A ROLE FOR POLYNUCLEOTIDE PHOSPHORYLASE IN PROTECTING CELLS AND CONTROLLING RNA QUALITY UNDER OXIDATIVE STRESS

By

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This dissertation was prepared under the direction of the candidate's dissertation advisor, Dr. Zhongwei Li, Department of Biomedical Sciences, and has been approved by the members of his supervisory committee. It was submitted to the faculty of The Charles E. Schmidt College of Science and was accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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ABSTRACT

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RNA damage occurring under oxidative stress has been shown to cause RNA dysfunction and must be detrimental to cells and organisms. We propose that damaged RNA can be removed by specific RNA surveillance activities. In this work, we investigated the role of polynucleotide phosphorylase (PNPase), a 3'->5' exoribonuclease, in protecting the cells against oxidative stress and eliminating oxidatively-damaged RNA. Previously, it was reported that *E. coli* PNPase has a higher affinity to poly(8-oxoG:A). We further confirmed that *E. coli* PNPase can specifically bind to an oxidized RNA with a high affinity. An *E. coli* strain deficient in PNPase (*pnp*) is hypersensitive to hydrogen peroxide (H_2O_2). Importantly, the level of H_2O_2-induced RNA damage, measured by the content of 8-hydroxyguanosine, increases significantly in the *pnp* mutant cells. Consistent

with the notion that PNPase plays a direct role in these processes, introduction of the *pnp* gene encoding *E. coli* PNPase can restore the viability and RNA oxidation level of the *pnp* mutant cells in response to H_2O_2 treatment. Interestingly, degradosome-association is not required for PNPase to protect cell against oxidative stress.

PNPase is evolutionary conserved in most of organisms of all domains of life. The human polynucleotide phosphorylase (hPNPase) localizes mainly in mitochondria and plays pleiotropic roles in cell differentiation and has been previously shown to bind 8-oxoG-RNA with a high affinity. Here we show that similar to *E. coli* PNPase, hPNPase plays an indispensable role in protecting HeLa cells against oxidative stress. The viability in HeLa cell and 8-oxoG levels in RNA are inversely correlated in response to H_2O_2-treatment. After removal of oxidative challenge, the elevated level of 8-oxoG in RNA decreases, suggesting the existence of surveillance mechanism(s) for cleaning up oxidized RNA. We have shown that hPNPase may be responsible for the surveillance of oxidized RNA in mammalian cells. Overexpression of hPNPase reduces RNA oxidation and increases HeLa cell viability against H_2O_2 insult. Conversely, hPNPase knockdown decreases the viability and increases 8-oxoG level in HeLa cells exposed to H_2O_2. Taken together, our results suggest that RNA oxidation is a challenging problem for living organisms, and PNPase may play an important role in protecting both prokaryotic and eukaryotic cells by limiting damage to RNA under oxidative stress.

To my parents

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INTRODUCTION

RNA plays fundamental roles in cellular processes. Messenger RNA, transfer RNA and ribosome RNA are the central players in the elaborate process of protein synthesis. RNA-based primer sequences are required for DNA replication. Moreover, many small, non- coding RNAs regulate a variety of other cellular processes including Small nuclear RNAs (snRNAs) in RNA splicing (removal of introns from hnRNA) (Valadkhan, 2005), Small nucleolar RNAs (snoRNAs) in RNA modifications at specific sites (Matzke et al. 2007). More recently, the exciting discovery of small interfering RNA (siRNA), microRNA (miRNA), and other small non-coding RNA and the elucidation of their roles in regulation of gene expression (Begemann, 2008) are important addition to our knowledge of RNA function. It is essential that cells maintain a functional RNA pool. Therefore, defect in RNA caused by oxidative stress or other reagents may present a challenging problem for normal cellular function.

Reactive oxygen species (ROS) was generated from normal cellular metabolism and exogenous resources like hydroxyl radicals produced from superoxide and peroxide by the Fenton reaction (Henle and Linn, 1997)**.** Reactive oxygen species (ROS) is a major source of damage to cellular components. Oxidative damage to DNA, proteins and lipids has proven deleterious to cells and organs, which is strongly implicated in the development of cancers, age-related disorders, and many other diseases. Much work is

Introduction part is mainly taken verbatim from a review article wrote by Zhongwei Li, Jinhua Wu and Christopher J. DeLeo (Li et al., 2006) except the first paragraph and subsection 1.5 and 1.6.

dedicated to advances in this field (reviewed by Beckman and Ames, 1998; Bohr, 2002; Moreira et al., 2005; Ungvari et al., 2005; Kang and Hamasaki, 2005). In contrast, RNA oxidation has not received much attention (Bellacosa and Moss, 2003). The lack of appreciation of RNA damage is presumably due to the assumption that damaged molecules do not accumulate due to the transient nature of RNA. However, damage by ROS occurs in only minutes, whereas most human mRNA stays in cells much longer, with half-lives varying from hours to days. Moreover, stable RNA species (mainly ribosomal RNA and transfer RNA) constitute the majority of cellular RNA and are turned over relatively slowly. Therefore, damaged RNA may present challenging problems to cells, and specific surveillance mechanisms may be used to protect cells and organisms from RNA damage under oxidative stress (Fig. 1). Similarly, RNA can be damaged by other insults such as exposure to carcinogens or chemotherapeutic agents (Bellacosa and Moss, 2003), which may also cause problems to cells. This article will focus on the discussion of oxidative stress damages.

Although RNA has a greater chemical oxidative stability than DNA(Thorp, 2000), RNA is likely more prone to oxidative damage than DNA for the following reasons: (1). RNA is mainly single-stranded and is easily accessible to ROS; (2). RNA has relatively less association with proteins; (3). RNA has an extensive sub-cellular distribution, and cytoplasmic RNA is in close proximity to mitochondria where the majority of ROS is generated; (4). In contrast to the active repair mechanisms for DNA, repair for oxidatively-damaged RNA has not been found; (5). Based on the chemical characteristics, ribose in RNA is less stable than deoxyribose in DNA. Additionally, RNA is multi-copied from each gene, making it more affordable to damage. A number of

reports have indeed demonstrated in various systems that the levels of oxidative damage in RNA are higher than those in DNA of the same source. For example, oxidative insults caused greater damage of RNA than DNA in rat liver (Fiala et al., 1989; Hofer et al., 2006). Studies of human leukocytes (Shen et al., 2000) and A549 lung epithelial cells (Hofer et al., 2005) revealed that oxidative stress causes greater oxidation in cellular RNA than in DNA.

Oxidative damage to RNA may affect its function. It is likely that oxidative lesions alter the structure of the RNA or interfere with the interaction between RNA and other molecules. This could result in loss of or altered RNA function. Although information related to such scenarios is extremely scarce, some studies indeed indicate that RNA oxidation is a legitimate challenge to its function. Oxidation is known to kill RNA bacteriophages and has been explored as an approach to inactivate RNA viruses (Schneider et al., 1993; Müller-Breitkreutz and Mohr, 1998). Oxidation of mRNAs including mRNAs encoding luciferase and enhanced green fluorescent protein reduces translation efficiency and produces abnormal proteins both *in vitro* and *in vivo* (Shan and Lin, 2006; Tanaka et al., 2007). RNA oxidation also causes ribosome dysfunction, which may play a role in the pathogenesis of Alzheimer's disease (Ding et al., 2005).

RNA damage under oxidative stress is potentially detrimental to cells and organisms (Bellacosa and Moss, 2003) (Fig.1). Evidence that oxidized RNA is related to the pathogenesis of degenerative diseases is mounting. Recently, remarkable increases in oxidized RNA products were reported in patients with multiple disorders, mostly age-related degenerations. These include Alzheimer's disease (Nunomura et al., 1999a, 1999b, 2001, 2004, and 2007; Abe et al., 2002; Honda et al., 2005), Parkinson's disease

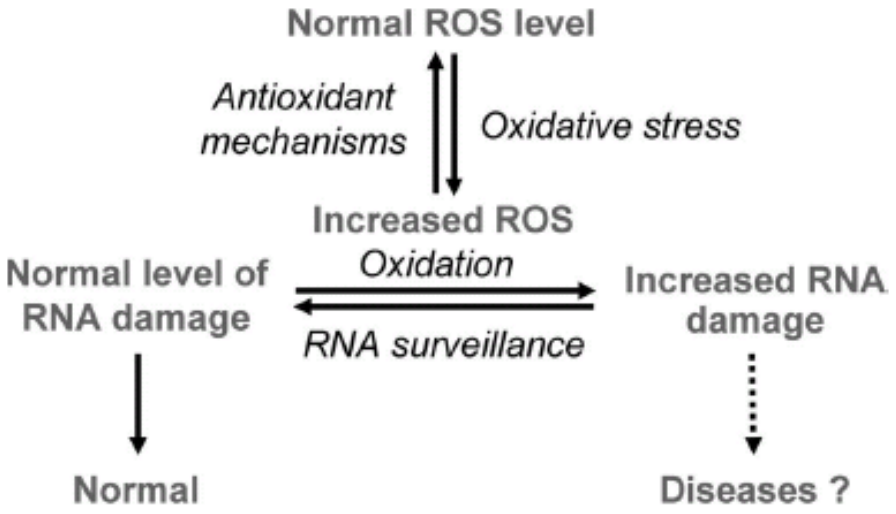


Fig. 1 RNA oxidative damage and defense mechanism

Reactive oxygen species (ROS) are produced by normal metabolism. Antioxidant mechanisms, such as superoxide dismutase, catalase, glutathione, etc., keep ROS levels low. ROS can be increased by exposure to environmental oxidants, or by reduction of antioxidants. Increased ROS in turn causes increase of the level of RNA oxidative damage. This diagram proposes that RNA surveillance mechanisms remove oxidatively-damaged RNA, reducing damage to normal level. In case RNA oxidation exceeds the capacity of RNA surveillance, or there exist defects in the surveillance pathways, damaged RNA may accumulate, resulting in dysfunction at molecular and cellular levels. This may eventually lead to disease.

(Zhang et al., 1999; Kikuchi et al., 2002), multiple system atrophy (Kikuchi et al., 2002), Down's syndrome (Nunomura et al., 1999a), dementia with Lewy bodies (Nunomura et

al., 2002), myopathies (Tateyama et al., 2003), and atherosclerosis (Martinet et al., 2004). An increase of oxidized RNA was also reported in aged rat models (Liu et al., 2002). In the early stages of Alzheimer's disease, the level of RNA oxidative damage is high (Abe et al., 2002) and ribosome function is affected (Ding et al., 2005), suggesting that RNA oxidation may contribute to the onset or development of the disease. RNA damage can be an initial causative factor, an enhancing factor, or simply a consequence of disease. This mystery is yet to be elucidated.

1.1 RNA oxidative damage and detection

RNA can be damaged by hydroxyl radicals produced from superoxide and peroxide by the Fenton reaction (Henle and Linn, 1997). More than 20 different types of oxidatively altered bases have been detected in DNA (Gajewski et al., 1990; Demple et al., 1994; Poulsen, 2005) and the RNA counterparts of these lesions must also be formed from oxidative damage of RNA (Bellacosa and Moss, 2003). It is currently-known that five types of base lesions in RNA are more abundant than others, including 8-hydroxyguanine, 8-hydroxyadenine, 2, 6-diamino-4-hydroxy-5-formamidopyrimidine (FapyGua) from guanine, 4, 6-diamino-5-formamidopyrimidine from adenine, and cytosine glycol (Fig 2) (Li et al., 2006). Among them, an oxidized guanine species, 8-hydroxydeoxyguanine (8-oxo-dG) in DNA or 8-hydroxyguanine (8-oxoG) in RNA, appears to be most deleterious (Kasai et al., 1986; Ames and Golden, 1991; Bessho et al., 1992) since 8-oxoG can incorrectly pair with adenine or thymine at about the same or at a higher efficiency than with cytosine, resulting in nucleotide mis-incorporation in reactions by DNA and RNA polymerases (Wood et al. 1990; Moriya et al., 1991;

Shibutani et al., 1991; Cheng et al., 1992; Furge and Guengerich, 1997; Taddei et al., 1997; Kim et al., 2004). Other damages occurring in DNA may also happen in RNA, including modification to other bases and to ribose, base excision, and strand break. Reverse transcription can be blocked by many of the oxidative damages to an RNA template (Rhee et al., 1995).

Several methods have been employed in the past to evaluate RNA oxidation levels, all by measuring 8-oxoG. To study DNA oxidative damage, Floyd and coworkers (Floyd et al., 1989) applied HPLC to separate deoxynucleosides. 8-oxo-dG was detected using an electrochemical detector with in-line detection of dG by a UV detector, which enables quantification of 8-oxo-dG levels per dG (Floyd et al., 1989). This method was adopted to detect 8-oxoG in RNA after digestion of RNA by Nuclease P1 and alkaline phosphatase (Fiala et al., 1989). RNA can also be hydrolyzed using formic acid at high temperature to generate nucleobases, and 8-oxo-guanine can be analyzed by HPLC equipped with ECD/UV detectors (Shen et al., 2000). ECD detects 8-oxoG or 8-oxo-dG at detection limit of ~20 fmol (Wang et al., 2005). Using tandem columns, these oxidative lesions of guanosine can be simultaneously detected in RNA and DNA (Hofer et al., 2006). Using HPLC coupled with electrospray tandem mass spectrometry detection, guanine and 8-oxo-guanine as a free purine in nucleosides and deoxynucleosides were simultaneously detected at the limit of 50 fmol for nucleobases and 12.5 fmol for nucleosides (Weimann et al., 2002).

As determined by HPLC-based analysis, the levels of 8-oxoG in RNA prepared from mammalian cell cultures or tissues without oxidative stress insults are about 1-2 8-oxoG/10^5 G. These levels increase up to 10 8-oxoG/10^5 G after oxidative stress, and are

10-25 times higher than those of 8-oxo-dG in DNA under the same conditions (Fiala et al., 1989; Shen et al., 2000; Hofer et al., 2005 and 2006). Since 8-oxoG is only part of oxidative lesions, total damage in RNA can be several times higher under both normal and oxidative stress conditions. Given that most mammalian mRNAs are in the length of kilobases, RNA oxidative lesions may be present in a low percentage of mRNAs under normal conditions, but can be found in a major fraction of mRNAs after oxidative stress. Consistent with this thought, it was reported that 8-oxoG is present in the majority of mRNA in the brain of Alzheimer's patients (Shan et al., 2006). Little is known about the effect of various lesions on RNA function. Some lesions in RNA may be fully functional.

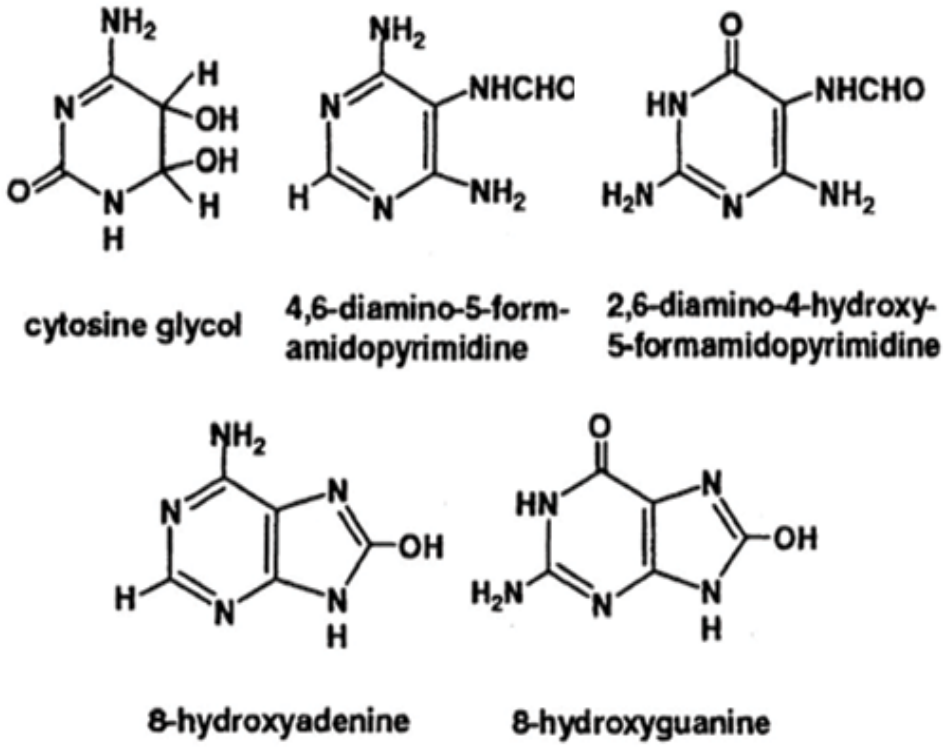


Fig. 2 The structure of five types of oxidative base modifications detected in RNA (drawn by ChemDraw Ultra 8.0)

Monoclonal antibodies against 8-oxo-dG have been developed (Park et al., 1992; Yin et al., 2005), and they recognize 8-oxoG in RNA as well as the DNA lesion. Various methods have employed such antibodies to measure 8-oxoG levels in RNA. Enzyme-linked Immunosorbent Assay (ELISA) was used to determine 8-oxo-dG in DNA (Yin et al., 2005), and may also potentially work for RNA. Nunomura and coworkers established an immunohistochemistry approach to detect *in situ* levels of 8-oxoG in brain sections from Alzheimer's patients (Nunomura et al., 1999a and 1999b). Anti-8-oxoG antibodies were also used in Northwestern blotting to quantify oxidized RNAs from brain tissues of Alzheimer patients (Shan et al., 2003). Antibody-based analysis is advantageous for detecting 8-oxoG in specific regions of tissue samples or in a subpopulation of total RNA.

RNA and DNA can be oxidized during preparation, storage and in reactions (*in vitro* oxidation), which may result in artificial levels of 8-oxoG and other oxidative lesions and account for the variations in reported levels of damage (de Souza-Pinto and Bohr, 2002). Chelators have been used to remove metal ions from solutions, glassware or plasticware, to reduce *in vitro* oxidation. Chelex 100 resin and chelators such as diethylenetriaminepentaacetate (DTPA) (Shen et al. 2000), desferrioxamine methansulphonate (DFOM) (Hofer et al., 2006), and ethylenediaminetetraacetic acid (EDTA) (Wang et al., 2005) have been used for this purpose. The addition of antioxidants or chelators to RNA samples also reduces the level of artificial oxidation. These include the antioxidants 2, 6-*tert*-Butyl-4-methylphenol (BHT), 8-hydroxyquinoline, and the iron chelator desferol (Shen et al., 2000 and Wang et al., 2005).

Damage of RNA by ROS seems to be random. The highly active hydroxyl radicals and other species may damage any molecule with which they collide. However, hot spots for damage may exist in RNA for the following reasons: (1). the four bases are chemically distinct and may have different reactivity to ROS; (2). local structure of RNA may protect certain regions from ROS; and (3). RNA sequences buried in RNA-protein complexes may be inaccessible to ROS. It has recently been reported that the steady-state levels of oxidative damage vary greatly among various mRNAs in Alzheimer's patients (Shan et al., 2003; Shan and Lin, 2006). It is interesting to wonder if levels of RNA damage also vary in different regions of a molecule or among different populations, such as rRNA, tRNA, and mRNA.

1.2 Quality control of RNA synthesis under oxidative stress

Oxidatively-damaged RNA can be generated by two ways: error incorporation of oxidized ribonucleotides into RNA during RNA synthesis and *in situ* RNA oxidation. The quality control mechanisms of RNA synthesis under oxidative stress is discussed in this section. Ribonucleotides can be oxidized by ROS directly or generated by the degradation of oxidized RNA. Increased 8-oxoG levels in the nucleotide pool were observed in leukocytes exposed to oxidative stress (Shen et al., 2000). If oxidized ribonucleotides are used in RNA synthesis, RNA damage levels would certainly increase.

Mechanisms have evolved in living systems to reduce incorporation of damaged ribonucleotides, as revealed by Mutsuo Sekiguchi and co-workers (Hayakawa et al., 1999; Shown in Fig. 3). In *E. coli* the MutT protein encoded by a mutator gene (Yanofsky et al., 1996) was found to degrade 8-oxo-dGTP, preventing incorporation of 8-

oxo-dGMP during replication (Maki and Sekiguchi, 1992). It was later shown that MutT also degrades 8-oxoGTP (Taddei et al., 1997), which can be generated by oxidation of GTP or by phosphorylation of 8-oxoGDP. In fact, its efficiency for degrading 8-oxoGTP is higher than that of 8-oxo-dGTP. An *E. coli* mutant devoid of MutT activity rescued LacZ protein at a much higher rate than would be expected by DNA mutations, suggesting increased errors in RNA synthesis in the mutant (Taddei et al., 1997). Therefore, degradation of 8-oxoGTP by MutT prevents incorporation of 8-oxoGMP into RNA during transcription. In addition, the *E. coli* RNA polymerase incorporates 8-oxoGMP from 8-oxoGTP into RNA at a much lower efficiency than incorporating GMP from GTP (Taddei et al., 1997). This intrinsic discriminative activity of RNA polymerase reduces 8-oxoG incorporation into RNA if 8-oxoGTP escapes MutT hydrolysis.

Hayakawa et al. found that a delicate quality control of ribonucleotide pool exists in human Jurkat cells for RNA synthesis (Hayakawa et al., 1999). Normally, GTP is produced through phosphorylation of GDP by nucleotide diphosphate kinase (ND Kinase). GDP in turn is produced by phosphorylation of GMP by guanylate kinase (GK). Human GK was shown to efficiently convert GMP to GDP, but it does not work on 8-oxoGMP, blocking its incorporation into RNA (Hayakawa et al., 1999). 8-oxoGMP is the product of degradation of 8-oxoG-containing RNA or 8-oxoGDP and 8-oxoGTP. GK is therefore a gatekeeper thwarting 8-oxoG incorporation during transcription (Fig. 3).

