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REVIEW

Defenses against Pro-oxidant Forces - Maintenance of Cellular and Genomic Integrity and Longevity

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There has been enormous recent progress in understanding how human cells respond to oxidative stress, such as that caused by exposure to ionizing radiation. We have witnessed a significant deciphering of the events that underlie how antioxidant responses counter pro-oxidant damage to key biological targets in all cellular compartments, including the genome and mitochondria. These cytoprotective responses include: 1. The basal cellular repertoire of antioxidant capabilities and its supporting cast of facilitator enzymes; and 2. The inducible phase of the antioxidant response, notably that mediated by the Nrf2 transcription factor. There has also been frenetic progress in defining how reactive electrophilic species swamp existing protective mechanisms to augment DNA damage, events that are embodied in the cellular "DNA-damage response", including cell cycle checkpoint activation and DNA repair, which occur on a time scale of hours to days, as well as the implementation of cellular responses such as apoptosis, autophagy, senescence and reprograming that extend the time period of damage sensing and response into weeks, months and years. It has become apparent that, in addition to the initial oxidative insult, cells typically undergo further waves of secondary reactive oxygen/nitrogen species generation, DNA damage and signaling and that these may reemerge long after the initial events have subsided, probably being driven, at least in part, by persisting DNA damage. These reactive oxygen/nitrogen species are an integral part of the pathological consequences of radiation exposure and may persist across multiple cell divisions. Because of the pervasive nature of oxidative stress, a cell will manifest different responses in different subcellular compartments and to different levels of stress injury. Aspects of these compartmentalized responses can involve the same proteins (such as ATM, p53 and p21) but in different functional guises, e.g., in cytoplasmic versus nuclear responses or in early- versus late-phase events. Many of these responses involve gene activation and new protein synthesis as well as a plethora of post-translational modifications of both basal and induced response proteins. It is these responses that we focus on in this review. © 2018 by Radiation Research Society

BACKGROUND

Oxidative stress, such as that invoked by exposing human cells to ionizing radiation or to various electrophilic/pro-oxidant chemicals or many xenobiotics, results in the formation of highly reactive intermediates. Some of the most powerful and pervasive of these are reactive oxygen and nitrogen species (ROS/RNS) that subsequently interact with cellular components to generate many types of biomolecular alterations, including DNA damage. The hydroxyl radical ($^{\bullet}OH$) is prominent among ROS, although hydrogen peroxide and the superoxide radical anion ($^{\bullet}O_2^-$) (*1*) can also cause many types of cellular damage. In addition, over the last decade, it has been shown that ROS/RNS play a role in metabolism and as essential mediators in signaling cascades and biochemical pathways. Human cells are endowed with a remarkable complement of enzymes that, on one hand aim to counteract such events, and on the other, aim to use them to generate stress responses. The differences in outcome depend on the magnitude of the change in redox status. The immediate phase of this response involves the engagement of basal factors that function to prevent these reactive species from damaging sensitive cellular targets (rather than relying on processing such damage once it has occurred). The second early phase of the cellular response to elevated levels of oxidative stress involves the rapid induction of a broad suite of antioxidant genes and proteins (*2*). The third phase, the classic DNA damage response (DDR), represents the multifaceted cellular response to DNA damage formed either directly or indirectly through reactive species that have evaded

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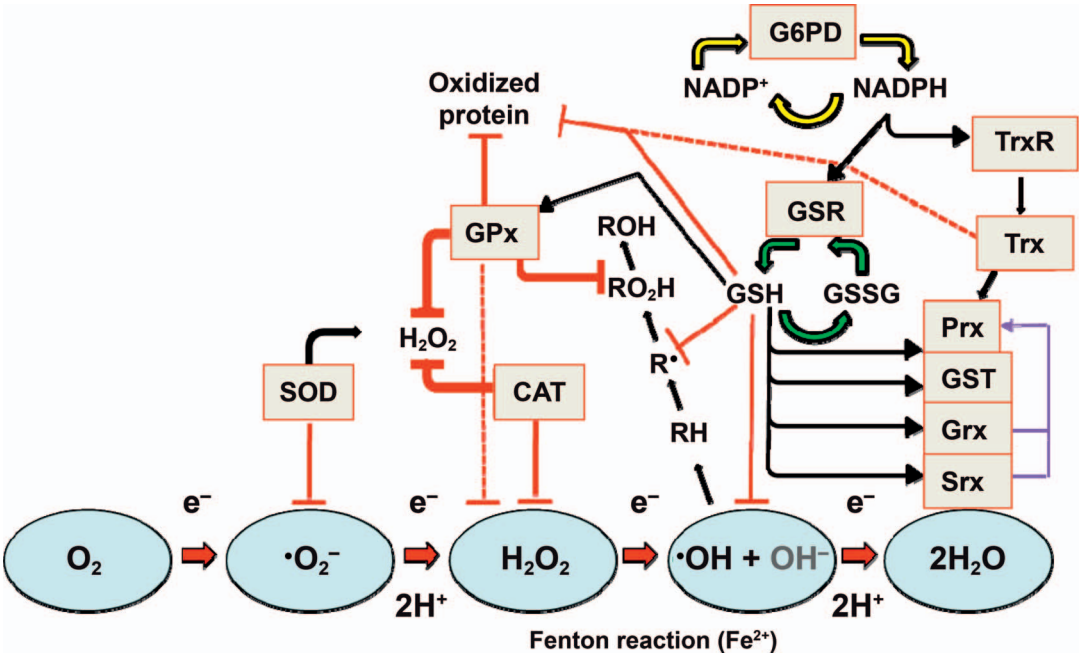


FIG. 1. Reactive oxygen species (ROS) formation and their removal in human cells. ROS are the intermediates in the reduction of molecular oxygen to water by sequential 1-electron transfers. The cellular oxidative stress caused by ROS is counteracted by numerous antioxidant enzymes that utilize the reducing equivalents of GSH generated by the GSH redox cycle (green arrows) or of NADPH generated by the G6PD-driven pentose phosphate cycle (yellow arrows). Antioxidant enzymes that utilize GSH include the GPx's, GSTs, Grxs and Prxs. The GSSG generated in such reactions can be recycled to GSH by the enzyme GSR using the reducing equivalents of NADPH. In addition to maintaining the GSH redox cycle via GSR, NADPH also maintains Trx reduction through the intermediacy of TrxR. Trx also regulates the Prxs. SOD and catalase eliminate superoxide ($^{\bullet}O_2^-$) and hydrogen peroxide, respectively. GSH itself is important for scavenging potentially harmful species such as the hydroxyl radical ($^{\bullet}OH$) and for restoring damaged biomolecules ($R^{\bullet}$) via hydrogen-atom/electron donation. CAT = catalase; GSH = glutathione; G6PD = glucose-6-phosphate dehydrogenase; GPx = glutathione peroxidase; GST = glutathione-S-transferase; Grx = glutaredoxin; GSSG = oxidized GSH (Disulfide): GSR = glutathione reductase; NADPH = nicotinamide adenine dinucleotide phosphate; Prx = peroxiredoxin; SOD = superoxide dismutase; Srx = sulfiredoxin; Trx = thioredoxin; TrxR = thioredoxin reductase.

antioxidant responses; the function of the DDR is either to restore the cell as closely as possible to its original pre-stress status or to eliminate it from the proliferating population if the level of unrepaired damage is excessive. Unfortunately, this is not always possible.

In this review, we endeavor to provide a high-level picture of the various cellular responses that abrogate these rapid chemical redox reactions. However, we must also consider the reality that further waves of secondary ROS will be generated as part of the cellular response to these early primary events, occurring long after the initial radiation/ROS exposure is over. Such secondary pro-oxidant cycles can be driven by a number of pathways, depending on the cell background and the type and magnitude of the stress, including oxidative injury to organelles such as nuclei, mitochondria, peroxisomes and proteasomes that may cause persistent or lethal damage [e.g., (*3, 4*) and references therein]. Activation of enzymes such as the NADPH oxidase (NOX) proteins, pro-inflammatory cytokine production, and activation of the pro-oxidant p53-inducible PIG/TP53I genes and their encoded proteins after high levels of damage can prolong the pro-oxidant state. Micronuclei and cytoplasmic DNA can signal through the cyclic GMP-AMP synthase (cGAS)-stimulator of interferon genes (STING) pathway to generate type 1 interferon production and trigger interferon response genetic programs. Oxidative damage to proteins disrupts their folding and maturation and slows their degradation by the proteasome. These proteins accumulate in the endoplasmic reticulum, producing a stress-signaling response known as the unfolded protein response that involves both cytoprotective and apoptotic/cell death pathways depending on the level of damage. Finally, persisting damage over many cell generations may continue to drive ROS production in a feed-forward response to cause further damage through chronic inflammation (*5*).

THE BASAL ANTIOXIDANT RESPONSE

Pivotal among the basal cellular antioxidants is glutathione (GSH), which is the most abundant nonprotein thiol compound in human cells (*6*) (Fig. 1). GSH, a tripeptide of glu-cys-gly, is synthesized by the sequential activities of the rate-limiting enzyme, γ-glutamylcysteine synthetase (γ-GCS; also known as glutamate cysteine ligase or GCL) and glutathione synthetase (GSS) enzymes. Because the glu-cys amino acid linkage involves the γ (rather than α) carboxyl group of glutamate, GSH is not readily hydrolyzed by cellular peptidases, which confers it with high stability, such that its levels typically reach ~5 m*M* in human cells.

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The intracellular antioxidant function of GSH is mediated by the thiol (SH) group of its cysteine residue, which acts as an ROS/electrophile scavenger and as an H-atom/electron donor to radicals formed by reaction of these ROS/electrophiles with biomolecular targets, resulting in chemical restoration of the target (assuming that there has been no intervening reaction of the radical other than migration). The GSH thiol group also maintains the activity of redox-sensitive proteins that have suffered oxidation of their cysteine residues, including some involved in DDR activities such as DNA repair and cell cycle checkpoint regulation, notably by restoring oxidized protein thiols to their reduced (active) state. GSH or glutathionylated biomolecules are released from cells after radiation exposure through membrane channels (*7*) and may influence the biology of bystander cells. A number of antioxidant enzymes utilize the reducing equivalents of GSH, including the glutathione peroxidase (GPx) family of selenocysteine proteins, of which eight human isoforms have been identified to date. The GPx's protect against oxidative damage by reducing molecules such as hydrogen peroxide (the preferred substrate of GPx1, the most abundant cytoplasmic form) and lipid hydroperoxides (the preferred substrate of GPx4).

