Evidence that G-quadruplex DNA Accumulates in the Cytoplasm and Participates in Stress Granule Assembly in Response to Oxidative Stress*

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Running title: G-quadruplex DNA in the cytoplasm

ABSTRACT

Cells engage numerous signaling pathways in response to oxidative stress that together, repair macromolecular damage or direct the cell towards apoptosis. As a result of DNA damage, mitochondrial DNA or nuclear DNA has been shown to enter the cytoplasm where it binds to “DNA sensors” which in turn, initiate signaling cascades. Here we report data that support a novel signaling pathway in response to oxidative stress that is mediated by specific guanine-rich sequences that can fold into G-quadruplex DNA (G4DNA). In response to oxidative stress, we demonstrate that sequences capable of forming G4DNA appear at increasing levels in the cytoplasm and participate in assembly of stress granules. Identified proteins that bind to endogenous G4DNA in the cytoplasm are known to modulate mRNA translation and participate in stress granule formation. Consistent with these findings, stress granule formation is known to regulate mRNA translation during oxidative stress. We propose a signaling pathway whereby cells can rapidly respond to DNA damage caused by oxidative stress. Guanine-rich sequences that are excised from damaged genomic DNA are proposed to enter the cytoplasm where they can regulate translation through stress granule formation. This newly proposed role for G4DNA provides an additional molecular explanation for why such sequences are prevalent in the human genome.

G4DNA is made of four guanines that form Hoogsteen hydrogen bonds in a planar ring referred to as a G quartet (Fig. 1A). Multiple stacks of these G quartets associate through pi bond interactions to form highly stable structures (4) (Fig. 1B). Further stabilization is supported through the coordination of monovalent ions in the central channel. Formation of quadruplex...
structures was suggested to mediate the biological response to telomeric oligonucleotides introduced into cells by transfection (5). Cells that undergo apoptosis have been suggested as a natural source of telomeric DNA presented to neighboring cells in order to reduce potential autoimmune responses (6). Thus far, evidence has not been presented for the appearance of endogenous G4DNA in the cytoplasm.

Recent reports have provided convincing evidence strongly supporting the formation of G4DNA in the nuclear and mitochondrial genomes of human cells. Antibodies to G4DNA have been used to visualize G4DNA in cells (7-10). Small molecules that are designed to interact specifically with G4DNA have been found to alter gene expression in a G4DNA selective manner (11,12). The Pif1 helicase was shown to localize to G4DNA sequence motifs and to bind and unwind G4DNA structures (13,14). G4DNA is proposed to play specific roles in DNA metabolism including replication (14), gene expression (15) and mitochondrial DNA metabolism (16).

In relation to human disease, G-quadruplexes have shown involvement in neurological disorders. Expansion of a DNA repeat sequence GGGGCC in the C9orf72 gene is associated with amyotrophic lateral sclerosis and frontotemporal dementia (17,18). Transcription of the repeat sequences results in RNA that can fold into a stable parallel G-quadruplex, leading to disruption of transcription and/or translation. The Fragile X Mental Retardation Protein (FMRP) binds specifically to G-quadruplex RNA through a conserved RGG motif (19). Studies on Fragile X syndrome indicate that repeat expansion of the CGG triplet can result in dysregulation of translation in neuronal cells through binding of transcripts by FMRP (20).

The in vivo existence and impact of G4DNA on normal and pathological processes is now accepted, but questions regarding the specific functions and mechanisms of action remain to be addressed. We report a new role for DNA sequences that have the capacity to fold into G-quadruplex structures. We used hydrogen peroxide (H₂O₂) to damage the DNA. Under oxidative stress conditions, we find that endogenous G4DNA sequences appear in the cytoplasm. G4DNA from this endogenous source can participate in assembly of stress granules which are known to alter mRNA translation. Stress granules are cytoplasmic aggregates of mRNA and proteins that regulate mRNA translation and decay (21,22). They have been proposed to function as sites of mRNA triage (23), which govern the composition and function of ribonucleoprotein complexes in order to determine whether individual mRNAs are stored, degraded, or translation is re-initiated. There are clear connections between stress granule function and the pathogenesis of cancer (24). The pathway proposed in this work provides a new mechanism by which cells can respond to the effects of DNA damage during oxidative stress.

RESULTS

Identification of G4DNA binding proteins—We performed quantitative proteomics analysis using G4DNA as bait to identify G4DNA binding proteins in WM266-4 melanoma cells (Fig. 1C). G4DNA is characterized by the sequence motif G₃N₁₋ₓG₃N₁₋ₓG₃N₁₋ₓG₃. However, G4DNA can vary in terms of the direction of each strand, the number of different strands, and the length of the DNA loops that occur between runs of guanines. A biotinylated DNA sequence based on a region of the c-MYC promoter that readily forms a stable, parallel quadruplex (18) bound to streptavidin Dynabeads (25) was incubated with human cell lysate to isolate G4DNA bound proteins. To account for non-specific interactions, a single-stranded DNA (ssDNA) was incubated with lysate in a separate experiment. Isolated proteins were identified by quantitative mass spectrometry to determine those proteins enriched upon binding to G4DNA (Fig. 1D, Supplementary Table 1, and Supplementary Fig. S1) (26).

The most highly enriched protein in the G4DNA sample was the DHX36/RHAU/G4R1 RNA helicase, which was previously identified as the major source of G4DNA binding and unwinding activity in the cell (27-29). DHX36 is a multi-functional enzyme involved in transcription (30), translation (31), and has been suggested to serve as a sensor for DNA in the cytoplasm (32). Surprisingly, other enriched proteins were ELAV1/HUR, YB-1/YBOX1, TIA1, and other proteins known to regulate translation and assemble into stress granules (21,24). Stress granules are cytosolic ribonucleoprotein aggregates of stalled translation.
complexes that form in response to various stresses. DHX36 has also been shown to be associated with stress granules (33). Formation of stress granules was recently shown to result upon introduction of exogenous G4DNA into cells (34). The proteomic results here support the hypothesis that endogenous G4DNA binds tightly to proteins that play important roles in translation, such as DHX36 and YB-1, thereby leading to assembly of stress granules.

The proteomic results do not serve as definitive proof that specific proteins do or do not bind to G4DNA. For example, nucleolin, a protein known to interact with G4DNA through its RGG motif (35) was not identified as a G4DNA interacting protein. However, nucleolin also binds tightly to ssDNA (36). Consistent with these results, we identified peptides from nucleolin in both the G4DNA and ssDNA samples. Although nucleolin was not classified as a G4DNA interacting protein due to its presence at similar levels in both the G4DNA and ssDNA samples, this does not exclude the possibility that it interacts with G4DNA; it only indicates that it did not interact with the G4DNA bait significantly more than the ssDNA bait. The most highly enriched protein in the ssDNA sample was Acetyl-CoA carboxylase 1, whose E. coli homolog is a known ssDNA and dsDNA binding protein (37). The identification of DHX36 as the most highly enriched protein in the G4DNA sample and nucleolin in both the G4DNA and ssDNA samples provides evidence that our pulldown and data analysis generally identified G4DNA interacting proteins. The results of the proteomics screen were also confirmed by incubating additional cell lysates with G4DNA or ssDNA and probing for DHX36 or TIA1 by western blotting (Fig. 1E). In both cases, the proteins were enriched in the G4DNA sample, consistent with the proteomics experiment.

The proteomics screen allowed us to develop the hypothesis that some cytoplasmic proteins involved in stress granule formation can bind to G4DNA in the cell. We surmised that G4DNA might enter the cytoplasm during episodes of DNA damage. We then developed a series of experiments to test this hypothesis working with the knowledge that DNA damage has been previously shown to increase the concentration of DNA in the cytoplasm.

