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P53 AND REACTIVE OXYGEN SPECIES: A CONVOLUTED STORY

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ABSTRACT OF THESIS

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The tumor suppressor p53 has a close relation with reactive oxygen species (ROS). As an indispensable component of the cellular redox system, ROS not only have been established to be involved in p53-dependent apoptosis, but also regulate p53 activity. Recent studies revealed several novel actions of p53, such as transactivation of antioxidative proteins, mitochondria translocation and inhibition of glycolysis. The fate of cells where p53 signaling pathways are initiated is either survival or death. In this review, we examine the hypothesis that ROS regulate cell fate through p53, in a way that physiological ROS levels trigger the protective pathways, while p53 behaves more like a cell killer under cytotoxic oxidative stress.

KEYWORDS: p53, reactive oxygen species (ROS), cell fate, redox proteins, mitochondria.

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May 24, 2007

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Bin Liu

The Graduate School
University of Kentucky
2007

P53 AND REACTIVE OXYGEN SPECIES: A CONVOLUTED STORY

THESIS

A thesis submitted in partial fulfillment of the
requirements for the degree of Master of Science in the
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at the University of Kentucky

By

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Lexington, Kentucky

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2007

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I. Introduction

Reactive oxygen species (ROS) are highly reactive small molecules containing oxygen. ROS include oxygen ions (such as hypochlorite ion, OCl^-), free radicals (such as hydroxyl radical, ·OH) and molecules (such as hydrogen peroxide, H_2O_2). ROS can be formed upon external stimulation such as ionizing radiation and as natural byproducts of cellular respiration. ROS are traditionally considered to damage DNA and to trigger apoptosis, but positive roles of ROS, particularly as signaling molecules, are also well documented in many areas of research, such as cancer, heart disease, neuroscience, and many others (for recent reviews, see [1-12]).

The interaction between tumor suppressor protein p53 and ROS has been the subject of many studies in recent years. It has been suggested that p53 and ROS affect each other, leading to a cascade of downstream events. p53 is one of the most critical and extensively studied regulators of cell fate. Once activated, p53 can lead to cell survival (either cell cycle arrest or permanent arrest, also known as senescence) or apoptosis, depending on the severity of damage and condition of the cells (for recent reviews, see [13-15]). More and more evidence supports the hypothesis that intracellular ROS levels regulate cell fate through p53. This article summarizes some research highlights of this issue, emphasizing the response of p53 to ROS stimulation under different stress conditions and corresponding events downstream of p53 activation.

II. Backgrounds

II.A. Functions, structure and properties of p53

p53 deficiency, which results in failure to control cell proliferation, is generally considered a significant genetic basis for cancer. It is estimated that functional mutation of TP53, the gene encoding tumor suppressor p53, occurs in 50% of human cancers, but this estimate may be far lower than actual incidence [16].

The human p53 gene (*TP53*) is located on the 17th chromosome (17p13.1) [17]. The p53 protein is a phosphoprotein consisting of 393 amino acids, with a molecular mass of 53 kDa, which gives the protein its name.

The p53 protein is made up of five domains. Localized at the N-terminus is the transactivation (TA) domain (amino acids 1-50). The TA domain binds to several components of the RNA transcription complex including the TATA-binding protein (TBP) and TBP-associated factors (TAFs), which regulate the transactivation capacity of p53 [18-21]. The ability to control transcription is essential to the role of p53 in inducting cell cycle arrest as well as apoptosis [22-24]. Amino acids in the transactivation domain are heavily phosphorylated upon radiation treatment, which is a sign of p53 activation. For instance, Ser6, Ser9, Ser15, Thr18, Ser20, Ser33, Ser37 and Ser46 undergo this modification [25]. Next to the transactivation domain is a polyproline domain (amino acids 63-97) that is necessary for transcription-independent apoptotic and growth

suppression functions [26-28]. There is a nuclear export signal (NES) in the N-terminus. In the middle part of the p53 protein lies the core domain (amino acids 100-300) which is responsible for the sequence-specific DNA binding activity of p53. Crucial to this important p53 function are four evolutionarily conserved regions within this domain [29]. Amino acids in the four regions have been identified in over 90% of the mutations found in human cancers [25]. Six amino acid residues that are essential to preserve the structure of the DNA binding motifs are the most frequently mutated (175 Arg>His, 245 Gly>Ser, 248 Arg>Trp, 249 Arg>Ser, 273 Arg>His, and 282 Arg>Trp), and they are often referred to as “hot-spot” mutations [25, 30]. A domain in the C-terminus (amino acids 323-356) helps p53 protein molecules to form tetramers, the optimized form for p53 to achieve its functions [31]. The C-terminus also plays an important part in the interaction of p53 with non-specific double stranded DNA, single stranded DNA and damaged DNA through a non-specific DNA binding domain (amino acids 363-393) [32-36].

Under unstressed conditions, the expression level of p53 is very low in most tissues because of a short protein half-life [37]. In the immortalized mouse 308 keratinocyte cell line, the half-life of wild-type (WT) p53 is only about 16 min [38]. At the physiological temperature of 37°C, WT p53 is highly unstructured (more than 50% unfolded) and shows low DNA-binding ability (75% loss after incubation) [39]. Several posttranslational mechanisms, such as phosphorylation, acetylation and sumoylation, contribute in great measure to the stabilization and activation of p53 (reviewed in [37]).

II.B. Types of DNA damage

DNA damage is the best known cause of p53 activation. DNA damage can be initiated by a number of endogenous (such as ROS) and exogenous (such as UV, radiation and chemicals) stimulations. Common types of DNA damage include covalent modification of bases (e.g., oxidation, alkylation and hydrolysis, Fig. 1), mismatch of bases (Fig. 2), breaks in the backbone occurring on one or both of the DNA strands, and crosslinks between bases on the same or opposite DNA strand and between DNA and protein molecules.

