCL pool that could carry out such a “moonlighting” function is revealed by results of biochemical fractionation experiments. We extend our discovery of the localization of oxidized RNA to human cells by showing that HeLa cells under arsenite-induced oxidative stress localize oxidized RNA to cytoplasmic foci which are neither SGs nor PBs. Our results begin to shed light on how oxidized RNA is managed in an algal chloroplast and suggest that the compartmentalization of oxidized RNA quality control is a general phenomenon.
RESULTS

Oxidized RNA localizes within the pyrenoid.

We characterized the distribution of oxidized RNA in Chlamydomonas cells by IF microscopy using a commercial antibody against 8-oxoG. The IF signal was specific to 8-oxoG because it was eliminated when the antibody was incubated with 8-oxoG prior to staining (Fig. 1 A and B).

Inspection of cell images revealed higher 8-oxoG IF signal in the chloroplast than in the central region with the nucleus and most cytosolic compartments (Fig. 1 A). The 8-oxoG IF signal was seen in two distinct patterns; throughout the pyrenoid and in punctate foci located in or near the chloroplast. In order to determine whether these patterns represent 8-oxoG in DNA or RNA, we used two approaches. First, we co-stained cells with DAPI, to visualize the nucleus and the chloroplast nucleoids (the latter contain the multicopy chloroplast genome). These DNA-containing structures only weakly IF-stained for 8-oxoG (Fig. 1 A) and were clearly distinct from the 8-oxoG IF-staining of the pyrenoid and foci. Therefore, most of the 8-oxoG detected with this method is not in the genomic DNA of either the nucleus or chloroplast. Second, we asked whether RNase or DNase treatment altered the 8-oxoG IF-staining of the pyrenoid, the foci, or both. Cells (fixed and permeabilized) were exposed to either RNase or DNase prior to staining with the 8-oxoG antibody and DAPI. The 8-oxoG IF signal in the pyrenoid was eliminated by treatment with RNase, but not DNase, revealing that it represents oxidized RNA (Fig. 1 C). By contrast the 8-oxoG IF signal in most punctate foci was eliminated by treatment with DNase, but not with RNase, revealing that it represents oxidized DNA (Fig. 1 D). These foci could be the DNA of mitochondria, which are localized adjacent to the chloroplast (Rasala et al., 2014). These foci were not explored further here. DNase treatment often generated a diffuse 8-oxoG IF signal throughout the chloroplast for unknown reasons (Fig. 1 D).
We focused on the 8-oxoG IF-staining of the pyrenoid because it represents oxidized RNA and was atypical; we did not see this pattern for any of the four chloroplast mRNAs or nine chloroplast proteins that we analyzed previously (not including RBCL and RBCS, which were seen in the pyrenoid) (Bohne et al., 2013; Schottkowski et al., 2012; Uniacke and Zerges, 2007; Uniacke and Zerges, 2008; Uniacke and Zerges, 2009). Therefore, 8-oxoG IF-staining of the pyrenoid represents the specific localization of oxidized RNA and is not due, for example, to nonspecific entry into the pyrenoid of RNA from the surrounding stroma (the chloroplast compartment analogous to the cytoplasm).

When cells were exposed to H$_2$O$_2$, a ROS used to induce oxidative stress, the percentage in which the pyrenoid IF-stained for 8-oxoG increased from 44% (n=215) to 64% (n=189). This result suggests that oxidized RNA localizes to the pyrenoid for quality control.

Oxidized RNA did not localize to cpSGs, which were IF-stained for two marker proteins; RBCL or a protein of the 30S subunit of the chloroplast ribosome (Fig. 1 E and F) (Uniacke and Zerges, 2008). cpSGs formed during exposure to H$_2$O$_2$, but the 8-oxoG IF signal remained diffuse throughout the pyrenoid (Fig. 1 E and F). This result suggests that cpSGs do not accumulate oxidized RNA.