8-oxoGDP and 8-oxoGTP are generated through the direct oxidation of GDP and GTP. 8-oxoGTP can also be produced through phosphorylation of 8-oxoGDP by ND Kinase (Hayakawa et al., 1999). *E. coli* RNA polymerase incorporates 8-oxoGTP into RNA at a rate ~10% that of GTP (Taddei et al., 1997), whereas human RNA polymerase

II incorporates 8-oxoGTP at a rate 2% of GTP incorporation (Hayakawa et al., 1999). Therefore, RNA polymerases play a pivotal role preventing oxidized ribonucleotides being used in RNA synthesis (Fig. 3).

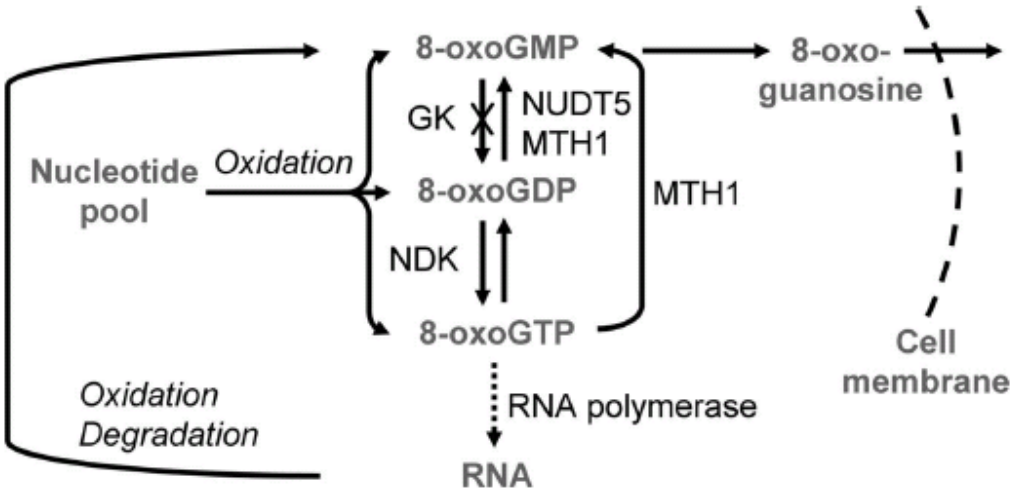


Fig. 3 Quality control of mammalian RNA synthesis from 8-oxoG nucleotides

8-oxoG nucleotides are generated by direct oxidation of guanine nucleotides by ROS. 8-oxoGMP is also produced by degradation of oxidized RNA. 8-oxoGTP, substrate for RNA synthesis, can be specifically hydrolyzed by the mammalian MTH1 protein to the mono-phosphate form. In addition, MTH1 and NUDT5 both specifically hydrolyze 8-oxoGDP to 8-oxoGMP (Maki and Sekiguchi, 1992; Yu et al., 2005). Remarkably, guanylate kinase (GK), an enzyme that converts GMP to GDP, does not act on 8-oxoGMP (Yu et al., 2005). 8-oxoGMP is therefore trapped and removed from the cell, possibly through dephosphorylation. RNA polymerases provide additional control: 8-oxoGTP escaping hydrolysis may be incorporated into RNA, however, at much lower efficiency than the incorporation of GTP shown in E. coli and human (Shibutan et al., 1991; Yu et al., 2005).

In addition, human cells have developed mechanisms to reduce the levels of oxidized GTP and GDP (Fig. 3). Three human proteins, MTH1, MTH2, and NUDT5, hydrolyze 8-oxo-dG-containing nucleotides in the DNA precursor pool, an activity similar to that of the *E. coli* MutT protein. It has been shown that MTH1 and NUDT5 are both active in the conversion of 8-oxoGDP to 8-oxoGMP (Ishibashi et al., 2005). In addition, MTH1 efficiently hydrolyzes 8-oxoGTP to 8-oxoGMP. These proteins also can suppress the mutator activity of the E. coli mutant lacking MutT (Ishibashi et al., 2005). Such findings are consistent with the notion that oxidized ribonucleotides can be eliminated from the RNA precursor pool. In harmony with the above analysis, Kajitani and co-workers demonstrated that knock-out of MTH1 in C57BL6/J mice increases the level of 8-oxoG in RNA in the hippocampal microglia during oxidative stress via kainite-treatment (Kajitani et al., 2006). This suggests that MTH1 can prevent the incorporation of oxidized guanosine into RNA *in vivo*, presumably by hydrolysis of 8-oxoG species.

1.3 RNA surveillance

Oxidative damage may cause RNA dysfunction and toxic to cells. Such RNA molecules are expected to be removed by cellular surveillance activities. In the past few years, surveillance mechanisms have been uncovered for RNA defects caused by mutations, errors in splicing or modification. It has been suggested that defective RNA is detrimental to cells and organisms. Hence, specific mechanisms have been evolved in various organisms to handle defective RNA. As discussed below, similar surveillance mechanisms may be responsible for removing oxidatively-damaged RNA.

Eukaryotic mRNAs with premature termination codons (PTC, or nonsense codons) can be generated by mutations of the gene, or abnormal splicing of the precursor RNA. Such mRNA may produce truncated proteins of altered or no function. They are degraded by a nonsense-mediated decay (NMD) pathway (Hentze and Kulozik, 1999; Baker et al., 2004). During the first round of translation, the ribosome stops at PTC of the nonsense mRNA, which triggers decapping and 5'->3' degradation of the mRNA. In addition, yeast mRNAs having "nonstop" mutations or those failing to be exported to the cytoplasm are recognized and bound by the Ski7p protein, and are subsequently degraded by the exosome complex (Hilleren et al., 2001; Van Hoof et al., 2002). This effectively prevents the expression of mRNAs that are prematurely polyadenylated within the coding region.

An interesting question is how an mRNA is switched from a state being actively translated to a state being rapidly degraded. Apparently, access of the degradation machinery to mRNA is a key step for such a switch. Recently, it has been discovered that non-translating mRNA is largely present in a microscopic cytoplasmic structure termed processing body (P-body) (Teixeira et al., 2005; Fillman and Lykke-Andersen, 2005). Decapping complex and Xrn1p enzyme are also present in P-body (Bashkirov et al., 1997; Ingelfinger et al., 2002; Lykke-Andersen et al., 2002; Van Dijk et al.,, 2002; Fenger-Gron et al., 2005). It is likely that recruitment of mRNA into P-body initiate decapping-dependent mRNA decay (Coller et al., 2005; Fillman and Lykke-Andersen, 2005). It is likely that defective or damaged mRNAs are recognized and sequestered in P-body by specific mechanisms.

Defective tRNAs were shown to be unstable (Tuohy et al., 1994; Li et al., 2002). In *E. coli*, a mutant $tRNA^{Trp}$ can be degraded depending on a 3'->>5' exoribonuclease, polynucleotide phosphorylase (PNPase), and poly(A) polymerase (Li and Deutscher, 1998; Li et al., 2002). The mutant $tRNA^{Trp}$ or its precursor accumulates to higher levels in the absence of PNPase and/or poly (A) polymerase. This suggests a model for surveillance of defective tRNA through 3'->5' exonucleolytic degradation. Polyadenylation may assist 3'->5' degradation by providing a platform for exoribonucleases to access the 3' end of the highly-structured RNA. Similarly, yeast tRNA precursor that are hypo-modified are degraded in a similar fashion in the nucleus (Kadaba et al., 2004). The exoribonuclease Rrp6, and Trf4p, a DNA polymerase with poly(A) polymerase activity for RNA, are responsible for degrading the hypo-modified tRNAs (Kadaba et al., 2004). In addition, tRNAs lacking nonessential modifications are degraded rapidly by a pathway independent of Trf4/Rrp6, and involves de-aminoacylation (Alexandrov et al., 2006). Various mutations in mitochondrial tRNA (mt-tRNA) cause human diseases (Florentz et al., 2003; Jacobs and Turnbull, 2005) by affecting maturation, modification, amino acylation or interaction with translational machinery (Levinger et al., 2004; Jacobs and Turnbull, 2005). It awaits see if such surveillance mechanisms exist for mutant tRNAs in mitochondria.

Alkylating agents, which add methyl or ethyl groups to the bases of nucleic acids, can modify both DNA and RNA. Enzymes such as *E. coli* AlkB and its human homolog hABH3 enzymatically repair alkylated bases in both DNA and RNA (Aas et al., 2003 and Lee et al., 2005). The fact that RNA is repaired demonstrates the importance of keeping the quality of RNA controlled.

1.4 The fate of oxidized RNA

Currently, little is known about how cells deal with oxidatively damaged RNA. One would expect oxidized or modified RNA to be removed from the functional RNA pool by cellular quality-control mechanisms. This thought is supported by recent observations that the levels of RNA oxidative damage drop sharply after removal of oxidative stress insult. Pulse exposure of cultured human lung epithelia cells to hydrogen peroxide caused a sharp increase in 8-oxoG levels, which was decreased to half of the highest level after 24 hours (Hofer et al., 2005). It was reported that RNA oxidation level in the CA3 sub-region of mice brain increased by 20% at 24 hours after the administration of kainate, an agonist of glutamate receptor that induces the production of ROS. After 72 hours, RNA oxidation levels returned to baseline (Kajitani et al., 2006). After a pulse treatment of HeLa cells with sub-millimolar levels of hydrogen peroxide, the increased levels of 8-oxoG in total RNA drops about 50% within 1 hour, followed by a slow decrease to baseline (Wu and Li, 2008). These results suggest that high levels of RNA oxidation are not tolerated by cells. Oxidized RNA is removed from total RNA, presumably by specific mechanisms.

One mechanism to remove oxidatively damaged RNA is selective degradation by ribonucleases (RNases). Some studies of this problem have been carried out in *Escherichia coli*. It is reported that the *E. coli* polynucleotide phosphorylase (PNPase), a 3'->5' exoribonuclease important for mRNA decay, binds an 8-oxoG-oligoribonucleotide specifically with a much higher affinity than the binding of a normal RNA of the same sequence (Hayakawa et al., 2001).However, *E. coli* mutant devoid of PNPase is hyper-resistant to paraquat, an oxidizing agent, suggesting that PNPase may be involved in

degrading oxidatively damaged RNA but its activity is not required for cell survival under paraquat treatment (Hayakawa et al., 2001). In a similar study, human PNPase, a protein sharing high similarity with the *E. coli* ortholog, was found to bind the 8-oxoG-oligoribonucleotide with high specificity (Hayakawa et al., 2006). RNase E is an endoribonuclease responsible for initiation of processing of stable RNA and degradation of mRNA and non-coding RNA species (Li and Deutscher, 2004). It was observed that active mutants of RNase E rescue *E. coli* cells from being killed by human Bax protein which induces oxidative stress (Nanbu-Wakao et al., 2000). Mutant RNase E proteins decrease ROS levels, but a direct examination of RNA oxidation or degradation was not reported.

Both PNPase and RNase E have been known to carry out other major functions of RNA degradation (Li and Deutscher, 2004). If they are responsible for selective degradation of oxidized RNA, they must be able to distinguish oxidized RNA from normal RNA. Such selective degradation activity has not been shown by any RNase, but PNPase has high affinity to 8-oxoG RNA. It is likely that RNA-binding proteins recognize or sequester oxidatively damaged RNA molecules before they are degraded or repaired. Collective studies suggest that such activity exists in various organisms, and recognition may happen during translation (Ohga et al., 1996; Hayakawa et al., 2001; Skabkin et al., 2001; Hayakawa et al., 2002; Bregeon and Sarasin, 2005). A hypothetical model for surveillance of oxidized mRNA in eukaryotes is presented in Fig. 4. The human YB-1 protein, a multi-function protein binding both DNA and RNA, was found to bind 8-oxoG-containing RNA oligonucleotide at high affinity (Hayakawa et al., 2002). When introduced into *E. coli*, the YB-1 gene confers high resistance of the bacterial cell

to paraquat treatment, suggesting a role for YB-1 in the metabolism of damaged RNA and cell survival under oxidative stress. However, the removal of oxidized RNA from such cells was not demonstrated. YB-1 has also been found to regulate mRNA stability and translation (Kohno et al., 2003), and to aid in the unwinding and winding of RNA duplexes (Skabkin et al., 2001). Therefore, YB-1 may work as an RNA chaperone to target damaged RNA to sequestration and/or degradation.

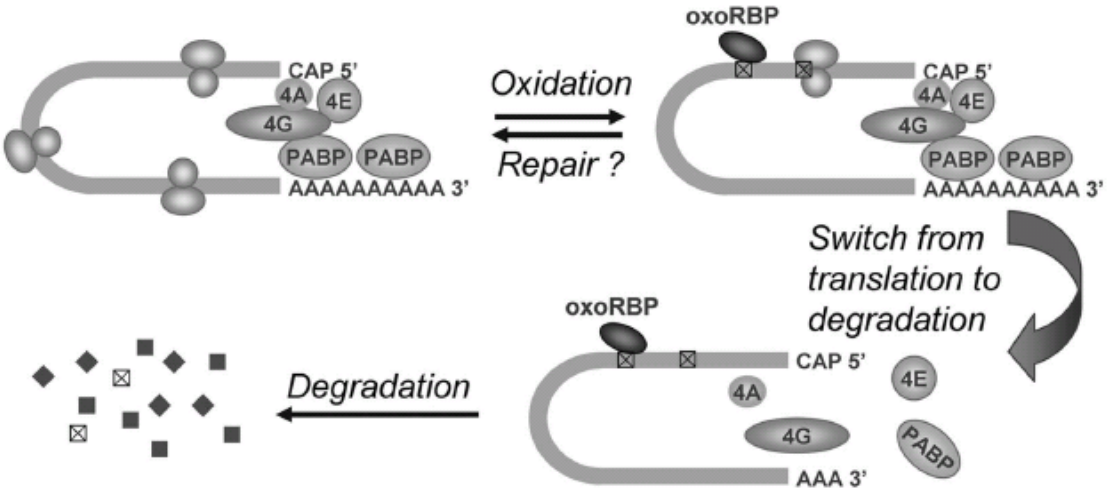


Fig. 4 Possible surveillance mechanisms for oxidized mRNA

Actively-translated mRNA (thick lines) are associated with ribosomes (double ovals), translation initiation factors (4A, 4E, and 4G), and poly(A)-binding proteins (PABP). mRNAs can be oxidized by ROS. Some oxidized mRNA may be repaired by unknown mechanisms and translation is continued. Oxidized residues in mRNA may be recognized by specific oxidized RNA-binding proteins (oxoRBP) or ribosome. Such recognition may trigger inactivation of translation, followed by degradation of the oxidized mRNA.

Work by Sandra Wolin and colleagues on the Ro autoantigen suggested that this RNA-binding protein may play a role in discarding defective or damaged RNA of similar nature (O'Brien and Wolin, 1994; Shi et al., 1996; Labbe et al., 2000). It was reported that Ro protein and its homologs specifically bind defective 5S rRNA precursors with mutations by recognizing a misfolded helix RNA (O'Brien and Wolin, 1994; Shi et al., 1996; Labbe et al., 2000). These defective pre-5S rRNA are inefficiently processed to mature 5S rRNA and are mostly degraded. Similarly, in mouse embryonic stem cell and Xenopus, Ro specifically binds variants of U2 spliceosomal snRNAs containing base mutations by the recognition of an alternative helix. Ro does *not* bind U2 with normal sequences (Chen et al., 2003). The ability of Ro to bind mis-folded RNAs is conferred by the central cavity structure (Stein et al., 2005). Importantly, the Rsr protein, an ortholog of Ro in *D. radiodurans*, contributes to the resistance of *D. radiodurans* to UV irradiation (Chen et al., 2003). Rsr levels increase during recovery from UV irradiation. Inactivation of the *rsr* gene renders *D. radiodurans* cells more sensitive to UV damage than the wild-type cells. Moreover, mouse ES cells lacking Ro showed decreased survival after UV irradiation (Chen et al., 2003). Finally, Ro knockout mice are sensitive to ultraviolet irradiation and are more susceptible to development of a lupus-like syndrome (Xue et al., 2003). The data strongly suggest a role for Ro and its homologues in repairing radiation damage to RNA.

It would not be surprising for the ribosome to plays a role in recognizing oxidatively-damaged mRNA during translation. The interactions between ribosomal RNA and proteins with mRNA may discriminate normal residues from oxidative lesions in mRNA (Fig. 2). Possible roles of ribosomal proteins in quality control of oxidatively-

damaged mRNA are suggested by previous studies. In drosophila, for example, the ribosomal protein S3 confers the N-glycosylase activity responsible for base excision and repair of 8-oxo-dG in DNA (Hegde et al., 2001 and 2004). The human S3 protein has a marginal N-glycosylase activity; nonetheless, it binds DNA oligonucleotides containing 8-oxoG or abasic residues and cleaves abasic DNA (Hegde et al., 2004). Moreover, structural studies revealed that upon entering the ribosome, mRNA is encircled by ribosomal proteins S3, S4, and S5. S3 and S4 are important for the processivity of the ribosome on mRNA (Yusupova et al., 2001). S3 may scan mRNA for oxidative lesions during the translation process and may play an important role in the inactivation or sequestration of oxidatively-damaged mRNA.

1.5 Polynucleotide phosphorylase (PNPase)

The fact that PNPase proteins from *E. coli* and human bind to 8-oxoG RNA suggests an important role for this enzyme in the control of oxidatively damaged RNA. Some details of these two proteins are introduced in this section.

1.5.1 *E. coli* polynucleotide phosphorylase (PNPase)

E. coli polynucleotide phosphorylase (PNPase) is an exoribonuclease degrading RNA in the 3' to 5' direction (Andoh et al., 1963). It is also a 3'->terminal oligonucleotide polymerase (Littauer and Kornberg, 1957; Gillam et al., 1978; Gillam and Smith, 1980). Its primary function *in vivo* is RNA degradation (Kinscherf and Apirion, 1975; Kaplan and Apirion, 1974 and 1975). It was well-known that *E. coli* PNPase is involved in various processes in mRNAs turnover, tRNA maturation and degradation (Li and

Deutscher, 1994; Mohanty and Kushner, 2003). Loss of PNPase leads to an increase in the steady-state levels of numerous mRNA species in the absence of the 3' exoribonuclease RNase II (Mohanty and Kushner, 2003), suggesting that PNPase is globally involved in mRNA decay.

A temperature-sensitive variant of $tRNA^{Trp}$ is unstable. Degradation of this tRNA depends on the activities of PNPase and poly(A) polymerase (Li et al., 2002). Additionally, ribosomal RNA fragments accumulate to high levels in a mutant lacking both PNPase and RNase R (Cheng and Deutscher, 2003). These data suggest that PNPase is an important enzyme responsible for the control of defective RNA.

E. coli PNPase also plays important roles in RNA degradation under the various stresses. PNPase plays specific roles in mRNA degradation during carbon starvation (Kaplan and Apirion, 1975). PNPase-mediated mRNA degradation is required for regulation of the response of *E. coli* cells to cold shock (Cairrao et al., 2003; Polissi et al., 2003). Therefore, *E. coli* PNPase may also play certain role in dealing with the oxidative stress in the cells.

E. coli PNPase is an important component of the degradosome, a multi-enzyme complex formed with the endoribonuclease RNase E, the ATP-dependent RNA helicase RhlB, and a glycolytic enzyme enolase (Py et al., 1994 and 1996; Carpousis et al., 1994). PNPase, RhlB and enolase are assembled on the C-terminal region (also is called the degradosome scaffold) of RNase E (Vanzo et al., 1998). In the degradosome, PNPase binds to the region of amino acids 844-1045 in RNase E and Rhl B and Enolase bind to the region immediately upstream of PNPase-binding site (Vanzo et al., 1998; Leroy et al., 2002). Polyphosphate kinase and the chaperone protein DnaK were also reported to

associate with the degradosome in substoichiometric amounts (Blum et al., 1997; Miczak et al., 1996). The N-terminal half of RNase E is not only the catalytic core of the enzyme (Taraseviciene et al., 1995; McDowall et al., 1996) but enables the degradosome protein complex to attach to the cytoplasmic membrane (Liou et al., 2001). Although the degradosome commonly has been regarded as an RNA decay machine (Li and Deutscher, 2004), degradosome components can also exist in a degradosome-dissociated form (Liou et al., 2001), and may function individually. Nearly 80~90% of enolase as well as RhlB are included in the membrane-bound degradosomes (Liou et al., 2001). In contrast, only ~30% of cellular PNPase are estimated to be present in the degradosome *in vivo* (Liou et al., 2001).

PNPase has also been shown to interact with Rhl B helicase independently of RNase E, or to exist in free form (Liou et al., 2002). The presence of PNPase in degradosome and the association of this RNase with RhlB are believed to make RNA degradation more efficient. Using ATP, RhlB opens structured regions of RNA which otherwise would inhibit degradation by PNPase (Khemici and Carpousis, 2004).

Poly(A) polymerase (PAP) is another enzyme facilitating degradation of structured RNA by PNPase and other exoribonucleases. The *pcnB* gene encoding PAP protein is not essential for growth (Li et al., 2004). PAP has been shown to account for at least 90% of the poly(A) tails found in *E. coli* RNA (Mohanty and Kushner, 1999 and 2006). Polyadenylation plays an important role in mRNA decay in prokaryotic cells (Mohanty and Kushner, 1999 and 2006). Poly(A) tails probably serve as a platform for recruiting the processive exoribonucleases. Consistent with this notion, RNA with 3' stem-loops are resistant to degradation by pure PNPase or whole degradosome *in vitro*, but addition of

even a short poly(A) tail overcomes this block (Lisitsky and Schuster, 1999). *In vivo*, PAP in concert with the degradosome is required for PNPase-mediated degradation of cistrons with 3' REP-stabilizers (Khemici and Carpusis, 2004). These results suggest that poly(A) polymerase I-catalyzed polyadenylation facilitates PNPase in RNA degradation, possibly by providing a better binding site for PNPase to initiate RNA degradation. Polyadenylation is also likely important for RNA degradation catalyzed by other exoribonucleases, such as RNase II and R. RNA decay intermediates accumulate at much higher level in a mutant lacking both PNPase and PAP than in the mutant lacking only PNPase (Li et al., 2004). The degradation of a defective tRNA requires both PNPase and polyadenylation poly(A) polymerase (Li et al., 2002). Under oxidative stress, polyadenylation catalyzed by poly(A) polymerase may facilitate the degradation of oxidatively-damaged RNA.