II.C. ROS and their reactions with DNA bases and proteins

Excessive ROS oxidizes DNA bases by means of breaking strands and modifying bases and nucleotides. The interaction of ROS and DNA varies according to type. Singlet oxygen ($^1O_2$) oxidizes guanine, yielding two 4R* and 4S* diastereomers of 4,8-dihydro-4-hydroxy-8-oxo-2'-deoxyguanosine as the main product when the guaninemoiety is oxidized within nucleosides, and 7,8-dihydro-8-oxoguanine as the main product when the oxidation occurs in double-stranded DNA [40]. ·OH is extremely unstable and easily attacks other molecules (usually at double bonds) because an unpaired electron exists in its outermost shell of electrons, resulting in the addition of a hydroxyl group. In addition, ·OH may remove a hydrogen atom from a carbon atom to form a H_2O molecule, leaving an unpaired atom on the carbon atom. When a molecule is attacked by ·OH, it becomes a radical and can initiate a chain reaction by subsequently changing

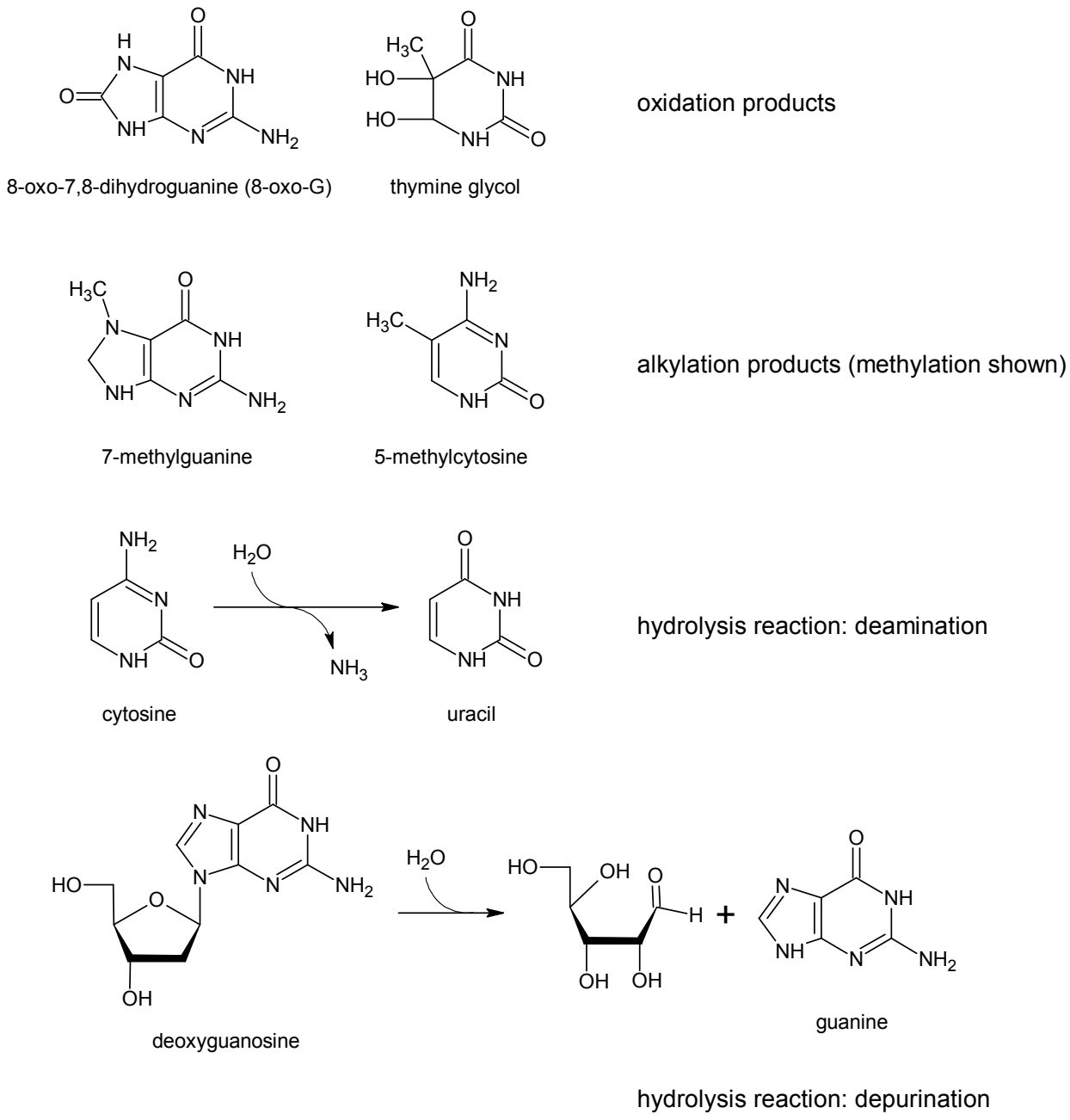

Figure 1. Examples of covalent modification of DNA bases

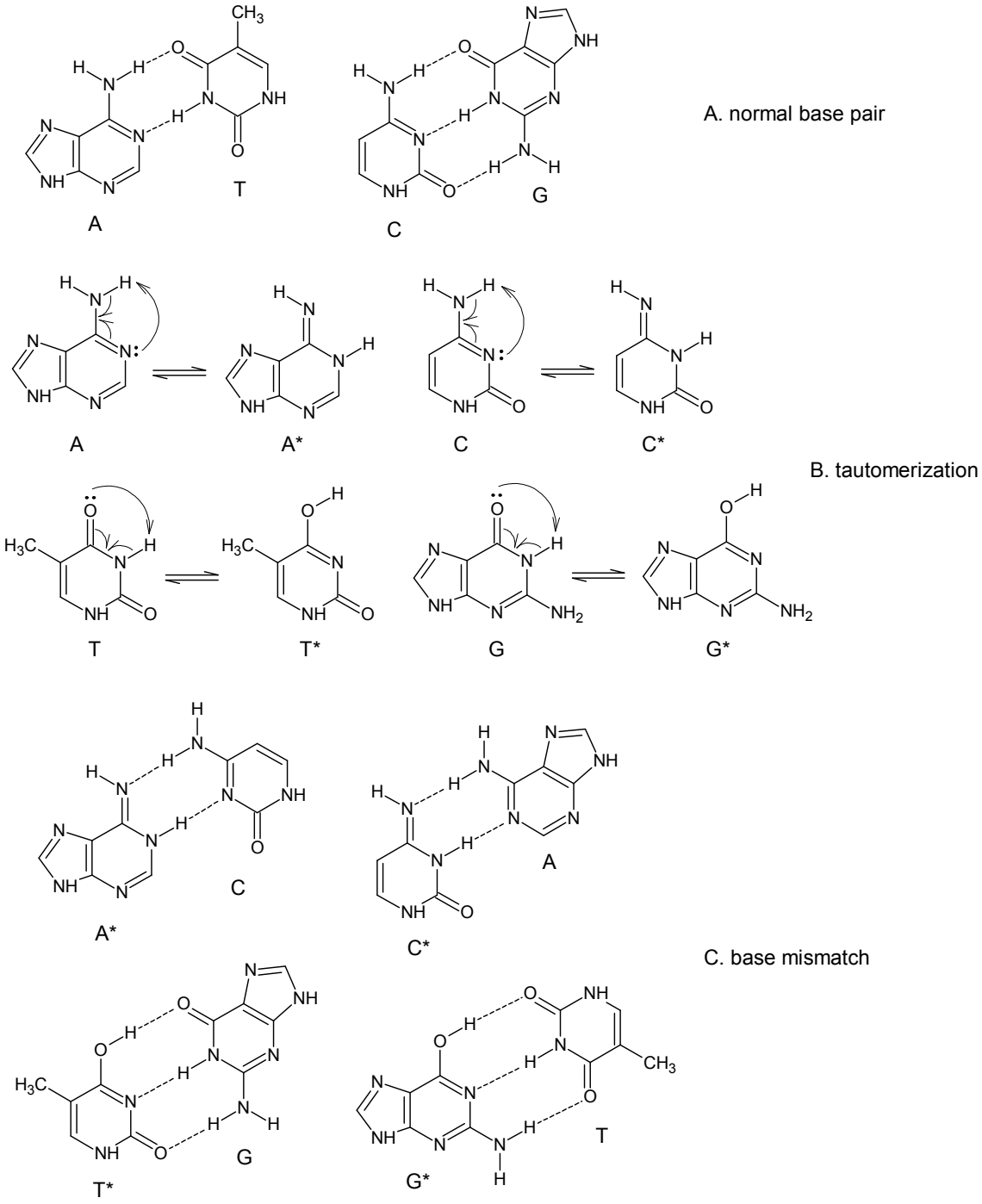

Figure 2. An example of DNA base mismatch. **A.** Normally, adenine (A) pairs with thymine (T) by two hydrogen bonds (dash line) and cytosine (C) pairs with guanine (G) by three hydrogen bonds. **B.** A and C undergo an amine-imine tautomerization, and T and G undergo an amide-iminol tautomerization. The tautomers are designated A*, C*, T* and G*. **C.** A*, C*, T* and G* can pair with C, A, G and T, respectively, causing base mismatches.

another molecule into a radical, until the two radicals use their unpaired electrons to form a covalent bond (Fig. 3). A unique property of ·OH is that it can react with all four kinds of DNA bases, yielding a variety of products [41] (Fig. 4). Although $O_2^{\cdot-}$ is both an ion and a radical, and thus is supposedly highly unstable, it cannot interact with DNA directly at physiological