**Exogenous G4DNA co-localizes with stress granule markers**—Initially, we wished to repeat previous work which indicated that exogenous G4DNA can induce stress granule formation in U2OS cells (34), so we conducted similar experiments by transfecting G4DNA into melanoma cells. Fluorescently labeled G4DNA was introduced into cells then visualized by fluorescence microscopy along with selected proteins identified from the proteomics screen. G4DNA was found predominantly in the nucleus, with some punctate staining in the cytoplasm (Fig. 2A). TIA1, a stress granule marker (38), was found to co-localize with G4DNA-induced stress granules in the cytoplasm (Fig. 2A). In contrast, introduction of fluorescently labeled ssDNA did not lead to stress granule formation as seen by the lack of TIA1 foci in the cytoplasm (Fig. 2B). Three different Cy3-ssDNAs were used for comparison: Cy3-T20 (Fig. 2B) since the G4DNA bait in the pulldown contained a single-stranded thymidine region; Cy3-Scr (Fig. 2C), a scrambled version of the G4DNA sequence, to account for any proteins that bind preferentially to G-rich DNA; and Cy3-PS-Scr (Fig. 2D). Since ssDNA is more sensitive than G4DNA to degradation by cytoplasmic nucleases such as TREX1, a scrambled sequence with phosphorothioate (PS) at the ends of the oligonucleotide to prevent degradation was also used for comparison. G4DNA is refractory to the nuclease activity of TREX1 (39). Although Cy3-ssDNA is visible in the cytosol, lack of cytoplasmic TIA1 foci indicates that stress granules are not present (Fig. 2B-D). Transfection efficiency was 100% for all the oligonucleotides (G4DNA, T20, ScrDNA and PS-ScrDNA) based on the counting the fractions of cells identified by DAPI stained nuclei that exhibit red fluorescence for at least 100 cells transfected with 500 nM Cy3-DNA.

Three additional proteins identified in the G4DNA proteomics screen that have been shown to associate with stress granules (33,34,40) were examined for co-localization with G4DNA-induced stress granules. DHX36 (Fig. 2E), YB-1 (Fig. 2F), and ELAV1/HuR (Fig. 2G) were also found in punctate regions of the cytoplasm which co-localize with DNA after transfection with G4DNA but not after transfection with ssDNA. In addition to DHX36, which is known to bind quadruplexes (27,41), YB-1 was recently reported...
to bind to G4RNA and G4DNA (34). Two stress granule markers that were not identified by the proteomics screen due to low mass spectral coverage of the proteins were G3BP and eIF3. However, both G3BP (Fig. 2H) and eIF3 (Fig. 2I) were also found in stress granules induced by G4DNA. The fraction of stress granule protein foci that contain G4DNA foci was determined to be 90%, 7%, 75%, 96%, 17%, and 83% of TIA1, DHX36, YB-1, ELAV1, G3BP, and eIF3 foci, respectively. The fraction of DHX36 and G3BP foci containing G4DNA was significantly lower than the other stress granule proteins. Although DHX36 is known to associate with stress granules (33), it also has other roles in the cytoplasm such as sensing double-stranded RNA (42). The cytoplasmic localization of G3BP combined with the inherent punctate staining observed with this antibody likely result in reduced co-localization of G4DNA with G3BP foci. Quantitation of cells with punctate foci containing both DNA and TIA1 indicated an increase in cells exhibiting stress granules with increasing G4DNA concentration (Fig. 3). Overall, the fluorescence microscopy results support the proteomics screen and a previous report (34) indicating that G4DNA introduced into cells can participate in stress granule formation.

*Endogenous DNA sequences capable of forming G4DNA structures accumulate in the cytoplasm during oxidative stress*—We next examined whether G4DNA from endogenous cellular sources could participate in stress granule formation. Stress granules are reported to form under conditions of oxidative stress (24,43,44). We reasoned that oxidative stress could damage DNA leading to excision of nuclear or mitochondrial DNA followed by transfer of the excised sequences into the cytoplasm.

DNA sequences containing runs of guanine are hot spots for oxidative damage (45-47), which could serve as an endogenous source of G4DNA after partial degradation or excision repair. We used H₂O₂ because it is known to cause oxidative damage to DNA (48-50). We applied a G-quadruplex structure specific antibody, BG4 (7,10,51) to determine if levels of endogenous G4DNA increased in the cytoplasm with oxidative stress. Cells were treated with H₂O₂, harvested and the cytosol was isolated. DNA from RNase treated cytosol was then passed through a nylon membrane using a dot-blot apparatus so that all DNA in the cytosol would bind to the membrane. The membrane was probed with the G4DNA antibody and the bound antibody was visualized by chemiluminescence. Cells treated with H₂O₂ for 2 hrs exhibited a 9-fold increase in G4DNA compared to untreated cells (Fig. 4A). Therefore, quadruplex DNA structures increase dramatically in the cytoplasm under conditions of oxidative stress.

The source of the G4DNA in the cytoplasm was investigated by selecting a few candidate sequences and performing quantitative PCR. The relative copy numbers of four G4DNA targets in the cytosol were examined; telomeric DNA and three sequences from mitochondrial DNA (mtDNA). These mtDNA sequences (KSS, HRCC, and PMPS) (52) and telomeric DNA (hTEL) (4) have been shown to form stable quadruplexes in vitro. The results from qPCR show a dramatic increase in telomeric DNA in the cytoplasm of treated cells (Fig. 4B) which is consistent with previous results showing that telomeres are shortened during treatment of cells with H₂O₂ (53). Small increases in the mtDNA sequences were also observed. Since the PCR reports only on the level of each DNA sequence observed in the treated cells relative to the untreated cells, not the absolute quantity of each DNA sequence present in the cytosol, this data does not suggest that most of the DNA is telomeric in origin, only that telomeric DNA sequences significantly increase in the cytosol in response to oxidative stress.

The appearance in the cytoplasm of endogenous G4DNA was next examined by fluorescence microscopy using BG4. This antibody reveals punctate fluorescence staining primarily in the nucleus after treatment with RNase (Fig. 4C), which is consistent with published results (7,10). Interestingly, upon treatment of cells with H₂O₂, there is an increase in punctate G4DNA fluorescent foci appearing in the cytoplasm and a reduction of foci in the nucleus (Fig. 4C). The fluorescence data are quantified in Fig. 4D. Control experiments confirm that the observed quadruplex signal in response to oxidative stress is indeed DNA. Inclusion of RNase treatment prior to BG4 antibody in all experiments excludes observation of G4RNA, and the addition of DNase eliminates
the observed G4DNA foci (Fig. 5). Additional control experiments lacking primary antibodies show the specificity of the antibodies (Fig. 5E).

Since there is a potential for antibodies that bind to a specific DNA conformation to induce folding of the recognized conformation, the effect of BG4 on folding of G4DNA was investigated. A quadruplex forming oligonucleotide with Cy5 and Cy3 at the termini was rapidly mixed with salt in a stopped flow apparatus and the increase in FRET due to G4DNA folding was monitored (Fig. 6A-B). Folding in the absence of BG4 and with an equivalent concentration of BG4 and DNA, and with an excess of BG4 (ten times the BG4 concentration used in the immunofluorescence experiments) all occurred at the same rate. When a Cy5 and Cy3 labeled scrambled DNA was mixed with salt, no increase in FRET was observed in the presence or absence of BG4. The lack of effect of BG4 on G4DNA folding indicates that BG4 does not cause quadruplex folding from unstructured DNA. Additionally, to confirm that the species detected in the cytosol of H2O2 treated cells is indeed a quadruplex, the localization of G4DNA in untreated and H2O2 treated cells was visualized using a different quadruplex specific antibody, 1H6 (Fig. 6C) (9). Similarly to what was observed with BG4, 1H6 detects G4DNA in the nucleus of untreated cells, but G4DNA appears in the cytosol after H2O2 treatment.