1.5.2 Human PNPase (hPNPase)

Polynucleotide phosphorylase (PNPase) is evolutionarily conserved in bacteria, fly, plant, animal and human (Leszczyniecka, et al., 2003b). The human PNPase (hPNPase) is highly homologous to *E. coli* PNPase with a similarity of 57.2% (Wu and Li, 2008). The gene encoding hPNPase was discovered in a genetic screening and has been shown responsible for cellular terminal differentiation and cellular senescence (Leszczyniecka et al., 2002). Interestingly, hPNPase is predominantly localized in mitochondria (Sarkar et al., 2005 and 2006). Human polynucleotide phosphorylase is also a type I IFN-inducible early response gene (Leszczyniecka et al., 2002, 2003a and 2003b; Sarkar et al., 2003).

Similar to *E. coli* PNPase, *hPNPase* is a 3', 5' exoribonuclease catalyzing mRNA decay in a 3'–5' direction (Leszczyniecka et al., 2002). Human PNPase, a type I IFN-inducible 3', 5' exoribonuclease involved in mRNA degradation, induces G_1 cell cycle arrest and eventually apoptosis by specifically degrading c-*myc* mRNA (Sarkar et al., 2006). Interestingly, like its *E. coli* homologue, hPNPase has also been shown to specifically bind 8-oxoG RNA with high affinity (Hayakawa et al., 2006). The above observation about hPNPase and our finding about *E. coli* PNPase's role under oxidative stress prompted us to further examine if hPNPase participates in surveillance of oxidized RNA.

1.6 Hypothesis and Rational of the project

We hypothesize that (1) RNA oxidation imposes challenge to cells and organisms and contributes to cellular dysfunction under oxidative stress; and (2) oxidized RNA is normally removed by RNA surveillance activities, and such activities protect cells under oxidative stress.

In this work, we proposed to investigate the roles of ribonucleases and other factors for RNA degradation in protecting cells against oxidative insults, and to examine if they affect the level of RNA oxidative damage. Our rational is that RNA degradation could play a major role in removing oxidized RNA. In a cell having the full reservoir of RNA surveillance activities, oxidized RNA is limited to achieve good viability under oxidative challenges. However, when some surveillance activity/activities for oxidized RNA is missing, damaged RNA may accumulate to higher levels and cells would become more susceptible to oxidative challenge. Therefore, we propose to examine cells lacking

particular RNA degradation activities to find out if their viability and RNA oxidation level are affected under oxidative stress. Conversely, we would introduce genes encoding such activities to see if the response to oxidative stress is reversed. My dissertation research focuses on the role of polynucleotide phosphorylase in *E. coli* and cultured human cells under oxidative stress conditions. As stated above, PNPase proteins from both organisms were shown to interact with oxidized RNA at higher affinity than they do to normal RNA. *E. coli* mutants lacking PNPase and other RNA degradation activities were previously shown hypersensitive to oxidative stress treatments (Shaohui Wu, Xin Gong and Zhongwei Li, unpublished observations). Therefore, it is very likely that these enzymes play a role in controlling the level of oxidized RNA *in vivo* in both *E. coli* and human cells. My results should be helpful to define the roles of these enzymes on RNA quality control and cell protection under oxidative stress. The work would be valuable to understand the problem in both prokaryotic and eukaryotic systems.

The specific aims of my dissertation research are: 1. to study the function of *E. coli* polynucleotide phosphorylase in protecting cells against oxidative stress and its possible roles in removing RNA containing oxidatively-damaged lesions; 2. to study the role of human polynucleotide phosphorylase in controlling oxidatively-damaged RNA and cell viability in human carcinoma HeLa cell treated under oxidative stress.

MATERIALS AND METHODS

Bacterial strains and plasmids

Escherichia coli K12 strain CA244 (*lacZ, trp, relA, spoT*) (Li and Deutscher, 1996) was used as wild type. Strains (CA244) lacking RNase R (*rnr*) (Cheng et al., 1998), II (*rnb*), PNPase (*pnp*) (Li and Deutscher, 1996), poly(A) polymerase (*pap*) were described in the related references. The strain lacking RNase I and II (*rna rnb*) was described previously (Cheng et al., 1998). The RNase and poly (A) polymerase mutants were all constructed by gene-interruption or deletion that completely abolished activity. Strain lacking methionine-sulfoxide reductase A (*msrA*) was constructed by transferring the mutant *msrA* allele (Moskovitz et al., 1995) to CA244 by bacteriophage P1 transduction. The strains AC21 (wild type) and AC24 (*rne*Δ*10*) (Leroy et al., 2002) were gifts from Dr. A. J. Carpousis. The *pnp* allele was transferred to AC21 and AC24 by P1 transduction. The plasmid pKAK7 harboring the *pnp* gene was provided by Dr. S. R. Kushner (Donovan and Kushner, 1986).

Material

A plasmid DNA Miniprep kit was purchased from Qiagen (Valencia, CA). Restriction enzymes and T4 DNA ligase were purchased from New England Biolabs (Ipswich, MA). Pfu Ultra Hotstart DNA polymerase was purchased from Stratagene (La Jolla, CA). Catalase and guanosine were the products of Sigma-Aldrich (St. Louis, MO).

8-hydorxyguanosine was purchased from Calbiochem (La Jolla, CA). [^{3}H]-labeled poly(A) is the product of Perkin-Elmer. All other chemicals are reagent grade.

HeLa cell was provided by Dr. Vijaya Iragavarapu-Charyulu. A plasmid pCMV-hPNP expressing hPNPase from the CMV promoter was purchased from Open Biosystems (Clone ID: 6062060). A control plasmid pCMV* was prepared by digesting pCMV-hPNP with *SalI* and *XhoI* to remove the *hPNP* DNA insert followed by self-ligation. An siRNA (5'-GAAACAGGUGUAACUAUUAdTdT-3') targeting the region of 1900-1918 of *hPNP* mRNA was chemically synthesized by Qiagen. AllStars Control siRNA provided in the RNAi Human/Mouse Starter Kit (Qiagen) was used as nonspecific control. Other materials include the plasmid DNA Miniprep and Maxiprep kits (Qiagen), restriction enzymes and T4 DNA ligase (New England Biolabs), *Pfu* DNA polymerase (Stratagene), guanosine (Sigma-Aldrich), 8-hydroxyguanosine (8-oxoG) (Calbiochem). All other chemicals are reagent grade.

PCR using intact cells

Fresh colonies of *E. coli* strains were dissolved in 50 µl water and boiled for 5 minutes. 5 µl of boiled colony solution was taken as a template for PCR amplification to confirm the genetic background of the strains.

RNA binding protein assay: immobilization of RNA on agarose beads and RNA affinity assays.

50 mer RNA oligo: GGAGAGUAAAAAUGAAAGUACGUGCUUCCGUGAAG UAAUUUUUUCGCAU (A, 32%; G, 24%; C, 14%); U, 30%] was used to prepared oxidized RNA. RNA and oxidized RNA are covalently linked to adipic acid dihydrazide agarose beads as previously described (modified from a published procedure) (Zahler et

al., 2004). 500 pmol of RNA was put into a 400 µl reaction mixture containing 100 mM sodium acetate (pH 5.0) and 5 mM sodium m-periodate (Sigma). Reaction mixtures were incubated for 1 h in the dark at room temperature. The RNA was then ethanol precipitated and resuspended in 500 µl of 0.1 M sodium acetate (pH 5.0). Four hundred microliters of 50% slurry of adipic acid dihydrazide agarose beads (Sigma) were washed four times in 10 ml of 0.1 M sodium acetate (pH 5.0) and pelleted after each wash at 300 rpm for 3 min in a clinical centrifuge. After the final wash, 500 µl of 0.1 M sodium acetate (pH 5.0) was added to the beads and the slurry was then mixed with the periodate-treated RNA and rotated for 12 h at 4°C. The beads with the bound RNA were then pelleted and washed three times in 1 ml of 2 M NaCl and three times in 1 ml of buffer D (20 mM HEPES-KOH [pH 7.6], 5% [vol/vol] glycerol, 0.1 M KCl, 0.2 mM EDTA, 0.5 mM dithiothreitol). The binding efficiency of RNA to the beads was between 70% and 80%, as determined with 5' ^{32}P-end-labeled RNA (the data not shown). The beads containing immobilized RNA were incubated in a reaction mixture containing 250 µl of *E. coli* cellular extract and 400 µl of buffer D for 20 min at 30°C. Beads were then pelleted by centrifugation at 106 x g for 3 min and washed four times with 1 ml of buffer D. After the final centrifugation, the proteins bound to the immobilized RNA were eluted by addition of a 60 µl of protein sample buffer. Eluted RNA-binding protein was analyzed by 12% SDS-Page to check the affinity of *E. coli* PNPase to oxidized RNA. Brief procedures are listed in Fig 5.

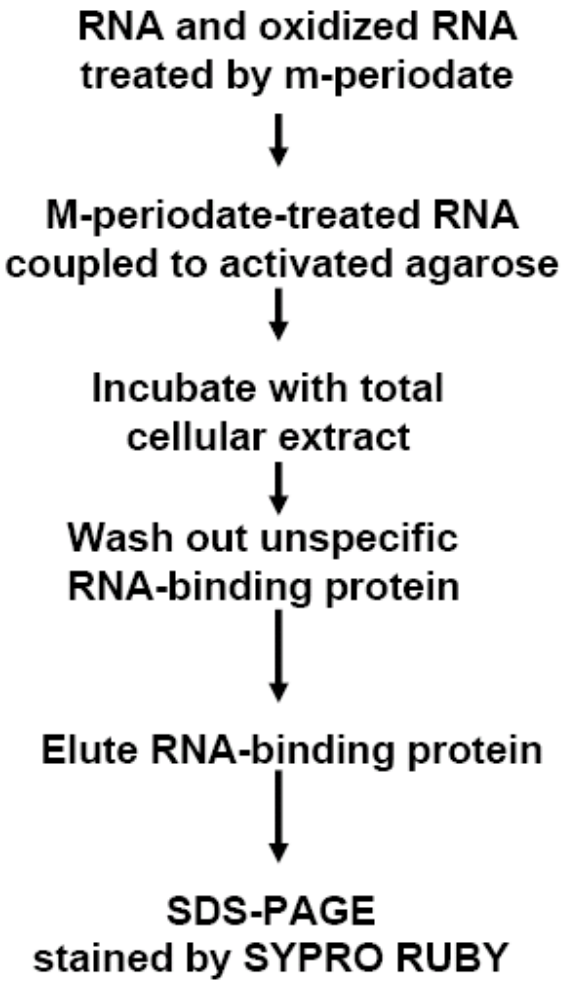


Fig. 5 The procedure for screening oxidized RNA-binding proteins

H_2O_2 degradation assay in *E. coli* culture

H_2O_2 concentration was assayed by Amplex Red kit purchase from Molecular Probe. *E. coli* cells was cultured till OD_{550nm}= 0.2. 180 µl culture was transferred in each well of 24 well plates and 20 µl of 5 mM H_2O_2 was respectively added into the well at 0, 15, 25, 28 and 30 minutes. At the time point of 30 minutes, 12.5 µl H_2O_2-treated culture,

37.5 µl Amplex Red reaction buffer and 50 µl Amplex Red working solution were mixed and incubated in the well in a 96 well plate at room temperature for 30 minutes. OD_{550nm} for each well standing for H_2O_2 concentration was recorded by a plate reader. Based on the standard curve of OD_{550nm} over H_2O_2 concentration, OD_{550nm} for H_2O_2-treated culture was converted to H_2O_2 concentration remaining in the cell culture. The curve of H_2O_2 degradation over the time was plotted.

***E. coli* RNA isolation for the analysis of RNA oxidation**

E. coli culture (OD_{550nm}=0.5) is treated with or without H_2O_2. After the treatment of H_2O_2 for 10 minutes, *E. coli* culture was precipitated at 10,000g for 1min, washed twice and suspended with prewarmed YT media, and then treated with 400 µg/ml of rifamycin to stop RNA synthesis. 1.5 ml of *E. coli* culture is pelleted at 10,000g for 1 min at the desired time points. 50 µl of 2X cell lysis buffer was used to lyse *E. coli* cell pellet (cell lysis buffer:10 mM Tris pH7.5, 10 mM EDTA pH8.0, 1%SDS, 10% Glycerol) at room temperature for 5 min, and the lysed *E. coli* sample was stored at -80^{0}C. When all of the samples were collected, 1 ml Tri-Reagent containing proper quantity of ion-chelators, 500 µM BHT and 100 µM Desferol, was added into every lysed *E. coli* sample, mixed well, and incubated at room temperature for 10 min. The mixture was added with 0.2 ml $CHCl_3$, mixed strongly for 15 seconds, incubated at RT for 10 min, and centrifuged at 12000 g at 4^{0}C for 15 min. Then the supernatant (about 50% volume of the quantity of Tri-Reagent, 0.5 ml) was transferred carefully to a 100 µM DTPA-treated tube, 0.5 ml Chelex-100 treated Isopropanol was added into the supernatant, the mixture was incubated at RT for 15 min, and finally the RNA pellet was spin down from the mixture at 20,000 g for 8 min and wash with 75% ethanol. The remaining ethanol of RNA pellet

was dried out by the vacuum for 2 min. RNA pellet was dissolved in 20 µl of chelex-treated dd-H_2O of a tube filled with argon.

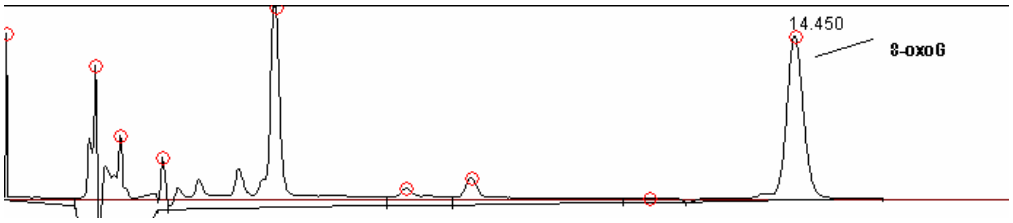
RNA oxidation analysis by reverse-phase HPLC

The reverse HPLC is in line with UV detector and electrochemical detector (EC detector).The contents of 8-oxo-Guanosine in hydrolysates were determined by using a Waters HPLC instrument equipped with electrochemical cells set to 600 mV. The contents of Guanosine in hydrolysates were determined by using a Waters HPLC instrument equipped with a variable UV-Vis detector. Samples were injected onto a Progel TSK ODS-AD TM column (5 µm, 4.6 × 250 mm) equilibrated in mobile phase: 12.5 mM Citric Acid, 25mM Sodium Acetate, 30 mM NaOH, 10 mM Acetic Acid, and 5% methanol. Peak identity was routinely established by demonstrating the appropriate retention time of standard 8-oxo-Guanosine and Guanosine. The content of guanine (G) was determined by UV monitoring at 254 nm. Authentic 8-oxoG and G standards were also used to generate external calibration curves. The RNA oxidation level is presented by the number of 8-oxoGuanosine per 10^5 Guanosine.

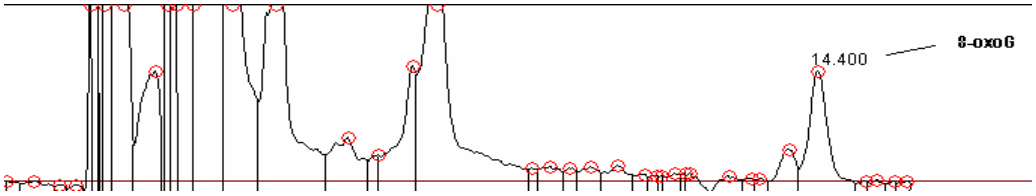
Based on Fiala's HPLC condition (Fiala et al., 1989), ~5 µg RNA samples were digested into nucleotides by 0.5 units of nuclease P1 in the 20 µl reaction volume of 100 mM Sodium acetate (pH5.2) for 1 hour at 37^0C. After addition of 8 µl of 0.4 M Tris-Cl, the mixture was further digested by 0.3 unit of bacterial alkaline phosphatase which was added into the reaction mixture for another 1 hour. The resulting hydrolysates were analyzed by HPLC-UV & ECD. According to the retention time of standard peaks of 8-oxoGuanosine (8-oxoG) and Guanosine (G), the peaks of 8-oxoG and G were identified (Fig 6.A and C); Based on the standard curve of 8-oxoGuanosine and Guanosine, the

quantity of 8-oxoGuanosine and Guanosine in total RNA (Fig 6.B and D) were calculated respectively; The RNA oxidation level was represented by 8-oxoG per 10^5 G in total RNA.

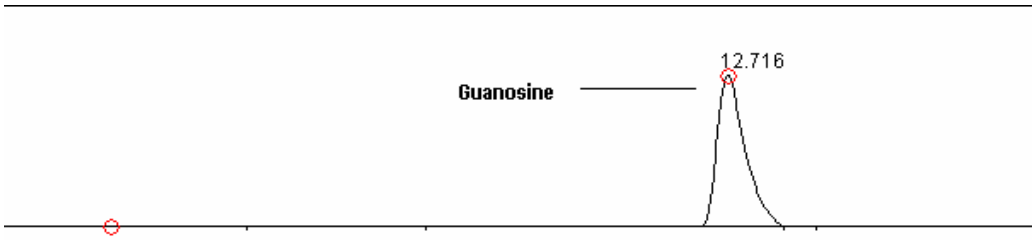
A.



B.



C.



D.

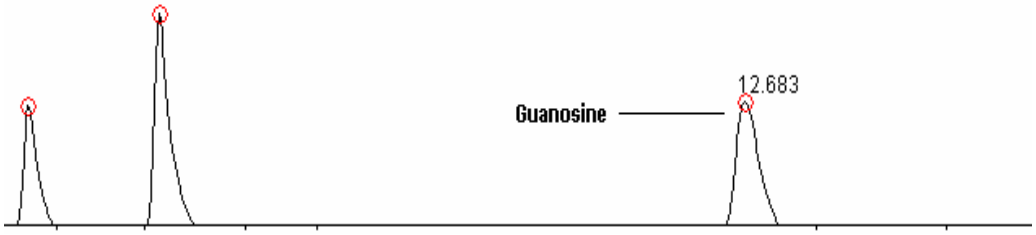


Fig. 6 8-oxoGuanosine electrochemical signal peak and guanosine UV absorbance peak of both the standard guanosine & 8-oxo-guansosine and RNA sample in HPLC

The number above the peak shows retention time (minutes) of the peak.

A. Electrochemical peak of standard 8-oxo-guanosine;

B. Ectrochemical peak of 8-oxo-guanosine in total RNA;

C. UV peak of standard guanosine;

D. UV peak of guanosine in total RNA

RNA oxidation *in vitro*

10 µg RNA is incubated in *in-vitro* oxidation buffer for 1 hour. *In vitro* oxidation buffer includes 300 mM PBS pH6.8, 30 mM Ascorbic Acid, and desired H_2O_2 concentration in 30 µl at 37^0C. After one hour's incubation, 0.3 mM Sodium Acetate is added into the reaction and 3 volume's ethanol is also added into the reactions. RNA is precipitated at 12000 g for 15 min, and is washed by 75% ethanol. RNA pellet is dissolved in 20 µl of DEPC-treated ddH_2O.

Treatment of E. coli cultures with H_2O_2

Overnight *E. coli* cultures of were diluted into fresh M9 medium and growth was continued until OD_{550} reaches ~0.5. The cultures were then divided into two parts, to which a series of H_2O_2 concentrations was added respectively. The cultures were incubated for different time. RNA was isolated as described above and the level of 8-oxoG is represented as number of 8-oxoG per 10^5 G.

Protein assay

Protein concentrations were determined by Bradford method (Bradford, 1976) using a protein assay kit from Bio-Rad. BSA (Bovine Serum Albumin) was used as protein standard.

PNPase degradation activity assay

Activity of PNPase in cell extracts was determined as the following. Cultures in YT medium were collected to make cell extract by sonication (Sonication buffer: 20 mM Tris-Cl pH7.5 and 0.1 mM DTT). Protein concentration was determined by the Bradford solution (Sigma-Aldrich, St. Louis, MO) using bovine serum albumin as standard. PNPase activity in cell extracts were assayed by the release of acid-soluble products from [^{3}H]-labeled poly (A). The reaction mixture contained 50 mM Tris-Cl, pH8.0, 5 mM $MgCl_2$, 160 mM KCl, 10 mM K_3PO_4, pH7.5, 40 µg [^{3}H]poly(A) (100 cpm/nmol), and ~6 µg of cell extracts. Acid-soluble radioactivity was measured as described (Cheng et al, 1998).

Isolation of RNA from cultured HeLa cell and analysis of 8-oxoG levels

Total RNA was isolated from cultured HeLa cells using TRI Reagent (Molecular Research Center). Guanosine and 8-oxoG in RNA were determined after HPLC separation according to conditions described previously (Fiala et al., 1989). The level of 8-oxoG is represented as number of 8-oxoG per 10^5 G.

Analysis of the level of hPNP mRNA

Total RNA samples were treated with the TURBO DNA-free Kit (Ambion) to remove residual DNA. Relative levels of *hPNP* mRNA were analyzed by semi-quantitative reverse transcription-PCR (RT-PCR) using human *GAPDH* (*hGAPDH*) mRNA as internal normalization control, as previously described (Kantorow et al., 2004; Nagaike et al., 2005). The *hPNP* primers and *hGAPDH* primers are listed in table 1. PCR products from desired cycles were separated on agarose gels and quantified after staining with ethidium bromide using the Epi Chemi II Darkroom (UVP Laboratory Products).