levels, probably due to its relatively weak reactivity and poor solubility in aqueous solution, which is also the case for H_2O_2. However, the addition of H_2O_2 to cells does cause DNA damage, possibly because H_2O_2 is converted to ·OH within nuclei by Fenton reactions [42]. Researchers have established patterns of DNA damage induced by some reactive species. Therefore, the reactive species causing the damage may be determined from the type of base damage [41].

ROS can also react with proteins. Most amino acids can be modified by endogenous ROS, and cysteine, methionine and tyrosine are the common residues modified by ·OH, $O_2^{\cdot-}$ and HOCl [43]. Upon oxidation, the sulfhydryl group (-SH) on cysteine can be converted to a sulfenic group (-SOH), which can then react with thiols or be further oxidized to sulfinic ($-SO_2H$) and sulfonic ($-SO_3H$) groups [44]. Another result of -SH oxidation is theformation of disulfide bonds (-S-S-). However, they can be easily reduced to -SH by antioxidative enzymes such as thioredoxin [45]. When oxidized, methionine residuesundergo a pathway different from that of cysteine. Methionine is converted to methionine sulfoxide (containing an -SO- group) and methionine sulfone (containing an $-SO_2-$ group) if further oxidized [46]. The process of conversion from cysteine sulfenic acid to sulfinic acid and sulfonic acid is irreversible, but the oxidation of methionine can be reversed by methionine sulfoxide reductase A (MSRA), an NADH-dependent enzyme

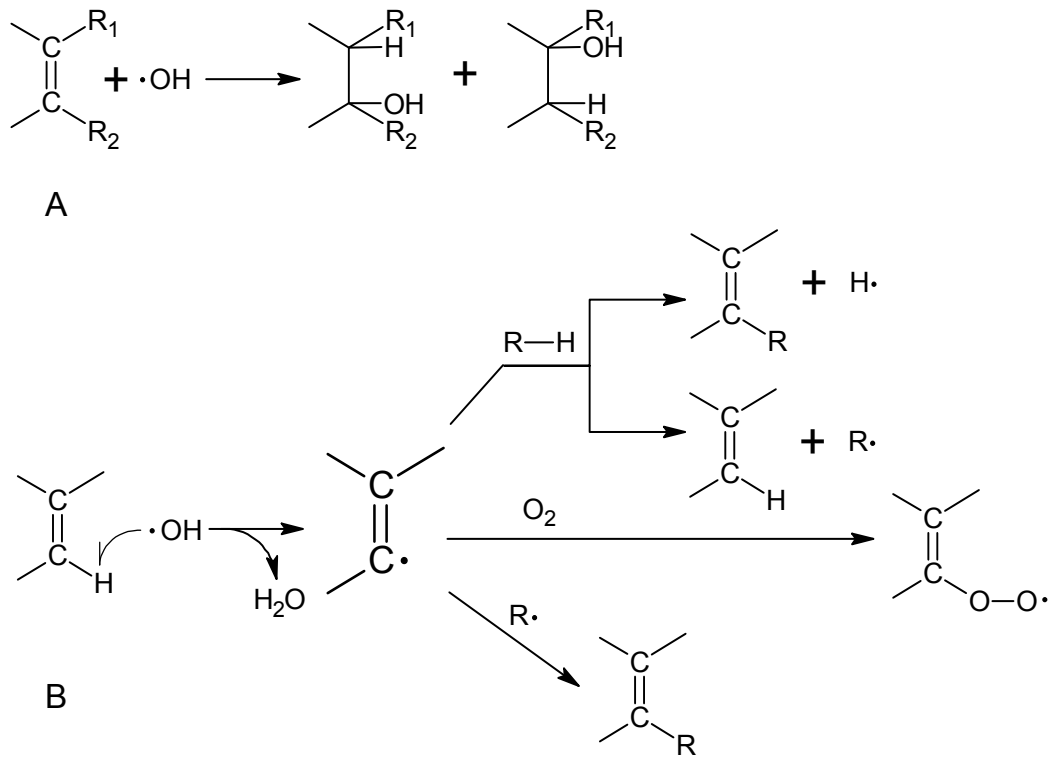

Figure 3. Reactions of Hydroxyl Radical (·OH). **A.** ·OH attacks a double bond, adding a hydroxyl to the molecule. **B.** ·OH strips a hydrogen bond from a molecule which then becomes a radical. The radical may: 1. react with another molecule to generate another radical; 2. form a larger radical with another molecule (e.g., react with O_2 to form a peroxide radical); 3. meet another radical to form a molecule.