These results suggest that DNA sequences that are capable of forming G4DNA structures as indicated by the BG4 and 1H6 antibodies accumulate in the cytoplasm. It is not clear from these data the specific source of the DNA, however the results are consistent with the data from the dot blot assay and the qPCR (Fig. 4). When the media containing H2O2 is removed and replaced with fresh media, G4DNA reappears in the nucleus within 6 hrs (Fig. 7A-B), suggesting that as the cell progresses through the cell cycle, G-quadruplexes reform. Some BG4 staining is still evident in the cytosol even 12 hrs after removing the H2O2. These results indicate that after folding, G4DNA has a relatively long lifetime in the cell, which is consistent with G4DNA serving as a signaling molecule. The appearance of G4DNA in the cytoplasm is not a consequence of apoptotic DNA fragmentation as H2O2 treatment under these conditions resulted in no detectable level of DNA fragmentation (Fig. 7C). Staurosporine, a common apoptosis inducer (54), was used as a positive control.

The increase in BG4 fluorescence in the cytoplasm upon oxidative stress was also observed in HeLa cells (Fig. 8A), and primary melanocytes (Fig. 8B) treated with H2O2. In order to generate oxidative stress through a different mechanism, melanoma cells that were treated with menadione (Fig. 8C). Menadione generates oxidative stress through an unstable semiquinone metabolite that redox cycles in the presence of oxygen to produce reactive oxygen species (55). Treatment with menadione produced cytoplasmic BG4 foci, consistent with what was observed with H2O2 treatment.

Endogenous G4DNA co-localizes with cytoplasmic stress granule proteins—To determine whether endogenous G4DNA can bind to stress granule proteins in the cytoplasm as was already shown for exogenous G4DNA (Figs. 2-3) (34), co-localization between the BG4 antibody and the stress granule protein TIA1 was examined. We chose TIA1 because it is found primarily in the nucleus prior to treatment with H2O2 and was identified as a G4DNA interacting protein (Fig. 1). Untreated cells exhibit no stress granule formation (Fig. 9A). Increasing amounts of G4DNA are observed in the cytoplasm after treatment with H2O2, and cytoplasmic stress granules, indicated by punctate staining with TIA1 antibodies, clearly co-localize with a subpopulation of the BG4 antibody after treatment (Fig. 9A, merged panels). The co-localization of a subset of the endogenous G4DNA and stress granule proteins was confirmed with G3BP, a stress granule marker which was not identified as a G4DNA binding protein in the proteomics experiment (Fig. 9B). Since stress granules contain both proteins and translationally stalled mRNAs, the presence of RNA in the granules was confirmed (Fig. 9C). Co-localization of BG4 with TIA1 was also observed in HeLa cells (Fig 10A) and primary melanocytes (Fig. 10B) treated with H2O2. In addition, melanoma cells that were treated with menadione also exhibited foci containing BG4 and TIA1 antibodies (Fig. 10C).

**DISCUSSION**

The data presented here support the conclusion that DNA sequences that can fold into
G4DNA structures accumulate in the cytoplasm as a result of oxidative stress. The G4DNA binds to proteins such as DHX36 and YB-1 and participates in formation of stress granules (Fig. 11). The source of the DNA is not known, but DNA sequences from nuclear or mitochondrial genomes are each implicated. Multiple results support the conclusion that the observed molecule which co-localizes with stress granule proteins after oxidative stress is indeed G4DNA; 1) the quadruplex specific antibodies BG4 and 1H6 bind to the molecule (Fig. 4 and 6), 2) The molecule is not sensitive to RNase (Figs. 4-10), 3) the molecule is sensitive to excess DNase (Fig. 5), 4) the reduction in nuclear foci with H$_2$O$_2$ treatment (Fig. 4C-4D) followed by re-emergence in the nucleus after removal of H$_2$O$_2$ (Fig 7) is consistent with DNA excision followed by DNA synthesis and chromatin reorganization, and 5) qPCR indicates an increase in specific G4DNA sequences in the cytoplasm (Fig 4B).

G4DNA structures from damaged G-rich sequences can provide a direct mechanism for the cell to sense oxidative stress-induced DNA damage (56). Formation of stress granules is known to modulate RNA translation during oxidative stress (24,57). Although the translation of some mRNAs is stalled by stress granule formation, other transcripts are actively excluded from stress granules (23), making effects of stress granule formation on individual protein levels difficult to predict.

It is not clear from the data whether oxidation of G4DNA sequences themselves results in the appearance of G4DNA in the cytoplasm or whether DNA damage in general up-regulates DNA repair, resulting in increased excision. It is known that DNA sequences containing runs of guanine residues are more reactive with hydroxyl radicals than guanine residues scattered throughout the genome (45,46,58). Guanine oxidation does reduce the thermal stability of G-quadruplexes; however, G4DNA structures can still form in the presence of 8-oxo-dG (59,60). Naturally occurring G4DNA forming sequences can have additional guanines that are not involved in quadruplex formation. In telomeres, formation of more stable G4DNA structures may occur by expelling of the damage-containing telomeric repeat from the quadruplex (60,61). Promoter G4 sequences often contain 5 tracks of guanines, leading to swapping of the spare guanine track for the track containing the damaged guanine (47). Additionally, it is known that DNA double-strand breaks occur more frequently at G4 sequences (62). During repair of dsDNA breaks, resection generates ssDNA regions in which quadruplexes could form when the appropriate sequences are present. Thus, dsDNA breaks could also lead to formation of G4DNA, and DNA damage in general may lead to an increase in G4DNA in the cytoplasm.

Sequences that can form G4DNA are found predominantly in mitochondrial DNA, telomeres, and promoters of proto-oncogenes. Hence, the appearance of G4DNA sequences in these regions provides an opportunity for an early alarm system for the DNA repair machinery to focus on these damaged regions. As shown in Fig. 4, the G4DNA that is observed in the nucleus is reduced within four hours of treatment with H$_2$O$_2$. A concomitant increase in G4DNA signal appears in the cytoplasm. We suggest that DNA excision repair of G-rich genomic DNA produces short segments of DNA that fold into stable quadruplex structures in response to episodes of oxidative stress and possibly other forms of DNA damage (Fig. 11). Although a clear reduction in nuclear G4DNA after H$_2$O$_2$ treatment is observed (Fig. 4C-D), the origin of the G4DNA in the cytoplasm is unknown. It is possible that these G4DNA sequences are excised from nuclear or mitochondrial genomes or that they are synthesized by telomerase in the cytoplasm. Although the source of the G4DNA (nuclear genome, mitochondrial genome, or synthesized in the cytoplasm) is not known at this time, it appears that these short strands of G4DNA serve as a signaling agent to inform the cellular machinery of DNA damage.

Numerous lines of evidence indicate that cells have evolved to respond to G-quadruplex structures. Several labs have reported that many cell types, usually cancer cells, spontaneously take up exogenous G4DNA (67-70). G4DNA taken up by cells causes various biological responses including apoptosis in tumor cells (69,71). The c-MYC quadruplex was recently shown to be readily
taken up by cells where it destabilized proteins that normally bind G4DNA in the cell (72). Indeed, the G4DNA oligonucleotide AS1411 has been developed as a possible treatment for cancer (67,73). G4 structures made up of DNA and RNA have also been proposed as important regulators of transcription (74-76). These facts suggest a fundamental biological response to G4DNA structures, even when excised from genomes. This work is the first to show G4DNA from an endogenous source can be observed within stress granules, thereby participating in a known response to cellular stress.