**Oxidized RNA localizes to foci in cultured mammalian cells.**

To determine whether the compartmentalization of oxidized RNA could be a general phenomenon, we asked whether it occurs in human cells. HeLa cells were treated with the oxidative stressor arsenite and then IF-stained for 8-oxoG. The 8-oxoG IF signal was detected in multiple cytoplasmic foci in more than 40% of these cells, as compared to 5% of untreated cells (Fig. 2 A). These foci were distinct from SGs and PBs, which were IF-stained for marker proteins specific to each of these RNA granule types (Fig. 2 A and B). They were also not within mitochondria and therefore could not be oxidized RNA or DNA of the mitochondrial genetic system (Fig. 2 C). Nuclei also had 8-oxoG-containing bodies, which appear to be nucleoli (Lee et al., 2014). RNA with 8-oxoG, and not DNA, was detected in these bodies,
both cytoplasmic and nuclear, because they did not stain for DNA with DAPI and they were not detected in RNase-treated cells (Fig. 2 A). These results reveal that mammalian cells compartmentalize oxidized RNA to cytoplasmic structures, which we name here “oxidized RNA bodies” (ORBs). They also substantiate the localization of oxidized RNA to nucleoli reported previously (Nunomura et al., 1999).

**RBCL affects the level of oxidized RNA in the chloroplast.**

Returning to the chloroplast; we hypothesized that RBCL functions in the quality control of oxidized RNA based on the localization of 8-oxoG RNA to the pyrenoid, the fact that RBCL is a major protein of the pyrenoid, and the evidence that RBCL has a dual function involving RNA during stress (Yosef et al., 2004). To test this hypothesis, we asked if an RBCL knockout mutant, MX3312 (hereafter “ΔrbcL”), has an elevated level of 8-oxoG in RNA. The results of immuno-slot-blot analyses revealed a significantly higher mean level of 8-oxoG in RNA from ΔrbcL than in RNA from the wild-type strain (Fig. 3 A). The elevated level of oxidized RNA in ΔrbcL is not due to Rubisco-deficiency because it was not detected in another Rubisco-deficient mutant, which lacks RBCS, but has RBCL (ΔRBCS-CAL005.01.13, hereafter “ΔRBCS”) (Fig. 3 A) (Dent et al., 2005). Therefore, our results support a role of RBCL in the management of oxidized RNA which either involves its known RNA-binding activity or is indirect, occurring via other factors that interact with RNA (Yosef et al., 2004). This oxidized RNA is probably in the chloroplast because RBCL is a chloroplast protein and most 8-oxoG RNA was detected in this organelle (Fig. 1).

We were surprised to find that RNA from ΔRBCS showed an even lower mean level of 8-oxoG than did RNA of the wild-type strain (Fig. 3 A). This phenotype is not due to genetic background because transformation of ΔRBCS with a wild-type copy of RBCS2 restored the mean level of 8-oxoG RNA to nearly that of the wild-type strain (Fig. 3 A). The low level of 8-oxoG in RNA from ΔRBCS is also not due to a deficiency for some unknown RBCS function because a double mutant lacking both RBCL and RBCS was found to have a high
mean level of 8-oxoG in RNA, similar to that of ΔrbcL (Fig. 3 A). Therefore, the low 8-oxoG RNA level in ΔRBCS is RBCL-dependent and does not reflect some unknown function of RBCS.

Exposure of the cells to H₂O₂ did not significantly change the mean level of 8-oxoG RNA from wild type, ArbcL, or ARBCS (Fig. 3 A). That these RBCL-dependent effects were detected in cells under non-stress or stress conditions supports a constitutive nature of the proposed moonlighting function of RBCL.

The moonlighting function of RBCL probably does not involve oxidized DNA because similar mean levels of 8-oxoG were detected in total DNA from ArbcL and the wild-type strain (Fig. 3 B). While the level of 8-oxoG was higher in DNA from ArbcL than in DNA from ΔRBCS, this difference was only 1.4-fold versus 10-fold for the same comparison of 8-oxoG in RNA (described above) (Fig. 3 A). Moreover, as stated above, chloroplast nucleoids did not IF-stain for 8-oxoG (Fig. 1 A and C). Treatment of cultures with H₂O₂ increased the variability in the level of 8-oxoG in DNA between biological replicate experiments but did not increase the mean levels relative to DNA from the non-treated cultures (Fig. 3 B). No differences in mean levels of oxidized protein were found between the wild-type strain, ArbcL, or ARBCS mutant strains, measured as carbonylated amino acid residues in total protein (Fig. 3 C). Therefore, our results do not support a function of RBCL related to oxidized DNA or oxidized protein.