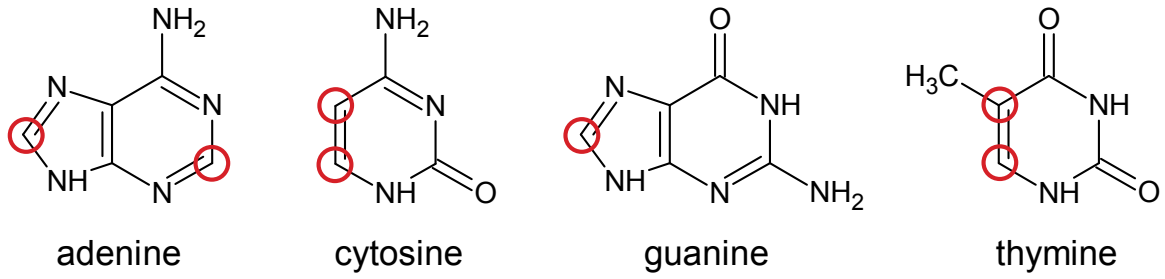

Figure 4. Sites of DNA bases where reaction with ·OH occurs (red circle)

that reduces methionine-R-sulfoxide to methionine [46] or selenoprotein R that reduces the S stereoisomer of methionine sulfoxide [47]. MSRA has been shown to be an antioxidant defense regulator protecting against oxidative damage in mouse and drosophila models [48, 49]. The aromatic amino acid residue phenylalanine can be oxidized by ·OH, yielding tyrosine. Tyrosine residues can be modified by HOCl and other oxidants, which cause their conversion to a series of products, including o,o'-dityrosine, 3,4-dihydroxyphenylalanine, 3-nitrotyrosine, and 3-chlorotyrosine [43, 50]. In addition to interacting with individual residues, ROS can directly damage the peptide backbone of proteins, resulting in the generation of protein carbonyls.

Redox signaling is the concept that free radicals, ROS, and other electronically activated species act as messengers in a biological system. ROS-protein interaction plays a key role in redox signaling. The primary effect of ROS in its capacity as a signal transduction messenger is the reversible oxidation of protein residues. By regulating the redox status of target proteins, ROS participate in a variety of signaling pathways. Examples of ROS-regulated proteins playing parts in signal transduction include transcription factors, protein tyrosine kinases (PTKs), and protein tyrosine phosphatases (PTPs) [51-60].

III. p53, ROS and redox proteins

It has long been suggested that ROS are downstream mediators of p53-dependent apoptosis [61, 62]. The work that provided convincing evidence used transgenic human and rat smooth muscle cells [63]. The researchers showed that, concomitantly with

increasing levels of p53 and onset of apoptosis, overexpression of p53 upregulates ROS levels in human smooth muscle cells, which are susceptible to p53-induced apoptosis. However, similar effects were not observed in rat smooth muscle cells, which are resistant to p53-induced apoptosis, although similar p53 levels were observed in the two cell lines. The genetic mechanism underlying the ROS regulation of p53 was subsequently elucidated. It was predicted that many genes induced by p53 expression before the onset of apoptosis encoded proteins that could generate or respond to oxidative stress [64]. The discovery confirmed a significant role of ROS in p53-induced apoptosis and indicated a possibility that p53 induces ROS production by transcriptionally activating various redox-related genes. A three-step model for p53-induced apoptosis was proposed: (i) the transcriptional induction of redox-related genes; (ii) the formation of ROS; and (iii) the oxidative degradation of mitochondrial components, culminating in cell death [64]. This model was later supported in human epithelial breast cancer MCF7 cells by a study using copper and zinc as ROS inducers [65]. In the following sections, several p53 targets related to oxidative stress are discussed.

III.A. p53-induced gene 3 (PIG3)

Polyak et al. transfected a replication-defective adenovirus encoding p53 (Ad-p53) into colorectal cancer cell line DLD-1 containing an inactive endogenous p53 gene. The patterns of gene expression were then analyzed, and a series of upregulated genes that followed p53 expression was revealed. These genes were named PIGs (p53-induced genes) and their functions were inferred. Interestingly, many PIGs members have a link

with oxidative stress (Table 1) [64]. Expression of PIG3 was shown to be correlated with the generation of ROS in the cell. The PIG3 gene is located on chromosome 2 (2p23.3) and includes 4 exons. The PIG3 promoter can be transactivated by WT p53 but not mutant p53. PIG3 expression in the absence of cellular stress is not sufficient to inhibit cell growth or induce apoptosis, but a role for PIG3 in p53-mediated apoptosis cannot be ruled out, because a p53-mediated elevation of PIG3 was observed after treating cells with the pro-apoptotic anthracycline quinone, adriamycin [66]. NAD(P)H quinone oxidoreductase 1 (NQO1), with which PIG3 shares sequence homology [64], was established as the catalyst of bioactivation of antitumor quinones [67-70]. Taken together, these findings suggested that PIG3 expression plays a role in increasing bioactivation of quinones, resulting in ROS production and apoptosis.

The mechanisms of how p53 regulates PIG3 expression were then studied in detail. Venot et al. reported that the proline-rich domain (see section I.A.) is necessary for PIG3 gene transactivation. This domain does not transactivate several promoters, such as WAF1, mdm-2 and BAX, but is responsible particularly for ROS production and sequence-specific transactivation of the PIG3 gene [27]. However, transactivation of the PIG3 gene by p53 seems not to follow ordinary mechanisms. Szak et al. demonstrated that p53 had a lower affinity for the consensus binding site in the PIG3 promoters compared to its counterparts in the p21 and mdm-2 genes, suggesting additional factors were required to stabilize the interaction of p53 with the PIG3 promoter [71]. Another group provided a possible explanation by showing that p53 does not interact with a p53 consensus binding site but with a pentanucleotide microsatellite sequence within the

Table 1. p53-induced genes (PIGs) (after [64])

Gene Name	Function/homology	Notes
p21	CDK inhibitor	
PIG1*	Galectin-7	Members of the galectin gene family can stimulate superoxide production.
PIG2	Guanidinoacetate N-methyl transferase	
PIG3*	Quinone oxidoreductase homologue	
PIG4*	Serum amyloid A	Can be induced by oxidative stress.
PIG5	Normal keratinocyte mRNA	
PIG6*	Proline oxidase homologue	
PIG7*	TNF-α induced mRNA	TNF-α is an inducer of oxidative stress.
PIG8*	Etoposide-induced mRNA	Etoposide is a quinone known to generate ROS.
PIG9	Tax1-binding protein	
PIG10	Actin-binding protein	
PIG11*	Sensitizes cells to arsenic-trioxide-induced apoptosis, a process in which ROS are also involved [72, 73].	
PIG12*	Microsomal glutathione transferase homologue	
PIG13	Unknown	

*: Genes that have a link with oxidative stress

PIG3 promoter to activate the promoter [74]. The microsatellite is polymorphic, with a varying number of pentanucleotide repeats directly correlated with the extent of transcriptional activation by p53. Supporting the existence of interaction between the p53 protein and the microsatellite, it has been suggested that the microsatellite polymorphism may be associated with a differential susceptibility to cancer [74].