Deletions of mtDNA and the resulting loss of mitochondrial function are thought to be a major cause of cancer, aging and genetic disorders (77). Human mtDNA contains 270 potential G4 forming sequences (52), many of which correlate with DNA breakpoints associated with diseases (52,78,79). It is of note that hypersensitive DNA cleavage sites have been described adjacent to G4DNA sequences in mitochondria (52,78,79) and proto-oncogenes (80,81). Recent results from the Balasubramanian lab support the conclusion that a very large number of DNA sequences throughout the genome can fold into quadruplex structures in addition to telomeric sequences (82). Hence, nuclear and mitochondrial human DNA has great potential for producing G4DNA during repair or excision of damaged DNA.

The signaling role proposed here for G4DNA, may partly explain why nature has preserved these sequences in such high propensity in the mitochondrial and nuclear genomes. The selection of G4DNA sequences for mitochondria, telomeres, and promoters of proto-oncogenes provides an additional survival advantage if these sequences and the resulting quadruplex structures play multiple roles, first in regulating DNA metabolism and then in alerting the cell to DNA damage of critical regions of the genome. Telomeres are reportedly favored targets of persistent DNA damage (83,84), which may be related in part to the high reactivity and chemical breakdown products observed for specific guanine residues in telomeric sequences (45). Oxidative stress can cause telomere shortening (53,85). Under conditions of mild oxidative stress, single strand breaks in telomeres accumulate (86) and the rate of telomere shortening can be altered by changes in the amount of oxidative stress (87).

As the name implies, stress granules are known to form in response to many different cellular stress events (88) through more than one mechanism. One route involves phosphorylation of eIF2α (reviewed in (21)). Assembly occurs through aggregation of proteins such as TIA1 or G3BP which can interact with mRNA leading to translationally stalled aggregates. The DDX3 RNA helicase can associate with stress granules and suppress translation through inhibition of eIF4E (89). Additionally, molecules that inhibit eIF4A can promote stress granule formation independently of eIF2α (reviewed in (90)). The observation that G4DNA can participate in assembly of stress granules suggests another mechanism for their formation. The fact that TIA1 and G3BP are found co-localized with cytoplasmic G4DNA supports this conclusion. We anticipate G4DNA acting through RNA binding proteins to promote stress granule formation.

A recent report found that oxidative stress due to H₂O₂ reduces stress granule formation due to TIA1 oxidation (91). However, others have reported that stress granules can form in the presence of H₂O₂ in an eIF2α independent manner (44,92). It is possible that the concentration and exposure time for H₂O₂ can modulate the appearance of stress granules. Work here used similar H₂O₂ concentrations and exposure times to (44), lower H₂O₂ concentrations than (92), and longer exposure times and higher concentrations than (91).

Stress granule formation reduces development of reactive oxygen species, thereby inhibiting apoptosis and favoring repair (93). The YB-1 protein has been proposed to play a key role in translation regulation by interacting with quadruplexes. tRNA-derived, stress-induced RNA (tRNA)-derived quadruplexes bind to YB-1, leading to YB-1 inhibition of cap-dependent translation initiation, thereby altering gene expression in response to cellular stress (34). Based on data here, it appears that endogenous G4DNA sequences (Fig. 11) can link oxidative stress and DNA damage directly to translation regulation through stress granule formation perhaps in part through YB-1 binding.

The DExD/H RNA helicase family has many members that play roles in cellular stress response (90). DHX36 is a member of this family
capable of binding and unwinding RNA which combined with its ability to be recruited to stress granules (33) likely plays a role in regulation of translation. It is an essential gene (94) and is the primary enzyme responsible for unwinding G4DNA in cells (27). Another cytosolic RNA helicase, DDX41 (95) can bind to DNA and activate STING, which ultimately leads to activation of NF-kB and interferon regulatory factor 3 (IRF3). G4DNA may serve as a signal through binding to multiple proteins that process RNA.

The proposed G4DNA signaling response to DNA oxidation reported here could be especially valuable to the cell by directly reporting the level of DNA damage to proteins that regulate translation (21,22,24). The fact that many cells can readily take up G4DNA indicates that this mechanism may also allow the cell to respond to its external environment as well as internal stress (67,72). While our results suggest important roles for G4DNA in regulation of cytoplasmic cellular processes, other studies have suggested roles for G4DNA in modulation of transcription through binding of helicases such as DHX36 (30,96,97), the RecQ family helicases (98,99), and XPB and XPD helicases (75). It is possible that any G4DNA binding protein may be regulated by freely diffusing G4DNA, leading to various cellular responses based on the large number of proteins that are reported to have G4DNA binding capacity (4,100-102). G4DNA has been proposed as a drug target for treatment of cancer and other diseases (74). This work will expand the rationale for targeting G4DNA and provides further support for G4DNA mimics as potential drugs (67,68).

**EXPERIMENTAL PROCEDURES**

**Oligonucleotides and Antibodies—**All oligonucleotides were purchased from Integrated DNA technologies and purified (103). Sequences were: Cy3-G4DNA (5'-T15 GAG GTT GGG TAG GGT TGT AAA-BioTEG-3'), 3'-Bio-ssDNA (5'-T15-BioTEG-3'), G4DNA (5'-T15 GAG GTT GGG TAG GGT TGT GGG TAA-3'), ssDNA (5'-T20-3'), Cy3-Cy5-G4DNA (5'-Cy5-T15 GAG GTT GGG TAG GGT TGT GGG TAA-3'), and Cy3-Cy5-Scr (5'-Cy5-TGT TGT TGT TGT TGT TGT TGT TGT TGT TAG TAG TAG TTG AAG-Cy3-3'). Intramolecular quadruplexes were formed as described (105) and structures were confirmed by circular dichroism of 10 µM DNA in 10 mM Hepes-Li, pH 7.5, 1 mM EDTA-Li, 100 mM KCl in a Jasco 715 spectropolarimeter (Fig. 1F-G). Sequences of PCR primers were: hTEL forward: 5'- GGT GGG TAA-BioTEG-3', reverse: 5' -TCC CCA TAT CCC TAT CCC TA-3' (modified from (106)), KSS forward: 5'- GAC AAT TAT ACC CTA GCC AAC C-3', KSS reverse: 5'- CTT GAT GTG AGG GGT GGG G-3, HRCC forward: 5'- CTA TGA ACC CCC CTC CCC-3', HRCC reverse: 5'- GTT GAC CAG GG TGG GTG GGG GA TA-3', PMP forward: 5'- CTA TGC ACC CTA TCC CCC CC-3', and PMP reverse: 5'-GGA AAA GAG GAC GCG GGG G-3'. pSANG10-3F-BG4 plasmid encoding the BG4 single chain antibody (7,10,51) was a gift from Shankar Balasubramanian (Addgene plasmid # 55756). Monoclonal antibody 1H6 was kindly provided by Peter Lansdorp (9). BG4 was used at 16 nM and was detected using rabbit anti-FLAG (1:800 dilution, 2368P, Cell Signaling) and DyLight 550 conjugated goat anti-rabbit (1:100 dilution, 84541, Thermo) antibodies or HRP conjugated goat anti-rabbit antibody (1:3000 dilution, NEF812, PerkinElmer). 1H6 was used at 1 µg/ml and was detected using Cy3 conjugated donkey anti-mouse antibodies (1:50 dilution, 715-165-151, Jackson ImmunoResearch). DHX36 was detected using rabbit anti-DHX36 (1:100 dilution, ab70269, Abcam) and Alexa Fluor 647 conjugated donkey anti-rabbit antibodies (1:100 dilution, 711-605-152, Jackson ImmunoResearch) or HRP conjugated goat anti-rabbit antibody (1:3000 dilution, NEF812, PerkinElmer). TIA1 was detected using goat anti-TIA1 (1:100 dilution for immunofluorescence, 1:1000 dilution for western blot, sc-1751, Santa Cruz) and Alexa Fluor 647 conjugated donkey anti-goat antibodies (1:100 dilution, 705-605-147, Jackson ImmunoResearch) or HRP conjugated...
donkey anti-goat antibody (1:5000 dilution, sc-2020, Santa Cruz). YB-1 was detected using mouse anti-YB-1 (1:50 dilution, sc-101198, Santa Cruz) and Alexa Fluor 647 conjugated donkey anti-mouse antibodies (1:50 dilution, 715-605-150, Jackson ImmunoResearch). ELAV1 was detected using mouse anti-HuR (1:50 dilution, sc-5261, Santa Cruz) and Alexa Fluor 647 conjugated donkey anti-mouse antibodies (1:50 dilution, 715-605-150, Jackson ImmunoResearch). G3BP was detected using mouse anti-G3BP (1:200 dilution, 611126, BD Transduction Laboratories) and Alexa Fluor 647 conjugated donkey anti-mouse antibodies (1:50 dilution, 715-605-150, Jackson ImmunoResearch).