**Biochemical evidence of a Rubisco-independent RBCL pool.**

Our evidence of a Rubisco-independent moonlighting function of RBCL suggests the existence of an RBCL pool that is distinct from the RBCL in the Rubisco holoenzyme. Therefore, we carried out biochemical fractionation experiments to identify such a pool. In addition to the major soluble RBCL form of the Rubisco holoenzyme, three forms of RBCL are known. Two insoluble forms of RBCL were detected in cells undergoing oxidative stress or senescence: one was in insoluble aggregates (Knopf and Shapira, 2005) and the other was
membrane-associated (Knopf and Shapira, 2005; Marin-Navarro and Moreno, 2006). In addition, RBCL that is newly synthesized and unassembled was proposed to autoregulate rbcL translation during oxidative stress through its RNA-binding activity (Cohen et al., 2005).

We developed a differential centrifugation scheme to separate soluble and insoluble proteins into three fractions (Fig. 4 B): S16 has soluble proteins; P16-TS has insoluble proteins that can be solubilized by Triton X-100, e.g. membrane proteins; and P16-TI has insoluble proteins that cannot be solubilized by Triton X-100. Analyses of fractions from the wild-type strain revealed that S16 had both RBCL and RBCS, representing Rubisco holoenzyme (Fig. 4 C). Detection of other RBCL forms in fractions of wild-type strains was hampered by contamination of most subcellular fractions by the Rubisco holoenzyme due to its extreme high abundance (Spreitzer, 2003). For example, fraction P16-TI from the wild-type strain contained both RBCL and RBCS, presumably in the Rubisco holoenzyme (Fig. 4 C). This contamination was not a problem with ΔRBCS because it lacks the Rubisco holoenzyme, it accumulates RBCL to only 1-10% of the wild-type level (Fig. 4 A), and it has an enhanced level of the RBCL function relating to oxidized RNA (Fig. 3 A). Therefore, we presumed that most or all RBCL in ΔRBCS represents a pool dedicated to this function. Analysis of fractions obtained from ΔRBCS cells revealed RBCL primarily in P16-TI and, as expected (because this mutant lacks soluble Rubisco holoenzyme), only in trace amounts in S16 (Fig. 4 D). The RBCL in P16-TI from ΔRBCS is not newly synthesized because this form is soluble and fractionated to S16, as revealed by 35S-pulse-labelling (Fig. 4 E). Furthermore, this RBCL is probably not insoluble due to membrane-association, because it was not with detergent-solubilized membrane proteins in P16-TS (Fig. 4 C and D). These results suggest that the major form of RBCL in ΔRBCS differs in physicochemical properties from the soluble RBCL pools of the Rubisco holoenzyme and the newly synthesized protein. Therefore, this RBCL could represent a pool of the protein that is dedicated to its function relating to oxidized RNA in the chloroplast.
To address the possibility that the RBCL in the P16-TI fraction from ΔRBCS represents the form in cpSGs, we asked whether P16-TI has another feature of cpSGs; enrichment in the 30S subunit of the chloroplast ribosome over the 50S subunit (Uniacke and Zerges, 2008). Indeed, results of immunoblot analyses revealed a greater proportion of the 30S subunit pool in P16-TI fractions, whereas the 50S subunit pool was not similarly enriched. This difference was observed in analyses of both ΔRBCS and the wild-type strain (Fig. 4 C, D). However, while stress induces the recruitment of RBCL and 30S subunits to cpSGs, neither shifted from the soluble pool (S16) to P16-TI when cells were exposed to H$_2$O$_2$ (Fig. 4 C and D). This result provides further support of a constitutive nature of the proposed moonlighting function of RBCL related to oxidized RNA, and raises the possibility that cpSGs are a manifestation of an RBCL-containing ribonucleoprotein particle that exists at the submicroscopic level under non-stress conditions (see Discussion).

Survival under oxidative stress inversely correlates with the level of oxidized RNA

To obtain evidence that the differential levels of oxidized RNA in the wild-type and mutant strains (Fig. 3 A) have relevance in vivo, we tested ΔrbcL for impaired tolerance to stress induced by exogenous H$_2$O$_2$. ΔRBCS was again used to control for the effects of Rubisco-deficiency. Cultures of the wild-type strain, ΔrbcL, and ΔRBCS were exposed to a toxic concentration of H$_2$O$_2$ (4.0 mM) and the percentage of live cells was monitored over 8 h (Fig. 5 A). The results revealed that ΔrbcL cells died significantly faster than did the wild-type cells. Therefore, the elevated level of oxidized RNA in ΔrbcL correlates with impaired H$_2$O$_2$ tolerance. ΔRBCS exhibited H$_2$O$_2$ hypertolerance, measured both as cell survival (Fig. 5 A) and in a more stringent assay of viability, as the percent initial colony forming unit concentration (Fig. 5 B). Therefore, in ΔRBCS, the low mean level of oxidized RNA and H$_2$O$_2$ hypertolerance are both opposite to the loss-of-function phenotypes for these traits in ΔrbcL. Similarly, the rescued ΔRBCS mutant (by transformation with RBCS2) showed wild-type H$_2$O$_2$ tolerance (Fig. 5 C) and a wild-type mean level of oxidized RNA (Fig. 3A).
Finally, like Δarbcl, the double mutant for both RBCL and RBCS showed impaired H$_2$O$_2$ tolerance and a high oxidized RNA level (Fig. 5 D). These differences in H$_2$O$_2$ tolerance did not reflect inherent differences in growth rate, transcript levels of oxidative stress marker genes, or the rate of H$_2$O$_2$ degradation in the medium (Fig. S1). Thus, biological relevance of the differential levels of oxidized RNA detected in vitro is supported by the role of RBCL in H$_2$O$_2$ tolerance in vivo.
DISCUSSION