The participation of GSH in antioxidant reactions, either chemically or enzymatically via GPx, results in its own oxidation to the disulfide form, GSSG; however, cells are equipped with another important enzyme, glutathione reductase (GSR), which uses the reducing equivalents of the critical antioxidant cofactor nicotinamide adenine dinucleotide phosphate (NADPH) to reduce GSSG back to two molecules of reduced GSH, which can then participate in further cycles of antioxidant reactions as above. Beyond this, GSH, through exerting redox control, critically regulates many cellular biochemical pathways, including metabolism, gene expression, cell cycle, DNA repair and cell death.

The glutathione-S-transferases (GST) are a family of phase II detoxifying enzymes that catalyze the conjugation of GSH to various electrophiles/xenobiotics, including lipid hydroperoxides, resulting in the elimination of such species; there are cytosolic, mitochondrial and microsomal GST families that act on a broad range of substrates. Of interest in the context of oxidative stress conditions is the ability of GSTπ to dissociate from complexes with jun N-terminal kinase 1 (JNK), resulting in activation of the JNK pathway that can lead to apoptosis (*8*); a similar scenario is apparent in the interaction of GSTμ with the apoptosis signal-regulating kinase 1 (ASK-1), which is upstream of JNK.

The glutaredoxins (Grx), also known as thioltransferases, are a family of small proteins whose catalytic site contains a disulfide bond (*9*). Like the GPx's, they utilize GSH thiol reducing equivalents to maintain a reduced/active form. Their antioxidant activities encompass the reduction of molecules such as dehydroascorbate (the oxidized form of the natural antioxidant ascorbic acid) and the peroxiredoxins (Prx). The human Prx's (*10*) are a family of six abundant proteins that again have a redox-active cysteine in their catalytic site; they reduce hydrogen peroxide and alkyl hydroperoxides as well as regulating cytokine-induced peroxide levels. The various family members can be reduced by thioredoxins (below) as well as by GSH, ascorbic acid, etc. Another enzyme, the oxidoreductase sulfiredoxin (Srx), can reactivate Prx's by reducing oxidized sulfinic acid ($Cys\text{-}SO_2H$) cysteine residues back to thiols (*11*).

The superoxide dismutase (SOD) family of enzymes represent another key element of the antioxidant defense in human cells by catalyzing the dismutation of two molecules of $^{\bullet}O_2^-$ to generate H_2O_2 and O_2, thus preventing $^{\bullet}O_2^-$ from reacting with critical cellular biomolecules (*12*). The hydrogen peroxide generated by this and other mechanisms is degraded by antioxidant enzymes such as catalase or GPx1, otherwise it could generate potentially harmful $^{\bullet}OH$ radicals via the Fenton reaction. Human cells have three forms of SOD: SOD1, which is predominantly cytoplasmic; SOD2, which is mitochondrial; and SOD3, which is extracellular. SOD1 and SOD3 contain copper (Cu) and zinc (Zn), whereas SOD2 has manganese (Mn) in its active site.

Another important antioxidant response involves the thioredoxin (Trx) system, which is comprised of Trx, thioredoxin reductase (TrxR) and NADPH (*13, 14*). The Trx family of small oxidoreductase proteins, which have intracellular concentrations of ~1 m*M*, help to maintain redox-sensitive proteins or other substrates in their reduced state through a cysteine thiol-disulfide exchange reaction involving a conserved CXXC motif (where C is cysteine and X is another amino acid). As noted above, Trx also regulates the enzyme activity of the Prx's. The Trx's functionally overlap with the related Grx's but are maintained in their reduced state by specific TrxRs via an NADPH-dependent process, rather than by GSH. The TrxRs are homodimeric selenocysteine-containing oxidoreductase flavoenzymes, each monomer of which contains an NADPH binding domain and an active site with a redox-active disulfide bond. Most of the cellular NADPH reducing equivalents are provided by the cytoplasmic pentose phosphate cycle (also known as the hexose monophosphate shunt), the activity of which can increase by up to 200-fold during oxidative stress and whose rate-limiting enzyme is glucose-6-phosphate dehydrogenase (G6PD) (*13, 15*). This pathway is strongly induced through the nuclear factor erythroid 2-like 2 (Nrf2) transcription factor that will be discussed later. As noted above, NADPH generated by this pathway is also used by cells to reduce GSH via GSR to maintain the antioxidant GSH redox cycle. NADPH:quinone oxidoreductase-1 (NQO1), a cytoplasmic 2-electron reductase, also acts as an antioxidant by preventing the generation of ROS via 1-electron reduction of quinones. Other biological antioxidants include ascorbic acid (vitamin C), vitamin E, melatonin and the metallothioneins (MT)

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(*16*), a family of small cysteine-rich proteins that localize to the membrane of the Golgi apparatus. In addition to protecting cells from toxic metals such as Zn, MTs protect against oxidative stress via their cysteine residues that scavenge ROS such as $^{\bullet}OH$ and $^{\bullet}O_2^-$ (*17*). The ferritin protein also contributes to cellular redox homeostasis by sequestering redox-active ferrous iron, such that it does not participate in the Fenton reaction that could otherwise result in the generation of highly-damaging $^{\bullet}OH$ radicals from hydrogen peroxide.

INDUCIBLE ANTIOXIDANT PATHWAYS

It is well known that many of the basal antioxidants in mammalian cells outlined above are induced in response to suprabasal levels of oxidative stress, e.g., as seen with GSH, SOD and GPx after irradiation in mouse models (*18, 19*). In the last decade the mechanisms by which such inducible responses occur have received considerable attention, particularly in the context of: 1. The ''adaptive response'' to radiation, in which exposure of a cell population to a low or ''priming'' dose of radiation (typically between ~0.5 and 20 cGy) can invoke increased resistance to a subsequent higher ''challenge'' radiation dose given several hours after the priming dose (*20*); 2. The more controversial phenomenon of radiation hormesis, in which low-dose radiation exposures have been suggested to provide a health benefit by inducing species such as antioxidants; and 3. The broader context of effects occurring across the range of doses and dose rates used in radiation therapy for cancer treatment.

p53 AND THE EARLY ANTIOXIDANT RESPONSE

Much has been written about the p53 tumor-suppressor protein, the ''guardian of the genome'' (*21*). Here we provide a brief overview of its diverse functions. In addition to its well-defined roles in activating DNA repair and cell cycle checkpoints, as will be discussed later, p53 also plays an even earlier role in preserving genome integrity either under normal physiological conditions or after low-moderate stress levels by upregulating a plethora of antioxidant genes, including SOD2, GPx1, catalase, p53-induced glycolysis and apoptotic regulator (TIGAR), which regulates glycolysis and apoptosis, and phosphate-activated glutaminase (GLS2), which encodes a mitochondrial enzyme that regulates the production of glutamate, an essential precursor for synthesis of the key antioxidant GSH (*22, 23*). Indeed, mitochondrial dysfunction and disruption of the electron transport chain is a major source of ROS (*24*). p53 also upregulates members of the sestrin protein family, including sestrin1 (also known as p53-regulated protein PA26), which protect against oxidative stress in part by enhancing the activation of the Nrf2 antioxidant transcription factor, which in turn activates a whole repertoire of antioxidant proteins (*11*), as discussed in some detail below; these include the enzyme Srx, which likely explains the reported association (*25*) between sestrins and Prx regeneration (*11*).

Ref1 INVOLVEMENT IN THE EARLY ANTIOXIDANT RESPONSE

A second important adaptive oxidative stress-sensing mechanism involves another multifunctional protein, redox factor-1 (Ref1, also known as apurinic/apyrimidinic endonuclease 1 or APE1). Ref1 subcellular localization in different cell types can be primarily nuclear, cytoplasmic, or both, with protein also being seen in the mitochondria (*26*). Ref1 activation in part involves its translocation from the cytoplasm to the nucleus, although Ref1 expression is induced by ROS, which function as redox coactivators of various transcription factors (*26–28*). Ref1 has primary roles in both DNA repair and redox control, and it also functions to control chronic oxidative stress-induced telomeric shortening, genomic instability and micronuclei formation. It functions in base excision repair (BER) by the endonuclease function that acts on abasic sites in the component of the DDR that removes oxidative DNA base damage. In cellular redox regulation it acts to inhibit ROS production. Thus, in response to oxidative stress, Ref1 (primarily via its Cys-65 residue, but with Cys-93 and Cys-99 also contributing) can act as a redox coactivator to activate a number of redox-sensitive/stress-inducible transcription factors, notably the pro-inflammatory nuclear factor κB (NF-κB), as well as activator protein-1 (AP-1), early growth response protein 1 (Egr-1), hypoxia-inducible factor-1α (HIF-1α), STAT3 and p53 (*26, 28*). This in turn stimulates the sequence-specific DNA binding activity of these transcription factors. Such effects typically involve the direct reduction by Ref1 of critical cysteine residues in the target transcription factors, with the reducing equivalents of molecules such as GSH and Trx being utilized to restore the reduced form of Ref1 (*26–28*). In addition to directly reducing transcription factors, Ref1 also appears to facilitate their reduction by cellular reductants such as GSH or Trx by serving as a redox ''chaperone'', a role that is independent of Ref1's cysteine residues but rather involves a direct interaction between Ref1 and the target transcription factor (*28*).

