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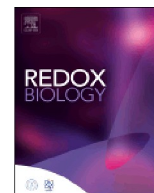
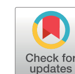


gRASping the redox lever to modulate cancer cell fate signaling



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ABSTRACT

RAS proteins are critical regulators of signaling networks controlling diverse cellular functions such as cell proliferation and survival and its mutation are among the most powerful oncogenic drivers in human cancers. Despite intense efforts, direct RAS-targeting strategies remain elusive due to its "undruggable" nature. To that end, bulk of the research efforts has been directed towards targeting upstream and/or downstream of RAS signaling. However, the therapeutic efficacies of these treatments are limited in the long run due to the acquired drug resistance in RAS-driven cancers. Interestingly, recent studies have uncovered a potential role of RAS in redox-regulation as well as the interplay between ROS and RAS-associated signaling networks during process of cancer initiation and progression. More specifically, these studies provide ample evidence to implicate RAS as a redox-rheostat, manipulating ROS levels to provide a redox-milieu conducive for carcinogenesis. Importantly, the understanding of RAS-ROS interplay could provide us with novel targetable vulnerabilities for designing therapeutic strategies. In this review, we provide a brief summary of the advances in the field to illustrate the dual role of RAS in redox-regulation and its implications in RAS signaling outcomes and also emerging redox-based strategies to target RAS-driven cancers.

1. Introduction

The first observation of RAS oncogene was reported as early as 1964 when inoculation of murine leukemic virus isolated from leukemic rats unintentionally induced sarcomas in newborn rodents [1]. This discovery eventually paved the way for the identification of acute tumor-inducing retrovirus and molecular characterization of its associated viral gene that confers its oncogenic potency. Despite these seminal

Abbreviations: AKT, Protein kinase B; ASK1, Apoptosis signal-regulating kinase 1; ATP, Adenosine triphosphate; Atg, Autophagy-related; BAX, Bcl-2-associated X protein; Ca^{2+}, Calcium (ii) ion; CuZnSOD/SOD1, Copper Zinc superoxide dismutase; Cu^{2+}, Copper (II) ion; DNA, Deoxyribonucleic acid; DPI, Diphenyleneiodonium; DRP1, Dynamin-related protein 1; DUOX, Dual oxidase; ECSOD/SOD3, Extracellular superoxide dismutase; EGF, Epidermal growth factor; ER, Endoplasmic reticulum; ERK, Extracellular signal-regulated kinase; ETC, Electron transport chain; Fe^{2+}, Iron (II) ion; FMN, Flavin mononucleotide; FOXO3A, Forkhead box O3a; G6PD, Glucose-6-phosphate dehydrogenase; GAP, GTPase-activating protein; GAPDH, Glyceraldehyde-3-phosphate dehydrogenase; GDP, Guanosine diphosphate; GEF, GTP exchange factors; GLUT1, Glucose transporter 1; GPx, Glutathione peroxidase; GSH, Glutathione; GSR, Glutathione disulfide reductase; GSSG, Glutathione disulfide; GTP, Guanosine triphosphate; •HO, Hydroxyl radical; H_2O_2, Hydrogen peroxide; HOCl, Hypochlorous acid; IMM, Inner mitochondrial membrane; JNK, c-Jun N-terminal Kinase; Keap1, Kelch-like ECH-associated protein 1; MAPK, Mitogen-activated protein kinase; MEK, Mitogen-activated protein kinase kinase; miR, Micro-Ribonucleic acid; MKLK, Mixed lineage kinase domain like pseudokinase; Mn^{3+}, Manganese (III) ion; MnSOD/SOD2, Manganese superoxide dismutase; MPK, MAPK phosphatase; mTOR, Mammalian target of rapamycin; mTORC1, Mammalian target of rapamycin complex 1; NADPH, Nicotinamide adenine dinucleotide phosphate; NF-κB, Nuclear factor kappa-light-chain-enhancer of B cell; NO, Nitric oxide; •NO_2, Nitrogen dioxide; NOS, Nitric oxide synthase; NOX, NADPH oxidase; Nrf2, Nuclear factor erythroid-related factor 2; NSCLC, Non-small cell lung cancer; O_2, Oxygen; $O_2^{•-}$, Superoxide anion; $ONOO^-$, Peroxynitrite; PDI, Protein disulfide isomerase; PI3K, phospoinositide-3-kinase; Pim1, Proto-oncogene serine/threonine-protein kinase Pim-1; PPP, Pentose Phosphate Pathway; Prx, Peroxiredoxin; PTEN, Phosphatase and tensin homolog; PTP, protein tyrosine phosphatase; Rac, Ras-related C3 botulinum toxin substrate; RAF, Rapidly Accelerated Fibrosarcoma; RAS, Rat sarcoma; RIPK, Receptor interacting protein kinase; ROS, Reactive Oxygen Species; RNS, Reactive nitrogen species; SASP, Senescence-associated secretory phenotype; SESN, sestrin; SH2, Src homology 2; SOD, Superoxide dismutase; TCA cycle, Citric acid cycle; TNF-α, Tumor necrosis factor-α; TNFR, Tumor necrosis factor receptor; TRAF, TNFR-associated factor; Trx, Thioredoxin; TrxR, Thioredoxin reductase; Zn^{2+}, Zinc (II) ion