Our results reveal that oxidized RNA is compartmentalized in the pyrenoid of an algal chloroplast and cytoplasmic ORBs in human tissue culture (HeLa) cells (Fig. 1 and 2). These findings in such phylogenetically distant genetic systems suggest that the compartmentalization of oxidized RNA has fundamental significance. Compartmentalization of DNA and protein quality control is well documented and believed to have several functions (Adjibade and Mazroui, 2014; Stoecklin and Bukau, 2013; Walters and Parker, 2014). The sequestration of damaged molecules prevents them from interfering with the processes in which they normally function. It prevents the degradation or attempted repair of undamaged substrates. Finally, compartmentalization could enhance local concentrations of substrate molecules and quality control factors to establish thermodynamic parameters that favor forward reactions, for example, in repair or degradation. Our results open avenues to study the role of compartmentalization in RNA quality control.

Our results show that RBCL has a moonlighting function related to oxidized RNA in the chloroplast (Fig. 3) and H$_2$O$_2$ tolerance (Fig. 5) and that this function is independent of its role as a subunit of the Rubisco holoenzyme. In ΔRBCS, the low level of oxidized RNA and high H$_2$O$_2$ tolerance could reflect an enhanced level of this moonlighting function because these phenotypes are RBCL-dependent. For example, more RBCL might available for the management of oxidized RNA when it cannot be assembled into the Rubisco holoenzyme. The Rubisco-independency of the proposed oxidized RNA-related function of RBCL could explain the evolutionary retention of RBCL in plant and algal lineages that have lost photosynthesis (Krause, 2008). We also identify an insoluble RBCL pool that is not part of the Rubisco holoenzyme and could carry out this dual moonlighting function (Fig. 4). Other evidence of a Rubisco-independent RBCL pool was reported in Chlamydomonas recently; RBCL was shown to accumulate several fold above the equal stoichiometric amounts with RBCS that are required in the Rubisco holoenzyme complex (Recuenco-Muñoz et al., 2015).
A Rubisco-independent pool of RBCL with a moonlighting function related to oxidized RNA could explain the non-colocalization of the IF signal from 8-oxoG RNA and the strongest patches of RBCL IF signal, which are probably the Rubisco holoenzyme (Fig. 1 E).

Our results and results reported previously support the inclusion of RBCL in a class of metabolic proteins with dual functions as RNA-binding proteins (Yosef et al., 2004). These proteins have been proposed to coordinate metabolism and gene expression and to enhance the functional diversity of proteomes (Hentze and Preiss, 2010). For only a few such proteins has evidence of the dual function been demonstrated in vivo, as we have done here for RBCL (Figs. 3 and 5).

The molecular mechanisms involved in the dual function of RBCL related to oxidized RNA remain to be determined. They could involve the protection of undamaged RNA from ROS or the selection of oxidized RNA for repair, degradation, or sequestration from the translated pool. That the IF signal from 8-oxoG RNA was not enriched in cpSGs (Fig. 1 E and F) is inconsistent with sequestration of oxidized RNA, but compatible with the other mechanisms. For example, cpSGs might not accumulate oxidized RNA if they protect non-oxidized RNA from ROS or if they rapidly degrade or repair oxidized RNA. Nevertheless, it remains to be determined whether or not cpSGs function in the quality control of oxidized RNA. It also remains to be determined whether RBCL controls the level of oxidized RNA directly or indirectly, i.e. via other factors and pathways.