Like many DDR/redox-regulatory proteins, Ref1 activity is fine-tuned by post-translational modifications such as phosphorylation and acetylation (*27, 29*). Ref1 may also downregulate its own expression (*30*). Crosstalk between Ref1 and p53 is clearly important in regulating redox homeostasis. Ref1 interacts with and enhances p53 binding to and translation of p53-regulated effector genes potentially through both redox-dependent and -independent mechanisms, but it can repress transcription of some genes (*26, 27, 31*). One mechanism by which Ref1 regulates p53 binding to its target genes is by promoting p53 tetramerization (*31*). The Trx-mediated redox regulation of Ref1 is required for

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the functional activation of p53 (*26*) and the AP-1 (*3*) transcription factor. A model for the latter signaling pathway after radiation exposure originates in the cytoplasm with activation of the pentose phosphate cycle (Fig. 1), which generates NADPH, which serves as the source of electrons for TrxR to in turn reduce Trx; Trx then translocates to the nucleus where it interacts with Ref1, leading to the activation of AP-1 and the transcription of AP-1-dependent genes (*3*). In each case, the sequential redox events that transmit the signal involve key cysteine residues (*3*). The Ref1-p53 interaction appears to be bidirectional, with p53 having been reported to negatively regulate Ref1 expression in response to DNA damage (*30*). p53 also interferes with the binding of Sp1 to the Ref1 gene promoter and thus its ability to transactivate its target genes (*30*).

Nrf2 INVOLVEMENT IN THE EARLY ANTIOXIDANT RESPONSE

The Nrf2 transcription factor plays a central role in the maintenance of cellular redox homeostasis via the coordinated transcriptional upregulation of numerous antioxidant proteins (*32, 33*). This includes more than 500 genes that are crucial to metabolize electrophilic drugs and toxins, protect against oxidative stress and inflammatory damage, and stabilize proteins and remove damaged proteins via proteasomal degradation or autophagy. Nrf2 belongs to the family of basic leucine zipper (bZip) transcription factors with a conserved cap 'n' collar (CNC) domain (*34*). Under basal/low-stress conditions, Nrf2 is sequestered in the cytoplasm by kelch-like ECH-associated protein 1 (Keap1), an adaptor for a Cul3-based E3 ligase that promotes constitutive proteasome-mediated Nrf2 degradation (*35, 36*). Keap1, however, by virtue of its numerous oxidizable cysteine residues (27 in human), is capable of responding to diverse forms of oxidative stress to generate a common output, namely the activation of Nrf2 (*37*). This involves an increase in the levels of the Nrf2 protein and its translocation from the cytoplasm to the nucleus where it binds to antioxidant response elements (AREs) located within gene promoters. Transcriptional activation at AREs is actually mediated by the binding of heterodimers of Nrf2 with the small MAF (musculoaponeurotic fibrosarcoma) or jun protein family members (*33*). The mechanisms of Nrf2 stabilization and nuclear translocation under increased oxidative stress and its reversal once homeostasis is re-established are complex (*33, 38, 39*) and beyond the scope of this review.

The Nrf2/ARE-dependent genes encode antioxidant proteins, such as GST, γ-GCS, GPx, Trx, TrxR1, G6PD, catalase, SOD1, SOD2 (Mn-SOD), heme oxygenase-1 (HO-1), Srx, sestrins, NQO1, UDP glucuronosyltransferases (UGT) and ferritin, which play diverse roles in the maintenance of cellular redox homeostasis, ROS/electrophile suppression, implementation of DNA repair, and inhibition of inflammatory cytokine production, as well as in tissue repair and functional recovery (*5, 11, 33, 40*). Nrf2 also activates multiple genes that drive the cytoplasmic pentose phosphate cycle mentioned earlier, and under some circumstances controls the production of proteasome subunits. The 26S proteasome is a redox sensor that, after oxidative stresses, including radiation, disassembles into intact 20S and 19S particles. At this time, even after low doses of radiation, proteasome activity is impaired (*41*), initially blocking removal of polyubiquitinated proteins, which may contribute to endoplasmic reticulum stress and activation of the unfolded protein response. Mitochondrial networks fragment and cellular ROS levels increase, as they do after proteasome inhibitor treatment (*42*). These multiple changes are important in signaling and cell death pathways as the 26S proteasome is the master controller of multiple cellular functions through controlled protein degradation. $NADH/NAD^+$ plays a key role in the proteasome function as NADH maintains normal proteasome levels (*43*). Nrf1 and Nrf2 are involved in the resynthesis of proteasome subunits after oxidative stress and their recovery. Nrf2 increases 20S proteasomes and their PA28αβ (11S) regulators, which are superior at degrading oxidized proteins and at stress resistance (*44*). Immunoproteasomes are also generated but are dependent on interferon regulatory factor 1 (IRF1), not Nrf2.

In cancer, mutually exclusive mutations in the Nrf2/Keap1 pathway are highly prevalent in the TCGA database of human squamous cell lung cancer, and are a likely marker of cancer-related oxidative stress. Nrf2 has also been reported to mediate induction of the anti-apoptotic B-cell lymphoma 2 (BCL-2) gene, which, like many Nrf2/ARE-regulated genes, can contribute to the resistance of tumors to therapy (*45*). In inflammation, Nrf2 activation inhibits pro-inflammatory cytokine production.

Nrf2 AND RADIATION EXPOSURES

In two articles published in 2010, it was reported that exposing mammalian cells and tissues to radiation activated the Nrf2-mediated transcription of various antioxidant proteins. Thus, Tsukimoto *et al*. (*46*) showed that exposure of murine Raw 264.7 macrophage cells to low-dose γ rays in the range 0.1–2.5 Gy caused an early (1–2 h) dose-dependent increase in cytoplasmic Nrf2 levels as well as its nuclear accumulation by 4 h and corresponding elevation of HO-1 levels by 24 h. McDonald *et al*. (*5*) similarly observed Nrf2/ARE-dependent gene induction after γ irradiation, albeit with markedly delayed kinetics, in several cell types. Although exposure of the stably expressing ARE-luciferase reporter cell line MCF7-AREc32 (derived from the MCF7 human breast cancer cell line) to either single dose of radiation in the range 0.05–10 Gy or to three daily fractions of 0.5, 2 or 4 Gy did not significantly increase luciferase expression at 24 h after completion of the irradiation, a dose-dependent activation was observed in cells receiving

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five daily fractions of 0.5, 1, 2, 3 or 4 Gy and evaluated at 3 h after the final dose (*5*). The delayed nature of this activation was confirmed by the observation that single doses of 2–8 Gy did indeed enhance the ARE-reporter signal at days 5–15 postirradiation before subsiding, suggesting that it represents a second-tier antioxidant response. Wild-type mouse embryo fibroblasts (MEFs), NIH-3T3 murine fibroblasts and DC2.4 murine dendritic cells (but not Nrf2-knockout MEFs) showed a similar pattern of delayed induction of mRNAs for two Nrf2-regulated genes, HO-1 and GSTA2, after single or fractionated doses. Corresponding increases in HO-1 protein levels were seen in wild-type MEFs, NIH-3T3 and primary murine bone marrow cells, but again not in Nrf2-knockout MEFs. Functional GSH activity at day 5 after a single 8 Gy dose increased by ~50% in wild-type (but not in Nrf2-knockout) MEFs. Similar responses were observed *in vivo* in C57BL/6 mice after five daily 2 Gy fractions, where splenic HO-1 (but not GSTA2) gene expression increased significantly. Nrf2-knockout MEFs and C57BL/6 mice showed increased radiosensitivity compared to their wild-type counterparts (*5*). Nrf2 activation in MEFs after 8 Gy irradiation was temporally correlated with delayed ROS production, peaking at ~5 days, with ROS induction greatly increased in Nrf2-knockout MEFs (*5*).

Subsequent reported studies have described phenotypic and mechanistic features of the Nrf2 response to radiation in various model systems in relationship to enhanced cell survival. Many of these studies were reviewed by Sekhar and Freeman (*37*). For example, activation of Nrf2/ARE signaling has been shown to lower intracellular ROS and confer radioresistance in fibroblasts, bronchial and breast epithelial cells, DU145 prostate cells, glioblastoma and squamous cell lung cancer cells. Knockdown or inhibition of Nrf2 in human cancer cell lines typically results in elevated ROS levels and radiosensitization, as it did in Nrf2-knockout MEFs. Collectively, these findings suggest that Nrf2 does indeed promote a pro-survival response in irradiated cells. Similar observations have been made in *in vivo* models, including the increased radiosensitivity of Nrf2-knockout mice (*37*). In addition to antioxidant responses, some Nrf2/ARE-regulated enzymes are involved in the repair of radiation/ROS-induced DNA damage, such as the BER protein 8-oxoguanine DNA glycosylase (OGG1) and the homologous recombination repair (HRR) protein RAD51 (*47*); furthermore, interactions between Nrf2 and factors such as p53-binding protein 1 (53BP1) and breast cancer 1 (BRCA1) protein may influence the cellular choice of double-strand break (DSB) repair pathway, i.e., non-homologous end joining (NHEJ) versus HRR (*37*). Sekhar and Freeman (*37*) also reiterate the importance of inflammatory cytokines in the radiation response of normal tissue and of Nrf2 in regulating cytokine expression, and they suggest that the pro-survival role of Nrf2 is related to its ability to modulate the pro-inflammatory cytokine response that can generate ROS as an effector against microbial challenge. Several published studies using various cell types [e.g., (*46, 48, 49*)] also indicate that radiation-induced Nrf2 activation and downstream effects can be suppressed by mitogen-activated protein kinase (MAPK)1/3 inhibitors such as U0126 or by shRNA MAPK knockdown, suggesting that this pathway might play a key role in this response.

The Nrf2-mediated induction of antioxidants has also been implicated in the above-mentioned adaptive response to radiation in which priming cells with a low dose of radiation can invoke resistance to a higher challenge dose delivered several hours later. However, defining the mechanism of this effect is complicated not only because it is not universally observed (*20*) but also because many contributing processes are likely to modify adaptive responses, including DNA repair, cell cycle checkpoints, stress chaperone proteins and intercellular signaling pathways involving, e.g., p53 and MAPK1/3 (*50*). Bravard *et al*. (*51*) examined the effect of γ rays on the activity and levels of various antioxidant proteins in AHH-1 human lymphoblast cells using an adaptive paradigm of a 0.02 Gy priming dose followed 6 h later by a 3 Gy challenge dose. Although the priming dose itself had little effect, adapted cells did exhibit slightly elevated activity/levels of SOD2, GST, GPx and catalase versus control cells at 3 h after the 3 Gy challenge dose, suggesting that such events may contribute to adaptation. Similarly, delivery of a priming dose of 5 cGy to AG1522 normal human skin fibroblasts caused Nrf2 translocation from the cytoplasm to the nucleus and induction of the HO-1 gene and protein, which presumably contributed to the observed adaptation to a subsequent 2 Gy challenge dose of X rays given 12 h later (*52*). In contrast, Miura (*50*) reported no changes in the activity of GST, GSR and catalase in rat glial cells receiving a 0.1 Gy priming dose 3 h prior to a 2 Gy challenge dose. Although in the latter two cases the phenotypic adaptive response was not demonstrated, there is evidence that Nrf2 responds to low-dose irradiation. For example, in human hematopoietic stem cells (HSCs), hypersensitivity to low doses of radiation was dependent on immediate increased levels of ROS that activated the Keap1-Nrf2 antioxidant pathway leading to autophagy (*52*).