Table 1 RT-PCR primers for quantification of hPNP mRNA

Primer	Sequence	Amplicon (bp)
hPNP forward_1	5'-TTGTTGGACCTGGTGGCTAT-3'	425
hPNP reverse_1	5'-TCTGACCACGGTTGTAGCTG-3'	
hPNP forward_2	5'-ACCCTTCATGGATCAGCATT-3'	349
hPNP reverse_2	5'- ATGCTAAACTTCCGCCACAT-3'	
hGAPDH forward	5'-CGGAGTCAACGGATTTGGTC-3'	516
hGAPDH reverse	5'-ACTGTGGTCATGAGTCCTTC-3'	

Treatment of HeLa cells with H_2O_2 and determination of cell viability

HeLa cells were cultured in a humidified atmosphere with 5% CO_2 at 37°C in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 4 mM L-glutamine, 1% penicillin and 1% streptomycin. According to a procedure described previously (Kantorow et al., 2004), HeLa cultures were treated with H_2O_2 for certain length of time prior to the MTS assay of cell viability using the CellTiter 96® AQ_{ueous} One Solution Cell Proliferation Assay kit (Promega). Relative cell viability in H_2O_2-treated cultures was presented as the percentage of the residual MTS-reducing activity.

Transfection of DNA or siRNA into HeLa cells

HeLa cultures were transfected with DNA constructs (2 μg/ml medium) using Lipofectamine 2000 (Invitrogen). siRNA constructs (5 nM) were introduced into HeLa cells using the RNAi Human/Mouse Starter Kit (Qiagen).

RESULTS

Section 1: The role of *E. coli* PNPase under oxidative stress

1.01 Polynucleotide phosphorylase is evolutionarily conserved

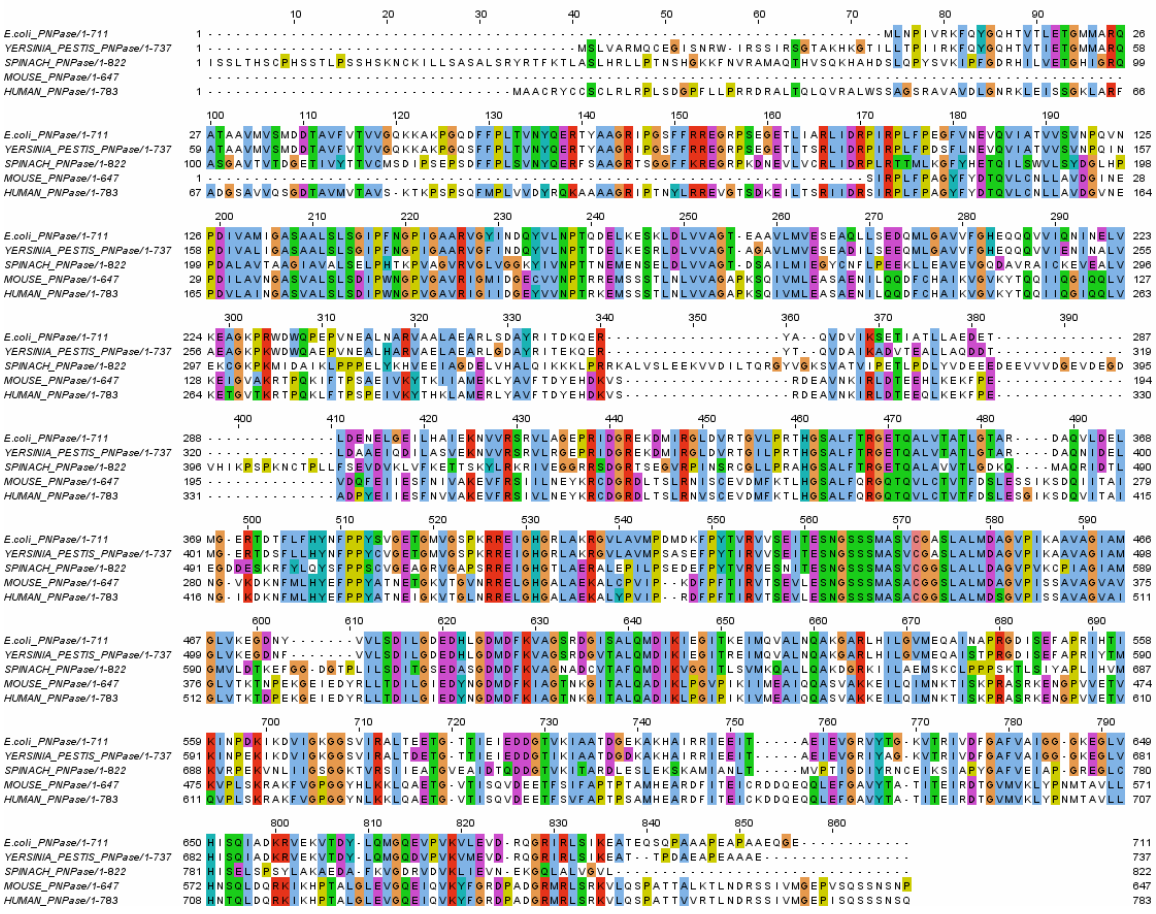


Fig. 7 Multiple sequence alignment of polynucleotide phosphorylase in five types of organisms including *E. coli*, *Yersinia pestis*, spinach, mouse, and human by software CLUSTAL W (1.83)(Chenna et al., 2003)

Genome sequence analysis revealed that PNPase gene is widely distributed in a variety of bacteria including aerobic, anaerobic, halophilic, and thermophilic bacteria, in most of single-celled eukaryotes, in all plants, and in all animals (Littauer, 2005). The PNPase gene is missing in all of *Archaea*-sequenced genomes, and it also doesn't exist in some single-celled eukaryotes like yeast (Littauer, 2005). As shown in Fig 7, the conservation of PNPase amino acid sequences among five organisms, including *E. coli, Yersinia pestis,* spinach, mouse, and human, was analyzed by multiple sequence alignment with Software Clustal. The results show that PNPase is highly evolutionarily conserved among these five organisms.

Table 2 The similarity of polynucleotide phosphorylase's amino acid sequences in five types of organisms including *E. coli, Yersinia pestis*, spinach, mouse, and human.

Species	Len(aa)	Species	Len(aa)	Identity	Similarity
E. coli	711	Y.PESTIS	737	88.4%	94.6%
E. coli	711	SPINACH	822	42.1%	59.2%
E. coli	711	MOUSE	647	39.7%	58.2%
E. coli	711	HUMAN	783	39.8%	57.2%

**Based on EMBOSS Local Pairwise Alignment Analysis from EBI, Matrix: Blosum62; Open gap penalty: 10; Gap extension penalty: 0.5(http://www.ebi.ac.uk/emboss/align/.)*

Identity and similarity of PNPase in these five types of organisms was further analyzed by local pairwise alignment as listed in table 2. The data not only indicated that PNPase is evolutionarily conserved, but also revealed that the conservation among prokaryotic organisms is significantly higher than that between prokaryotic organisms and eukaryotic organisms.

1.02 *E. coli* PNPase specifically binds to oxidized RNA

To understand RNA surveillance mechanisms in *E. coli* under oxidative stress, an RNA-binding proteins assay was carried out to search oxidized RNA-binding proteins (oxoRBPs). Identification of proteins binding specifically to oxidized RNA can lead to the comprehension of their roles *in vivo* and contribute to the discovery of oxidized RNA metabolism. We screened oxidized RNA-binding proteins (oxoRBPs) on a large scale using a RNA-binding assay (Zahler et al., 2004). This RNA-binding assay uses an unoxidized/oxidized oligoribonucleotide attached to agarose beads, the cellular extract is incubated with oligoribonucleotide attached to the agarose beads, and the proteins binding specifically to the beads are eluted and separated by SDS-PAGE. Finally, those proteins are identified by mass spectrometry.

The 50-mer oligoribonucleotide was used to search for oxidized RNA-binding proteins. RNA can be oxidized *in vitro* by H_2O_2 and the higher the concentration of H_2O_2, the higher the level of RNA oxidation. The level of RNA oxidation is dependent on the concentration of H_2O_2 in the range of 0.1~1 mM H_2O_2 (data not shown). As shown in Fig 8, one major protein (black arrow), which comes from the cellular extract of wild type cells, has a higher affinity to 0.5 mM H_2O_2-oxidized RNA than to normal RNA. In

contrast, there is no major protein in the corresponding position in the gel when the cellular extract, which is made from *E. coli* double mutants lacking PNPase and RNase G, is used to screen oxidized RNA-binding protein using RNA-binding assay. More importantly, the intensity of that protein is dependent on H_2O_2-treatment when only cellular extract from the wild type cells is used for the screening of oxoRBPs (lane 3 and lane 4 in Fig 8). These results suggest that that major protein has a higher affinity to oxidatively-damaged RNA. Compared to the protein size marker (not shown), the molecular weight of that protein, is estimated to be ~80 kDa, which is close to *E. coli* PNPase's MW, 77 kDa. That major protein also co-migrates with the purified *E. coli* PNPase (arrow) in the gel as shown in Fig 9. Mass-Spectrometry analysis (MS-ESI) of that protein band indicates that *E. coli* PNPase protein with a highest score of 715 exists in that protein band. This further suggests that *E. coli* PNPase is one of the proteins, which can specifically binds to oxidized RNA protein. These data are consistent with a previous report that PNPase has a higher binding activity to a poly (8-oxoG:A) (Hayakawa et al., 2001) though oxidatively-damaged natural RNA is used here. All of the above data demonstrate that *E. coli* PNPase, a major 3'->5' exoribonuclease, has a higher affinity to oxidatively-damaged RNA than normal RNA. *E. coli* PNPase may function *in vivo* in discriminating the oxidatively-damaged RNA from RNA pool and degrading it by itself or targeting it for the degradation by other RNases' activity/activities.

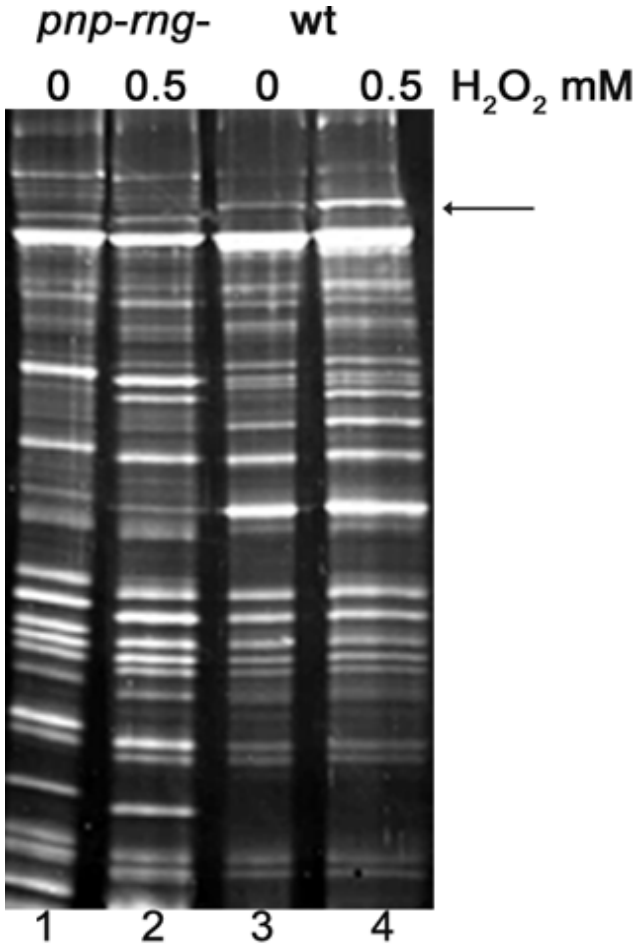


Fig. 8 *E. coli* PNPase specifically binds to oxidized RNA

According to RNA-binding protein assay, total cellular extracts from the wild type cells and double mutant cells lacking both PNPase and RNase G were respectively incubated with 50 mer RNA oligo which is in-vitro oxidized by 0 or 2.0 mM H_2O_2. Eluted RNA-binding proteins were separated in SDS-PAGE gel. One of oxidized RNA-binding proteins is shown by black arrow (Generated by Dr. Zhe Jiang).

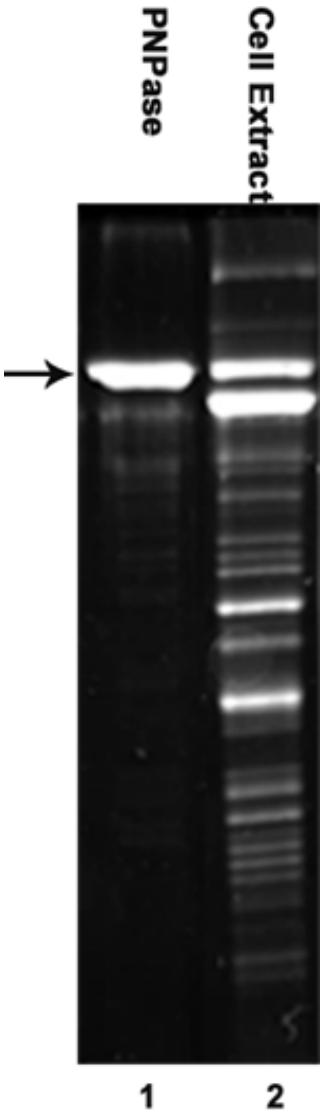


Fig. 9 The oxidized RNA-binding protein (black arrow) in cellular extract co-migrates with the purified *E. coli* PNPase

Purified E. coli PNPase is loaded in lane 1 of SDS-PAGE gel; Total cellular extract is loaded in lane 2 of SDS-PAGE gel (Generated by Dr. Zhe Jiang).

1.03 An *E. coli* mutant lacking PNPase is hypersensitive to hydrogen peroxide

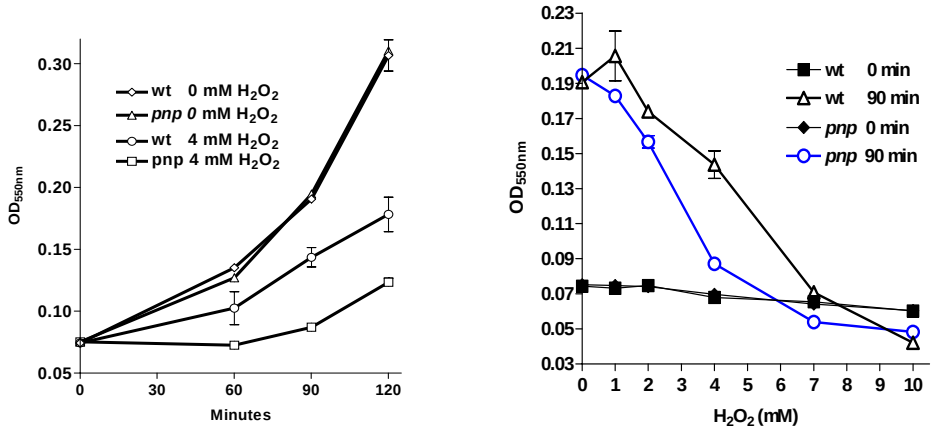


Fig. 10 *E. coli pnp* mutant is more sensitive to hydrogen peroxide than the wild type cell in liquid culture

E. coli culture (OD_{550nm}=0.2) is treated with a series of H_2O_2 concentrations, and then 100 ul of E. coli culture per well was immediately transferred into 96-well plate and cultured at 37°C, 150rpm. At different time points, OD_{550nm} value was determined by plate reader, and the growth curve was plotted with the time (left panel) or with the dosage of H_2O_2 (right panel). Sample number is 4.

E. coli PNPase can specifically bind to oxidized RNA (Fig. 8 and Hayakawa et al., 2001), so *E. coli* PNPase may play an important role in protecting the cells against oxidative stress by discriminating oxidatively-damaged RNA from RNA pool. To examine this possibility, the viability of *E. coli* wild type and *pnp* mutant cells was examined under H_2O_2-treatment with. A *pnp* gene-deletion mutant, in which the wild type *pnp* gene was completely replaced by a kanamycin gene cassette , was confirmed by both PCR amplification (data not shown) and PNPase's activity for the degradation of ^{3}H-

poly(A) (data not shown). Cell viability was determined by three types of growth experiments under the challenge of H_2O_2 in the following descriptions.

In liquid culture, *E. coli* culture was challenged with a series of H_2O_2 concentrations for different time in a 96-well plate. Under normal conditions, the wild-type cells and the mutant cells grow at a similar rate. However, under the challenge of H_2O_2, the *pnp* mutant has a significant growth deficiency compared with wild type cell (Fig 10; the results of only 4 mM H_2O_2 treatment for different time are shown in the left panel of Fig 10 and the results of different concentrations of H_2O_2 -treatment only for 90 minutes are shown in the right panel of Fig 10).

Growth experiments in liquid culture can give us a rough idea about the sensitivity of *E. coli pnp* mutant to H_2O_2, it is still not very clear because optical density (OD) can not clearly differentiate live *E. coli* cells from dead cells. Additionally, cell size may also affect the OD values of the cell culture. Therefore, the H_2O_2-sensitivity of *E. coli pnp* mutant was further determined by the ability to forming colonies. Exponentially-growing cultures(OD_{550nm}=0.2) of *E. coli* wild type and *pnp* mutant cells were challenged with the indicated concentrations of H_2O_2 for 90 min, the treated cell cultures were diluted by the ratio of 1:1000, 1:5000, and 1:20,000 respectively and finally the diluted culture was evenly plated on the plates. The plates were incubated overnight at 37^{o}C and colony-forming units (CFU) plotted for H_2O_2-treated cultures are expressed as a percentage of the CFU values for untreated cultures.

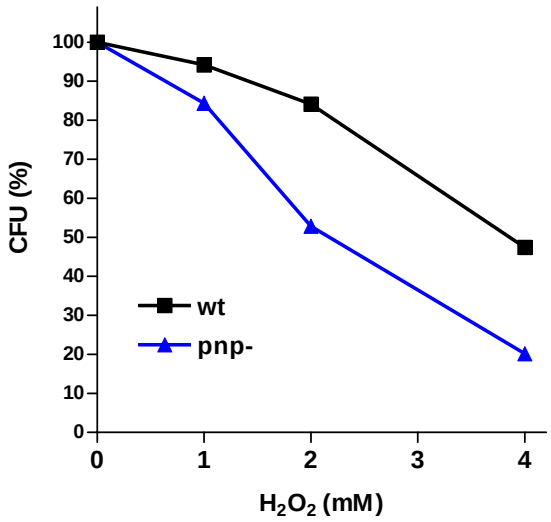


Fig. 11 *E. coli pnp* mutant has a less ability to form colonies than wild type cell under H_2O_2 treatment

Exponentially growing cultures of E. coli wild-type (wt, filled squares) and pnp mutant (pnp, filled triangle) were challenged with the indicated concentrations of H_2O_2 for 90 min and then plated on YT medium plate. Colony-forming units (CFU) plotted are expressed as a percentage of the CFU values for untreated cultures.

As shown in Fig 11, the *pnp* mutant cells has a less ability to form colonies thatn wild type cells under H_2O_2. 1 mM H_2O_2 concentration resulted in a 16% loss of colonies in the wild type cells comparing to a 6% loss for the wild-type strain. Using a 2 mM H_2O_2 concentration, a 16% loss of colonies was observed for the wild-type strain, whereas the ability to form colonies dropped 47% in the *pnp* mutant. Under the treatment of 4 mM H_2O_2 concentration, a 53% and 80% loss of colonies were observed for the wild type and the *pnp* mutant strains respectively. This result further confirmed that the *pnp* mutant is hypersensitive to H_2O_2.

The hypersensitivity of the *pnp* mutant to H_2O_2 was further confirmed by a spot assay. As shown in Fig 12, in the presence of 0.6 mM and 0.8 mM H_2O_2, the *pnp* mutant shows increased sensitivity to H_2O_2. On the YT agarose plate containing 0 mM and 0.4 mM H_2O_2 (data are not shown), the growth of wild-type cells and the *pnp* mutant has no apparent difference. Growth of wild-type cells and the *pnp* mutant were completely inhibited on the YT agarose plate containing 1 mM H_2O_2 (data are not shown).

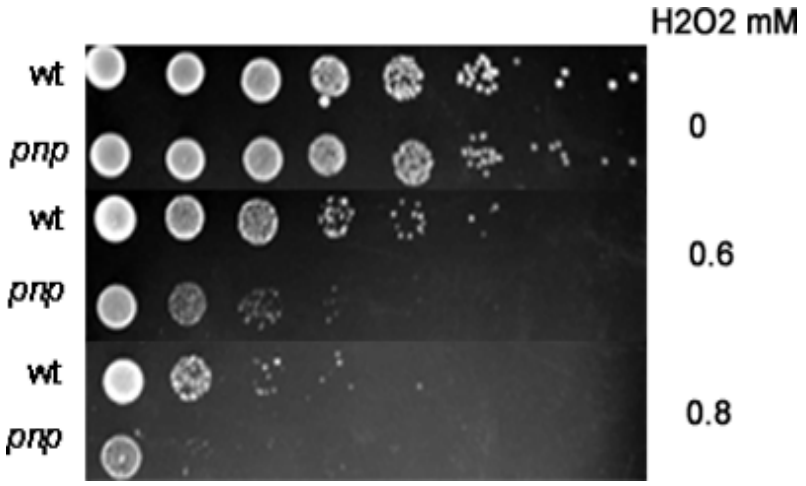


Fig. 12 *E. coli pnp* mutant is more sensitive to H_2O_2 than the wild type cell in a spot assay

2 ul of gradually-diluted E. coli cultures (dilution ratio is 1:5) were spotted on YT medium plates containing the indicated concentrations of H_2O_2. The E. coli spots were observed after the incubation for 14~16 hour. The experiment was repeated twice.

It is well known that methionine sulfoxide reductase A (MsrA) can rescue the cells under oxidative stress by repairing the oxidized methionine in the protein (Weissbach et al., 2005). *E. coli msrA* mutant displayed enhanced sensitivity to oxidative stress and lower survival rates (Moskovitz et al., 1995). Here the sensitivity of *E. coli pnp* mutant

and *E. coli msrA* mutant to H_2O_2 was compared. As shown in Fig 13, an *E. coli pnp* mutant even had a little more sensitivity to H_2O_2 than the *msrA* mutant.