**BG4 purification**—BL21(DE3) E. coli containing the pSANG10-3F-BG4 plasmid were grown and BG4 expression was induced at 25 °C overnight with 1 mM IPTG. Cells were pelleted at 4500×g for 1 hour at 4 °C. The cell pellet was suspended in TS buffer (50 mM Tris-Cl, pH 8.0, 20% sucrose) then placed on ice for 15 min followed by microfluidization. Lysate was centrifuged at 8000×g for 30 minutes at 4 °C followed by centrifugation at 110000xg for 1.5 hours at 4 °C. Imidazole was added to the supernatant to 25 mM, followed by incubation with Ni²⁺ Sepharose resin overnight at 4 °C. The slurry was loaded into a column, washed with TS buffer containing 25 mM imidazole and bound proteins were eluted with elution buffer (PBS, pH 8.0 with 250 mM imidazole). Purified BG4 was dialyzed to PBS, pH 8.0, 20% glycerol and stored in aliquots at -80 °C.

**Cell culture**—WM-266-4 (melanoma; ATCC CRL-1676) cells or HeLa (cervical adenocarcinoma; ATCC CCL2) were cultured in Dulbecco’s Modified Eagle Medium (DMEM), 10% fetal bovine serum, 1X MEM non-essential amino acids (Life Technologies) and 100 U/ml penicillin/streptomycin at 37 °C with 5% CO₂. Adult primary melanocytes (ATCC PCS-200-013) were cultured in Dermal Cell Basal Medium (ATCC) supplemented with an Adult Melanocyte Growth Kit (ATCC) and 100 U/ml penicillin/streptomycin at 37 °C with 5% CO₂. Cells treated with H₂O₂ were cultured in serum free media for 30 min before addition of 0.5 mM H₂O₂. Cells treated with menadione were treated with 30 µM menadione in serum free DMEM for 2 hours. For comparison, experiments with 30 µM menadione in DMEM containing 10% serum produced similar results. Some cells were transfected for 6 hrs with Cy3-G4DNA or Cy3-ssDNA using 0.8 µl Lipofectamine 2000 in 200 µl serum free media.

**Conjugation of DNA to Dynabeads**—M-280 Streptavidin Dynabeads (Life Technologies) were conjugated with 3'-biotinylated oligonucleotides (3'-Bio-G4DNA or 3'-Bio ssDNA). Beads were washed and resuspended in 5 mM Tris-Cl pH 7.5, 0.5 mM EDTA, 150 mM KCl, followed by addition of DNA (at the ratio of 1 mg of beads per 200 pmol of DNA) and incubated at 25 °C for 20 min with gentle rotation. The beads were washed 3 times with the binding and washing buffer followed by a final wash in protein binding buffer (300 mM KCl, 20 mM Tris, 0.1% Tween, pH 7.5).

**Affinity purification of proteins for mass spectrometry**—WM266-4 were grown to approximately 80% confluence in 75 cm² flasks and harvested by centrifugation at 225×g for 5 min. Cell pellets were resuspended in lysis buffer (300 mM KCl, 20 mM Tris, 0.1% Tween, pH 7.5; in the proportion of 1 ml per 200 mg of cells) and sonicated at 4 °C using Bioraptor UCD-200 sonicator at the power of 200 W for 15 minutes. Mammalian protease inhibitor cocktail was added to the cell lysates at 1:166 dilution (Sigma-Aldrich). Cell lysates were incubated with DNA-conjugated Dynabeads (5 ml/mg) at 4 °C with gentle rotation for 4 hours. Beads were collected with magnets and washed four times with protein binding buffer. Proteins were eluted by boiling in Laemmli sample buffer for 5 minutes and resolved on 4-15% Mini-PROTEAN TGX precast gels (BioRad).

**LC-MS analysis**—Protein gel bands were excised and in-gel trypsin digested (107). Tryptic peptides were separated using a nanoAcquity UPLC system (Waters) coupled to a LTQ Orbitrap Velos mass spectrometer (Thermo) (107,108). Proteins were identified by database search using PEAKS Studio v7 (Bioinformatics Solutions). Data Refine routine was performed with the following parameters: no merge, corrected precursor, charge options — no correction, filter.
quality >0.65. Denovo and Peaks database searches were done with 10 ppm parent mass error tolerance and 0.85 Da fragment mass error tolerance. Methionine oxidation, asparagine deamidation, glutamine deamidation, and pyro-glutamine formation were set as variable modifications. Cysteine carbamidomethylation was set as fixed modification. Glutamine was set to equal to Lysine. Leucine was set to equal to Isoleucine. The searches were done against the reviewed Swiss-Prot human proteome database (20,187 total accessions), which was downloaded from the uniprot web-site at http://www.uniprot.org/. Peaks PTM and Spider searches were performed, with the maximum number of PTMs per peptide set to three. Semi-tryptic peptides were included. Peptide-to-spectrum match false identification rate was set at 1%, which corresponded approximately to -10LogP score of 15. Proteins identified by only one unique peptide were included in the identification results, but the protein hit -10LogP threshold was set to 30. Proteins that had no unique peptides were reported as a group. Proteins, which were subsets without unique peptides were not reported. G4DNA interacting proteins were determined using spectral counting (26,109,110) by comparing spectral counts (SPCs) in the G4DNA-bound sample, to the ssDNA-bound sample. For quantification by spectral counting, each accession was scored for total SPCs, unique SPC (uniquely matching to an accession), and adjusted SPC. The latter assigns shared peptides to accessions in proportion to their relative abundance using unique SPCs for each accession as a basis. Proteins with adjusted p-values less than 0.05 were called significant (p-values were derived from G-test of independence and adjusted for multiple hypotheses testing using Bonferroni correction). Zero spectral count values that indicated absence of a protein in a given condition were replaced with 0.1 SPCs to allow log operation to be performed and G-statistics to be derived. Significant proteins, which were enriched at least 4 times in the G4DNA-bound sample compared to ssDNA-bound and were represented by at least 14 adjusted SPCs, were considered to be G4DNA interacting proteins. The particular cutoff of 14 for adjusted SPCs was chosen based on the results of the confidence testing by G-test; namely we found that given the absence of protein in one condition 14 or more SPCs are required in the other condition to claim significant over accumulation with 95% confidence (after Bonferroni correction for multiple hypothesis testing). The Perl script for the calculations of adjusted SPCs as well as the Excel template for the calculation of G-statistics and P values are available from the authors by request.