The adaptive response to radiation is clearly multifactorial and extensive mechanistic discussion is beyond the scope of this review. Here, we consider two recently published studies that shed some light on the role of Nrf2 in such responses. First, the studies described so far typically involved low-LET beams (X rays or γ rays). In their published study, Chen *et al*. (*53*) examined whether high-LET α particles evoked a similar adaptive response in A549 human lung adenocarcinoma cells and, if so, whether the Nrf2-mediated induction of antioxidants might play a role therein. A clear adaptive response to α particles (increase in cell survival) was apparent in cells receiving a priming dose of 5 cGy delivered 6 h prior to a challenge dose of 75 cGy. Nrf2 elevation and accumulation in the nucleus as well as

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transcriptional activation of its target gene HO-1 were seen at 6 h after 5 cGy irradiation. Also, DSB levels (γ-H2AX foci) at 3 h after the 75 cGy challenge dose were decreased in cells that received the 5 cGy priming dose compared to non-primed cells, presumably reflecting enhanced DNA repair. Knockdown of Nrf2 using shRNA suppressed the adaptive response, as did the MAPK1/3 inhibitor U0126. The autophagy inhibitor 3-methyladenine and the ROS scavenger N-acetyl cysteine also blocked the increase in Nrf2 and HO-1 levels and the adaptive response, with the latter also blocking the autophagy response. Collectively, these observations suggest that the adaptive response to α particles in A549 cells is mediated by ROS elevation, autophagy and activation of the Nrf2 antioxidant pathway, which is very similar to findings in human HSCs (*52*). Second, there appears to be a more general aspect of Nrf2-mediated adaptation insofar as a whole-body priming X-ray dose of 7.5 cGy given to C57BL/6J diabetic mice in 1 or 3 daily fractions (but not 1×2.5 cGy) was able to protect against manifestations of diabetes injury that are related to excessive ROS generation in the kidney (*54*). The priming dose upregulated Nrf2 expression in the kidney at 3–6 h postirradiation as well as Nrf2 function, as reflected by levels of its downstream antioxidants (NQO1 at 3–6 h and HO-1 at 3–9 h); it also attenuated various manifestations of diabetes-induced oxidative damage to the kidney (inflammation, dysfunction).

The cellular regulation of Nrf2 is actually more complex than outlined above. Indeed, there is evidence of crosstalk between the Ref1 and Nrf2 oxidative stress-sensing proteins. For example, Ref1 has been reported to negatively regulate Nrf2 in a variety of cell types via its redox function (*55*). The sestrin proteins described earlier have been suggested to stimulate the Nrf2-mediated antioxidant response by enhancing the disruption of the Keap1-Nrf2 interaction via the p62/SQSTM1 (sequestosome 1) protein, thereby promoting the autophagic degradation of Keap1 (*56*). Both the p62 and sestrin2 genes are themselves transcriptional targets of Nrf2, resulting in a positive feedback loop. Also worth noting is that the cellular homeostatic response to radiation/ROS-mediated stress involves a critical element of crosstalk between the Nrf2 and p53-p21^{WAF1} pathways. Such interactions will be discussed later.

A final but important point relates to the kinetics of Nrf2 induction by radiation in various model systems. Although the two initial reports of this effect (*5, 46*) both highlighted the importance of Nrf2 induction in the cellular response to radiation, they also indicated very different activation kinetics. Whereas a rapid ($\leq$24 h) induction of Nrf2/ARE-dependent events by exposure was observed by Tsukimoto *et al*. (*46*) in mouse Raw 264.7 macrophage cells and in subsequent studies with a variety of human cell lines, McDonald *et al*. (*5*) found that induction was minimal at less than 48 h in several cell types and required a delay of ~5 days to fully manifest. However, Rodrigues-Moreira *et al*. (*52*) suggest a recurring state of persisting oxidative stress that might drive delayed waves of Nrf2 induction. This may be driven by waves of further cell death and micronuclei formation and be dependent on cell line differences related to cell fate; e.g., the late activation seen by McDonald *et al*. (*5*) appears to reflect an enzyme-mediated delayed ROS production related to radiation-induced senescence (a form of cell growth arrest that will be discussed below), which Nrf2 generally inhibits. In keratinocytes, for example, radiation-induced senescence is associated with increased ROS levels at four days postirradiation due to activation of enzymes such as the NOX proteins, events that are blocked by the B lymphoma Mo-MLV insertion region 1 homolog (Bmi-1) polycomb complex protein that, among other activities, regulates mitochondrial oxidative stress levels and increases radioresistance (*57*). Senescence can also trigger cell fate decisions associated with reprogramming in which Nrf2 appears to play a critical role. In particular, Nrf2, perhaps induced by late ROS production, orchestrates the metabolic shift from oxidative to glycolytic energy production that is associated with reprogramming of induced pluripotent stem cells (*58*) and drives anabolic pathways essential for metabolic reprogramming through enhanced glucose utilization and the pentose phosphate cycle (*59–61*), which is also associated with increased radioresistance. Although the focus here has been on Nrf2's role in regulating redox homeostasis, these additional roles in regulating a variety of metabolic enzymes that contribute to the rapid synthesis of macromolecules (e.g., via activation of enzymes involved in nucleotide synthesis) and to cell proliferation are clearly of relevance in the cellular response to oxidative stress. Also relevant is the role of Nrf2 in the early replacement of proteasome subunits after the rapid radiation-induced disassembly of the 26S proteasome, which is cell line dependent, presumably being regulated by redox and metabolic status (*62*). Indeed the early Nrf2 response could be for this very purpose.

IMPLICATIONS OF Nrf2/ARE-MEDIATED RESPONSES FOR THE TREATMENT OF NSCLC?

Targeting proteins involved in antioxidant/redox homeostatic pathways as an approach to tumor radiosensitization in clinical radiation therapy is an active area of research inquiry (*63*). Interest in targeting Nrf2 in this context has been driven by repeated observations that Nrf2 is overexpressed/dysregulated/mutated in some cancer cell lines and tumors and that this dysregulation appears to contribute to diminished responses to radiation therapy as well as some chemotherapeutic agents. Several excellent reviews on this topic have been published (*37, 64–67*), and we will only briefly highlight the more pertinent points here. Squamous non-small cell lung cancer (NSCLC) has been the subject of particular interest for several reasons: 1. the high frequency of Keap1 and Nrf2 mutations seen in this

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disease, in the region of ~25%; 2. the correlation between high Nrf2 levels/activity/mutations and poor treatment outcome, and the similar, mutually exclusive scenario for mutations of Keap1, the natural negative regulator of Nrf2; and 3. genetic or pharmacological targeting of Nrf2 in NSCLC cells typically evokes sensitivity to radiation and chemotherapy drugs. Among the Nrf2-inhibitory compounds identified for potential application as enhancers of radiation therapy in cancers with constitutive activation of the Keap1-Nrf2 pathway is IM3829 (4-(2-cyclohexylethoxy)aniline), which was shown to enhance the effect of radiation against human lung cancer cell lines and xenograft models (*68*). In addition, many oncogenic signaling pathways have been reported to crosstalk with the Keap1-Nrf2 pathway (*66*). Indeed, loss of Keap1 has been associated with resistance to targeted therapeutics directed against kinases such as epidermal growth factor receptor (EGFR) in lung cancer cell lines (*69*). As noted above, pharmacological and genetic inhibitors of MAPK1/3 can block radiation-induced Nrf2 activation and enhance the radiosensitivity of some cell types [e.g., (*46, 48, 49*)] and may thus represent a potential approach to radiosensitizing tumors with EGFR mutations that are in turn associated with activation of MAPK1/3 and Nrf2 (*69*).

A final caveat is that clinical exploitation of inhibitors of the Keap1-Nrf2 pathway will require differential effects on cancerous versus normal cells. This may be in terms of its elevation in cancer, but may also reside at the level of induction. A difference between normal and cancer cells in this regard is illustrated by the observation that normal murine splenic lymphocytes and EL-4 murine T-cell lymphoma cells exhibited quite distinct Nrf2-dependent responses to radiation, presumably related to tumor cells being subjected to elevated oxidative stress and consequently having antioxidant profiles different from those of normal cells (*49*).

THE DNA DAMAGE RESPONSE (DDR)

As outlined in the preceding sections, much of the potential for cellular injury after exposure of human cells to radiation and other genotoxic stresses can be prevented by the early antioxidant responses that can intercept species such as ROS before they can react with biomolecular targets. On the other hand, reactive species that have evaded elimination can lead to damage to these targets. Any damage to genomic DNA will then be subject to processing by the complex network of pathways collectively known as the DDR. The DDR can be subdivided into ''early'' responses, notably the concerted activation of cell cycle checkpoints and DNA repair pathways that function to maintain genome integrity and thus promote cell survival, and ''late'' responses that serve to eliminate or proliferatively disable cells that have developed genome instability (*70*). Normal cells that undergo significant levels of unrepaired DNA damage may either die (e.g., through apoptosis or necrosis) or they may undergo an extended proliferative arrest that is often referred to as stress-induced premature senescence (SIPS), or continue to progress through the cell cycle checkpoints with damage. Cells with DDR defects (e.g., many p53-mutant tumor cell lines) may execute aberrant mitosis despite having highly-damaged genomes and give rise to polyploid offspring, some of which may retain viability and potentially become even more malignant (*70, 71*). A detailed discussion of the various modes of cell death that can occur after irradiation is beyond the scope of this review, but the reader is referred to one of the many reviews on this topic (*72*).