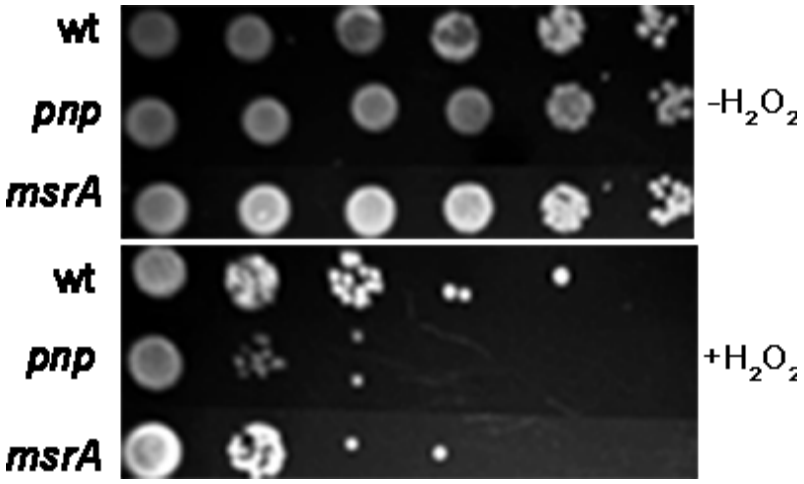


Fig. 13 Comparison of cell viability among wild type, the *pnp* mutant and the *msrA* mutant after H_2O_2 treatment

2 ul of gradually-diluted E. coli cultures (dilution ratio is 1:5) were spotted on YT medium plate containing the indicated concentrations of H_2O_2. The E. coli spots were observed after the incubation for 14~16 hour. The experiment was repeated twice. The msrA mutant was constructed by transferring the mutant msrA allele, which is kindly provided by Dr. Herbert Weissbach (Moskovitz et al., 1995).

In all the growth experiments shown above, H_2O_2 consistently caused more cell death to the *pnp* mutants than to wild-type cells, though the relative hypersensitivities of the mutants varied slightly from one experiment to another. It indicates that the 3'->5' exoribonuclease PNPase is involved in protecting cells against oxidative stress by a mechanism that has not been described previously. These results suggest that the absence of the exoribonuclease PNPase causes elevated cellular damage under H_2O_2 treatment. Deficiency in the PNPase's activities may directly or indirectly affect cell's ability to

remove oxidatively-damaged RNA, perhaps causing RNA dysfunction and further increasing growth arrest or cell death.

1.04 PNPase deficiency does not affect the hydrolysis of H_2O_2 in the cells

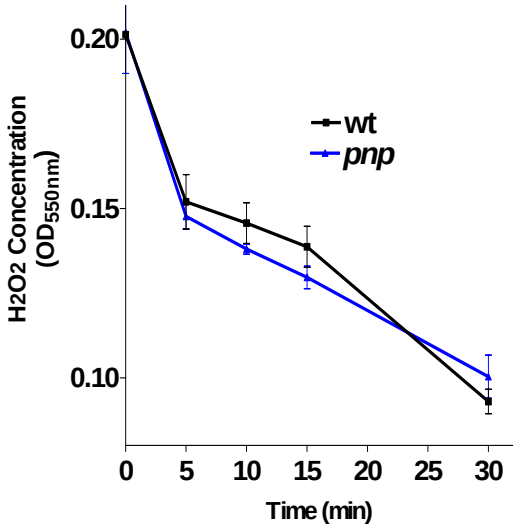


Fig. 14 Comparison of H_2O_2 hydrolysis rate in the culture of wild type and *pnp* mutant

The proper quantity of H_2O_2 was put into the culture with an OD_{550} of 0.5 to form a final concentration of 0.5 mM H_2O_2 in YT medium. Samples were taken at different time points and assayed for the content of H_2O_2 using Amplex Red H_2O_2 Kit.

Under normal conditions, there is a well-balanced antioxidant-oxidant system to maintain cell's normal function. Any disruption of this system may change the cells' ability to survive against oxidative challenge. As a 3'->5' exoribonuclease, *E. coli* polynucleotide phosphorylase normally plays a major role in mRNA decay. PNPase-deficiency may change the expression levels of some genes such as the genes encoding the enzymes superoxide dismutase (SOD) and catalase, which work as an immediate

antioxidant system. The phenotypes of the *pnp* mutant under oxidative stress is likely caused by a deficiency in the removal of H_2O_2 in the culture. In order to examine this possibility, the hydrolysis rate of H_2O_2 is compared in the cultures of *E. coli* wild type cells and the *pnp* mutant cells. As shown in Fig. 14, H_2O_2 is degraded rapidly in the cultures of both the wild type and the *pnp* mutant strains without significant difference. This data suggests that *E. coli* PNPase does not affect the hydrolysis of H_2O_2 and *E. coli* PNPase most likely plays a role in directly controlling the quality of RNA under oxidative stress.

1.05 8-oxoG-containing RNA is specifically removed *in vivo* depending on PNPase

E. coli PNPase has a higher affinity to oxidized RNA than normal RNA and the mutant lacking *E. coli* PNPase is more sensitive to H_2O_2. These results prompt us to hypothesize that *E. coli* PNPase, a major 3'->5' exoribonuclease, may firstly discriminate oxidatively-damage RNA by specific recognition and further eliminate oxidized RNA in order to protect the cells against oxidative stress. To examine this hypothesis, 8-oxoG containing RNA was tracked in wild type and *pnp* mutant cells under H_2O_2-treatment. 0.2 ml of overnight *E. coli* cell culture including wild type and *pnp* mutant cells was inoculated into 25 ml culture media and was cultured at 37^0C till an OD_{550nm} of 0.5. The cultures were continually treated with 0 and 0.5 mM H_2O_2 for 15 minute respectively. At the time point of 15 minutes, 400μg/ml rifamycin was added into the culture to immediately stop the synthesis of new RNA. 1 ml of the culture was taken out for the analysis of RNA oxidation at the indicated time points of 0, 15, 30, 60, 90 minutes, The levels of RNA oxidation, the number of 8-oxoG per 10^5G, was determined by HPLC.

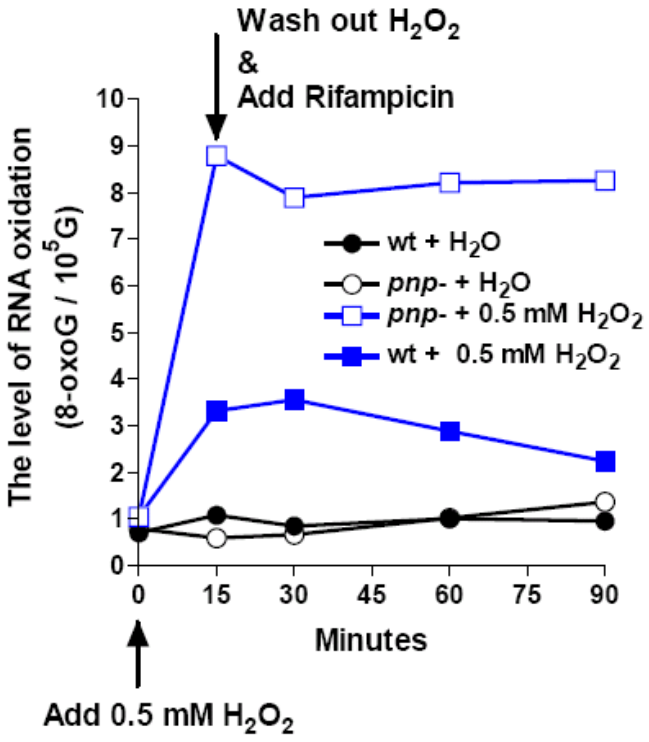


Fig.15 8-oxoG-containing RNA is specifically removed *in vivo* depending on PNPase

Cultures of E. coli wild type and pnp mutant cells were treated with and without 1 mM H_2O_2 respectively. After 15 minutes, the residual H_2O_2 was removed and rifamycin of 200μg/ml was added to stop RNA synthesis. RNA was isolated from the culture samples taken at the indicated time points, and the levels of 8-oxoG in RNA were determined by HPLC. The experiment was repeated for 3 times (Nithya Bhagavatula contributed part of this work).

As shown in Fig. 15, under normal conditions, the basal levels of RNA oxidation in both *E. coli* wild type and *pnp* mutant cells are almost unchanged in 90 minutes and the basal levels of RNA oxidation in *E. coli* wild type cells is similar to that in the *pnp* mutant. After exposure to 0.5 mM H_2O_2, both wild type and *pnp* mutant have an elevated RNA oxidation level. However, there is a significantly higher level of RNA oxidation in the *pnp* mutants than in wild type cells after treatment with 0.5 mM H_2O_2. The level of 8-

oxoG in total RNA increase to the highest one from 15 minutes to 30 minutes, then decreases gradually after 30 minutes, and almost goes back to the basal level at 90 minute in wild type cells after H_2O_2-treatment for 15 minutes. This data suggest that there are specific mechanisms to eliminating oxidatively-damaged RNA inside *E. coli* cells. However, the level of 8-oxoG in total RNA dramatically increases to 8 8-oxoG/10^5G, then decreases a little bit from 15 minutes to 30 minutes, and is almost unchanged from 30 minutes to 90 minutes in *pnp* mutant cells after H_2O_2-treatment for 15 minutes. These data suggest that *E. coli* PNPase plays a critical role in removing oxidatively-damaged RNA in the cells.

1.06 Cloned PNPase gene rescues the *pnp* mutant under oxidative stress by reducing RNA oxidation level and increasing cell viability

To examine whether PNPase activity is directly co-related to H_2O_2-hypersensitivity and the level of RNA oxidation in the cell, the *pnp* gene encoded by a medium-copy plasmid (10~20 copy number) (Donovan and Kushner, 1986), was introduced into wild type and the *pnp* mutant cells.

Wild type and the *pnp* mutant cells were transformed with pKAK7 (pnp+) and the control vector pBR322 respectively. As shown in Fig. 16, the expression of the plasmid-born PNPase was confirmed by PNPase's poly(A) degradation activity assay. The cellular extract from the *pnp* mutant transformed with control vector, as a negative control, only shows a little poly(A) RNA degradation activity, which is regarded as background activity and is set to 0 (Fig. 16). The cellular extract from wild type cells transformed with control vector was considered to have normal PNPase degradation activity. The cellular extracts from wild type cells and the *pnp* mutants transformed with

pKAK7 (pnp+) display 2.5 and1.5 fold of normal PNPase activity respectively (Fig. 16). These four strains were also confirmed by both PCR amplification in the *pnp* gene and plasmid map analysis (data not shown).

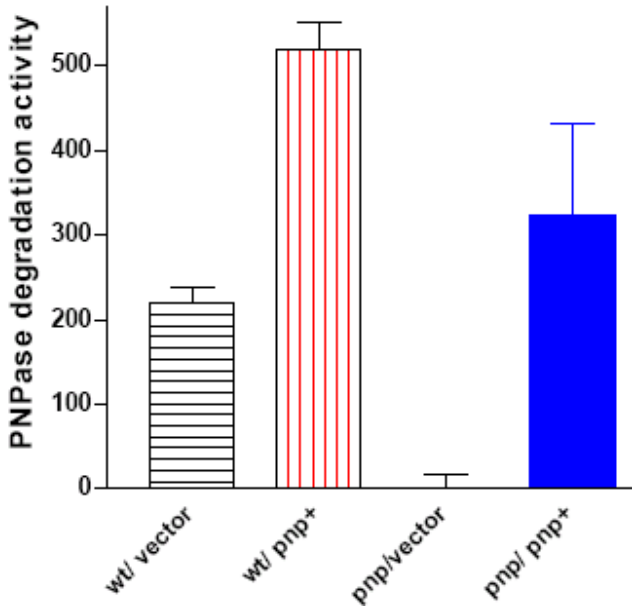


Fig.16 The *pnp* gene complementation in the *pnp* mutant was confirmed by poly(A) RNA degradation activity

The control vector means plasmid pBR322 and pnp$^+$ means plasmid pKAK7 encoding E. coli PNPase. The plasmids pBR322 and pKAK7 were respectively transformed into wild type cell and the pnp mutant to generate wt/vector, wt/pnp+, pnp/vector, and pnp/pnp+.

After validating the strains and plasmids, we examined whether cloned PNPase can rescue the cell viability of a *pnp* mutant under a H_2O_2 challenge. As shown in Fig. 17, wild type cells with a PNPase expression plasmid shows only a little growth defect under oxidative stress conditions and it could be due to the toxicity of over-expressed *E. coli* PNPase; the *pnp* mutant with control vector is hypersensitive to H_2O_2, which is consistent

with the result of Fig. 12. Importantly, compared to the vector control, introduction of the cloned *pnp* gene into *pnp* mutant cells almost completely restored cell viability when treated by H_2O_2. This restoration was consistent with the finding that the *pnp* mutant with the overexpression of *E. coli* PNPase has 150% of the endogenous PNPase activity.

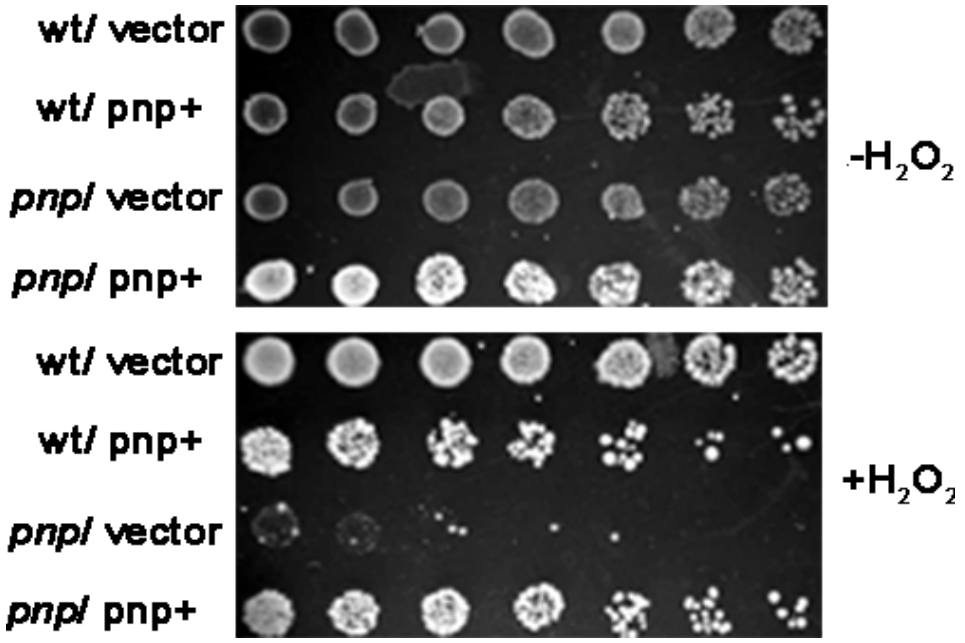


Fig. 17 Cloned PNPase gene restores cell viability of *E. coli pnp* mutant under H_2O_2 treatment

The control vector means plasmid pBR322 and pnp+ means plasmid pKAK7 encoding E. coli PNPase. Plasmids pBR322 and pKAK7 were respectively transformed into wild type cell and the pnp mutant to generate wt/vector, wt/pnp+, pnp/vector, and pnp/pnp+.

To further examine whether PNPase activity is directly related to the level of RNA oxidation in the cell, the levels of RNA oxidation were determined and compared among the wild type cells and *pnp* mutant cells, respectively, transformed with control vector

and PNPase expression plasmid after the culture was treated with 0.5 mM H_2O_2 for 15 minutes. As shown in Fig. 18, the basic levels of RNA oxidation are similar and H_2O_2-treated cells always have a higher RNA oxidation level than H2O-treated cell in each of four strains. Under the treatment of H_2O_2, introduction of cloned *pnp* gene into wild type cells didn't cause the significant change of RNA oxidation level compared to the vector control. However, *pnp* mutant cells transformed with control vector had a significantly-elevated RNA oxidation level. More importantly, compared to the vector control, introduction of the cloned *pnp* gene into *pnp* mutant cells almost completely decreased the level of RNA oxidation when treated by 0.5 mM H_2O_2.

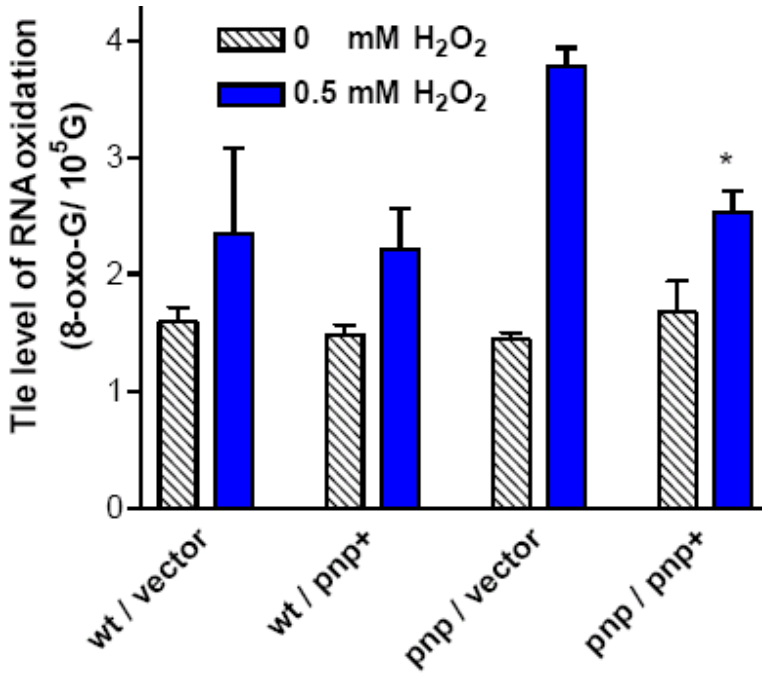


Fig. 18 Cloned PNPase gene reduced the elevated levels of RNA oxidation in pnp mutant under H_2O_2 treatment

The control vector is plasmid pBR322 and pnp$^+$ means plasmid pKAK7 encoding E. coli PNPase. Plasmid pKAK7 encoding E. coli PNPase and empty pBR322 vector were transformed into wild type and pnp mutant respectively.

In a conclusion, both the restoring of cell viability and the reduction of 8-oxoG levels in the *pnp* mutant under the treatment of H_2O_2 can be reasonably explained by the recovered *E. coli* PNPase activity after the introduction of cloned PNPase gene into the *pnp* mutant cell.

1.07 *E. coli* PNPase facilitates the degradation of ompF mRNA under oxidative stress

Elevated level of RNA oxidation in total cellular RNA in an *E. coli pnp* mutant can be restored by introduction of cloned PNPase gene. This strongly suggested that *E. coli* PNPase plays a significant role in removing oxidatively-damaged RNA. If *E. coli* PNPase really plays an important role in removing oxidized RNA, oxidized RNA in a *pnp* mutant should accumulate under H_2O_2 treatment. To examine this hypothesis, the quantity of higher abundant mRNAs including *ompF*, *rpsA*, and *tufA,* which were shown to be degraded under H_2O_2 treatment (Chang et al., 2002), was determined by northern-blotting analysis in wild type strain and the *pnp* mutant under normal conditions and after H_2O_2 treatment.

As shown in the Fig 19, the levels of the full length mRNAs of *ompF* were down-regulated in the cells under the treatment of H_2O_2, which is consistent with microarray data generated from (Chang et al., 2002). More importantly, we discovered that compared with wild type cells, the full length *ompF* mRNA significantly accumulated under H_2O_2 treatment of in the *pnp* mutant cells. One possible explanation is that oxidized *ompF*

mRNA can be specifically degraded by *E. coli* PNPase in wild type cells but it can't be in the *pnp* mutant cells. As shown in Fig 19, *ompF* mRNA accumulation in *pnp* mutant cells was quantified under the treatment of H_2O_2. In the *pnp* mutant, other mRNAs including fusA and rpsA also accumulated after treatment with H_2O_2, though they have less accumulation than *ompF* mRNA (data not shown).

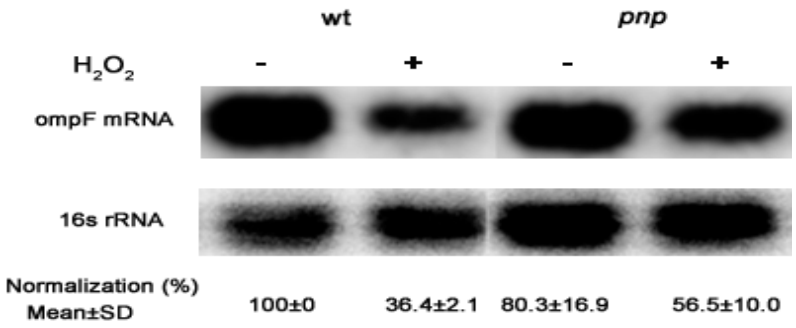


Fig. 19 The accumulation of *ompF* mRNA in the *pnp* mutant under H_2O_2 treatment

Both wild type and pnp mutant strains were grown to exponential phase and the cultures were treated with either 1.5 mM H_2O_2 (oxidized) or with H_2O (unoxidized) for 10 minutes. The culture were collected and lysed by lysis buffer (see methods). Total RNA in the cell lysate was separated on 1.0% agarose gels, blotted onto the nylon membrane, and hybridized with the ^{32}P-labelled probes. ompF mRNA was probed with internally ^{32}P-labelled DNA probe and 16S rRNA, as an internal control, was probed with 5' end-^{32}P-labelled DNA probe. Northern blotting revealed the band corresponding to full-length ompF mRNA as a sharp band. The experiment was repeated twice. The signal intensity for both full-length mRNA band and 16S rRNA was determined of by UVP. The signal of mRNA is normalized by the signal of 16S rRNA in the same loading sample. In

wild type cells without H_2O_2-treatment, the relative intensity signal ratio of ompF mRNA and 16srRNA was set to 100%. (Generated by Gayatri Kollipara)

1.08 Association with the degradosome is not required for *E. coli* PNPase to protect cells against oxidative stress

Since about 30% of total *E. coli* PNPase exists in the RNA degradosome (Liou et al., 2001), we were prompted to ask if integration of *E. coli* PNPase into the degradosome is important for its protective role under oxidative stress. To answer this question, we made use of an RNase E construct lacking the PNPase binding domain (RneΔ10 lacking amino acids 844-1045, Leroy et al., 2002). This truncated RNase E is expected to interact with other members of the degradosome except with *E. coli* PNPase. Cells substituting the wild type RNase E with RneΔ10 grow relatively normally (Leroy et al., 2002; Fig. 20, upper panel).