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discoveries, enthusiasm in RAS oncogene only heightened upon the discovery of its cellular homologues and its high prevalence of mutations in cancer [2–6]. Research efforts spanning over half a century eventually led to the discovery of numerous signaling pathways associated with RAS activation. Not only do these signaling pathways contribute to mitogen-independent growth of tumors, they are also involved in acquisition of other established cancer hallmarks such as cell survival, motility and angiogenesis as defined by Hanahan and Weinberg [7].

Emerging alongside these discoveries, studies have also implicated the diverse physiological functions of reactive oxygen species (ROS) in cell survival and death signaling as well as their pathological relevance to cancer initiation and progression, underscoring the potential cross-talk between oncogenes and ROS in facilitating the acquisition of cancer hallmarks [8–16]. To that end, recent studies provide overwhelming evidence linking oncogenic signaling to an altered redox-milieu that favors cell survival and promotes transformation and carcinogenesis. In a reciprocal manner, ROS are also able to regulate the activities of oncoproteins. In this review, we attempt to summarize experimental evidence demonstrating the interplay between oncogene RAS and cellular redox status in the context of carcinogenesis as well as present evidence, where relevant, of existing strategies and potential therapeutic avenues targeting the RAS-ROS signaling network(s).

2. Intracellular generation of ROS

ROS are a heterogenous group of chemically reactive ions and molecules derived from the reduction of molecular oxygen, O_2, via various cellular processes. The group encompasses both, oxygen-derived radicals such as superoxide anion ($O_2^{\bullet-}$) and hydroxyl radical (HO•), and also non-radical species such as hydrogen peroxide (H_2O_2) and hypochlorous acid (HOCl), which despite their non-radical properties, are powerful oxidizing agents. Beside its known deleterious effects, ROS are able to regulate protein activity through reversible oxidative modification of redox-sensitive thiol/cysteine groups into sulfenic, sulfinic, sulfonic or S-glutathionylated forms, thereby, altering the function and activity of proteins. It is also worth mentioning that Reactive Nitrogen Species (RNS), such as peroxynitrite ($ONOO^-$), derived from the reaction between nitric oxide (•NO) and $O_2^{\bullet-}$ can also react directly with proteins and modify its activity and function. However, due to its tendency to act in unison with ROS to induce cellular effects, it is often loosely termed as ROS as well. Among all ROS, superoxide anion and hydrogen peroxide remain the most extensively studied. These two entities remain the most critical determinant of oxidative stress and cell fate with specialized cellular antioxidant systems evolved to buffer and/or counteract their harmful effects.

The major source of intracellular ROS is the membrane bound NADPH-oxidase complexes which consist of NOX family of enzymes and its regulatory phox subunits and/or Rac GTPase (Fig. 1C). The NOX family comprises seven isoforms (NOX1–5, DUOX1 and DUOX2), which catalyzes NADPH-dependent one electron reduction of O_2 to $O_2^{\bullet-}$. Among which, NOX1-NOX3 requires Rac GTPase for its activation [17–19]. Similarly, NOX5, DUOX1 and DUOX2 activation requires binding of calcium (Ca^{2+}) to its calcium-binding domains and are usually activated by upstream signaling pathways that increases cellular Ca^{2+} levels [20,21]. Interestingly, NOX4 differs from the other members of NOX family enzymes in that it is constitutively active and produces predominantly H_2O_2 owing to its high affinity towards O_2 [22]. DUOX enzymes, characterized by a defining N-terminal peroxidase-like domain, also produces both $O_2^{\bullet-}$ and H_2O_2 [23]. However, the ability for its peroxidase-like domain to catalyze conversion of $O_2^{\bullet-}$ to H_2O_2 remains inconclusive [24]. Initially discovered as a membranous protein required for 'oxidative burst' in phagocytes for antimicrobial defense, NOX expression was found to be far more ubiquitous than presumed [25]. Although $O_2^{\bullet-}$ production by NOX complexes are directed towards extracellular space, studies have suggested that extracellular $O_2^{\bullet-}$ can diffuse back into the cell via anion channels to serve as intracellular messengers [26,27]. Importantly, the functional relevance of NOX-produced ROS in promoting cancer cell survival and proliferation has been demonstrated in various cancer cell types [28–30].