III.B. Redox proteins as p53 targets

Some other genes involved in intracellular ROS regulation were subsequently identified as p53 target genes. Glutathione peroxidase (GPX) is a primary antioxidant enzyme family catalyzing the reduction of hydroperoxides to their corresponding alcohols and the conversion of free H_2O_2 to water, which are accompanied by the oxidation of glutathione (GSH). Members of the GPX family include GPX1, GPX2 (gastrointestinal), GPX3 (plasma), GPX4 (phospholipid hydroperoxidase), GPX5 (epididymal androgen-related protein), GPX6 (olfactory) and GPX7 (also olfactory). The promoter of the GPX1 gene was found to be transactivated by endogenous WT p53 activated by etoposide, a topoisomerase II inhibitor and a p53 activator [75]. The relationship between p53 and mitochondrial enzyme manganese superoxide dismutase (MnSOD), a significant ROS scavenger inside mitochondria, is an interesting one. On one hand, MnSOD is subject to p53-mediated transcriptional repression at the promoter level; on the other hand, MnSOD overexpression decreases p53-gene expression at the promoter level [76]. Catalase (CAT), another antioxidative enzyme catalyzing the decomposition of H_2O_2 into H_2O and O_2, does not, however, increase upon p53 activation, while CAT overexpression inhibits

p53-mediated apoptosis [77]. Mitochondrial ferredoxin reductase (FR) is a mitochondrial flavoprotein that initiates electron transport to cytochrome P450 from NADPH and also contributes to tumor growth suppression and cell viability. The gene (FDXR) encoding FR is significantly induced by p53 in human colon cancer cells treated with 5-fluorouracil (5-FU), an effective adjuvant therapy for patients with colon cancer and a potent apoptosis inducer [78]. Triphenylmethyl phosphonium (TPMP)-vitamin E (MitoVitE) and TPMP-ubiquinol (MitoQ), two antioxidants specifically targeted to mitochondria, completely prevent 5-FU-induced apoptosis, whereas their non-targeted counterparts are inactive, indicating that 5-FU induced apoptosis is dependent on ROS generation [78].

A recent report indicated that a common mechanism of p53 regulation on antioxidant responsive genes is that p53 directly suppresses the NF-E2-related factor (Nrf2)-dependent transcription of antioxidant response element (ARE)-containing promoters, which can be triggered by DNA damage [79]. Examples of genes regulated by p53 through this mechanism include x-CT (a subunit of the cystine/glutamate transporter), glutathione S-transferases (GSTs), and NADH quinone oxidoreductase 1 (NQO1) [79].

III.C. Interaction between PTEN and PIG3

The phosphatase with tensin homology PTEN is a lipid phosphatase that negatively regulates the phosphatidylinositol 3-kinase (PI3K) pathway [80]. By counteracting the PI3K pathway, which is a potent stimulator of cell proliferation and survival, PTEN acts

as a tumor suppressor. Moreover, the PI3K pathway sits at the junction of several other important tumorigenic signaling pathways (reviewed in [81]). Like p53, inactivating mutations of the PTEN gene are found in a wide range of common human cancers [82-84]. Interestingly, just opposite p53, PTEN deletion upregulates PIG3 alongside several other p53 effectors [85]. Given the fact that p53 transactivates PTEN [86], how PIG3 expression is balanced between p53 and PTEN regulation is a topic needing further elucidation.

IV. Events downstream of ROS

What happened after p53 induction of ROS? An obvious consequence was apoptosis, but the exact mechanisms remained unclear. The work by Li et al. suggested that mitochondria played a role. Using HeLa cells, they showed that, after p53 induction of ROS, the mitochondrial membrane potential ($\Delta\Psi_m$) increases, activating the caspase cascade [87]. Downstream of caspase activation, $\Delta\Psi_m$ decreases, and the alternation of $\Delta\Psi_m$ induced by p53 can be prevented by Bcl-2 downstream of ROS [87]. Cytochrome c is not involved in this model of p53-induced apoptosis [87]. ROS were then established to be mediators of senescence, and a rise in intracellular ROS may be an important signal that triggers senescence (reviewed in [88, 89]). Mitochondrial permeability transition pore (MPTP) is a mitochondrial protein complex assembled at contact sites between the inner and outer membranes. Because MPTP was suggested to be a key determinant of the severity of mitochondria-mediated apoptosis [90], the interaction between permeability transition and p53 became a hot research subject. Various studies suggested that, upon

various kinds of stimulation related to p53, permeability transition inhibitor cyclosporin A blocks ROS generation [91], increases $\Delta\Psi_m$ [91], releases cytochrome c into cytosol [91-93] and induces DNA fragmentation [94], but does not block caspase activation [95]. These data validate the roles of ROS and MPTP in p53-mediated pathways.

V. Regulation of p53 by redox system components

Given the multiple ways p53 may regulate ROS levels and redox protein expression, it is of great interest whether these components of the redox system have regulatory effects on p53. This section is a summary of some notable research addressing the effects of ROS and redox proteins on p53.

V.A. Effects of ROS on p53

Similar to many positive and negative feedback loops in p53 signaling pathways involving multiple proteins (reviewed in [96]), ROS can regulate p53 through many pathways, and significant evidence supports this hypothesis. First, ROS-treated cells show increased p53 stability and activity. In human umbilical vein endothelial cells, H_2O_2 was shown to induce p53 phosphorylation, which required transactivation of platelet-derived growth factor-β receptor and subsequent ataxia telangiectasia mutated kinase activation [97]. H_2O_2-treated human hepatoma cells demonstrate increased apoptosis [98] and p53 mutation in a specific exon [99]. ROS are required for p53 activation in leukemia cells and normal lymphocytes [100] and stabilization of p53

during hypoxia in mammary breast cancer cells MCF-7 [101]. Second, cells lacking antioxidant defense demonstrate increased p53 expression. In human lung cancer cells in which peroxiredoxin (Prx) I activity was lowered, ROS increased, upregulating p53 expression [102]. Similarly, in human B lymphoma cells treated with L-buthionine sulphoximine, an inhibitor of γ-glutamyl cysteine synthetase which is essential in gluathione biosynthesis, a truncated form of p53 is expressed after ROS production increases [103]. Third, antioxidants inhibit p53 activity. Two potent antioxidants, N-acetyl-cysteine and glutathione, strongly suppressed p53-mediated apoptosis in human hepatoma cells [104]. Fourth, there are reports that suggest that nuclear factor-kappa B (NF-κB) [105] and Fas [99, 106] play a role in ROS regulation of p53, but some other evidence suggests that ROS and p53 may not be required for Fas-induced apoptosis [107, 108]. The source of stress may be the key determinant of which pathway is involved [107, 108]. In summary, ROS may have a positive feedback effect on p53 expression and activation, but the detailed mechanisms have not been clarified yet.