The most deleterious DNA lesions induced by radiation, from a cell death or proliferative disablement perspective, and thus of greatest relevance to its therapeutic use in the treatment of cancer, are the DSBs that involve local scission of both strands of the DNA helix. Many of these DSBs are presumed to reflect complex clustered lesions arising from the spatial and temporal coincidence of direct damage to the DNA strands caused by fast secondary electrons and indirect damage to the DNA mediated by ROS. The normal cellular response to DSBs is affected by an ordered sequence of steps involving damage sensing, signal transduction and enlistment of effector proteins whose collective function is to return the cell as closely as possible to its preirradiation state (*73*). From the broader perspective, radiation exposure results in oxidative stress not only via the initial primary ROS resulting from rapid ionization events but also via the delayed generation of secondary ROS related to the perturbation of intracellular metabolic redox status (*3, 4*). These secondary ROS are generated by processes such as oxidative injury to organelles, notably mitochondria and peroxisomes, by activation of pro-inflammatory cytokines such as tumor necrosis factor α (TNF-α) family members, by pro-oxidant responses triggered by p53 after high levels of damage (as will be discussed below), as well as in the context of enzymatically generated ROS, e.g., in cells undergoing senescence, and persistent cytosolic DNA including micronuclei that may undergo chromothripsis. It appears likely that the latter will occur only after mitosis, which is required for micronuclei formation. Whereas DSB induction will be a low-frequency but biologically important feature in the initial early response to radiation, the subsequent delayed phases of secondary ROS generation will cause a higher proportion of simple oxidative types of DNA damage such as single-strand breaks and base/sugar modifications versus complex DSBs. An interesting consequence of this scenario should be that the engagement of the various DNA repair pathways will also evolve with time. In addition to the oxidation of critical cellular components, including the genome, ROS also activate signal-transduction pathways that control cell proliferation, survival and transformation, and that can thus lead to malignancy when dysfunctional (*23*).

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THE ATM PROTEIN

A key signaling protein in the cellular response to DSB/oxidative injury is the ataxia-telangiectasia mutated (ATM) serine-threonine kinase (*73, 74*). Activation of ATM in response to DSBs is associated with an early autophosphorylation between ATM dimer partners at serine-1981 and the generation of ATM monomers (*75*), although the actual mechanism of activation remains obscure (*76*). At the DSB site, activated ATM monomers interact with the Mre11-Rad50-Nbs1 (MRN) damage-sensing complex and subsequently transmit damage signals by phosphorylating hundreds of target proteins. These include effectors of responses such as DNA repair, cell cycle checkpoint activation, apoptosis, autophagy and senescence, as well as the detoxification of potentially harmful reactive species. They include the variant histone H2AX, the CHK2 checkpoint kinase, and the wild-type p53 protein that coordinates the downstream aspects of the DDR network as well as other stress responses (*70, 77, 78*). Cells from ataxia-telangiectasia patients who have ATM mutations are markedly deficient in activating p53 and implementing early p53-mediated responses after irradiation, such as DNA repair (*74, 79*) and cell cycle checkpoints (*73, 74*), and display greatly increased radiosensitivity in the clonogenic survival assay (*80*).

Whether the initial rapid activation of ATM in response to DSBs occurs primarily in the nucleus, as has been widely assumed based on a number of reports that ATM in non-stressed cells is primarily nuclear [e.g., (*81*)], or whether it occurs in the cytoplasm (*82*), requires clarification. Of note, ATM also undergoes additional post-translational modifications in the course of the DDR, including acetylation by Tip60 (HIV-1 Tat interacting protein 60 kDa), which links the DDR to the chromatin remodeling that is important for repair (*83*).

Although much of the focus on ATM has involved its nuclear role in DSB repair, its function clearly extends beyond this well-defined DDR activity. Indeed, ATM's extranuclear activities may be important in the early response to radiation because irradiated cells will be subjected to both DNA damage in the nucleus and to ROS accumulation in all cell compartments, and they will therefore be responding to all of these stresses concomitantly. For example, mitochondrial ATM may help to maintain redox homeostasis in the face of mitochondrial dysfunction (*74*) and thus, its exacerbation after irradiation. Cytoplasmic ATM has also been implicated in maintaining redox homeostasis by acting directly as a ROS sensor via its oxidation at cysteine residues (*84*). Indeed, cells lacking ATM exhibit elevated levels of ROS and are hypersensitive to ROS-generating agents (*73, 85*). Among the downstream responses to cytoplasmic ATM activation by ROS that contribute to redox homeostasis after irradiation/oxidative stress (and indeed other types of stress, such as nutrient/energy deficiency) is activation of the TSC2 (tuberous sclerosis complex 2) component of the TSC1-TSC2 complex via the liver kinase B1 (LKB1)-AMP-activated protein kinase (AMPK) pathway (Fig. 2), which results in inhibition of the mammalian target of rapamycin complex 1 (mTORC1) protein complex, a critical regulator of the balance between cell proliferation and autophagy (*84*). This pathway is a key sensor of redox/nutrient/energy stress. Repression of mTORC1 by the LKB1-AMPK pathway suppresses cell growth and promotes induction of autophagy, notably the selective autophagy of damaged mitochondria (mitophagy) and peroxisomes (pexophagy) which, when dysregulated, can generate high levels of secondary ROS (*23, 73, 84, 86–88*). Of note, cells lacking ATM function display aberrant mitophagy (*73*).

In an intriguing study (*85*), ATM activation in fibroblasts subjected to ROS elevation via hydrogen peroxide treatment was found to be associated with the direct oxidation of the Cys-2991 residue of ATM, leading to the formation of disulfide-crosslinked ATM homodimers; this activation was independent of DSBs and of the MRN complex, but *was* accompanied by ATM serine-1981 phosphorylation. Whether this mechanism occurred in the cytoplasm, nucleus or both, was not specified.

Cytoplasmic ATM also regulates ROS levels after genotoxic stress by enhancing the pentose phosphate cycle and thus cellular levels of the antioxidant cofactor NADPH (*86*). This involves phosphorylation of heat shock protein 27 (HSP27) by ATM which then stimulates the activity of G6PD, promoting NADPH production, nucleotide synthesis and DSB repair (*86*). The ability of the ATM-p53 axis to inhibit glycolytic metabolism (a major source of ROS) through TIGAR may also contribute to redox homeostasis by decreasing ROS production (*86*).

ATM also participates in other signaling responses to radiation and to ROS in general, including activation of the NF-κB transcription factor and its translocation from the cytoplasm to the nucleus, where it binds to target genes involved in cell survival (e.g., anti-apoptotic genes such as BCL-2, BCL-xl, XIAP, MCL-1 and survivin), cytokine signaling, and inflammatory and immune responses (*89*). ATM also appears to crosstalk with the transforming growth factor β (TGF-β) pathway, which plays an important role in the cellular and tissue response to radiation (*90, 91*). It is also a pathway to type I interferon production through IRF1 (*91*). The ATM substrate, BH3-interacting domain death agonist (BID), has also emerged as an important mediator of stress responses, including the regulation of mitochondrial metabolism (*73*). Another target of the activated ATM kinase after genotoxic stress, p53-induced death domain containing protein (PIDD), regulates a binary signaling switch between pro-survival (NF-κB) and pro-death (caspase-2) pathways (*92*).

Thus, ATM in various subcellular compartments appears to be activated by different types of stress through a variety of mechanisms that appear to direct its kinase activity to different sets of target proteins/pathways involved in

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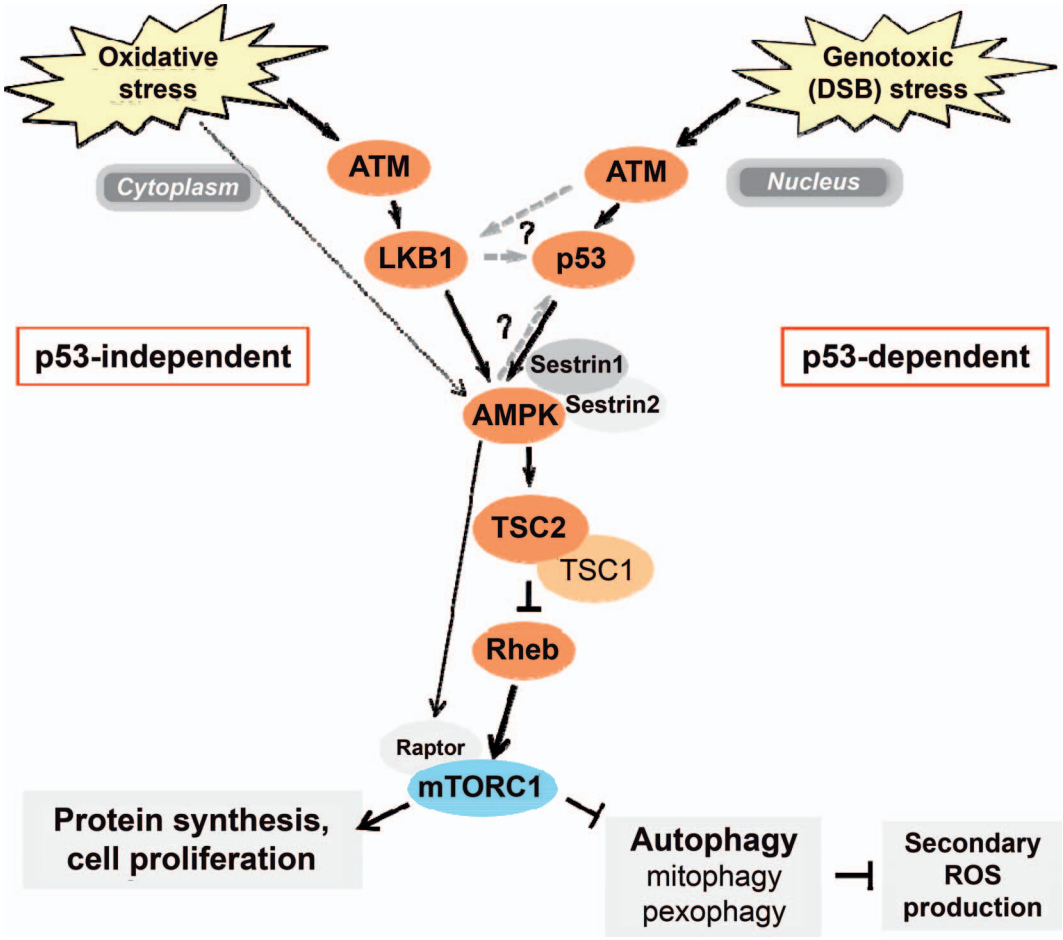