Our findings have potential relevance to stress granules and processing bodies. These RNA granules have been implicated in fundamental cell biological processes, but their functions and physicochemical properties are only partially understood. Our results reveal an Rubisco-independent pool of RBCL which is insoluble, a feature that might be expected of cpSG proteins because cpSGs are aggregates of RNA and protein and insolubility in Triton X-100 is a property of PB proteins (Teixeira et al., 2005). Furthermore, the P16-TI fraction with this RBCL pool, like cpSGs, has an excess of the 30S subunit of the chloroplast ribosome.
over the 50S subunit (Fig. 4). Even under non-stress conditions, when most cells do not have cpSGs, RBCL was detected in the insoluble form and shown to affect the level of oxidized RNA in vivo (Fig. 3). Together, these results suggest that RBCL carries out its moonlighting function constitutively, and at the submicroscopic level, i.e. independently of cpSGs. Whether or not SGs and PBs also function as submicroscopic RNP assemblies under non-stress conditions remains to be determined.

Oxidized RNA arises under optimal growth conditions and the mean level did not to increase during stress (Fig. 3). RNA oxidation might result from ROS produced as a byproduct of photosynthesis, which occurs under both non-stress and stress conditions (Foyer and Noctor, 2009). Moreover, a presumed detrimental nature of oxidized RNA in the chloroplast seems at odds with the wild-type growth rate ΔrbcL despite its having an elevated level of oxidized RNA (relative to wild-type) (Fig. 3 A and Fig. S1 A). Our results are consistent with a certain level of oxidized RNA being benign under optimal conditions (Fig. S1 A) and becoming detrimental in the presence of H$_2$O$_2$ (Fig. 5). For example, oxidized bases in chloroplast mRNAs could result in the synthesis of aberrant iron-binding proteins with Fenton activity (conversion of H$_2$O$_2$ to the highly toxic hydroxyl radical) and thereby potentiate the toxicity of H$_2$O$_2$ (Foyer and Noctor, 2009).
MATERIALS AND METHODS

Culturing of Chlamydomonas. The wild-type strain was 4A+ (CC-4051); ΔrbcL was CC-4696 (MX3312) (Dr. Genhai Zhu, Pioneer Hi-Brid) (Satagopan and Spreitzer, 2004); ΔRBCS was CAL005.01.13 (dim1) (Dent et al., 2005). ΔrbcL and ΔRBCS are non-photosynthetic, but fully viable under heterotrophic conditions (Satagopan and Spreitzer, 2004). Because Rubisco mutants are highly light-sensitive (Johnson, 2011), all cultures were grown and tested under heterotrophic conditions; in the dark (on Tris-acetate phosphate (TAP) medium (Gorman and Levine, 1965), at 24°C, with orbital shaking). To generate the complemented ΔRBCS strain, the wild-type RBCS2 gene (on plasmid pSS2) (Khrebtukova and Spreitzer, 1996) was introduced into ΔRBCS by glass bead-mediated transformation as described previously (Purton, 2007). The double mutants for rbcL and the RBCS locus (RBCS1 and RBCS2) were obtained from a cross between ΔrbcL (mt+) and ΔRBCS (mt-). It was critical to use cultures in exponential growth phase and in the density range of 2-4 x 10⁶ cells/ml.

C. reinhardtii microscopy and IF-staining. The IF protocol for Chlamydomonas was reported previously (Uniacke et al., 2011). To induce cpSG formation, live cells were treated with 2.0 mM H₂O₂ for 15 min prior to fixation. Where indicated, fixed and permeabilized cells were treated for 1h at 37°C with 10 µg/ml RNase A (Fermentas) or 50 µg/ml DNase I (Invitrogen). 8-oxoG was immunodetected with a monoclonal mouse antibody (QED Bioscience Inc., clone 15A3). The specificity of this antibody in situ was confirmed by incubating with its antigen 8-hydroxy-2′-deoxyguanosine (1.0 mg/mL) for 2 h before IF-staining. Rabbit antibodies were used to immunodetect RBCL (Agrisera AS03037) and the 30S subunit chloroplast ribosomal-protein (S-20) (Randolph-Anderson et al., 1989). Secondary antibodies were: Alexa Fluor 488 goat anti-mouse IgG and Alexa Fluor 568 goat anti-rabbit IgG (Invitrogen Inc.). Images were acquired by epifluorescence microscopy using a Leica DMI 6000 microscope (Leica Microsystems) with a 63X/1.4 objective, a Hamamatsu OrcaR2 camera, and Volocity acquisition software (Perkin-Elmer). Z-stacks were taken by
series capture at a thickness of 0.2 µm per section. Stacks were deconvoluted with AutoQuant X3 (Media Cybernetics Inc.) (Abramoff et al., 2004).