Interestingly, the strain carrying RneΔ10 grows better than the wild type in the presence of H_2O_2 (Fig. 20, lower panel). Consistent with cell viability, cell extract made from the *rneΔ10* cultures have a slightly higher PNPase activity than the wild type extract (Fig. 21). We have introduced the *pnp* null mutation to the *rneΔ10* strain. Remarkably, the *rneΔ10, pnp* mutant shows a H_2O_2-sensitivity similar to the *pnp* mutant (Fig. 20, lower panel). This result suggests that under H_2O_2 treatment, *E. coli* PNPase protects *rneΔ10* cells in the same way, if not better, as it does on cells harboring the wild type RNase E. The data is consistent with the notion that association of *E. coli* PNPase with

the degradosome is dispensable for the protective function of *E. coli* PNPase under oxidative stress.

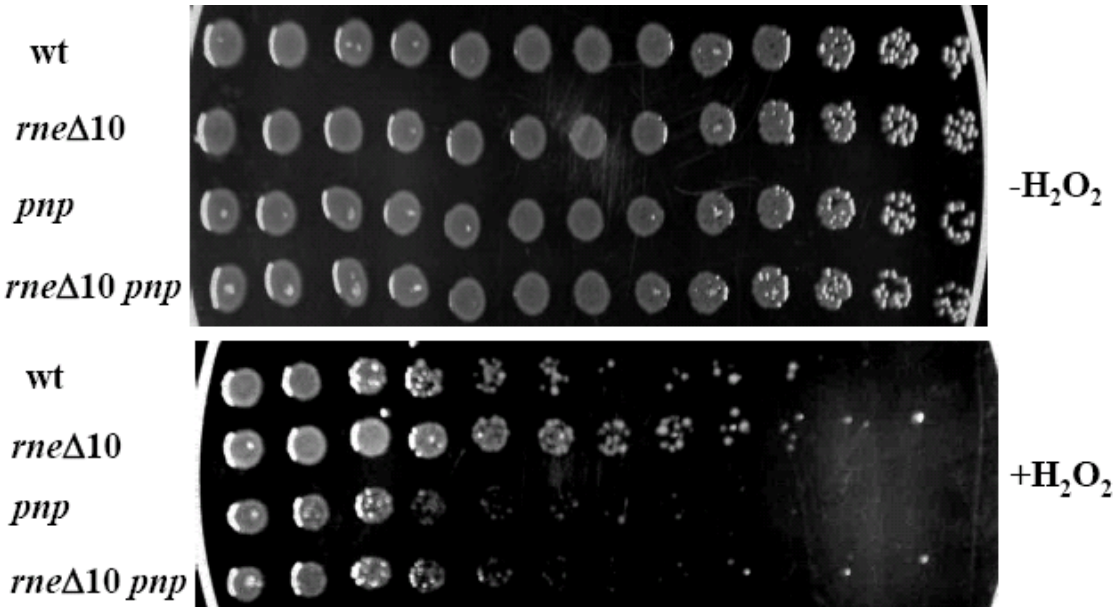


Fig. 20 Association with the degradosome is not required for *E. coli* PNPase to protect cells under H_2O_2 treatment

E. coli cultures with an OD_{550nm} of 0.05 were gradually-diluted at the ratio of 1:5 and 2 ul of diluted cultures was spotted on YT medium plates containing the indicated concentrations of H_2O_2. The E. coli spots were observed after the incubation for 14~16 hour. The experiment was repeated twice. wt, wild type cells; pnp, a mutant lacking E. coli PNPase; rneΔ10, a rne mutant which encodes a truncated RNase E with the loss of the PNPase binding domain of amino acids 844-1045; rneΔ10 pnp, a double mutant lacking both E. coli PNPase and the above-mentioned truncated RNase E. Here wild type strain and the rneΔ10 mutant strain, whose original names are AC21 and AC24 respectively, was nicely offered by Dr. A. J. Carpousis.

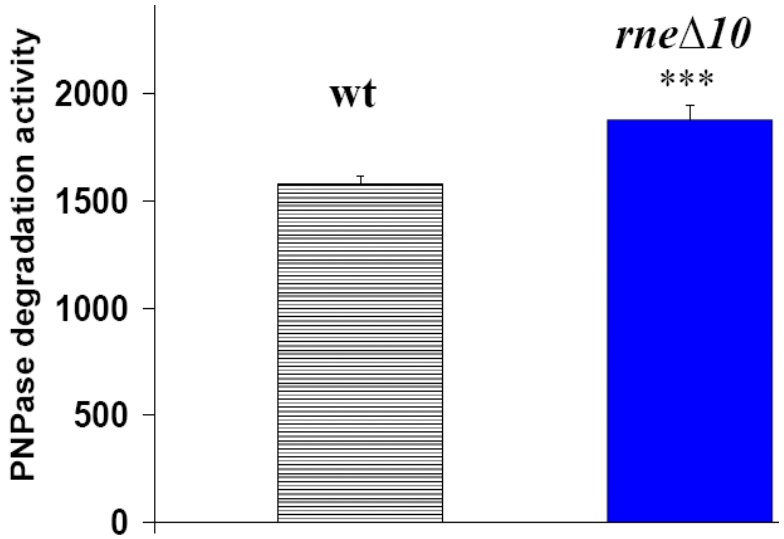


Fig. 21 The *rneΔ10* mutant has more PNPase degradation activity than wild type cell.

The *rneΔ10 mutant is an rne mutant which encodes a truncated RNase E lacking amino 844-1045.*

1.09 *E. coli* poly(A) polymerase facilitate polynucleotide phosphorylase in protecting the cells against oxidative stress and removing oxidized RNA

In the pathway of mRNA degradation, poly(A) polymerase I assisted *E. coli* PNPase to degrade RNA by the polyadenylation of RNA decay intermediates (Khemici and Carpusis, 2004; Li et al. 2002), so polyadenylation may also facilitates *E. coli* PNPase to selectively degrade oxidized RNA and protect the cells against oxidative stress. To examine this possibility, a double mutant *pcnB pnp* lacking both *E. coli* PNPase and *E. coli* poly(A) polymerase I was generated by introducing a *pnp* gene-deletion mutation into a *pcnB* mutant lacking *E. coli* poly(A) polymerase I. *pcnB* gene

encoding poly(A) polymerase I is not an essential gene and the growth rate of cells lacking poly(A) polymerase I is not affected (Li et al., 2004). The H_2O_2-sensitivity of wild type cells, *pcnB* mutants, *pnp* mutants, and *pcnB pnp* double mutants was measured by a spot assay. As shown in Fig 22, *pcnB* single mutant's sensitivity to H_2O_2 is similar to wild type, the *pnp* single mutant is more sensitive to H_2O_2 than wild type, and the double mutant *pcnB pnp* is much more sensitive to H_2O_2 than the *pnp* single mutant. Apparently, the absence of poly(A) polymerase accentuates the sensitivity of the *pnp* single mutant to H_2O_2 though *pcnB* single mutant's sensitivity to H_2O_2 is similar to wild type. These data suggests that *E. coli* PNPase is assisted by poly(A) polymerase in protecting the cells under oxidative stress.

We further ask how *E. coli* poly(A) polymerase facilitates PNPase to protect the cells against oxidative stress. In mRNA decay, poly(A) polymerase I facilitates polynucleotide phosphorylase to degrade RNA decay intermediates through polyadenylation. Possibly, poly(A) polymerase I also helps PNPase to selectively degrade oxidatively-damaged RNA. 8-oxoG-containing RNA was tracked in all of four strains of wild type, *pcnB, pnp,* and the double mutant of *pcnB pnp* after treatment with 1 mM H_2O_2 for 15 minutes. As shown in Fig 23, without H_2O_2-treatment, there was no significant difference in the levels of RNA oxidation among these four strains; With the exposure to 1 mM H_2O_2, the level of RNA oxidation in the *pnp* single mutant is higher than of wild type cells, and the RNA oxidation level of *pcnB pnp* double mutant is much higher than of wild type cells and higher than pnp single mutant though the level of RNA oxidation in the *pcnB* single mutant is similar to that of wild type cells. These results

suggest that poly(A) polymerase facilitate PNPase to selectively degrade oxidized RNA possibly by the polyadenylation of oxidatively-damaged RNA.

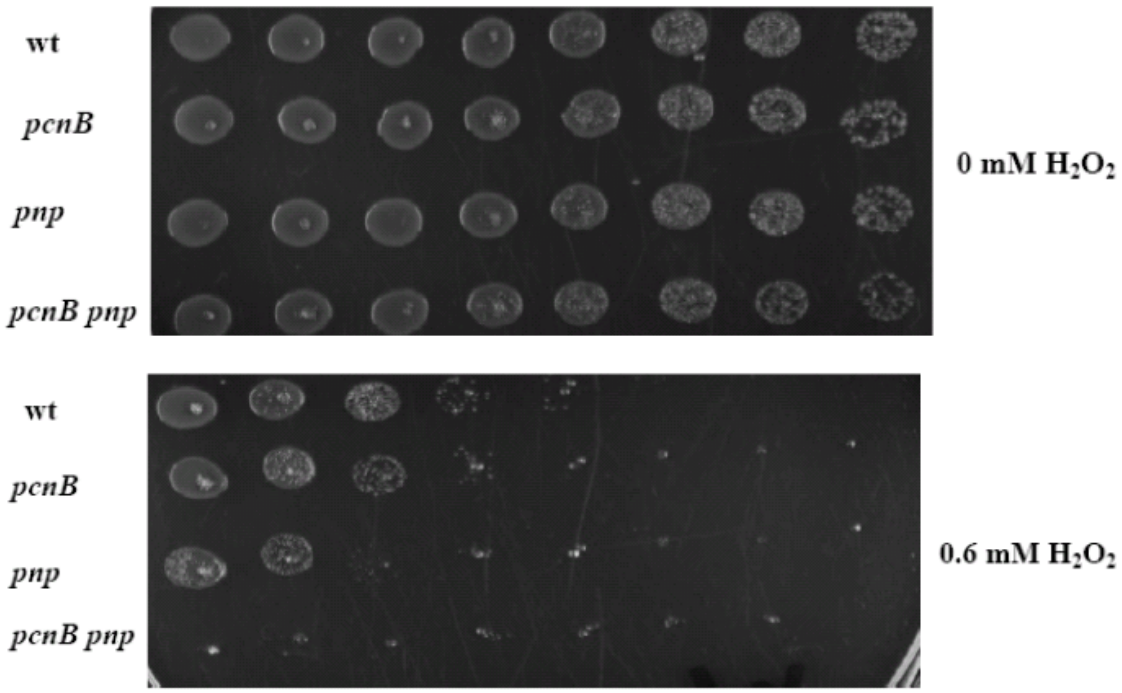


Fig. 22 Comparisons of the H_2O_2-sensitivity among wild type, the *pcnB* mutant, *the pnp* mutant, *and* double mutant *pcnB pnp*

The gene pcnB encodes E. coli poly(A) polymerase I and both the pcnB mutant and the pnp mutant are gene-deletion mutants. The double mutant pcnB pnp is generated by introducing a pnp gene-deletion mutation into a pcnB gene-deletion mutant.

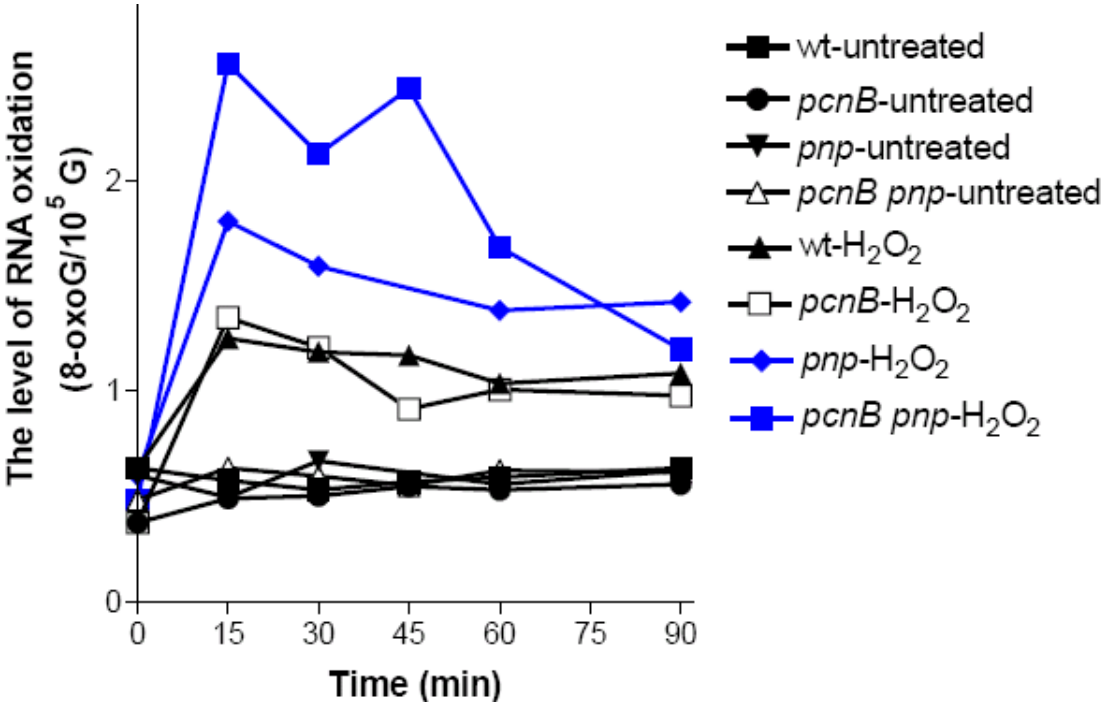


Fig. 23 The fate of oxidized RNA in wild type, *pcnB* mutant, *pnp* mutant and *pcnB pnp* double mutant

The gene pcnB encodes E. coli poly(A) polymerase I and both the pcnB mutant and the pnp mutant are gene-deletion mutants. The double mutant pcnB pnp is generated by introducing a pnp gene-deletion mutation into a pcnB gene-deletion mutant. After the cells was treated with 1 mM H_2O_2 for 15 minutes, oxidized RNA was tracked by monitoring the levels of 8-oxoG/105G in total RNA (Generated by Xin Gong).

1.10 The *E. coli pnp* mutant lacking PNPase is hyper-resistant to paraquat

Since it was shown that a *pnp* mutant strain is hyper-resistant to paraquat (Hayakawa et al., 2001), we examined the growth of the wild type and *pnp* mutant strain in the presence of various concentrations of paraquat. The cultures were serially diluted and plated as spots onto YT agar with or without paraquat. Growth was recorded after overnight incubation. As shown in Fig. 24, the growth of wild type cells is inhibited by

paraquat partially at 0.4 mM, and almost completely at 0.6 mM. In contrast, the growth of *pnp* mutant was reproducibly better, demonstrating higher viability of the *pnp* mutant cells than the wild type against paraquat treatment. Our results completely agree with the previous observations (Hayakawa et al., 2001).

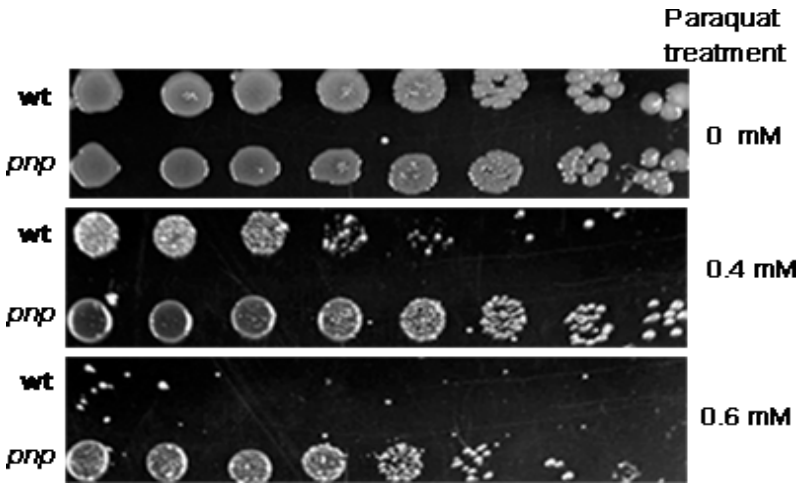


Fig. 24 *E. coli pnp* mutant is more resistant to paraquat than the wild type cell.

E. coli cultures (OD_{550nm}=0.002) gradually-diluted in the ratio of 1:5. Two µl of the diluted cultures were inoculated spotted on YT agar plates containing 0, 0.4 or 0.6 mM paraquat. The E. coli spots were observed after the incubation for 18 hours.

We then asked if paraquat causes RNA oxidation. RNA samples were prepared from cultures of wild type cells before or after a 60 min treatment with 0, 0.5 or 1mM paraquat. The level of 8-oxoG was determined. As shown in Fig. 25, 8-oxoG level in RNA does not increase one hour after the addition of paraquat at a concentration of 0.5 and 1 mM. In contrast, treatment with H_2O_2 induces remarkable increase in RNA 8-oxoG (Fig. 15).

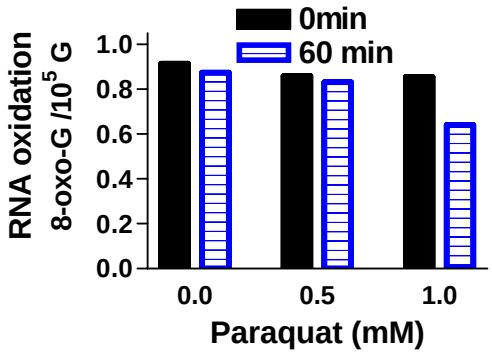


Fig. 25 The level of RNA oxidation is not elevated in *E. coli* wild type cells after treatment with paraquat

The culture of E. coli wild type cells in YT medium was treated with 0, 0.5 or 1 mM paraquat for 60 minutes. Total RNA was isolated, and assayed for the level of 8-oxoG in RNA by HPLC.

In a conclusion, our results suggest that *E. coli* PNPase plays an important role in both protecting the cells against oxidative stress and removing the oxidized RNA and association with the degradosome is not required for *E. coli* PNPase to protect cells under oxidative stress.

Section 2: The role of human PNPase under oxidative stress

2.01 H_2O_2 treatment reduces viability of HeLa cell and increases oxidation of the cellular RNA

Addition of exogenous H_2O_2 has been widely used as a convenient method to increase cellular ROS levels. Here we show that HeLa cell viability reduces dramatically in the presence of sub-millimolar concentrations of H_2O_2 (Fig. 26). Similar responses of HeLa cell to this concentration range of H_2O_2 have been previously reported (Wiese et al., 1995). As expected, cell viability decreases depending on H_2O_2 dosage, especially between 0.2 to 0.5 mM. Viable cells drop below 20% under 0.5 mM or higher H_2O_2. Note there is no change, or sometimes a modest increase, in cell viability when 0.1 mM H_2O_2 was added (Fig. 26), consistent with similar mitogenic response described previously (Wiese et al., 1995). Treatment for 2 to 25 hours caused no significant difference in relative cell viability (data not shown), presumably due to the degradation of H_2O_2 after 2 hours' incubation.

We have examined if RNA damage occurs when exposed to H_2O_2. We detected no difference in RNA intactness in cultures treated without or with 0.1 or 0.5 mM H_2O_2 (Fig. 27, lanes1-3). RNA degradation was observed only when a much higher concentration of

Result section 2 is mainly taken verbatim from a published article wrote by Jinhua Wu and Zhongwei Li (Wu and Li, 2006).

H_2O_2 (10 mM) was applied (Fig. 27, lane 4). Therefore, RNA fragmentation can be induced by H_2O_2 but it is not a detectable damage at the low H_2O_2 dosages enough to cause significant cell death.

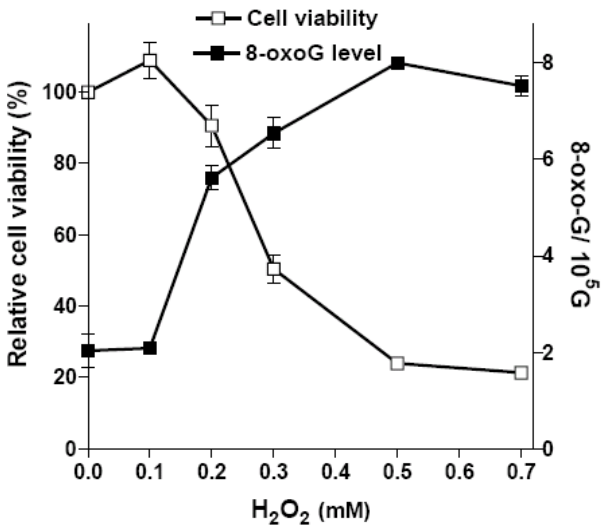


Fig. 26 HeLa cell viability and RNA damage in response to H_2O_2 treatment

Cell viability was measured in cultures grown in 96-well plates and treated by indicated concentrations of H_2O_2 for 2 hours. The mean ± SD of five replicates were plotted. To measure 8-oxoG level, cells grown in 6-well plates were treated with indicated concentrations of H_2O_2 for 2 hours before RNA isolation. The mean and SEM of triplicates were plotted.

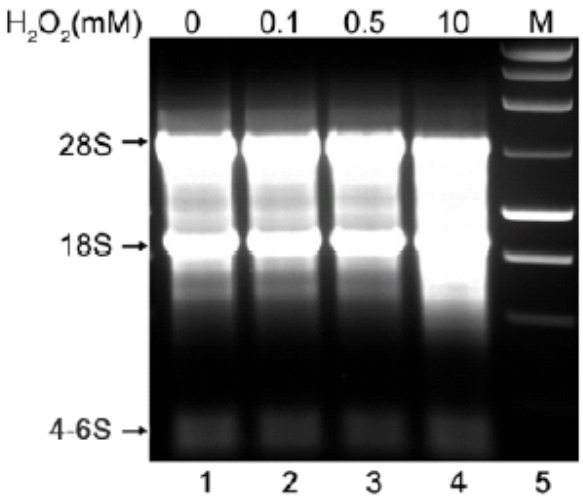


Fig.27 RNA intactness in HeLa cell cultures treated with H_2O_2

Total RNAs prepared from cells underwent 2 hours H_2O_2-treatment were separated on 1.2% agarose gels and stained with SYBR Gold (Invitrogen). The sizes of known RNA species are marked on the left. A DNA size marker was included in lane 5.