FIG. 2. Cellular oxidative and genotoxic stress responses converge on the AMPK/mTORC1 pathway. A major pathway regulating cellular homeostasis decisions after various stress situations involves the mTORC1 complex. Activation of the ATM kinase in the cytoplasm in response to oxidative stress leads to phosphorylation of the LKB1 tumor suppressor protein and engagement of the AMPK pathway. AMPK in turn activates the TSC2 protein and thereby negatively regulates mTORC1 signaling, thus inhibiting cell growth while promoting autophagy (*84*). AMPK also inhibits mTORC1 by phosphorylating the raptor protein. This cytoplasm-derived AMPK-mTORC1 response does not require p53 function. Activation of ATM in response to genotoxic stress also engages the AMPK pathway, although this activation is p53-dependent and redox-independent and is mediated by the p53-regulated sestrins 1/2, but again results in the inhibition of mTORC1 (*23, 87, 111*). AMPK also regulates cytoplasmic stress granule formation after oxidative stress (*148*). AMPK = AMP-activated protein kinase; ATM = ataxia-telangiectasia mutated; LKB1 = liver kinase B1; mTORC1 = mammalian target of rapamycin complex 1; TSC1/2 = tuberous sclerosis complex 1, 2; DSB = DNA double-strand break; Rheb = Ras homolog enriched in brain; raptor = regulatory-associated protein of mTOR.

various aspects of redox homeostasis (*73*). As discussed in the next section, in addition to these direct roles in cellular redox signaling ATM also impacts on redox homeostasis via its downstream target p53.

THE p53 PROTEIN

In addition to its early role in activating the antioxidant response (above), the multifunctional p53 protein acts at the very hub of the DDR network, and indeed, of the cellular response to many types of stress, including oxidative stress and metabolic stress, e.g., nutrient or glucose deprivation, where it is activated by kinases such as ATM and AMPK (*23*). In general, p53 modulates the activity of other DDR proteins either by direct interaction (*93*) or by transcriptional regulation (*94*).

In cells that undergo relatively low levels of stress, p53 functions largely as a protective/pro-survival factor, in part by directing the abrogation of ROS or other electrophilic species before they can react with their cellular targets, including the genome, and in part by promoting the removal of DNA damage that does occur by activating DNA repair and cell cycle checkpoints (*77*). p53 has in fact been reported to influence the activity of the entire cellular repertoire of DNA excision- and recombination-repair systems (*93, 95*). When the stress level and resulting unrepaired damage level is severe, p53 can trigger pro-oxidant responses that result in apoptosis in some genetic

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backgrounds or in growth arrest/senescence (SIPS) in others (*96, 97*). Under some conditions p53 can trigger apoptotic signaling directly through its polyproline-rich region (*98*) or indirectly by transcriptionally upregulating pro-apoptotic proteins such as BCL-2-associated X (BAX), p53 upregulated modulator of apoptosis (PUMA), and NOXA or downregulating anti-apoptotic proteins such as survivin and BCL-2 (*100*). It can also transcriptionally activate anti-apoptotic proteins such as $p21^{WAF1}$, 14-3-3δ, DNAJ homolog subfamily B member 9 (DNAJB9) and wild-type p53-induced phosphatase 1 (WIP1) (*70, 100*). Whether anti- or pro-oxidant responses are triggered likely depends on the sensitivity of the various promoters to p53, and thus on the extent of damage and degree of p53 activation (*22, 23, 73*).

The rapid activation of p53 after irradiation involves its phosphorylation, primarily by ATM but also by other kinases (e.g., CHK2, p38 MAPK), as well as other post-translational modifications, including acetylation, ribosylation, SUMOylation and O-glycosylation. These modifications collectively result in p53's transient stabilization, nuclear accumulation and biochemical activation, e.g., as a transcription factor for many genes encoding downstream effector proteins (*70, 101*), including $p21^{WAF1}$ which, in addition to its well-known roles in cell cycle checkpoint activation and SIPS, mediates p53's ability to *repress* some genes (*102*). They also affect p53's interactions with other proteins and with the transcription machinery.

Turnover of p53 is mediated by several ubiquitin ligases, primarily murine double minute-2 homologue (MDM2) but also MDM4 (*103*). Ordinarily, in non-stressed cells, MDM2 maintains p53 at a low level by targeting it for poly-ubiquitylation and proteasomal degradation, as well as mono-ubiquitylation and sequestration in the cytoplasm. MDM2 can also bind to the N-terminal domain of p53, thereby preventing its interaction with the basal transcriptional machinery and transcriptional co-activators such as the CREB-binding protein (p300-CBP) complex (*70*). In cells subjected to genomic injury, the p53-MDM2 interaction is disrupted by the phosphorylation of both p53 and MDM2 by kinases such as ATM, which results in the nuclear accumulation of p53 and activation of its transcriptional function. Radiation-induced suppression of 26S proteasome function (*41*) therefore represents another mechanism of p53 stabilization. Another key participant in the p53-regulatory circuitry is $p14^{ARF}$, which binds to MDM2 in the nucleolus, thereby preventing it from binding to nuclear p53 and flagging it for degradation (*104*).

As noted above, p53 facilitates the suppression of ROS accumulation after low-moderate stresses via the intermediacy of a variety of antioxidant mechanisms. In contrast, under severe or prolonged oxidative stress conditions p53 exhibits the opposite function and instead triggers a pro-oxidant response mediated by another set of p53-regulated genes, leading to the accumulation of ROS and favoring the death of damaged cells by apoptosis or their proliferative disablement by SIPS (*22*, *23*, *96, 97, 105–107*). These pro-oxidant proteins include several of the thirteen p53-inducible PIG/TP53I proteins identified by Polyak *et al.* (*105*), which include: PIG1/galectin-7, which can stimulate $^{\bullet}O_2^-$ production as well as regulate apoptosis through JNK activation and mitochondrial cytochrome c release (*108*); PIG3/TP53I3, which is closely related to several NQOs associated with ROS production and apoptosis, as well as being a component of the DDR pathway (*109*), likely by maintaining the levels of proteins such as DNA-dependent protein kinase, catalytic subunit (DNA-PKcs) and ATM (*110*); PIG4/TP53I4 (SAA1), a serum amyloid protein associated with inflammation; PIG6, a homolog of proline oxidoreductase; and PIG7, which is induced by TNF-α. Other p53-regulated pro-oxidant proteins include BAX and PUMA (*22*), although to what extent ROS generation by some of these pro-oxidant proteins reflects a cause or a secondary effect of biological responses such as apoptosis is not always apparent; however, the PIG proteins were clearly induced by p53 prior to the onset of apoptosis (*105*).

As noted earlier, p53 is widely held to be activated in the nucleus in response to radiation-induced DSBs primarily by the ATM kinase which, among its many targets, phosphorylates p53 at serine 15. Similarly for oxidative stress, treatment of fibroblasts with hydrogen peroxide (under conditions that did *not* induce measurable DSBs) resulted in ATM activation (albeit by a very different mechanism) that was again associated with phosphorylation of p53 at serine 15 (*85*). As for the above-mentioned cytoplasmic response to oxidative stress in which ATM was identified as a ROS sensor leading to inhibition of mTORC1 via the LKB1-AMPK-TSC1/2 pathway, this response appeared *not* to depend on p53 (*84*) (Fig. 2). In contrast, the genotoxic stress-mediated inhibition of mTORC1 and the resulting inhibition of cell growth and activation of autophagy in MEFs and in human cancer cells treated with the drug etoposide (which *does* induce DSBs) *was* reported to be mediated by p53 activation of AMPK-TSC1/2 signaling (*87*). The p53-dependent inhibition of mTORC1 via AMPK seen in various cell types in response to genotoxic stress was suggested to be mediated by the p53 target genes, sestrin1 and sestrin2, in a redox-independent pathway that required the TSC1/2 complex (*111*) (Fig. 2). As noted above, the sestrins also protect against oxidative stress by enhancing the Nrf2/ARE-regulated antioxidant pathway (*11*).

As with ATM, p53 has well-defined nuclear roles in the DDR after its accumulation and activation in the nucleus, but the cytoplasmic pools of p53 also display a number of functional associations with various cytoplasmic proteins after oxidative stress, such as interaction with pro- and anti-apoptotic members of the BCL-2 family, thereby regulating the mitochondrial apoptotic response (*112, 113*). The outcome again is cell type dependent. In addition to its roles in the cellular DDR, p53 is also an important regulator of the intercellular communication that plays a critical role

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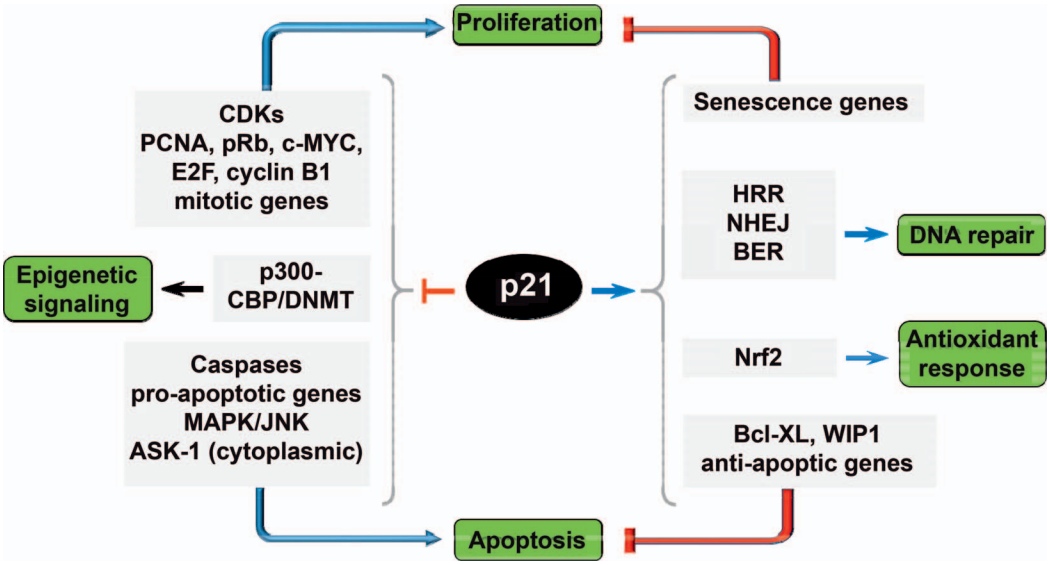