**Western blot**—Proteins were separated by 10% SDS-PAGE and transferred to PVDF. Membranes were blocked with 5% nonfat dry milk (DHX36) or 5% chicken ovalbumin (TIA1) in TBS-T. Membranes were probed with antibody dilutions in 1% nonfat dry milk or ovalbumin in TBS-T. Proteins were detected using horseradish peroxidase conjugated secondary antibodies and Amersham ECL Plus (GE Healthcare). Blots were imaged using a LAS4000 (GE Healthcare).

**Immunofluorescence**—WM266-4 cells, unless otherwise stated, grown on chamber slides were fixed with 3.7% formaldehyde for 15 min at room temperature followed by 10 min with ice cold methanol at -20 °C. Slides were blocked with 2 mg/ml chicken ovalbumin, 0.02% sodium azide, in PBS overnight at 4 °C. Cells were further permeabilized with a buffer containing 0.2% Triton X-100, 2 mg/ml chicken ovalbumin, 0.02% sodium azide, in PBS. Before staining with BG4, cells were treated with 0.1 mg/ml RNase A for 30 min as described (7-10) so only DNA quadruplexes would be detected. After staining with antibodies, coverslips were mounted with 2.5 mg/ml DABCO (1,4-diazobicyclo[2.2.2]octane), 0.1 µg/ml DAPI (4′,6-diamidino-2-phenylindole), in 25% PBS, 75% glycerol.

**RNA Labeling**—RNA was fluorescently labeled with Alexa Fluor 488 using the Click-iT RNA Imaging kit (Invitrogen) according to the manufacturer’s instructions. Briefly, WM266-4 cells were grown on an 8 chamber slide and incubated for 24 hours in complete media containing 1 mM 5-ethynyl uridine (EU) to allow incorporation of the nucleoside analog into cellular RNA pools. The cells were pulsed for 10 minutes with serum-free media containing 2 mM EU, followed by the addition of an equal volume of serum free media with or without 1 mM H₂O₂ resulting in the final concentrations of 1 mM EU and 0.5 mM H₂O₂. Cultures were incubated at 37°C for an additional 2 hours, washed with PBS,
and fixed for 15 minutes in 3.7% formaldehyde. For the labeling of modified RNA, the cells were washed with PBS and incubated with 1× Click-iT reaction buffer containing 4 mM CuSO₄ and Alexa Fluor 488 azide for 30 minutes at room temperature. Cells were rinsed with Click-iT reaction rinse buffer and twice with PBS followed by staining for TIA-1 as described above. Nuclear DNA was stained with Hoechst 33342 for 15 minutes, and slides were mounted with a 75% glycerol/PBS solution containing 2.5 mg/ml DABCO.

**Fluorescence microscopy**—Images were captured using an Olympus IX81 confocal microscope at room temperature with a 60x PLAPON60XO objective (1.42 numerical aperture) using FV10-ASW 3.1a software. DAPI was excited at 405 nm and fluorescence was measured after a 430-470 nm bandpass filter. Alexa Fluor 488 was excited at 488 nm and emission was measured after a 505-605 nm bandpass filter. Alexa Fluor 647 was excited at 635 nm and emission was measured after a 560-605 nm bandpass filter. Alexa Fluor 488 was excited at 488 nm and emission was measured after a 430-470 nm bandpass filter. Images were acquired with an Olympus FV1000 camera. Fluorescence was quantitated using ImageQuant software.

**Quantitative PCR (qPCR)**—DNA was isolated from cytosolic fractions by phenol-chloroform extraction and ethanol precipitation. qPCR was performed using Sso Advanced Universal SYBR Green Supermix (Bio-Rad) with primers listed above. DNA was amplified with 40 cycles of 10 s at 90 °C and 30 s at 50 °C in a MiniOpticon (BioRad). P values were calculated using a two-tailed paired t test.

**DNA fragmentation**—Approximately 2×10⁶ cells were harvested after treatment with 0.5 mM H₂O₂ or 1 μM staurosporine. Both the media and the adherent cells were collected to ensure that both live and dead cells were retained. Cell pellets were resuspended in PBS. Proteinase K and RNase A were added to 6.7 mg/ml each and samples were incubated at room temperature for 2 minutes followed by addition of an equal volume of lysis buffer AL (Qiagen). Samples were incubated at 56 °C for 15 minutes and 1 μg of DNA was loaded in each lane of a 1.5% agarose gel containing 0.5 μg/mL ethidium bromide.

**G4DNA folding**—All concentrations listed are final, after mixing. Cy3-Cy5-G4DNA or Cy3-Cy5-Scr in Tris-OAc, pH 7.2 was mixed with 135 mM KOAc, 4 mM KCl, 12 mM NaHCO₃, and 0.8 mM Mg(OAc)₂ with or without BG4 in an SX.18MV stopped flow reaction analyzer (Applied Photophysics). The samples were excited...
at 550 nm and FRET was measured after a 665 nm cut-on filter (Newport Corporation, #51330). Data was fit to a sum of two exponentials to obtain rate constants for folding.

**Supplementary data:** Supplementary Data are available at JBC Online.

**Acknowledgements:** The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE (112) partner repository with the dataset identifier PXD003501. Reviewer login details (Username: reviewer68172@ebi.ac.uk; Password: hhXEbWFK). We thank Shankar Balasubramanian, University of Cambridge, for the gift of the pSANG10-3F-BG4 plasmid encoding the BG4 single chain antibody. Monoclonal antibody 1H6 was kindly provided by Peter Lansdorp, European Research Institute for the Biology of Ageing, University of Groningen. We thank Timothy Chambers for helpful discussions and Andrea Edwards for technical assistance.

**Conflict of interest:** The authors declare that they have no conflicts of interest with the contents of this article.

**Author contributions:** K.D.R., R.L.E, G.B., and A.K.B. designed and supervised research. B.L.Z. and J.G. performed and B.L.Z, M.J, and S.G.M. analyzed data for proteomics experiments. A.K.B., L.M., J.C.M., M.R.B, M.R.R, and S.C. conducted immunofluorescence experiments. A.K.B. performed circular dichroism, DNA fragmentation, G4DNA folding, dot-blot and qPCR experiments. W.C.G. purified BG4. A.K.B., B.L.Z., L.M., J.G., J.C.M., A.M.M., G.B., R.L.E., and K.D.R. interpreted data. K.D.R., A.K.B., B.L.Z., and J.C.M prepared the manuscript. All authors edited the manuscript.

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FOOTNOTES

*This work was supported by National Institutes of Health Grants R01 GM098922 and R01 GM117439 to K.D.R.; and the Arkansas Biosciences Institute, the major research component of the Arkansas Tobacco Settlement Proceeds Act of 2000. The UAMS Proteomics Core is supported by the Arkansas IDeA Network for Biomedical Research Excellence (National Institutes of Health P20GM103429); the UA Center for Protein Structure and Function (National Institutes of Health P30GM103450); the UAMS Center for Microbial Pathogenesis and Host Inflammatory Responses (National Institutes of Health P20GM103625); and the UAMS Translational Research Institute (National Institutes of Health UL1TR000039). The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

2The abbreviations used are: G4DNA, G-quadruplex DNA; FMRP, Fragile X Mental Retardation Protein; SPCs, spectral counts; ssDNA, single-stranded DNA; PS, phosphorothioate; mtDNA, mitochondrial DNA.
FIGURE LEGENDS

FIGURE 1. Proteomics screen for G4DNA binding proteins. (A) Structure of a single G-quadruplex tetrad. (B) Stacks of tetrads, usually three, form a G-quadruplex which is stabilized by monovalent cations (blue) which bind in the central channel. (C) Strategy for proteomics screen for binders to G4DNA. To determine enrichment of proteins with the G4DNA, a separate experiment was conducted with single-stranded DNA as the bait. Cell lysates were incubated with G4DNA or ssDNA followed by SDS-PAGE and then LC-MS. Relative quantities of proteins were determined by spectral counting. (D) Proteins previously found in stress granules that were identified in the G4DNA proteomics screen (24) (full list in Supplementary Table 1). Data are the result of a single experiment in WM266-4 cells. (E) Western blots of cell lysates incubated with G4DNA or ssDNA. The G4DNA and ssDNA affinity purifications contained identical quantities of the cell lysate. 30% of the cell lysate volume was affinity purified on ssDNA or an identical concentration of G4DNA coated beads as described in experimental procedures. 20% of the elution volume was loaded in each lane for SDS-PAGE. Circular dichroism indicates that the G4DNA oligonucleotides used in the proteomics screen and western blot (F) and for immunofluorescence (G) form parallel quadruplexes while the ssDNAs do not form quadruplexes.