V.B. Redox proteins regulate p53 activity

V.B.1. WOX1

The genomic organization patterns of human and murine WW domain-containing oxidoreductase (WOX1) are almost identical [109]. The human WOX1 gene encodes a protein that contains two WW domains responsible for protein-protein interactions and a short-chain dehydrogenase (SDR) domain. It has been suggested that WOX1 is involved

in sex steroid metabolism and signaling pathways [110, 111]. This gene has been reported to behave as a suppressor of tumor growth in human breast and prostate, and in ovarian, oral squamous, pancreatic and hepatocellular cell lines [111-118]. Murine WOX1 was shown to enhance TNF cytotoxicity in L929 cells by downregulating apoptosis inhibitors Bcl-2 and Bcl-xL and upregulating pro-apoptotic p53 by the ADH domain [119]. Specifically, Tyr33-phosphorylated WOX1 directly binds and stabilizes Ser46-phosphorylated p53 [120].

V.B.2. NQO1

NQO1 is a flavin adenine dinucleotide (FAD)-binding protein that forms homodimers and reduces quinones to hydroquinones. The protein's enzymatic activity prevents the one electron reduction of quinones that results in the production of radical species. It has been demonstrated that NQO1 stabilizes hot-spot p53 mutant proteins in cancer [121]. This role of NQO-1 was further confirmed by an in vivo study that suggests that the sensitivity of NQO1-null mice to benzo(a)pyrene-induced skin cancer is increased due to lower induction of p53 and decreased apoptosis [122].

V.B.3. Ref1

Another gene drawing much attention is redox factor-1 (Ref1). The Ref1 protein is both a regulator of the redox state of a number of proteins and a DNA repair (A/P) endonuclease [123]. It functions in the process of base excision repair (BER) to cleave DNA 5' to

abasic sites, as well as to load DNA polymerase γ onto DNA [124]. The redox activity of Ref1 is mediated by ROS and protein kinase C (PKC) phosphorylation in response to DNA damaging agents such as oxidizing agent hypochlorite [125]. PKC had been known to phosphorylate p53 [126, 127] and thus increase p53 activity in cell cycle control, particularly the induction of the G1/S growth arrest [128, 129].

There is some evidence demonstrating that Ref1 is able to stimulate p53 activity. While Refl was observed to repair inactivated (oxidized) forms of both full-length and carboxy-terminally truncated p53, it potently stimulated full-length but not truncated p53 in the presence of reducing agents in vitro and in vivo [123]. Consistent with this, reduction of residues Cys275 and Cys277 of p53 by selenomethionine (the major dietary source of selenium) caused p53 to recruit Ref1 and activate DNA-repair machinery, suggesting that Ref1 is required in the activation of p53 by selenomethionine [130]. An in vivo study further revealed that Ref1 enhances the ability of p53 as a transcription factor and an apoptosis inducer [131]. Interestingly, Ref1 and thioredoxin reductase cooperate in the regulation of basal p53 activity, but not in p53 induction by DNA damage [132].

V.B.4. Azurin

Azurin is a redox protein involved in a series of electron-transfer reactions with cytochrome C551 and nitrite reductase [133]. Found to be localized in the cytosol and the nuclear fractions, azurin was determined to induce apoptosis in J774 murine macrophages through complex formation and stabilization of p53 and ROS generation [134]. In human

cancer (melanoma UISO-Mel-2) cells, azurin forms a complex with p53, thereby stabilizing it and raising its intracellular levels, and hence apoptosis is triggered through the mitochondrial pathway [135]. A redox-negative mutant form of azurin (M44K/M64E) demonstrates much less cytotoxicity, fails to form a complex with p53 and shows less efficiency in stabilizing p53 than WT azurin, suggesting that the functional site of azurin involved in p53 regulation might be at those two amino acid residues [135]. The same group later confirmed that the M44/M64 residues are responsible for p53 regulation by showing that WT and M44K/M64E mutant azurin contradictorily affect p53 function [136]. The former induces apoptosis but minimally inhibits cell-cycle progression, whereas the latter is incapable of inducing apoptosis but mediates strong inhibition of cell-cycle progression [136].

A good example of a p53-binding protein is mdm2 [137]. The mdm2-p53 interaction has been extensively studied (reviewed in [138]). Briefly, the hydrophobic residues of Phe19, Trp23 and Leu26 of the transactivation domain of p53 bind a deep hydrophobic binding pocket on the mdm-2 surface, forming an amphiphilic α-helix (literature cited in [138]). A comparison of the mechanisms shows how the interaction of the redox proteins mentioned above and mdm2 with p53 may be beneficial. Studies of this aspect of NQO1 have been extensive. NQO1 does not inhibit p53 degradation mediated by mdm-2 [139], but hsp90 stabilizes mutant p53 by inhibiting mdm-2 [140]. NQO1 keeps p53 from proteasomal degradation through an mdm-2 and ubiquitin-independent mechanism [141]. Accordingly, NQO1 does not associate with mdm-2 co-immunoprecipitated with p53 [142]. Also, an inhibitor of NQO1, dicoumarol, is more effective in decreasing WT p53

levels and apoptosis in normal thymocytes than hsp90 inhibitors are, which performs better in myeloid leukemic cells [139]. These findings suggest that NQO1 stabilizes p53 through a distinct pathway, particularly in response to oxidative stress [139]. Ref1 regulates p53 by promoting p53 tetramerization in two ways, thereby enhancing p53 binding to target DNA [143]. First, Ref1 increases the association of p53 dimers into tetramers, the optimized form for p53 activity [143, 144]. Second, Ref1 contributes to the de-stacking of higher oligomeric forms of p53 into the tetrameric form in vitro [143]. For the other two proteins, the detailed mechanisms remain unclear, even though the amino acid residues by which WOX1 and azurin interact with p53 have been identified.