FIG. 3. Multiple functions of p21 in the cellular response to oxidative stress and associated injury to the genome. The p21 protein inhibits cell cycle progression by inhibiting cyclin/CDK complexes and by interfering with other drivers of the cell cycle, including PCNA, pRb, c-MYC, E2F and cyclin B1, as well as downregulating genes required for mitosis. p21 also promotes DNA repair, including the HRR and NHEJ pathways that process DNA DSBs as well as the BER pathway that processes various types of base damage. p21 also promotes the cellular early response to oxidative stress by facilitating the activation of the Nrf2 antioxidant transcription factor. p21 is an important inhibitor of the apoptosis pathway of cell death through multiple mechanisms. Another key activity of p21 in the DDR relates to the implementation and maintenance of the prolonged senescence-like SIPS response which again involves multiple mechanisms. DNMT = DNA methyltransferase; CBP = CREB-binding protein; ASK-1 = apoptosis signal-regulating kinase 1; WIP1 = wild-type p53-induced phosphatase 1; CDK = cyclin-dependent kinase; PCNA = proliferating cell nuclear antigen; pRb = retinoblastoma protein; MAPK = mitogen-activated protein kinase; JNK = c-jun N-terminal kinase; BCL-XL = B-cell lymphoma-extra large; HRR = homologous recombination repair; NHEJ = non-homologous end joining; BER = base excision repair; Nrf2 = nuclear factor erythroid 2-like 2.

in determining cell and tissue responses after irradiation (*114*).

THE p21^{WAF1} PROTEIN

Another major decision-making hub in the DDR activated by DNA-damaging agents, including radiation and oxidative stress in general, involves the p21^{WAF1} (CDKN1A) protein (henceforth referred to simply as p21). p21 is best known as a universal inhibitor of the cell cycle by binding to and inhibiting cyclin/cyclin-dependent kinase (CDK) complexes (*115, 116*), although it can also influence cell cycle progression in a number of additional ways (Fig. 3). After exposure of cells to radiation or oxidants such as hydrogen peroxide, p21 is transcriptionally upregulated by p53 and transiently activates cell cycle checkpoints, ostensibly to allow for DNA repair factors to process their substrates unimpeded by competing DNA transactions. Alternatively, it can be regulated at the post-transcriptional level. The proteasome activator PA28γ, the alternative 11S cap to PA28αβ, regulates p21 and p53 expression levels, and its inhibition leads to cell cycle arrest (*117*). In addition to cell cycle regulation, p21 plays a number of other key roles in the DDR relevant to the maintenance of genome stability (Fig. 3). These include the promotion of DNA repair pathways such as BER and the HRR and NHEJ pathways for DSB repair (*116*) and inhibition of apoptosis through a variety of mechanisms that result in the downregulation/inhibition of pro-apoptotic genes/proteins or the upregulation of anti-apoptotic genes [e.g., (*71, 78, 115, 116*) and references therein]. A functional illustration of these diverse roles of p21 in the DDR as both an inhibitor of apoptosis and a stimulator of DSB rejoining *in vivo* is seen in the observed correlation between the radioresistance of mouse Langerhans cells and their high levels of p21 (*118*). Specifically, irradiated wild-type cells (in contrast to their p21-knockout/radiosensitive counterparts) display robust p21-mediated G_1 checkpoint activation and rapid DSB rejoining and consequently undergo limited apoptosis after irradiation, with some survivors then being able to migrate to the skin-draining lymph nodes where they cause an expansion of immune-suppressive T-regulatory cells.

Another important role for p21 is in triggering the SIPS response. Many human cell types with wild-type p53, such as fibroblasts and solid tumor-derived cancer cell lines, primarily activate the prolonged growth-arrested senescence-like SIPS program (rather than undergoing rapid cell death) after exposure to moderate levels of DNA-damaging agents, with sustained upregulation of p21 being crucial to implementing this response (*70, 119*). Although cells in

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SIPS shut down DNA synthesis, they remain viable and can secrete growth-/tumor-promoting and pro-inflammatory factors, the so called ''senescence-associated secretory phenotype'' or SASP (*120*). The ability of p21 to evoke SIPS relates both to its ability to inhibit CDKs and to regulate gene expression (Fig. 3) [e.g., (*77, 121*) and references therein]. Like p21, $p16^{INK4A}$ is best known as a CDK inhibitor but has also emerged as a multifunctional protein and may substitute for p21 in driving the SIPS phenotype in some cell backgrounds or complement p21 in others (*122*).

The diverse functions of p21 are hugely influenced by its subcellular localization, which in turn is dictated by post-translational modifications such as phosphorylation (mediated by AKT and several other kinases) and ubiquitination (*115, 116*). In general, nuclear p21 appears to function as a tumor suppressor through its roles as a transcription factor and in regulating cell cycle checkpoints and DNA repair, whereas cytoplasmic localization of p21 appears to favor its oncogenic functions via inhibition of apoptosis and promotion of actin-mediated cell motility, which may contribute to tumor invasion and metastasis (*116*).

p21, MITOCHONDRIA, ROS AND SENESCENCE

In our laboratory, we typically see biomarkers of SIPS emerging at ~3 days after 8 Gy irradiation, with a delayed but sustained wave of p53 and p21 activation just preceding these changes (*77*). One interpretation of these findings is that the sustained wave of p53/p21 activation is triggered by a constitutively activated DDR signal [e.g., (*119*)] reflecting ongoing DNA damage. Indeed, a number of studies have related SIPS to secondary ROS production, with damaged mitochondria being a major anticipated source thereof. There is an increasingly well-documented relationship between the maintenance of SIPS and elevated levels of both ROS and p21 [e.g., (*123, 124*)]. Passos *et al.* (*124*) reported that a feedback loop between the DDR and ROS production contributes to the long-term maintenance of the SIPS phenotype in 20 Gy irradiated MRC5 human fibroblasts. The sustained DDR and activation of p21 in senescent cells was shown to cause mitochondrial dysfunction that in turn drives ROS generation via a signaling pathway that likely involves p53, p21, GADD45A, p38α MAPK14, GRB2, TGFBR2 and TGFβ. These ROS in turn induce additional DNA damage and thus a further round of DDR, and so on, generating a self-sustaining positive-feedback loop (*124*). Failure of the cell to resolve these events within ~1–2 days was presumed to activate a cellular program that drives the long-term generation of ROS and thus continued ROS-mediated DNA damage, perpetuating the SIPS phenotype; suggested contributory mechanisms include p16 activation, SASP-related autocrine signaling and chromatin remodeling (*124, 125*). Interestingly, when irradiated cultures that were within 9 days of having entered SIPS were treated with either the MAPK14 inhibitor SB203580, the free radical scavenger α-phenyl-N-*tert*-butyl nitrone or the antioxidant N-acetylcysteine, the ability to proliferate was reinstated in some of the cells (*124*).

Skinner *et al.* (*126*) also showed that secondary ROS (likely generated in the mitochondria) play a causative role in radiation-induced SIPS in head and neck cancer cell lines. In that study, ROS levels broadly correlated with p21 expression, SIPS biomarkers and cellular radiosensitivity. The tenet that secondary ROS was the primary trigger for these events was supported by the observation that adding N-acetyl cysteine 2 h *after* irradiation markedly decreased SIPS biomarkers in cells with wild-type or non-disruptive mutation of p53. These investigators (*127*) subsequently showed that p21 (rather than other downstream targets of p53) had a direct influence on ROS levels in irradiated cells. For example, when p21 was knocked down using shRNA in p53 wild-type tumor cells, the extent of SIPS and sustained elevation of ROS ($^{\bullet}O_2^-$) were both greatly decreased. Precisely how p21 regulates ROS levels remains an open question. However, these authors speculated that it may relate to the known ability of p21 to influence gene expression (Fig. 3), with a possible contributor being the PIG3/TP53I3 pro-oxidant protein discussed above in the context of its regulation by p53, but that was shown earlier by Macip *et al.* (*128*) to also be upregulated by p21 (but not by p16). Indeed, the latter authors (*128*) noted that SIPS and ROS accumulation by human cell lines were directly related to the levels of p21, independent of p53. Similarly, Westin *et al.* (*129*) reported that depletion of p21 (but again, not p16) using shRNA abrogated both $^{\bullet}O_2^-$ production and the onset of replicative senescence in cells from patients with the premature aging disorder dyskeratosis congenita that is associated with shortened telomeres, implying a causal role for p21 in these phenotypes.

The Passos group (*130*) subsequently showed that mitochondria play a major role in implementing SIPS after exposure of Parkin-expressing MRC5 fibroblasts to X rays (10 or 20 Gy) or oxidative stress (e.g., hydrogen peroxide). This was done by treating cells that had already entered SIPS with an oxidative phosphorylation inhibitor, carbonyl cyanide *m*-chlorophenyl hydrazone, which targets the Parkin ubiquitin ligase to the mitochondria, promoting their proteasomal and autophagic degradation. Mitochondrial ablation greatly reduced the levels of SIPS biomarkers, but for yet-unknown reasons it did not restart the cell cycle even though the cells showed elevated glycolysis and ATP generation. The study also identified ATM-AKT-mTORC1 signaling to peroxisome proliferator-activated receptor γ co-activator 1β (PGC-1β), a transcriptional co-regulator of genes involved in mitochondrial biogenesis, as an important participant in SIPS. The authors acknowledge that mitochondrial ablation is an extreme manipulation and that other events may contribute to these effects (*130*).