**Mammalian tissue culture.** HeLa cervical cancer cells were obtained from the American Type Culture Collection (Manassas, VA; ATCC). Cells were cultured at 37°C in DMEM (Sigma-Aldrich, St. Louis, MO) supplemented with 10% fetal bovine serum, penicillin and streptomycin (all from Sigma-Aldrich). Fixed and permeabilized cells were treated with 10 µg/ml RNase for 1h, where indicated. 1.0 mM sodium arsenite treatments of live cells were for 1.0 hr under the conditions described above.

**Mammalian cell IF-staining.**

The protocol was described previously (Fournier et al., 2013). Primary antibodies were; αG3BP1 from Dr. Imed Gallouzi (McGill University), αRCK and αTOM20 (FL-145) from Santa Cruz Biotechnology, and α8-oxoG from QED Bioscience Inc. (clone 15A3). The secondary antibodies were Alexa Fluor 488 goat anti-mouse IgG, Alexa Fluor 598 goat anti-mouse IgG, and Alexa Fluor 568 goat anti-rabbit IgG (Invitrogen Inc.). IF signals were visualized by an LSM 700 confocal laser scanning microscope (Zeiss), controlled with 2009 ZEN software for image acquisition and analysis. Images were acquired using the following settings: 63X oil objective (zoom 1.0), 0.06 µm for pixel size, and 1.00 airy units as pinhole.

**Analyses of oxidized RNA, DNA and Protein.** In each biological replicate experiment, RNA, DNA, and protein were isolated from the same culture. Where indicated, live cells were exposed to 2.0 mM H₂O₂ for 15 min. Total RNA was extracted using TRI-reagent (Sigma-Aldrich) according to the manufacturer’s protocol. RNA was shown to be free of DNA and quantified by analysis with a 2100 Bioanalyzer (Agilent). Total DNA was extracted using hexadecyltrimethylammonium bromide, as described previously (Murray and Thompson, 1980). DNA preparations were rid of RNA by treatment with DNase-free RNase (10 µg /ml, Fermentas) at 37 °C for 1 hour, followed by precipitation of the DNA from RNA fragments with polyethylene glycol (Sambrook and Russell, 2001). DNA concentrations were quantified
by UV spectrophotometry, respectively. Total RNA (5 µg) and DNA (1 µg) samples were transferred to a nitrocellulose membrane with a Minifold-II slot blot system (Schleicher & Schuell). Membrane filters were reacted with the commercial antibody against 8-oxoG (QED Bioscience Inc., clone 15A3) overnight, at 4°C (Sambrook and Russell, 2001). A goat anti-mouse secondary antibody (KPL) was used and ECL detection was performed with a commercial kit (Millipore). To isolate total protein, cells were pelleted (3,000 x g, 3 min) and broken in 50 mM Tris-Cl pH 8.0, 1 mM EDTA, 50 mM NaCl, 1 mM PMSF, 100 mM DTT by bead-beating (Hopkins, 1991). Protein samples (20 µg) were analyzed with the OxyBlot Kit according to the manufacturer’s protocol (Millipore). Total protein concentration was determined as described previously (Smith et al., 1985). Quantification was carried out as described previously (Bohne et al., 2013). For statistical analyses, each biological replicate was compared to a corresponding ΔrbcL strain prior to the addition of H2O2, which was designated as 100% oxidation. Statistical differences were determined in each case using a one-sample t-test.

**Differential Centrifugation.** Cells from 75 ml cultures were pelleted by centrifugation (5,000 x g, 5 min) at room temperature and resuspended in 5.0 ml ice cold MKT-buffer (25 mM MgCl2, 20 mM KCl, 10 mM Tricine pH 7.5, 1.0% (v/v) protease inhibitor cocktail (Sigma-Aldrich)). Cells were broken by three passages through a chilled French pressure cell at 1,000 psi. The cell lysate was centrifuged at 3,200 x g for 1 min to pellet unbroken cells. The supernatant was collected and centrifuged at 16,000 x g for 20 minutes at 4°C. The resulting supernatant was S16. The pellet (P16) was resuspended in MKT-buffer with 2% (v/v) Triton X-100 and incubated for 15 minutes at room temperature with gentle agitation to solubilize membranes. These samples were then centrifuged at 16,000 x g for 20 minutes at 4°C. The supernatant was P16-TS. The pellet (P16-TI) was resuspended in MKT-buffer. Protein samples from each fraction were subjected to SDS-PAGE and immunoblot analysis. Protein loading was based on equal proportions of each fraction. The antisera were: αRBCL
(Agrisera, AS03037), αRBCS (Dr. Robert Spreitzer, University of Nebraska), αCP43
(Agrisera, 111787) αS-20 (30S r-protein), αL-30 (50S r-protein) (Randolph-Anderson et al.,
1989), and αHSP70B (Schroda et al., 1999). Secondary staining used goat anti-rabbit antibody
(KPL) incubated for 1 h at room temperature.