We then examined if oxidized nucleotide lesions elevate in RNA upon H_2O_2-treatment. Interestingly, 8-oxoG level increased remarkably in RNA in response to the oxidative insult. As shown in Fig. 26, the level of 8-oxoG increased 3-4 folds in cells treated with 0.2-0.5 mM H_2O_2 in a concentration-dependent manner. The increase in 8-oxoG is associated with a sharp decrease in cell viability. Treatment with 0.1 mM H_2O_2 caused no increase in 8-oxoG level and no decrease in cell viability. Likewise, H_2O_2 at 0.5 and 0.7 mM resulted in similar reductions in cell viability and increases in 8-oxoG level. Therefore, RNA can be damaged in the form of oxidatively-modified bases at lethal concentrations of H_2O_2 though fragmentation is undetectable. Importantly, the level of 8-oxoG is inversely correlated to the level of cell viability.

2.02 H_2O_2-induced 8-oxoG is specifically removed from RNA in HeLa cells

We further examined if 8-oxoG level declines after removal of oxidative challenge. HeLa cell cultures were treated with or without H_2O_2 followed by washing and incubating with H_2O_2-free medium, or treated with H_2O_2 continually. Samples were withdrawn in a time course to analyze 8-oxoG content. As shown in the left panel of Fig. 28, 8-oxoG level remains low in control cultures. As expected, there is a quick increase in 8-oxoG content after addition of H_2O_2. The level of 8-oxoG remains high in the period of time tested when H_2O_2 was continually present. In the cultures pulse-treated with H_2O_2, the increased 8-oxoG level drops shortly after H_2O_2 is removed, by nearly half of the initial elevation after 30 minutes. This reduced level, still higher than the level at start, remains in the later time points. The removal of 8-oxoG in RNA most likely occurs also in the control cultures and cultures continually treated with H_2O_2. In both cases, 8-oxoG production and removal may become close to equilibrium, resulting in relatively steady levels of this lesion in RNA in the later time points.

We then tried to see if extended incubation after a pulse H_2O_2 treatment helps to further remove the residual 8-oxoG in RNA. An increase in the level of 8-oxoG after adding H_2O_2 and a decrease shortly after removal of H_2O_2 were observed (Fig. 28, right panel), similar to the result shown in the left panel of Fig. 28. The remaining half of the H_2O_2-induced 8-oxoG was completely removed after 24 hours. A similar observation was described previously in human lung epithelial cell (Hofer et al., 2005). In that case, 8-oxoG in RNA returned to basal level 24 hours after removal of oxidative challenge.

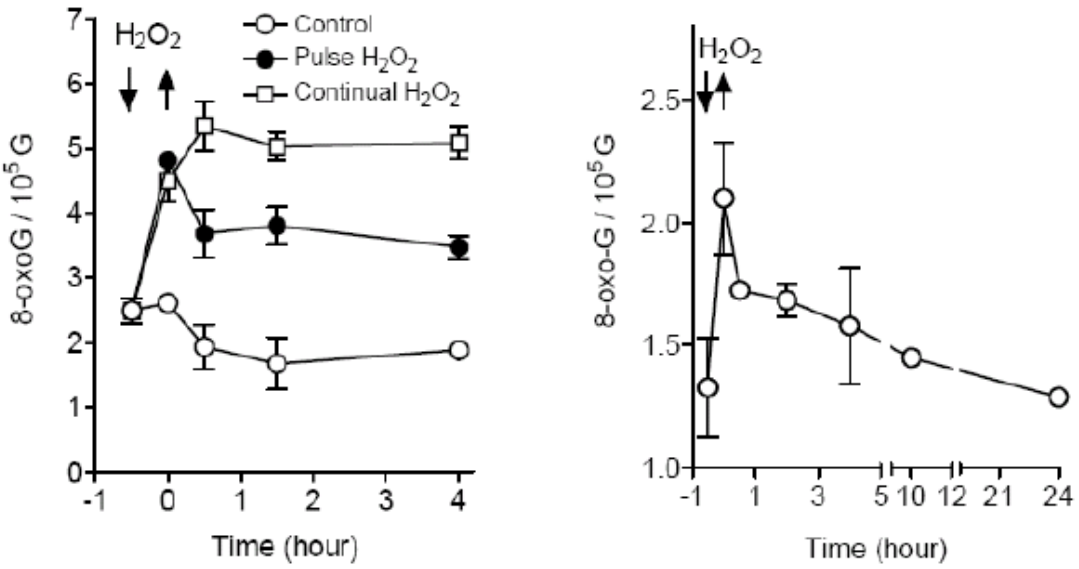


Fig 28 The fate of oxidatively-damaged RNA in HeLa cells

RNA 8-oxoG levels were determined in cells exposed to a pulse or a continual treatment of 0.2 mM H_2O_2. In the pulse treatment, H_2O_2 was added and removed at the time points indicated by the arrows. Cells were washed once and incubated with pre-warmed H_2O_2-free medium in the remaining part of the time course. The control culture was also treated with washing and medium change. The mean ± SD from triplicates were plotted. An extended time course after pulse treatment was plotted in a separate graph on the right.

2.03 hPNPase overexpression increases cell viability and reduces RNA oxidation in response to H_2O_2-treatment

A plausible mechanism for eliminating 8-oxoG-containing RNA may involve recognition and degradation of such RNA. Both *E. coli* PNPase and hPNPase were

shown to bind 8-oxoG RNA with specificity (Hayakawa et al., 2001 and Hayakawa and Sekiguchi, 2006). We have observed that PNPase protects *E. coli* cells against oxidative stress, possibly by degrading oxidized RNA (Jinhua Wu, Xin Gong, Shaohui Wu and Zhongwei Li, unpublished observations). Human PNPase is highly homologous to *E. coli* PNPase (Fig. 29), sharing 39.8% identity and 57.2% similarity in their sequences (based on an analysis using EMBOSS Pairwise Alignment from EBI, http://www.ebi.ac.uk/emboss/align/). These enzymes may share similar function in degrading defective or damaged RNA. Therefore, we studied the possible role of hPNPase in controlling oxidatively damaged RNA.

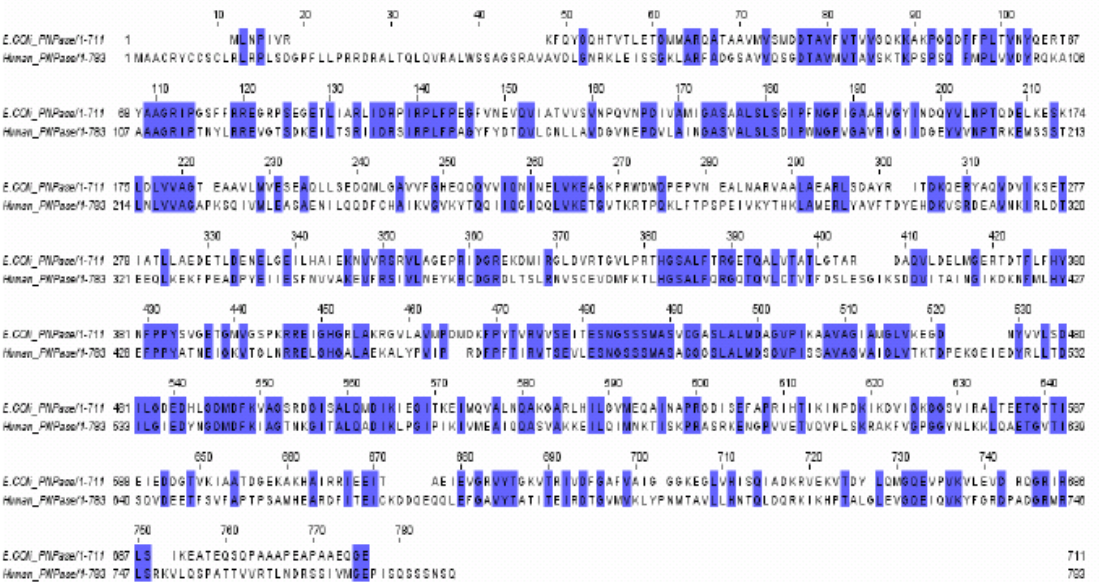


Fig. 29 Human PNPase is significantly homologous to *E. coli* PNPase

Multiple sequence alignment between E. coli Polynucleotide phosphorylase and human PNPNase was analyzed by the software CLUSTAL W (1.83) (Chenna et al., 2003).

The plasmid pCMV-hPNP carrying *hPNP* gene was introduced into HeLa cell to examine the effect of hPNPase overexpression. Equimolar pCMV* DNA was transfected into control cultures. As shown in Fig. 30, *hPNP* mRNA is moderately overexpressed from pCMV-hPNP. At 48 hours after transfection, the normalized level of *hPNP* mRNA in cultures with pCMV-hPNP is 2 to 3 fold of that in the controls.

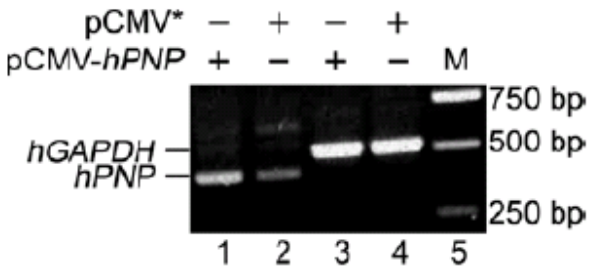


Fig. 30 *hPNP* mRNA is overexpressed in HeLa cells transfected with pCMV-hPNP

The level of over-expression of hPNP mRNA was determined by semi-quantitative RT-PCR after transfection of pCMV-hPNP and the control plasmid pCMV. RT-PCR products (marked on the left) from RNA isolated 48 hours after transfection with indicated DNA are shown together with a DNA size marker ("M").*

H_2O_2-treatment was carried out at 48 hours after transfection. As shown in Fig. 31, the viability of control cells decreases depending on the dosage of H_2O_2, in a manner similar to that shown in Fig. 26. Interestingly, in the presence of 0.3-0.7 mM H_2O_2, cell viability was significantly improved by the introduction of *hPNP* gene (Fig. 31). Overexpression of *hPNP* causes 25-35% reduction in cell death in this range of H_2O_2 concentration (Table 3). This rescue effect was not observed at higher H_2O_2

concentration when few cells survived. These data clearly demonstrate a protective role of overexpressed hPNPase against oxidative stress.

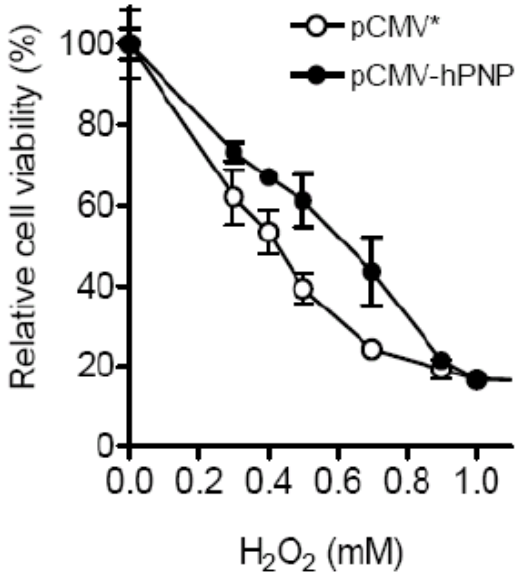


Fig. 31 hPNPase overexpression increases viability of HeLa cells under H_2O_2 treatment

Relative viability of HeLa cell in response to treatment with indicated concentrations of H_2O_2 at 48 hours after transfection of pCMV-hPNP and the control plasmid pCMV. The mean ± SD from 4 replicates were plotted.*

Table 3 Restoration of HeLa cell viability by hPNP overexpression against H_2O_2-treatment

H_2O_2 (mM)	pCMV*		pCMV-hPNP		Rescue by *hPNP* overexpression	
	Rel. Viability (Mean ± SD)	*a*. Decrease (%)	Rel. Viability (Mean ± SD)	*b*. Decrease (%)	*c*. Rel.Viability Rescued (*a*–*b*)	% Rescued ((*c*/*a*)x100)
0.3	62.1 ± 6.9	37.9	73.3 ± 2.4	26.7	11.2	29.5
0.4	53.4 ± 5.4	46.6	67.0 ± 1.8	33.0	13.6	29.3
0.5	39.4 ± 3.6	60.6	61.1 ± 6.5	38.9	21.8	35.9
0.7	24.3 ± 2.1	75.7	43.5 ± 8.4	56.5	19.3	25.5
0.9	19.4 ± 2.5	80.6	21.5 ± 0.7	78.5	2.2	2.7

**Data from Fig 31 were analyzed.*

In order to examine if 8-oxoG production and removal in RNA are affected by *hPNP* overexpression, 8-oxoG level was analyzed in transfected cells pulse-treated with H_2O_2. Interestingly, overexpression of hPNPase significantly reduced the level of 8-oxoG in RNA in the entire time course (Fig. 32). Before H_2O_2-treatment, the level of RNA 8-oxoG in cultures overexpressing *hPNP* is about 30% lower than that in control cultures. Similar differences were observed after H_2O_2 addition. However, the 8-oxoG curves from the transfected cells are different from those shown in Fig 28, presumably due to the differences in culture and treatment conditions. This behavior prevented us from

identifying any change in the pattern of 8-oxoG removal following a pulse H_2O_2 treatment. Nevertheless, these data indicated that *hPNP* overexpression decreases the level of 8-oxoG in RNA, which is coupled to a partial rescue of HeLa cell.

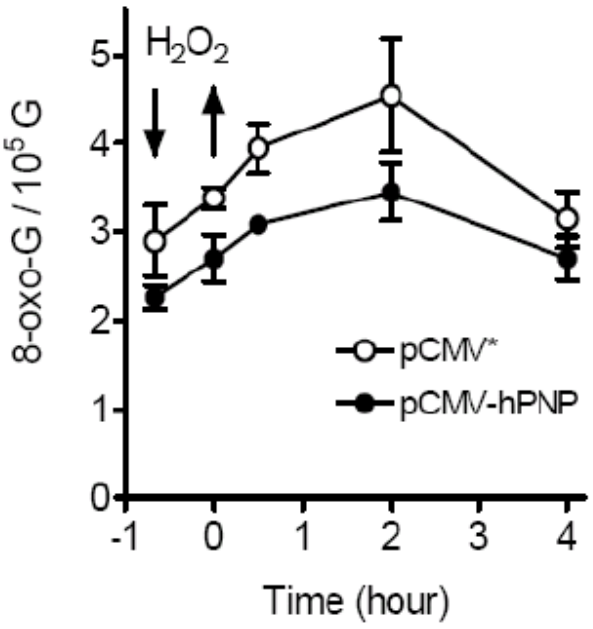


Fig. 32 hPNPase overexpression reduces the levels of RNA oxidation in HeLa cells treated with H_2O_2

Levels of 8-oxoG were determined in RNA isolated from cells pulse-treated with 0.3 mM H_2O_2 at 48 hours after transfection of pCMV-hPNP and the control plasmid pCMV. The mean ± SD of triplicates were plotted.*

2.04 Knockdown of hPNPase increases H_2O_2-induced RNA oxidation and cell death

To further examine the effect of hPNPase reduction, we have introduced a siRNA to knockdown this enzyme. The siRNA sequence was depicted from a construct previously shown to knockdown both *hPNP* mRNA and hPNPase's poly(A) degradation

activity in HeLa cells (Nagaike et al., 2005). As shown in Fig. 33, *hPNP* mRNA is down-regulated by at least 80% at 24, 48 and 72 hours after the introduction of the *hPNP* siRNA, compared to the level in the control. In contrast, *hGAPDH* mRNA was unaffected by the siRNAs. Similar knockdown efficacy by this *hPNP* siRNA was reported previously (Nagaike et al., 2005).

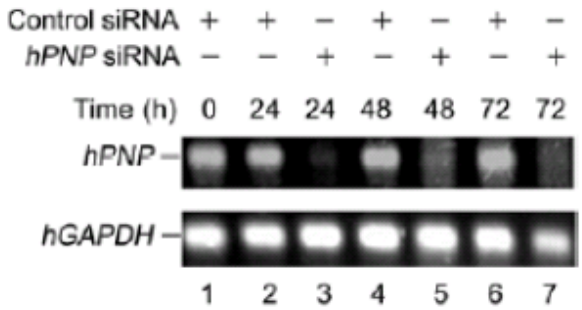


Fig. 33 hPNP mRNA is knocked-down by RNA interference in HeLa cells

At indicated time points after transfection of 5 nM hPNP siRNA or control siRNA, the levels of hPNP mRNA were determined by RT-PCR using hGAPDH as internal normalization control.

HeLa cell viability was determined at various H_2O_2 concentrations at 48 hours after siRNA introduction. As shown in Fig. 34 *hPNP* knock-down significantly decreased the viability of HeLa cells after exposure to 0.4 and 0.5 mM H_2O_2. We noted that the response of the siRNA-transfected cells to various dosages of H_2O_2 is different from those shown in Fig 26 and Fig 31, presumably due to variations caused by transfection. Nevertheless, the significant reduction in cell viability by *hPNP* siRNA further supports the notion that hPNPase is important in protecting cells against oxidative stress.

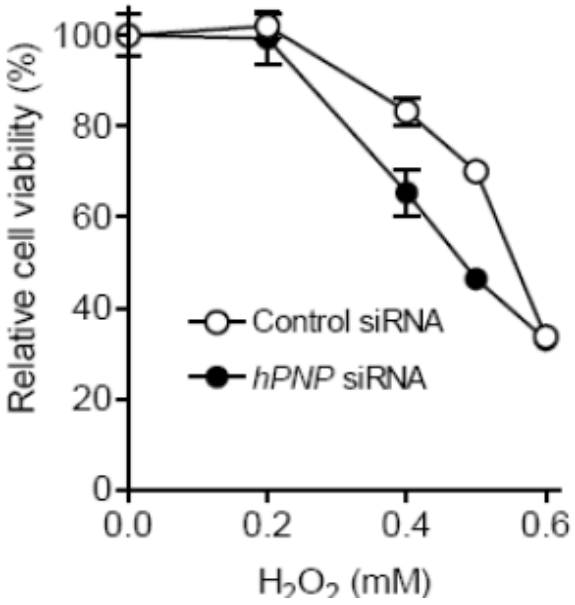


Fig. 34 hPNPase knock-down by RNA interference decreases viability of HeLa cells treated with H_2O_2

HeLa cell cultures 48 hours after transfection of 5 nM hPNP siRNA or control siRNA were exposed to indicated concentrations of H_2O_2 and relative viability levels was determined as in Fig. 26. The mean ± SD from 5 replicates were plotted.

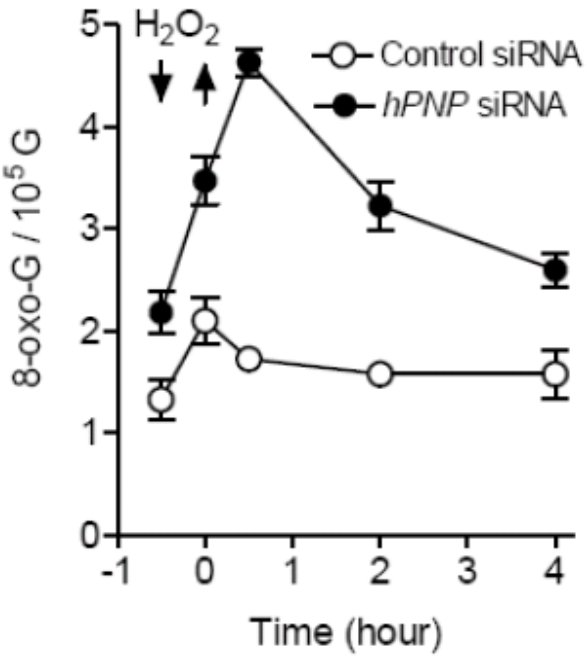


Fig. 35 hPNPase knock-down by RNA interference increases the levels of RNA in HeLa cells treated with H_2O_2

Cells transfected with 5 nM hPNP siRNA or control siRNA for 48 hours were pulse-treated with H_2O_2 and 8-oxoG levels were determined as described in Fig. 6. The mean ± SD of triplicates were plotted.

A pulse H_2O_2-treatment was conducted to HeLa cells at 48 hours after siRNA transfection to study the effect of *hPNP* knockdown on 8-oxoG level and its removal. In cultures with the nonspecific siRNA, the level of RNA-borne 8-oxoG first increased to nearly 2 fold after H_2O_2-addition, then decreased in the next 30 minutes after removal of H_2O_2 (Fig. 35), a response similar to that of non-transfected cultures (Fig. 28). Importantly, cells transfected with *hPNP* siRNA contains higher basal levels of 8-oxoG-

containing RNA than those with the control siRNA, suggesting that *hPNP* knockdown causes an increase in RNA oxidation even under normal aerobic growth (Fig. 35). Upon pulse H_2O_2-treatment, 8-oxoG level increases dramatically in *hPNP* siRNA-transfected cells, with a delayed and elevated peak after removal of H_2O_2. Compared to controls, *hPNP* knockdown causes accumulation of higher 8-oxoG in RNA at all time points.