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ROLE OF p21 IN THE REGULATION OF Nrf2

Crosstalk between the Nrf2 and p53-p21 pathways has emerged as an important element of the cellular homeostatic response to oxidative stress. As already noted, cytoplasmic Nrf2 levels are normally constrained by Keap1; oxidation of Keap1's cysteine residues during oxidative stress moderates its interaction with Nrf2, resulting in Nrf2 stabilization and translocation to the nucleus where it activates the transcription of ARE-containing antioxidant genes. Zhang and colleagues (*131*) used the HCT116 cell line and its p21-knockout derivative, as well as wild-type and Nrf2-knockout MEFs, to show that p21 can augment this response by binding to Nrf2, competing with Keap1, and thus interfering with the Keap1-dependent ubiquitination and degradation of Nrf2, thereby enhancing Nrf2 antioxidant signaling under both basal and stress conditions. The effect of Nrf2-p21 crosstalk is anticipated to be supra-additive during oxidative stress because both pathways individually are induced under these circumstances. Nrf2 was actually *required* for p21 to execute its role as an antioxidant (*131*); thus, ectopic expression of p21 was able to protect against hydrogen peroxide-mediated injury in wild-type but not in Nrf2-knockout MEFs. A diminished Nrf2 activation and antioxidant response was also seen in the livers of p21-deficient mice challenged with tert-butyl-hydroxyanisole under conditions that caused oxidative stress, supporting an extension of the *in vitro* findings to the *in vivo* environment (*131*).

The above study related to cells undergoing moderate levels of oxidative stress and showed that under such conditions the p53-p21 axis facilitates upregulation of the protective/pro-survival Nrf2-activated antioxidant response. As noted above, very different responses have been observed under severe stress conditions where p53 instead triggers pro-oxidant responses that result in outcomes such as apoptosis or SIPS. The possibility that Nrf2-mediated antioxidant responses might be deactivated in concert with the activation of pro-oxidant responses under such conditions was suggested by earlier observations from the Zhang group showing that, whereas treatment of human cell lines with low/moderate concentrations of oridonin, an ROS-generating diterpenoid compound, caused a cytoprotective Nrf2-induction response, Nrf2 protein levels actually *decreased* after high concentrations or oridonin and consequent high levels of oxidative stress (*132, 133*), implying that the resulting increase in ROS levels after antioxidant suppression should facilitate the commitment of cells with high-damage levels to undergo p53-dependent apoptosis. A follow-up study (*107*) detailed the Nrf2 response in human renal mesangial cells exposed to increasing levels of hydrogen peroxide or etoposide. Consistent with the low-stress/antioxidant, high-stress/pro-oxidant model of p53 activity outlined above, a biphasic regulation of Nrf2 expression was observed in these cells: whereas low to moderate levels of stress (and thus of p53 activation) enhanced Nrf2 protein levels and transcription of its target genes through p21, decreasing ROS formation and favoring cell survival, high levels of stress (and thus of activated p53) inhibited the Nrf2-mediated survival response, favoring initiation of cell death or proliferative disablement.

SUMMARY

In this work we have reviewed some of the pertinent features of the cellular antioxidant and DDR responses evoked by oxidative stress/radiation exposure in human cells. The cellular response to oxidative stress represents an elaborate and compartmentalized network, many aspects of which remain undefined. The initial oxidative insult is followed by waves of secondary ROS generated by events such as damage to the mitochondria and peroxisomes, proteasome disassembly, persistent cytoplasmic DNA, activation of oxidases and pro-inflammatory cytokines and of p53-activated pro-oxidant proteins in heavily-damaged cells. Nonetheless, some understanding of how these responses might be integrated is beginning to emerge. One thing that has become apparent is that many proteins best known for their roles in the DDR, notably ATM, p53, p21 and Ref1, also exert major earlier roles in the antioxidant response, suggesting that they might play a broad temporal coordinating role across the entire cellular response to oxidative stress. Another key finding has been that the multiple activities of many of these proteins are highly dependent on their post-translational modification and subcellular localization, e.g., the distinct nuclear versus cytoplasmic roles of ATM, p53 and p21, as well as on the temporal characteristics of these proteins which, for proteins such as p53 and p21 in p53 wild-type cells, exhibit a wave-like response after exposure to moderate doses (5–10 Gy) of γ rays (*77, 134*). The ability of Nrf2 to moderate oxidative stress may also extend beyond its well-described role as an antioxidant transcription factor in the nucleus; it was recently reported that, in cardiomyocytes, Nrf2 can localize to the mitochondrial outer membrane in response to hydrogen peroxide treatment, suggesting that it has a novel function in protecting the mitochondria from oxidative injury (*135*).

Many facets of the crosstalk between components of these individual pathways remain to be deciphered. Such is the case in regard to the reported crosstalk between Ref1 and Nrf2 and the potential involvement of p53-p21 signaling therein. In one published study, it was shown that Ref1 positively influences Nrf2 activity (*136*), as might be expected if these antioxidant pathways were coordinately upregulated after moderate levels of stress. In a more recently reported study however, Ref1 was observed to *suppress* Nrf2 levels and activity in several cell types and in patient-derived samples by altering Nrf2 mRNA and protein levels and by a direct interaction between the two proteins at the promoters of Nrf2 target genes (*55*). As we already noted, p53 may influence this dialogue because it engages in bidirectional crosstalk with Ref1, such that p53 might indirectly influence Nrf2 activity. However, the authors of the latter study (*55*) noted that Ref1 can also repress p21 gene expression (*137*); considering the above-noted report that

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the p21 protein can augment Nrf2 antioxidant signaling (*131*), especially after its induction by p53 under stress conditions, it may be reasonable to speculate that the p53-p21 axis may also be involved in the regulatory crosstalk between these two key antioxidant proteins via such mechanisms. It is unclear how these interactions might change at low-, moderate-, high- or severe-stress levels, in part because the degree of stress has rarely been quantitated and is difficult to compare across studies, but it may explain several discrepancies in the literature noted here. The mechanism of Nrf2 degradation at high stress levels is similarly unclear. An early study showed that p53 can inhibit the transcription of Nrf2 target genes, possibly by binding to a sequence near the ARE and repressing Nrf2-dependent transcription (*138*). In a subsequently published study (*107*) a similar inhibition of Nrf2-mediated responses was noted after high levels of stress but this was likely *not* a result of p53 interaction with the AREs; rather, this effect was interpreted as involving the intermediacy of the p53-regulated p21 protein. Again, though, the relative levels of stress in these studies are difficult to compare.

The forkhead box O (FoxO) transcription factors, and potentially Nrf2, are also targets of the AMPK kinase (*139, 140*). FoxOs, when activated by various kinases (including AMPK) in response to high levels of ROS, translocate to the nucleus and transcriptionally activate antioxidant genes such as GPx1, Prx3, SOD2, GSTμ1, catalase and sestrins, as well as genes involved in GSH and NADPH generation. In addition to antioxidant functions, FoxO target proteins influence cell cycle arrest, apoptosis, autophagy and DNA repair and suppress mTOR activity (*140, 141*). Interestingly, exposure to radiation was reported to upregulate FoxO3a protein levels and functional responses (nuclear translocation, target gene activation) in Saos2 human osteosarcoma cells (*142*). A role for FoxO3a in the DDR, suggested in the findings, was that it could directly modulate ATM kinase activity (*143*) and that this was important for full activation of the ATM/Chk2/p53-mediated response to DSBs (*144*), apparently by bridging the ATM protein to the above-mentioned Tip60 acetyltransferase (*145*). FoxOs also engage in crosstalk with the sirtuin family proteins. For example, sirtuin 3 (SIRT3), a protein deacetylase, is an important regulator of mitochondrial oxidative metabolism after exposure to radiation and other oxidative stresses both *in vitro* and *in vivo* (*146*). It abrogates mitochondrial $^{\bullet}O_2^-$ production by regulating SOD2 expression transcriptionally through FoxO3a as well as via deacetylation of SOD2 at lysine residues, which results in increased SOD2 activity (*146*). These events also appear to crosstalk with p53 signaling insofar as SIRT3 may further promote cell survival after irradiation/oxidative stress by deacetylating the pool of p53 that translocates to the mitochondria in response to increased $^{\bullet}O_2^-$ generation, thereby enhancing p53 degradation (*146*). That a failure of cells to regulate $^{\bullet}O_2^-$ can have major *in vivo* functional consequences is clearly illustrated by its direct association with functional liver injury in irradiated SIRT3-knockout mice (*146*). In another intriguing study, donor age-related changes in mitochondrial oxidative metabolism leading to $^{\bullet}O_2^-$ accumulation in human fibroblasts were associated with increased sensitivity to radiation and chemotherapy (*147*).

Another area that requires clarification relates to the role of LKB1 in the nuclear/p53-dependent response to DNA damage (Fig. 2). p53 has been variously shown to associate with LKB1 and to activate AMPK after DNA damage, while LKB1 has also been reported to be phosphorylated by ATM in response to DNA damage, e.g., after irradiation. ATM may also phosphorylate AMPK independently of LKB1 in the DDR. Both LKB1 and AMPK may phosphorylate p53 under some conditions. ROS also activate AMPK directly (*148, 140*). Additional redox-sensing systems of interest, which we have not had the opportunity to cover, include that involving the sumoylation of tumor protein p53-induced nuclear protein 1 (TP53INP1), which is an important enhancer of the p53 response (*149*).

Going forward, the model in which p53-mediated responses are largely cytoprotective at low-to-moderate-stress levels but switch to cytostatic or cytotoxic at high-to-severe-stress levels provides an important framework. In only a few published studies have researchers looked in detail at stress-level thresholds in regards to where the various molecular and phenotypic responses are triggered. Macip *et al*. (*106*) suggest that the main discriminator between a SIPS and apoptotic response may be as simple as the level of p53 itself, such that apoptosis should be the default after a severe stress for many cell types. For example, we noted earlier that Nrf2 is eliminated by the cell at the same time that the pro-oxidant state is being activated, presumably to generate a powerful signal when the cell needs to be proliferatively disabled or executed. Whether there is a definable threshold for the transition between prolonged proliferative arrest/SIPS and apoptosis as the primary outcome in terms of high versus severe damage levels is not clear but is very likely, although both of these outcomes are characterized by high levels of ROS accumulation. Indeed, it has been suggested that the level of ROS accumulation *per se* may be another factor in determining cell fate decisions (*123*). The size of the radiation dose/oxidative stress level and the cell type are of course critical variables in all of these responses.

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