**In vivo** $^{35}$S-pulse-labeling of proteins. $^{35}$S-pulse-labeling reactions were performed with
$^{35}$SO$_4$ as described previously (Uniacke and Zerges, 2007). Cells (c.a. 1.2 x 10$^7$ per sample)
were then pelleted by centrifugation (5,000 x g, 5 min), washed once with 500 μL 50mM
Tris-Cl pH 7.4, resuspended in 80 μL MKT-buffer, and broken by bead-beating (Hopkins,
1991). The cell lysates were centrifuged (16,000 x g, 5 min), and the pellet and supernatant
fractions were subjected to SDS-PAGE (7.5-15% acrylamide, 6.0 M urea). $^{35}$S-pulse-labelled
proteins in dried gels were visualized with a phosphoimager (Typhoon, GE Healthcare).

**Survival and viability**

Cell survival and viability were assayed following addition of H$_2$O$_2$ to 4.0 mM. Cell survival
was determined by counting the proportion of live cells with the Trypan blue exclusion assay
(Sigma-Aldrich). Viability was determined as colony forming units on agar-solidified TAP
medium. For statistical analyses of survival and viability, a mixed analysis of variance
(ANOVA) was conducted with strain as a between factor and time as a within factor. When
appropriate, post hoc analyses were conducted using Tukey’s HSD.
ACKNOWLEDGEMENTS

We thank A. Piekny and C. van Oostende for assistance with microscopy, W. Brake for assistance with statistical analyses, M. Schroda for the αHSP70B antiserum; I. Gallouzi for the αG3BP1 antiserum; G. Zhu, and Pioneer Hi-Bred (Redwood City, Ca) for ΔrbcL-MX3312, and R. Spreitzer for the pSS2 plasmid and the αRBCS antiserum. This work was carried out, in part, in the Centre for Microscopy and in the Centre for Structural & Functional Genomics (Concordia University).

FUNDING

This work was funded by Natural Sciences and Engineering Council of Canada grants 217566 (WZ) and MOP-702406 (RM) and a Canadian Institutes of Health Research New investigator Scholarship award (RM).

AUTHOR CONTRIBUTIONS

Y.Z, J.D., P.A., and J.U. performed the research. Y.Z, J.D., P.A., J.U., R.M. and W.Z. designed the research, analyzed the data, and wrote the paper. Y.Z. and J.D. contributed equally to this study.

ABBREVIATIONS LIST

8-oxoG, 8-hydroxyguanine; cpSG, chloroplast stress granule; H₂O₂, hydrogen peroxide; IF, immunofluorescence; PB, processing body; SG, stress granule; ROS, reactive oxygen species; RBCL, Rubisco large subunit; RBCS, Rubisco small subunit; Rubisco, ribulose bisphosphate carboxylase/oxygenase.
REFERENCES