2.05 Partial complementation of decreased cell viability in hPNP siRNA-treatment by over-expressing hPNPase in HeLa cells under H_2O_2 treatment

The above data indicated that, in HeLa cells, over-expressed human PNPase increased cell viability and decreased the level of RNA oxidation and, conversely, knock-down human PNPase decreased cell viability and increased the level of RNA oxidation under oxidative stress. Based on these results, a conclusion may be drawn that human PNPase plays an important role in cell viability and specifically removing oxidized RNA in HeLa cell under oxidative stress. If over-expressing human PNPase can complement the decrease of cell viability in HeLa cells transfected with hPNPase siRNA, this conclusion will be further solidified. As shown in Fig 36, under the treatment of H_2O_2, cell viability in the cells transfected with vehicle plasmid and hPNP siRNA was significantly less than in the cells transfected with vehicle plasmid and nonsilencing siRNA; Cell viability in the cells transfected with hPNPase-encoding plasmid and hPNP siRNA was significantly higher in the cells transfected with vehicle plasmid and hPNP siRNA but still less than in the cells transfected with vehicle plasmid and nonsilencing siRNA. This data demonstrated that over-expressed hPNPase in the expression vector pCMV-SPORT6 can

partially complement the decrease of cell viability in HeLa cells under the treatment of H_2O_2.

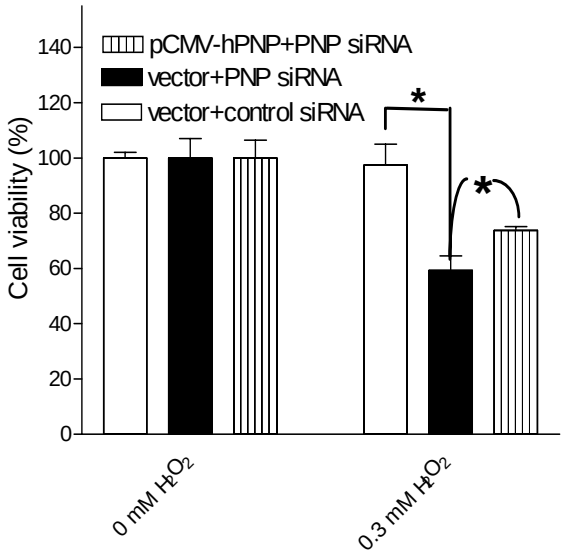


Fig. 36 Partial complementation of the decrease of cell viability in hPNP siRNA-transfected HeLa cells by over-expressing hPNPase under the treatment of H_2O_2

HeLa cells with the 60% confluence were respectively transfected by vehicle plasmid and plasmid pCMV-hPNPase expressing hPNPase and after 24 hour of plasmid transfection, nonsilencing siRNA and hPNP siRNA were respectively transfected into HeLa cells. After 36 hour of siRNA transfection, the cells were treated by 0 and 0.3 mM H_2O_2 for 2 hours and after another 24 hours cell viability was determined by MTT assay.

In a conclusion, our results suggest that human PNPase may play an important role in both protecting the cells against oxidative stress and removing the oxidized RNA in HeLa cells, though we still have no direct proof to indicate how human PNPase protects the cells against oxidative stress.

DISCUSSION

In this work, we have shown that *E. coli* polynucleotide phosphorylase plays important role in protecting *E. coli* cells under oxidative stress. Cells lacking PNPase are hypersensitive to H_2O_2 and contain a higher level of 8-oxoG in RNA. The viability and RNA oxidation of the *pnp* mutant strain are restored by the introduction of the plasmid-borne *pnp* gene. Interestingly, the protective role of PNPase is independent of its association with the RNA degradosome. We have also shown that HeLa cell viability is inversely correlated with RNA oxidation when exposed to H_2O_2. While overexpression of hPNPase reduces the level of 8-oxoG in RNA and improves cell viability against H_2O_2-treatment, knock-down of hPNPase causes converse effects. Our observations suggest an important biological role for polynucleotide phosphorylase under oxidative stress.

Under oxidative stress, cell viability decreases and the level of oxidative damage in cellular RNA increases in the absence of PNPase and other RNA degradation activities, suggesting that RNA oxidation is detrimental to cells. The activities described in this work, which protect both *E. coli* and human cells under oxidative stress, all work on RNA, but not on other cellular components. In addition, deficiency in *E. coli* PNPase causes the accumulation of higher levels of 8-oxoG in RNA when treated with H_2O_2, suggesting a direct role for this enzyme in removing oxidized RNA, most likely by degradation. We conclude that RNA damage is a challenging problem for both bacteria and mammalian cells and RNA surveillance plays pivotal roles in protecting cells. Our results support the hypothesis of this work stated in the Introduction section.

The binding of *E. coli* PNPase to normal vs. oxidized RNA

Both *E. coli* PNPase and human PNPase were previously shown to have a higher affinity to poly(8-oxoG:A) than poly(G:A)(Hayakawa et al., 2001; Hayakawa and Sekiguchi, 2006). In this work, we confirmed that, using an RNA-binding assay, *E. coli* PNPase also preferentially binds to RNA that is oxidized by H_2O_2 treatment. In *E. coli* PNPase, there are two conserved RNA-binding domains (RBDs), the KH and S1 motives, located in the C-terminal region of PNPase protein (Matus-Ortega, et al., 2007). KH domains can bind single-stranded RNA and are found in a wide variety of proteins including ribosomal proteins, transcription factors and post-transcriptional modifiers of mRNA; S1 domain is ribosomal protein S1-like RNA-binding domain (Matus-Ortega, et al., 2007). Removal of one or both RBDs greatly reduces the binding and moderately affects the 3'->5' degradation of an RNA substrate *in vitro* (Stickney et al., 2005). Intriguingly, the oxidized RNA substrate used in our experiment is covalently cross-linked to agarose beads at the 3' end. Consequently, the preferential binding of PNPase must occur to the oxidized RNA independent of a free 3' end. It remains to be elucidated if the binding involves the conserved RBDs or other unidentified RNA-binding domains of *E. coli* PNPase.

The surprising hyper-resistance of the *pnp* mutant cells to paraquat

Although the *pnp* mutant cells are hypersensitive to the oxidants H_2O_2, they are much more resistant than the wild type cells to paraquat, another widely used oxidative reagent. The apparent discrepancy prompted us to ask if paraquat causes oxidative damage to *E. coli* cytoplasmic components. According to a previous report, paraquat

generates ROS mainly outside the cell, presumably causing primary oxidative damage to periplasmic components (Sion et al., 1989). In this work, we have confirmed the hyper-resistance of our *pnp* mutant cells to paraquat (Fig. 24). However, we have observed no increase in RNA 8-oxoG levels upon treatment with paraquat (Fig. 25). In contrast, treatment with H_2O_2 induces a remarkable increase in RNA 8-oxoG (Fig. 15). Therefore, paraquat may kill *E. coli* cells by a process unrelated to RNA oxidation, and the hyper-resistance of *pnp* mutant cells to paraquat is likely caused by some other processes such as altered expression of the outer membrane proteins in this mutant (Andrade and Arraiano, 2008).

Does *E. coli* PNPase degrade oxidized RNA?

Compared with wild type cells, there is an elevation in the level of RNA oxidation in the *pnp* mutant after H_2O_2 treatment (Fig 15). Introduction of *pnp* gene restores the viability and reduces level of RNA oxidation in *pnp* mutant cells (Fig 17 and Fig 18). The most straightforward explanation of these observations is the specific removal of oxidized RNA by this enzyme. However, a role for PNPase in directly degrading oxidized RNA cannot be easily established. First of all, *E. coli* PNPase has been reported to bind but not to efficiently degrade poly (8-oxo-G:A) (Hayakawa et al., 2001). However, in that case, poly (8-oxoG:A) may not be a suitable substrate for *E. coli* ribonucleases since one half of nucleobases in this RNA is 8-oxoG, which may render the RNA an unnatural structure and non-degradable. Secondly, oxidatively-damaged RNA generated by incubation with H_2O_2 *in vitro* appears not to be preferentially degraded over untreated RNA when incubated with *E. coli* cell extracts containing *E. coli* PNPase (Xin Gong and

Zhongwei Li, unpublished observation). Further studies are required to better understand the exact role of hPNPase under oxidative stress.

Antioxidant activity: RNase E, PNPase or the degradosome in *E. coli*

Interestingly, the association of *E. coli* PNPase with the RNA degradosome is not required for the function of this enzyme under oxidative stress though nearly 30% of the total quantity of *E. coli* PNPase associates with the degradosome (Liou et al., 2001). This is not surprising since the degradosome may only play a limited role in the degradation of certain mRNA species or structures (Bernstein et al., 2004; Khemici and Carpousis, 2004). In fact, the strain carrying the truncated RNase E lacking *E. coli* PNPase binding site is resistant to H_2O_2 treatment (Fig 20) and has higher PNPase activity *in vitro* (Fig. 21). Consistent with our observation, an *E. coli* mutant containing a transposon insertion that blocks the expression of the C-terminal half of RNase E was shown to be hyper-resistant to oxidative stress generated by the introduction of the human Bax protein (Nanbu-Wakao et al., 2000). It is likely that the truncated RNase Es confer anti-oxidant activities. Alternatively, these mutant RNase Es or the PNPase-dissociated degradosome may caused an elevation in the expression or activity of PNPase, resulting in a more protective effect on the viability of *E. coli* cells under oxidative stress.

Here are several possible reasons why the *rne* mutant lacking the PNPase-binding domain in RNase E protein grows better than wild type cells. Firstly, it is less likely that truncated RNase E without the PNPase-binding domain confer anti-oxidant activities. In this work, a double mutant is made by introducing the *pnp* null mutation into a *rne* mutant lacking the PNPase-binding domain in RNase E protein (lacking amino acids 844-

1045, Leroy et al., 2002). That mutant shows a H_2O_2-sensitivity similar to the *pnp* null mutant (Fig. 20) while an *rne* mutant lacking the PNPase-binding domain in RNase E protein is resistant to H_2O_2 (Fig. 20). It is more likely that RNase E mutant lacking the PNPase-binding domain or the PNPase-dissociated degradosome may cause an elevation in the expression or activity of PNPase, resulting more protective effect on the viability in *E. coli* cells under oxidative stress; Secondly, it is also possible that this mutated RNase E without a PNPase-binding domain or the PNPase-dissociated degradosome cause an elevation in the expression of PNPase, resulting in a more protective effect. Conversely, the quantity of *E. coli* PNPase mRNA was down-regulated by 19% in an *E. coli* degradosome-disassembled mutant on the basis of original microarray data (Bernstein et al., 2004) though down-regulation of *E. coli* PNPase mRNA needs further confirmation by quantitative RT-PCR.

Lastly, so far no evidence eliminates the possibility that it is an elevation in the activity of PNPase that makes the mutant having PNPase dissociation from the degradosome grow better than wild type cells under oxidative stress. It is a traditional strategy used in cells that a protein's activity is activated or inhibited by another protein's binding or unbinding. Therefore, it is possible that the RNA degradation activity of *E. coli* PNPase is enhanced by RNase E's release and further makes more cells viable under oxidative stress. More studies are needed to support this hypothesis.

Additional activities for RNA degradation may be involved in protecting *E. coli* cells against oxidative stress

It has been believed that RNA degradation in *E. coli* is initiated by the cleavages catalyzed primarily by endoribonuclease RNase E following 3'->5' exonucleolytic degradations catalyzed by polynucleotide phosphorylase, RNase II or RNase R, the three exoribonucleases that rapidly and processively degrade RNA (Kushner, 2002). The involvement of *E. coli* PNPase in eliminating oxidatively-damage RNA of cellular processes was strongly implied by the findings that *E. coli* PNPase protein specifically binds to 8-oxoguanine-containing RNA, that *pnp* mutants are hypersensitive to H_2O_2, and that the level of RNA oxidation is elevated in the *pnp* mutant after treatment with H_2O_2. These results suggest that, under oxidative stress, the *E. coli* PNPase protein is capable of discriminating 8-oxoguanine-containing RNA from normal RNA and then eliminating oxidatively-damaged RNA within cells. Under oxidative stress, *E. coli* PNPase protein probably functions in the following ways: specifically binding 8-oxoguanine-containing RNA, which would remove oxidized RNA from the translational machinery, and finally targeting the oxidized RNA to the RNA degradation pathway. This is the first demonstration that, under oxidative stress, the catalytic phosphorylase activity and the binding capacity for 8-oxoguanine-containing RNA are closely associated.

In addition to PNPase, RNase II and R were also found to protect cells and control oxidatively damaged RNA under oxidative stress (unpublished observations, Xin Gong, Jinhua Wu, Shaohui Wu and Zhongwei Li). In this preliminary work, RNase II was also shown to be important in cell viability and the resistance to oxidative stress, and introduction of the *rnb* gene encoding RNase II restores the viability of the *rnb* mutant

cells under oxidative stress (Jinhua Wu and Zhongwei Li, unpublished observation). This result is consistent with the notion that multiple RNases are involved in removing damaged RNA.

Of particularly interest are the potential roles of the helicase RhlB, an integral member of the RNA degradosome, and RNase R, an exoribonuclease shown to degrade structured RNA with high efficiency (Cheng and Deutscher, 2003). I have observed that while the mutant lacking one enzyme is normal (*rhlB*) or only moderately hypersensitive (*rnr*) in response to H_2O_2 insult, a mutant lacking both RNA helicase B and RNase R becomes extremely sensitive to H_2O_2 (Jinhua Wu and Zhongwei Li, unpublished observation). These results suggest that RNA helicase B may facilitate RNase R to protect *E. coli* cells against oxidative stress. Currently, there is no knowledge about the physical and functional interaction of RNA helicase B and RNase R. Therefore, the observations about the double mutant *rnr rhlB* under oxidative stress raises a question about the physical and functional interaction of these two enzymes in RNA degradation under normal conditions, specifically, whether RNA helicase B can facilitate RNase R in normal RNA degradation pathways in *E. coli* and whether RNA helicase B can interact with RNase R.

Translation mediated by tmRNA is an important mechanism for mRNA surveillance in prokaryotes. tmRNA encoded by the *ssrA* gene functions as $tRNA^{Ala}$ and mRNA coding a short peptide. During translation of certain abnormal mRNA species, such as mRNA without stop codons, or ribosomes halted at the 3’ end of mRNA can be rescued by recruiting tmRNA to continue translation on the short open reading frame encoded by tmRNA. As a result, the ribosome can terminate translation and be released

normally. The peptide is tagged with the tmRNA-encoded sequence at the C-terminus which targets the peptide to degradation. The aberrant mRNA is released from the ribosome and is degraded (Burton et al., 2003; Yamamoto et. al., 2003). In this work, it was found that the *ssrA* mutant cells are hypersensitive to H_2O_2 and that introduction of a plasmid-born *ssrA* gene rescues the *ssrA* mutant cells under oxidative stress (Gayatri Kollipara, Jinhua Wu and Zhongwei Li, unpublished observations). These results indicate that tmRNA is also involved in surveillance mechanism in the cells under oxidative stress.

The fate of oxidized RNA in human cells

Our data show that there exists a specific RNA surveillance mechanism in HeLa cells under oxidative stress (shown in Fig 28): after 15-minute H_2O_2 treatment, the levels of RNA oxidation are gradually decreased and finally go back to the basal level in HeLa cells after 25 hours. Our results are consistent with previous observations that oxidative lesions in total RNA decreased to basal oxidation levels in 24–72h after removal of oxidative stress in mammals (Hofer et al., 2006; Kajitani et al., 2006). All of these findings suggest the existence of important control mechanisms for oxidized RNA in the cell.

After the removal of oxidative challenge, 8-oxoG-containing RNA appears to be reduced in a biphasic pattern: quick degradation and slow degradation (shown in Fig 28). The analysis for the degradation speed of oxidatively-damage RNA suggests that different control mechanisms might be involved in the degradation of oxidatively-damage RNA. It is known that the half life of stable RNA including ribosomal RNA and transfer RNA is much longer than that of unstable RNA mainly referring to mRNA. Since there

are different RNA degradation pathways to take care of stable RNA and unstable RNA, different surveillance mechanisms may also be responsible for the degradation of oxidatively-damaged stable RNA and unstable RNA respectively. Therefore, the quick degradation stage and slow degradation stage of oxidatively-damage RNA may be attributed to the degradation of oxidized unstable RNA and unstable RNA respectively. This possibility can be examined by quantification of the levels of RNA oxidation in individual RNA classes including stable RNA (rRNA and tRNA) and unstable one (mRNA) in the future.

Our results suggest an important role for hPNPase in limiting 8-oxoG in RNA (Fig 32 and Fig 34). Currently, it is not clear if hPNPase functions in any specific part of the biphasic removal of 8-oxoG. Oxidative damage is expected to occur randomly in RNA. Various damaged RNA species may be removed by different mechanisms which remain to be elucidated.

Human PNPase may play a role in HeLa cell similar to its *E. coli* homologue under oxidative stress

Being a 3'->5' exoribonuclease, hPNPase was shown to preferentially bind 8-oxoG RNA (Hayakawa and Sekiguchi, 2006). However, its role in directly degrading oxidized RNA has not been reported. Human PNPase is induced by type I interferons and contributes to cell terminal differentiation and senescence (Sarkar et al., 2004, 2005, 2006a, and 2006b). It was further shown that hPNPase plays roles in tumorigenesis (Sarkar et al., 2006a), the cellular response to viral infection (Sarkar et al., 2006b) and maintaining mitochondrial homeostasis (Chen et al., 2006). As a 3'->5' exoribonuclease, hPNPase is responsible for the degradation of the cytosolic c-myc mRNA and for the

processing and adenylation of mitochondrial RNA (Sarkar et al., 2003; Slomovic et al., 2008). However, a link of hPNPase's diversified roles to its RNase activity has been only suggested in limited cases (Sarkar et al., 2005 and 2006a).

The situation is more controversial when hPNPase, initially found mainly in mitochondria, was recently shown to localize in the intermembrane space of mitochondria (Chen et al., 2006) where no RNA is present. Therefore, certain functions of hPNPase may be indirect or independent of its activities on RNA (Slomovic et al., 2008). In the case of controlling RNA oxidation levels, it is unlikely that hPNPase works as a general antioxidant since its overexpression increases ROS levels in cell (Sarkar et al., 2004). Oxidized RNA may be selectively degraded by any residual hPNPase present in the matrix of mitochondria or in the cytoplasm. Alternatively, hPNPase may regulate other activities that are directly involved in eliminating oxidatively damaged RNA. Further studies are required to better understand the role of hPNPase under oxidative stress and in other physiological processes.

The fact that cell viability and RNA oxidation are correlated, and that hPNPase helps in both reducing RNA damage and increasing cell viability, suggests a causative relationship of RNA oxidation and cell death under oxidative stress. Damage of RNA is obviously as deleterious as the damage of other macromolecules. However, evidence of cell death caused by RNA damage has been missing. The extent of cell death caused by RNA oxidation remains to be examined by future studies. We have shown that moderate overexpression of hPNPase reduces nearly 30% of 8-oxoG in RNA (Fig. 32), and rescues 25-35% of total cells at various H_2O_2 concentrations (Table 3). Though the effect is moderate, hPNPase must play an important role in controlling damaged RNA, a

complicated process in which the involvement of multiple overlapping activities is expected.

RNA-borne 8-oxoG is much more abundant than its DNA counterpart in the urine and plasma of human and rat (Park et al., 1992), suggesting that 8-oxoG derived from degradation of oxidized RNA may serve as a promising biomarker for cellular oxidative stress. The possible role of human PNPase in releasing 8-oxoG from RNA and in controlling its level in biological fluids remains to be elucidated.

Future Directions

Although we have known that *E. coli* PNPase can specifically bind oxidized RNA and *E. coli* PNPase have two RNA-binding domains including KH domain and S domain, it is still interesting to know which motif makes *E. coli* PNPase is most important for binding to oxidized RNA. Different truncated *E. coli* PNPase can be constructed in order to study which domain decides *E. coli* PNPase's higher affinity to oxidized RNA.

The degradosome is not required for *E. coli* PNPase in protecting the cells against oxidative stress. However, we still do not know what the functional relationship between *E. coli* PNPase and the degradosome under normal conditions and oxidative stress. Further genetic studies and biochemistry can be done to understand the functional relationship between *E. coli* PNPase and the RNA degradosome under normal conditions and oxidative stress. A plasmid pET-RneCTH, encoding C-terminal domain of the RNase E protein, was obtained from Dr. Agamemnon J. Carpousis's lab with the help of Dr. Chris Burns. In my preliminary work, a new expression plasmid pET-RneCTH-*PBD*, which encodes C-terminal domain with the deletion of PNPase binding domain (PBD,

amino acids 842-1044) of the RNase E protein, was constructed by reverse PCR. Both pET-RneCTH and pET-RneCTH-*PBD* can be transformed into BL21 *E. coli* wild type strain and *rne* mutant only lacking PNPase-binding domain (amino acid 844-1045) in order to overexpress RNase E's C terminal fragment and truncated RNase E's C terminal fragment lacking the PNPase-binding domain respectively. Comparison of H_2O_2-sensitivity among these strains will provide us some useful information in understanding the functional relationship between *E. coli* PNPase and the RNA degradosome under the oxidative stress condition. Using purified C terminal fragments of the RNase E protein with or without the PNPase-binding domain, we can also examine if C-terminal domain of the RNase E protein binding to *E. coli* PNPase affects normal RNA degradation activity of *E. coli* PNPase, which will be helpful to analyze the functional relationship between *E. coli* PNPase and the degradosome under the normal condition.

Rhl B is an RNA helicase having both ATPase activity and RNA helicase activity and so far it was reported that Rhl B only can interact with both PNPase and RNase E. My preliminary data suggest that RNA helicase B can facilitate RNase R to protect *E. coli* cells against oxidative stress. Based on this, we can further explore the interaction of RNA Helicase B with RNase R under normal conditions. If the interaction of RNA Helicase B with RNase R is confirmed, we can study the role of RNA Helicase B in facilitating RNase R in RNA maturation, RNA processing, and RNA quality control, specifically for the degradation of the intermediate RNAs containing REP-stabilizers.

CONCLUSION

To conclude, the results presented in this dissertation clearly show that *E. coli* polynucleotide phosphorylase plays an important role in protect the cells against oxidative stress and removing oxidatively-damaged RNA; Additionally, association with the degradosome is not required for *E. coli* PNPase to protect cells under oxidative stress; More importantly, human polynucleotide phosphorylase, homologous to *E. coli* polynucleotide phosphorylase, also be proven to have a protective effect on the viability in human HeLa cells and to eliminate oxidatively-damaged RNA.

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