FIGURE 2. G4DNA introduced into cells co-localizes with stress granule markers in WM266-4 cells. (A) Transfected Cy3-G4DNA induces stress granule formation and co-localizes with TIA1 (90% of TIA1 foci contain G4DNA, indicated by arrows). (A) Three different Cy3-ssDNAs were used for comparison: Cy3-T<sub>20</sub> (B) since the G4DNA bait in the pulldown contained a single-stranded thymidine region; Cy3-Scr (C), a scrambled version of the G4DNA sequence, to account for any proteins that bind preferentially to G-rich DNA; and Cy3-PS-Scr (D), which contains phosphorothioate linkages at each end of the oligonucleotide to prevent degradation by cellular nucleases. Transfected Cy3-ssDNA does not cause stress granule formation. Transfected G4DNA co-localizes with additional stress granule proteins. DXH36 (E), YB-1 (F), ELAV1 (G), G3BP (H), and eIF3 (I) co-localize with transfected Cy3-G4DNA in the cytosol (indicated by arrows) but not Cy3-Scr DNA. Quantitation of foci that contain G4DNA resulted in 7% of DHX36 foci, 75% of YB-1 foci, 96% of ELAV1 foci, 17% of G3BP foci, and 83% of eIF3 foci contain G4DNA. Bar equals 10 µm.

FIGURE 3. Increasing quantities of G4DNA introduced into cells increase the quantity of stress granules formed. (A) G4DNA (125 nM, 250 nM, 500 nM, or 750 nM) was transfected into WM266-4 cells. Increasing quantities of transfected Cy3-G4DNA (red) induce more stress granule formation, determined by TIA1 foci (green). Merged images indicate co-localization as marked by arrows. The fraction of TIA1 foci that contain G4DNA was 81%, 80%, 81%, and 84% for cells transfected with 125 nM, 250 nM, 500 nM, and 750 nM G4DNA, respectively. (B) The plot indicates that the fraction of cells which contain cytoplasmic TIA1 foci co-localized with DNA increases as the concentration of transfected G4DNA increases (red bars). For comparison, the fraction of cells containing TIA1 foci co-localized with transfected ssDNA is shown (blue bars). Experiments were performed three separate times in WM266-4 cells. Bar equals 10 µm.

FIGURE 4. Treatment of cells with hydrogen peroxide leads to accumulation of G4DNA in the cytosol. (A) Dot blot of cytosolic fractions with BG4 indicates G4DNA in the cytosol increases 8.7 ± 1.8 fold after 2 hrs H<sub>2</sub>O<sub>2</sub> and 16.9 ± 3.5 fold after 4 hrs H<sub>2</sub>O<sub>2</sub> (mean ± s.d. of biological triplicates) in WM266-4 cells. (B) The relative copy numbers of four quadruplex forming sequences in the cytosol were determined by qPCR. Results shown are the relative to untreated cells for each template. qPCR indicates a dramatic increase of telomeric DNA in the cytoplasm upon H<sub>2</sub>O<sub>2</sub> treatment (mean ± s.d. of biological triplicates). (C) BG4 indicates that G4DNA is present in the nucleus in untreated cells, but appears in the cytoplasm in the presence of oxidative stress. Cell morphology changes upon treatment with 0.5 mM H<sub>2</sub>O<sub>2</sub>, consistent with previous reports (113). (D) Quantitation of BG4 fluorescence localization in panel (C).
values were calculated using a two-tailed paired t test. Plot shows the mean of 60 cells per condition from three or more independent biological experiments per condition in WM266-4 cells. Bar equals 10 µm.

FIGURE 5. Control experiments to confirm the quadruplex is DNA. In WM266-4 cells treated with RNase A, only G4DNA in the nucleus is detected by BG4 in cells without H$_2$O$_2$ treatment (A). In WM266-4 cells treated with H$_2$O$_2$ for 2 hrs, G4DNA is visible throughout the cell in RNase A treated cells (B). When cells are treated with both RNase A and DNase I, little G4DNA is detected in either untreated cells (C) or cells treated with H$_2$O$_2$ for 2 hrs (D). (E) Control experiments lacking primary antibodies. WM266-4 cells were treated with 0.5 mM H$_2$O$_2$ for 2 hrs then fixed and stained with secondary antibodies without primary antibodies. Bar equals 10 µm.

FIGURE 6. G4DNA folding. (A) Folding of Cy3-Cy5-G4DNA can be monitored by the increase in FRET upon addition of salt. (B) Folding of 50 nM Cy3-Cy5-G4DNA upon addition of salt was measured in the absence of BG4 (orange), in the presence of 50 nM BG4 (green), and in the presence of 160 nM BG4. Data were fit to a sum of two exponentials and the rate constants were 0.083 s$^{-1}$ and 0.032 s$^{-1}$, 0.067 s$^{-1}$ and 0.025 s$^{-1}$, and 0.084 s$^{-1}$ and 0.029 s$^{-1}$ for 0 nM, 50 nM, and 160 nM BG4, respectively. Folding of a Cy3-Cy5-ScrDNA was not observed in the absence of BG4 (red) or in the presence of 50 nM (blue) or 160 nM BG4 (black). (C) Staining with another quadruplex specific antibody, 1H6, indicates that G4DNA is present in the nucleus in untreated cells, but appears in the cytoplasm in the presence of oxidative stress.

FIGURE 7. Removal of hydrogen peroxide from media leads to reappearance of G4DNA in the nucleus. (A) After 2 hrs of treatment with 0.5 mM H$_2$O$_2$, the media was removed and replaced with fresh media for 6 to 12 hrs before fixing cells. (B) Quantification of BG4 fluorescence localization in (A). P values were calculated using a two-tailed paired t test. Plot shows the mean of 30 cells per condition from three independent biological experiments per condition in WM266-4 cells. Bar equals 10 µm. (C) DNA from untreated and 0.5 mM H$_2$O$_2$ treated cells runs as a large species on an agarose gel whereas DNA from cells treated with 1 µM staurosporine for 24 hrs shows some fragmentation, indicative of apoptosis.

FIGURE 8. G4DNA appears in the cytosol of cells during oxidative stress. (A) Treatment of HeLa cells with 0.5 mM hydrogen peroxide leads to accumulation of G4DNA in the cytosol, similar to WM266-4 cells. Experiments were performed two times. Bar equals 2 µm. (B) Treatment of primary melanocytes with hydrogen peroxide leads to G4DNA accumulation in the cytosol. Bar equals 10 µm. (C) Treatment of WM266-4 cells with 30 µM menadione for 2 hours results in the appearance of G4DNA in the cytosol. Bar equals 10 µm.