VI. An antioxidative function of p53

It was surprising that p53 was found to have an antioxidative function. Although it has been reported that p53 activates GPX [75], it could be argued that GPX induction and ROS generation occur at different time points dowstream of p53 activation, with ROS generation occurring later [145]. Aldehyde dehydrogenase 4 (ALDH4), a mitochondrial-matrix NAD^+-dependent enzyme that catalyzes the second step of the proline degradation pathway [146], was found to be directly transactivated by p53 [147]. ALDH4 appears to be an antioxidative protein, because human lung carcinoma H1299 cells transformed to overexpress ALDH4 showed significantly lower intracellular ROS than control cells after H_2O_2 or UV treatment [147]. The sestrin gene family is another antioxidative protein family revealed to be a p53 target. In an attempt to use cDNA microarray in order to identify novel genes participating in cellular responses to

prolonged hypoxia in cancer cells, the gene designated Hi95 was found to be p53-inducible in human glioblastoma cell line A172 and to prevent apoptosis induced by ischemia or H_2O_2 in human breast carcinoma cell line MCF7-tet-off [148]. Later, Hi95 was put into the sestrin gene family [149]. The antioxidative role of sestrins was confirmed by evidence demonstrating that after a peroxide burst, sestrins substantially increase the rate of recovery of overoxidized Prxs [150].

This leads to the question of how prooxidants and antioxidants regulate p53. A promising answer has been given by Sablina et al. [151]. By examining the response of p53 under different severities of oxidative stress, they showed that p53 decreases ROS after mild stress (0.2 mM H_2O_2) but increases ROS after grave stress (1 mM H_2O_2). Antioxidant genes SESN2, SESN1 and GPX1 were rapidly activated by p53 at both low and high concentrations of H_2O_2 (<0.2 h and <2 h, respectively), while prooxidant genes TP53I3 and BBC3 were activated more slowly (>0.4 h at low H_2O_2 concentration and >6 h at high H_2O_2 concentration). The researchers suggest that whether p53 is inclined to yield antioxidative or prooxidative effects depends on the stress condition. In stress-induced apoptosis, p53 functions as a prooxidant, resulting in release of mitochondrial ROS. In non-stressed or physiologically stressed cells, the antioxidant function of p53 is mediated through a set of antioxidant gene products, which are responsive to lower levels of p53. Under mild conditions, the antioxidant function of p53 decreases the probability of genetic alterations and assists the survival and repair of cells in a nonfatal way, without triggering the apoptotic pathway. The phenomena described by Tan et al. [75], that p53 quickly upregulates GPX expression, agrees with this model.

However, it is hard to distinguish a benign stress condition from a devastating one. First, it is difficult to determine real-time ROS concentration in living cells. As Nicholls and Budd have pointed out [152], major impediments include the rapidness and complexity of intracellular ROS production and conversion, non-quantitative assays due to competition between dyes and redox enzymes, and non-specific fluorescent ROS probes which may respond to other biochemical changes such as pH and $\Delta\Psi_m$. However, this issue will hopefully be partially resolved by the emergence of new probes capable of accurately detecting natural signaling levels of H_2O_2 generated by living cells [153]. Second, it is still under debate whether exogenous addition of ROS (commonly H_2O_2) can mimic an actual physiological or pathophysiological condition. Given the difficulty in measuring real-time ROS levels, it is hard to estimate how much ROS should be added. Therefore, results of current studies utilizing exogenous ROS are sometimes questioned, although some evidence suggests that H_2O_2 in signaling pathways may be produced exogenously under biological conditions [154]. Finally, experiments employing known concentrations of ROS yield puzzling results. Some reports, previously cited, show that 1-6 h treatment with 0.1-0.2 mM H_2O_2 effectively induces apoptosis [97-99, 146], while in another research project significant apoptosis onset was not observed under similar treatment [151]. While cells may have different sensitivities to oxidative stress, a concentration "threshold" of H_2O_2 to induce the antioxidative effect of p53 without initiating the p53-induced apoptotic pathway might not exist. Under mild conditions, mitochondrial apoptosis may still progress, and the progression seems to become apparent as treatment time lengthens or oxidant concentration increases. Whether and to what extent p53 can counteract the apoptotic trend in various cell types are questions demanding further research.

VII. ROS levels: a change in the faces of p53?

A more generic question than the ability of ROS to choose between antioxidant and prooxidant functions of p53 is whether ROS levels can decide cell fate through p53. To address this question, a summary of some related p53 functions and characteristics is necessary.

p53 is always called “the guardian of the genome.” not only because it is extremely sensitive to DNA damage [155], but also because it interacts with a variety of DNA double-strand break sensing and repair proteins and it can serve as a 3'-5' exonuclease [25]. A recent report suggested that p53 may even contribute to mitochondrial DNA (mtDNA) repair by interacting with DNA polymerase γ [156]. The stability of both nuclear and mitochondrial DNA leads to cell survival.

A small fraction of p53 has been proved to translocate to mitochondria at the onset of p53-dependent apoptosis [157]. The primary signal that causes localization of p53 to mitochondria is mitochondrial ROS generation, which preferentially induces oxidative mitochondrial DNA damage [156]. Mitochondria-translocated p53 can trigger transcription-independent apoptosis by direct suppression of the antiapoptotic Bcl-xL and Bcl2 proteins [158] and activation of the proapoptotic Bax [159, 160] and Bak [161]. Mitochondrial p53 accumulation happens soon after stress stimulation, and before its transcriptional effect [162]. In accordance with this, mitochondrial p53 translocation occurs before the nuclear translocation of p53, and p53 targets MnSOD and directly

inhibits MnSOD activity when translocating to mitochondria, indicating a novel pathway by which p53 regulates ROS levels [163]. Some efforts have been made to find the force that drives p53, normally localized in the nucleus, to mitochondria. Loss of Bax completely protects human colon carcinoma cells (HCT116) against p53-induced, transcription-independent apoptosis, and reexpression of Bax restores p53 sensitivity in HCT116 cells, indicating that Bax may be able to bring p53 to mitochondria [159]. Bad, a positive regulator of cell death which can be transactivated by p53, can also direct nucleus-localized p53 to the mitochondria and form a complex with p53 there, promoting apoptosis via activation and oligomerization of Bak [164]. Endogenous p53 was found to be cleaved by caspases (specifically, caspase-3, -6, and -7, but not caspase-8) during apoptosis induced by several anticancer drugs, and some caspase-cleaved p53 fragments localized to mitochondria [165]. Since caspase activation has been well documented as a marker of the apoptosis process, this finding suggests a positive feedback loop in p53-induced apoptosis. Details on how p53 translocates to mitochondria and what other functions mitochondria-localized p53 possesses are attractive topics.