Fig. 1. The *in situ* distribution of 8-oxoG RNA in *Chlamydomonas*. A) Wild-type cells were IF-stained for 8-oxoG (green) and co-stained with DAPI to visualize DNA. The pyrenoid is seen in differential interference contrast (DIC) images. A cell illustration (right-hand most image in A) shows the locations of the nucleus (N), cytosol (Cy), and chloroplast (Cp), wherein the pyrenoid
(P) is surrounded by a starch sheath (white) and contains cpSGs (red). B) The IF signal from 8-oxoG was eliminated by pre-incubating the primary antibody with 8-oxoG. C) The 8-oxoG IF signal in the pyrenoid is sensitive to RNase (10 μg/ml) treatment. D) DNase (50 μg/ml) treatment did not reduce the 8-oxoG signal in the pyrenoid but did in the punctate foci. E and F) In cells that had been treated with 2.0 mM H₂O₂ to induce cpSG formation, the 8-oxoG IF signal (green) was seen throughout the pyrenoid and not in cpSGs (white arrows), which were IF-stained (red) for either RBCL (E) or the protein of the 30S chloroplast ribosomal subunit (F). Size bars = 5.0 μm.
Fig. 2. In cultured human (HeLa) cells, oxidized RNA localizes to cytoplasmic ORBs during arsenite-induced oxidative stress. Cells were IF-stained for 8-oxoG (green) and DNA was co-stained with DAPI (blue). A) Foci of 8-oxoG were not seen in 95% of cells prior to arsenite treatment. In 40% of arsenite-treated cells, the 8-oxoG IF signal was seen in cytoplasmic foci (white arrows) which are distinct from SGs (seen as foci of the red G3BP1 IF signal). ORBs were not seen in arsenite-treated cells which were treated with RNase after fixation. B) ORBs are also distinct from PBs (seen as foci of the red RAK IF signal). C) ORBs are not mitochondria (seen as the red TOM20 IF signal). n = 250 cells for each. Size bars = 20 µm.
Fig. 3. RBCL affects the level of oxidized RNA, but not oxidized DNA or protein. A) Levels of 8-oxoG in RNA from the wild-type strain, Δrbcl, ΔRBCS, the rescued-ΔRBCS, and the double
mutant for \( rbcL \) and the \( RBCS \) locus are shown under non-stress conditions (dark bars) and from cells exposed to 2.0 mM H\(_2\)O\(_2\) (for the wild-type strain, \( \Delta rbcL, \Delta RBCS \)) (grey bars). Bar height represents the percentage of 8-oxoG ECL signal of the \( \Delta rbcL \) strain before stress (white bar; 100\%). B) The relative levels of 8-oxoG in DNA from the wild-type strain, \( \Delta rbcL \), and \( \Delta RBCS \) are presented as described for RNA in panel A. C) Levels of carbonylated amino acid residues in protein from the wild-type strain, \( \Delta rbcL \), and \( \Delta RBCS \) are presented as described for RNA in panel A. Results were analyzed and are presented as described in Materials and Methods. Error bars indicate the standard error of the mean. Asterisks indicate a significant difference from \( \Delta rbcL \), as determined by one-sample t-tests (\( p \leq 0.05 \)).
Fig. 4. A Rubisco-independent pool of RBCL. A) The relative levels of RBCL in crude lysates of wild-type, ΔRBCS and ΔrbcL cells were determined by SDS-PAGE and immunoblot analysis. Dilutions of the wild-type cell lysate were supplemented with lysate from ΔrbcL to maintain a constant amount of total protein in each lane. The band attributed to RBCL was not a cross-reacting protein as it was not detected in ΔrbcL. The signal from a protein of the 30S subunit
the chloroplast ribosome (30S) was used as a loading control. Black bars indicate adjoined non-
adjacent lanes from a single exposure. B) The fractionation scheme is illustrated. C and D) RBCL fractionation during differential centrifugation and solubilisation with Triton X-100 was revealed by immunoblot analyses for C) the wild-type strain and D) ΔRBCS. Proteins analyzed were RBCL, RBCS, a soluble protein of the chloroplast stroma (HSP70B), an integral thylakoid membrane protein (CP43), and ribosomal proteins of chloroplast ribosome subunits (30S and 50S). Where indicated, fractions were prepared from cells that had been exposed to 2.0 mM H_2O_2. E) ^35S-pulse-labelled (newly synthesized) RBCL in the insoluble (P16) and soluble (S16) fractions from the wild-type strain and ΔRBCS was revealed by SDS-PAGE and phosphoimaging. As a negative control, fractions from ΔrbcL were shown to lack the ^35S-pulse-labelled band assigned to RBCL. An unidentified ^35S-pulse-labelled protein was detected in both fractions of all three strains (asterisk). Black bars indicate adjoined non-adjacent lanes from a single exposure.
Fig. 5. RBCL functions in H$_2$O$_2$ tolerance. A, C, D) The effects of H$_2$O$_2$ on cell survival were measured by Trypan blue exclusion and graphed as the mean percentages of the initial values (immediately prior to H$_2$O$_2$ exposure). B) The effects of H$_2$O$_2$ on viability were measured as colony-forming unit concentration and graphed as the mean percentages of the initial values (immediately prior to H$_2$O$_2$ exposure). The strains analysed are indicated in each panel. Asterisks indicate a significant difference from the wild-type strain, as determined by mixed analysis of variance (ANOVA) (p ≤ 0.05).