FIGURE 9. Endogenous G4DNA co-localizes with stress granule markers with H$_2$O$_2$ treatment. The G4DNA binding antibody, BG4, co-localizes with TIA1 (A) and G3BP (B) in stress granules after treatment with H$_2$O$_2$ (indicated by arrows). For TIA1, 79% of TIA1 foci co-localize with BG4 after 2 hrs of H$_2$O$_2$ treatment and 81% co-localize after 4 hrs of H$_2$O$_2$ treatment. For G3BP, 76% of G3BP foci co-localize with BG4 after 2 or 4 hrs of H$_2$O$_2$ treatment. (C) Cytosolic TIA1 positive foci that form in response to H$_2$O$_2$ treatment also contain RNA (96% of TIA1 foci contain RNA, indicated by arrows). Images are shown for treatment with 0.5 mM H$_2$O$_2$ in WM266-4 cells. Bar equals 10 µm.

FIGURE 10. Endogenous G4DNA co-localizes with TIA1 in different cell types with oxidative stress. The G4DNA binding antibody, BG4, co-localizes with TIA1 (indicated by arrows) in HeLa cells (A) and primary melanocytes (B) treated with 0.5 mM H$_2$O$_2$. In HeLa cells, 68% of TIA1 foci co-localize with BG4 after 2 hrs of H$_2$O$_2$ treatment and 71% co-localize after 4 hrs of H$_2$O$_2$ treatment. In primary melanocytes, 68% of TIA1 foci co-localize with BG4 after 2 hrs of H$_2$O$_2$ treatment and 72% co-localize after 4 hrs of H$_2$O$_2$ treatment. (C) Treatment of WM266-4 cells with 30 µM menadione for 2 hours
results in co-localization of BG4 with TIA1 in the cytosol. Treatment with menadione for 2 hrs results in 77% of TIA1 foci co-localized with BG4. Bar equals 10 µm.

**FIGURE 11.** Model for G4DNA signaling. DNA repair and excision of oxidatively damaged DNA can release short segments of DNA, some of which can fold into quadruplex structures which are resistant to nuclease activity and can diffuse or be transported out of the nucleus. Quadruplex folding may occur before excision, after excision in the nucleus, or in the cytosol after transport out of the nucleus. Cytoplasmic G4DNA binds to proteins that assemble into stress granules such as DHX36 and YB-1 (33,34), thereby regulating translation. Excised G4DNA in the nucleus is available to bind to and potentially regulate any other G4DNA binding protein, thereby impacting additional biological pathways.
Figure 1

**D**

**Stress granule proteins identified from the G4DNA proteomics screen**

| Name      | Description                           | ss | G4 | \(P\) value |
|-----------|---------------------------------------|----|----|------------|
| DHX36     | ATP-dependent RNA helicase DHX36      | 0.1| 528| 8.7E-168   |
| ELAV1     | ELAV-like protein 1                   | 26 | 471| 3.2E-113   |
| HNRPM     | Heterogeneous nuclear ribonucleoprotein M | 94 | 531| 5.6E-81    |
| TIAR      | Nucleolysin TIAR                      | 0.1| 221| 5.6E-71    |
| TIA1      | Nucleolysin TIA-1 isoform p40         | 0.1| 159| 2.6E-51    |
| HNRPD     | Heterogeneous nuclear ribonucleoprotein D0 | 27 | 138| 9.5E-21    |
| YBOX1     | Nuclease-sensitive element-binding protein 1 | 26 | 111| 5.3E-15    |

**E**

[Image of gel electrophoresis with bands for Marker, G4DNA, and ssDNA, with DHX36 and TIA1 highlighted]

**F**

**G**

[Graphs showing ellipticity vs. wavelength for different samples, including 3'-Bio-G4DNA, 3'-Bio-T_{15}, 3'-Cy3-G4DNA, 3'-Cy3-T, and 3'-Cy3-PS-Sr]
Figure 2
Figure 3

A

125 nM G4DNA  TIA1  Merge

250 nM G4DNA  TIA1  Merge

500 nM G4DNA  TIA1  Merge

750 nM G4DNA  TIA1  Merge

B

Cells with Cy3 positive SG, %

G4DNA  ssDNA

125  250  500  750

Cy3 DNA, nM
Figure 4

A

RNase A

| Time (hr) | 0 hr | 2 hr | 4 hr |
|-----------|------|------|------|
| BG4       | 0    | 0    | 0    |

RNase A + DNase 1

| Time (hr) | 0 hr | 2 hr | 4 hr |
|-----------|------|------|------|
| BG4       | 0    | 0    | 0    |

B

RNase A treated cells

| Relative enrichment | hTEL | KSS | HRCC | PMPS |
|--------------------|------|-----|------|------|
| 0 hrs H₂O₂         | 0    | 0   | 0    | 0    |
| 2 hrs H₂O₂         | 0    | 0   | 0    | 0    |
| 4 hrs H₂O₂         | 0    | 0   | 0    | 0    |

C

RNase A treated cells

DAPI | BG4 | Merge

| Time (hrs) | DAPI | BG4 | Merge |
|------------|------|-----|-------|
| 0 hrs      | Blue | Red |       |
| 2 hrs H₂O₂ | Blue | Red |       |
| 4 hrs H₂O₂ | Blue | Red |       |

D

Fraction nuclear BG4

| H₂O₂ treatment, hrs | 0   | 2   | 4   |
|---------------------|-----|-----|-----|
| BG4                 | 0.1 | 0.2 | 0.4 |

* * * *
Figure 5

**Untreated cells**

|     | DAPI | BG4 | DAPI/BG4 |
|-----|------|-----|----------|
| A   | RNase A |     |          |

|     | DAPI | BG4 | DAPI/BG4 |
|-----|------|-----|----------|
| B   | RNase A |     |          |

|     | DAPI | BG4 | DAPI/BG4 |
|-----|------|-----|----------|
| C   | RNase A + DNase I |     |          |

|     | DAPI | BG4 | DAPI/BG4 |
|-----|------|-----|----------|
| D   | RNase A + DNase I |     |          |

|     | DAPI | BG4 | DAPI/BG4 |
|-----|------|-----|----------|
| E   | DAPI | AF647 anti-goat (TIA1 and eIF3 secondary) | AF647 anti-mouse (YB1, ELAV1, and G3BP secondary) |

DyLight550 anti-rabbit (BG4 secondary)
Figure 6

A. Low FRET and High FRET

B. Fluorescence vs. Time

C. DAPI, 1H6, Merge

- Untreated
- 2 hrs H₂O₂
Figure 7

A. DAPI/BG4

B. Fraction nuclear BG4

C. Untreated, 2 hrs H₂O₂, 4 hrs H₂O₂, 12 hrs staurosporine, 24 hrs staurosporine
Figure 8

A 0.5 mM H₂O₂ in HeLa cells

0 hrs

2 hrs

4 hrs

B 0.5 mM H₂O₂ in primary melanocytes

0 hrs

2 hrs

4 hrs

C 30 μM Menadione in WM266-4 cells

0 hrs

2 hrs
G-quadruplex DNA in the cytoplasm

Figure 9

A

B

C

0 hrs

Molecst

RNA

TIA1

RNA/TIA1

0 hrs

BG4

TIA1

BG4/TIA1

0 hrs

Background

BG4

G3BP

BG4/G3BP

0 hrs

2 hrs

4 hrs

2 hrs

4 hrs

2 hrs
Figure 10

A 0.5 mM H$_2$O$_2$ in HeLa cells

B 0.5 mM H$_2$O$_2$ in primary melanocytes

C 30 µM Menadione in WM266-4 cells
G-quadruplex DNA in the cytoplasm

Figure 11

G4DNA binds to DHX36 and YB-1 leading to assembly of stress granules.

Damage to DNA leads to production of strands of ssDNA, some of which can fold into G4 structures.
Evidence that G-quadruplex DNA Accumulates in the Cytoplasm and Participates in Stress Granule Assembly in Response to Oxidative Stress

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J. Biol. Chem. published online July 1, 2016

Access the most updated version of this article at doi: 10.1074/jbc.M116.718478

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