The mitochondrial metabolic activity is another important source of intracellular $O_2^{\bullet-}$. During oxidative phosphorylation, adenosine triphosphate (ATP) is generated as electrons shuttle through the mitochondrial electron transport chain (ETC), comprising of four multiprotein complexes named I – IV, located on the inner mitochondrial membrane (IMM). However, during electron shuttling, 1–2% of electrons inevitably leak out from the ETC leading to the reduction of O_2 to $O_2^{\bullet-}$ [31,32]. (Fig. 1A). Notably, mitochondrial complex I and III are the two major sites within the ETC responsible for the by-production of $O_2^{\bullet-}$ [33–37]. Besides the mitochondrial ETC, production of ROS by other mitochondrial enzymes involved in the citric acid (TCA) cycle such as pyruvate dehydrogenase and α-ketoglutarate dehydrogenase has been reported, but the mechanism(s) remain unclear [38].

The endoplasmic reticulum (ER) is a site of critical redox-reactions that assist in proper folding and maturation of proteins, resulting in the generation of ROS [39]. The oxidative folding of proteins is catalyzed by the oxidation of dithiol groups of targets by protein disulfide isomerase (PDI), during which, disulfide bond of PDI enzyme is reduced (Fig. 1B). PDI is reverted to its oxidized from via ER oxidoreductase-1 (ERO1) and results in the reduction of terminal electron acceptor, O_2 to H_2O_2 [40,41]. Further substantiating the role of ER as a ROS-producing organelle, NOX4 has been shown to localize to the ER and increase in the in situ generation of ROS upon stress [42].

Last but not least, Nitric Oxide Synthases (NOSs) produces nitric oxide, which is sometimes considered ROS due to the presence of an oxygen moiety. Briefly, synthesis of nitric oxide by NOS involves single electron reduction of O_2 followed by its incorporation into guanidine nitrogen of L-arginine to form •NO and L-citrulline [43]. The electron in this reaction is derived from NADPH and transferred to the heme catalytic center of NOS via a flavin mononucleotide (FMN)-prosthetic group located on the C-terminal end of NOS [44]. Despite being a radical species with an unpaired electron, •NO exhibits low reactivity towards biological molecules and is able diffuse across cell membrane to neighboring cells, allowing it to serve as a signaling agent [45]. However, under high ambient $O_2^{\bullet-}$ concentrations, •NO can react readily with $O_2^{\bullet-}$ to generate $ONOO^-$, a strong oxidizing/nitrating agent [46]. $ONOO^-$ has been reported to damage DNA, proteins and lipids through its oxidative/nitrative properties [47–51].

Other metabolic enzymes such as xanthine oxidase, cytochrome P450, aldehyde oxidase have also been reported to produce ROS as by-products [52–54]. However, they are not known to significantly influence disease initiation and/or progression of RAS-driven cancers. Thus, they are beyond the scope of this review.

3. Cellular antioxidant defense systems

Uncontrolled increase in intracellular ROS could be detrimental to cells and tissues, resulting in cell death and tissue damage. To that end, cells employ various antioxidant enzymes and non-enzymatic systems to maintain redox homeostasis and prevent oxidative stress-induced injury/damage.

Nuclear factor erythroid 2 (NFE2)-related factor 2 (Nrf2) is a major antioxidant transcription factor and one of the first responders towards increased oxidative stress [55]. Briefly, activation of Nrf2 involves the dissociation of its negative regulator, Kelch-like ECH-associated protein 1 (Keap1), from Nrf2, allowing Nrf2 to translocate into the nucleus to transcribe antioxidant genes [56,57]. The dissociation of Keap1 from Nrf2 in response to oxidative stress involves the modification of redox-sensing cysteine residues on Keap1 which presumably alters its structure and disrupts the Keap1/Nrf2 interaction [57,58]. Other mechanisms of Nrf2 activation has also been reported and reviewed in [59].