The discovery of two new p53 targets has revealed another unexpected aspect of p53 function, the ability to regulate glucose metabolism. In a study to provide a genetic explanation of the Warburg effect (that cancer cells preferentially utilize glycolytic pathways for energy generation while down-regulating their aerobic respiratory activity [166]), synthesis of the cytochrome c oxidase 2 (SCO2) gene, whose translational product is a critical regulator of the cytochrome c oxidase complex (also known as Complex IV), which is the major site of oxygen utilization in the eukaryotic cell, was shown to be

p53-transactivated [167]. It was demonstrated that a p53-induced gene, TP53-induced glycolysis and apoptosis regulator (TIGAR), controlled glucose metabolism through the pentose phosphate pathway by lowering fructose-2,6-bisphosphate levels in cells, resulting in an inhibition of glycolysis and an overall decrease in intracellular ROS levels [168]. These studies reveal that inhibition of glycolysis, the dominant way cancer cells generate power to sustain themselves, is a new weapon for tumor suppression by p53. It should be noticed that AMP-activated protein kinase (AMPK) induces p53 phosphorylation [169] on Ser15 [170] and results in cell cycle arrest [170, 171], suggesting that metabolic status has a reverse impact on p53.

The answer to how ROS can affect these issues may again be that physiological and cytotoxic ROS levels will lead p53 to different pathways. Once stimulation causes ROS concentrations to become abnormally high but not critical, p53 may first try to repair nuclear and mitochondrial DNA and transactivate antioxidant enzymes to counteract increased ROS production. However, if ROS levels continue to rise, p53 may turn into a killer by inducing apoptosis through both mitochondrial and nuclear pathways. A recent report described how different levels of ROS result in different cell fates, using bladder (EJ) and prostate (PC3) tumor cell lines as examples [172]. When a small increase of p53 levels was induced, cells showed characteristics of senescence with a small increase in ROS levels. However, with a large increase in p53 levels, cells demonstrated apoptosis accompanied by a greater increase in ROS levels. Moreover, addition of exogenous ROS could convert a senescent response induced by relatively low levels of p53 into apoptosis.

Related events occurring in ROS regulation of cell fate mediated by p53 are summarized in Fig. 5.

VIII. Concluding Remarks

While ROS have long been established as a mediator of p53-induced apoptosis, recent research projects have revealed the impact of ROS on p53. ROS levels have been proven to regulate the redox functions of p53, and to have the potential of determining cell fate through p53. Nonetheless, there are still many unanswered questions, some of which follow.

1. Are there thresholds of ROS levels and/or stimulation time length for p53 to switch from functioning to sustain cell survival to triggering apoptosis? More generally, is there a clear line between physiological and toxic ROS levels?

2. If a clear line exists, what are the thresholds in different types of cells and how do characteristics of specific cells influence the thresholds? If there are no clear boundaries between physiological and toxic ROS levels, how do cells choose their fate under various conditions?

3. More and more new unanticipated properties of p53 have been discovered. For example, literature suggests that for p21, PIG3 and Bax, p53 demonstrates a hierarchy in its transactivation activity and subsequent pathway activation by preferentially

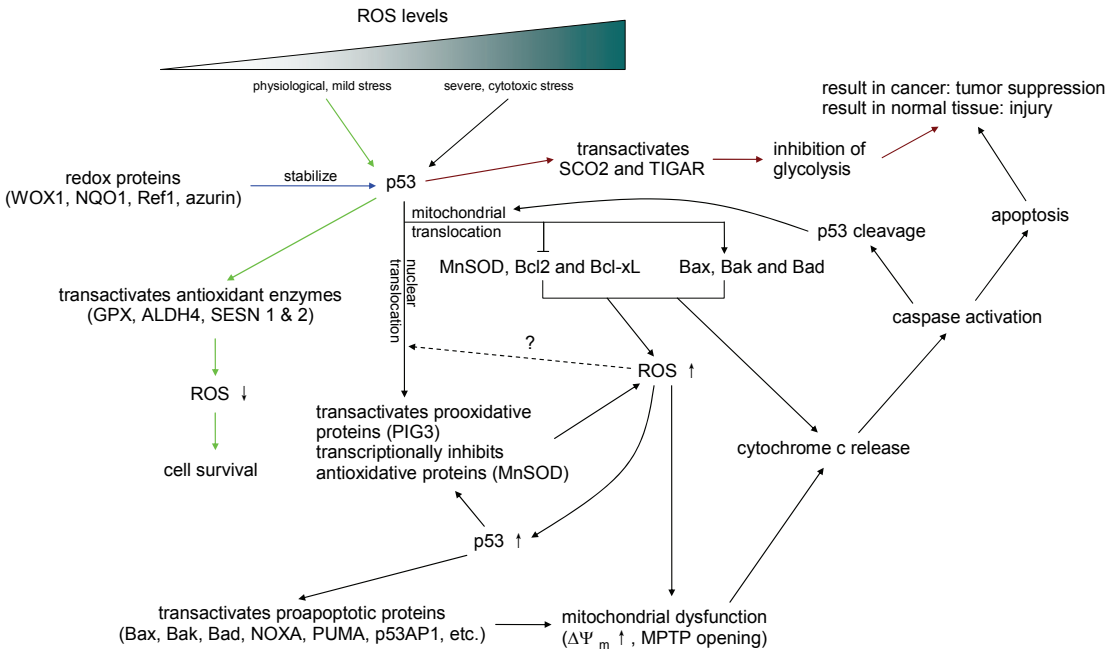

Figure 5. Proposed network of ROS regulation on cell fate through p53. Different colors of arrows indicate different pathways. **Blue arrow:** Some redox proteins contribute to the stabilization of p53. **Green arrows:** Under physiological ROS levels, oxidative stress is mild. p53 transactivates antioxidant enzymes, resulting in lower ROS levels and cell survival. **Black arrows:** Under high ROS levels, oxidative stress is cytotoxic. p53 translocates to mitochondria first. In the mitochondria, p53 directly inhibits MnSOD, Bcl2 and Bcl-xL and activates Bax, Bak and Bad. These events lead to ROS increase and cytochrome c release which trigger caspase cascade and apoptosis. p53 later translocates to the nucleus, where it transactivates prooxidative proteins and transcriptionally inhibits antioxidative proteins, inducing more ROS increase. The ROS increase may upregulate p53 furthermore, thus the positive feedback loop. More p53 results in transactivation of multiple proapoptotic proteins, which cause mitochondrial dysfunction. The mitochondrial apoptosis pathway is then initiated. Downstream of cytochrome c release, caspase activation brings about p53 fragments, parts of which are found to translocate to the mitochondria. Whether ROS increase contributes to the nuclear translocation of p53 remains undetermined. **Red arrows:** p53 can inhibit glycolysis by transactivation of SCO2 and TIGAR. The final effects of pathways indicated by black and red arrows pathways are tumor suppression if the pathways take place in cancer cells, and tissue injury if the pathways happen in normal cells.

transactivating p21 over the other two [173-175]. What additional functions may the tumor suppressor have? How are they regulated?

ROS, the inevitable byproduct of the oxidative phosphorylation process, may be able to do more than what is currently known. Further research of the regulatory function of ROS on p53 will contribute to a greater understanding of mechanisms that regulate cell fate as well as to developing therapies for cancer.

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