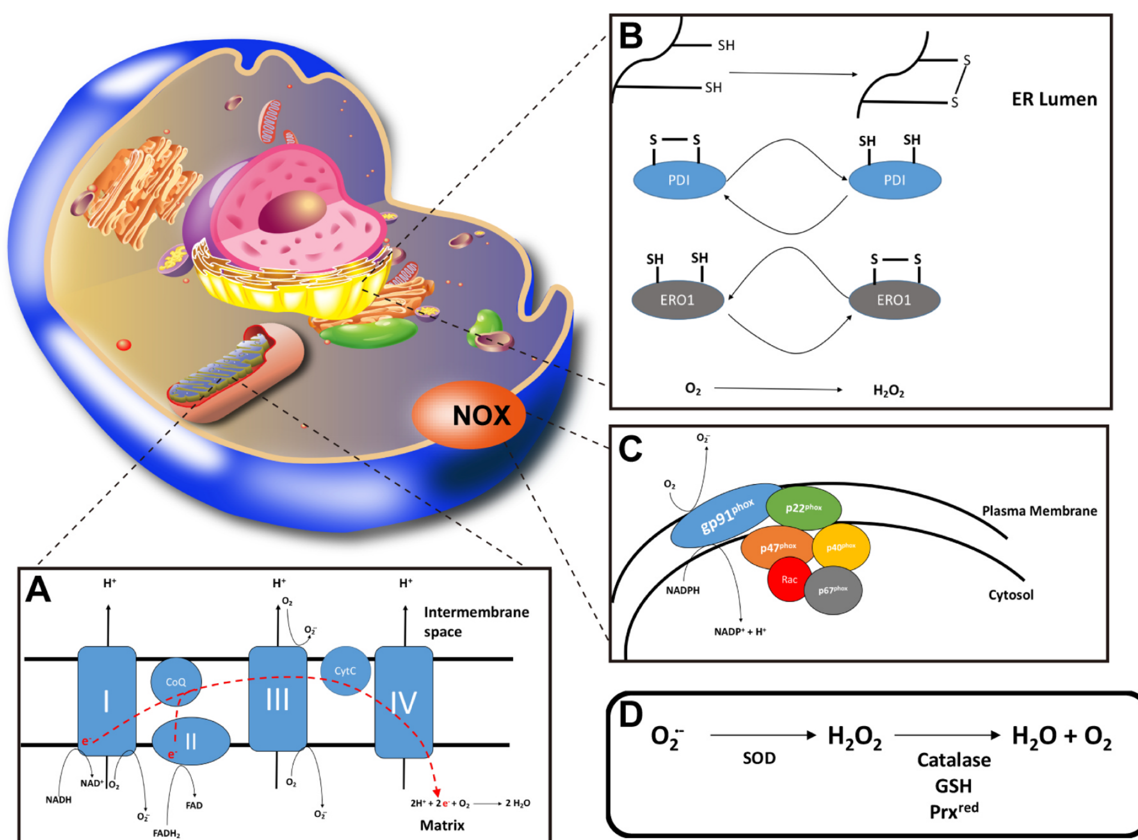

Fig. 1. Cellular sources of ROS. ROS are inevitable byproduct of cellular metabolism and are generated in various cellular compartments. (A) The mitochondrial ETC generates $O_2{\bullet}^-$ as electrons are shuttled from complexes I and II to III and IV through a series of redox-reactions. Electrons are derived from the oxidation of NADH to NAD^+ at complex I and oxidation of $FADH_2$ to FAD^+ at complex II. The inevitable leakage of electrons causes the reduction of O_2 to $O_2{\bullet}^-$. Complex I and III are major sites of $O_2{\bullet}^-$production within the ETC. (B) ER is another site of ROS production. PDI catalyzes disulfide bond formation, required for the maturation of its protein substrate. During which, PDI reduces its own disulfide bond to two dithiol groups. The restoration of PDI activity involves oxidation of dithiol group by ERO1 with $FADH_2$ as a cofactor and results in reduction of terminal electron acceptor, O_2 to H_2O_2. (C) NOX complexes (NOX2 depicted in figure) consists 6 subunits. Namely, $gp91^{phox}$, $p22^{phox}$,$p47^{phox}$, $p40^{phox}$, $p67^{phox}$ and Rac. Electrons released from the oxidation of NADPH reduces extracellular O_2 to $O_2{\bullet}^-$. NOX4 and DUOX produces both $O_2{\bullet}^-$ and H_2O_2. (D) $O_2{\bullet}^-$ are dismutated into H_2O_2 by SOD enzymes. H_2O_2 is further decomposed into H_2O and O_2 by catalase, GSH or Prx. There are other sources of ROS stated in-text that were not depicted within this figure.

The target genes of Nrf2 includes, but are not limited to, glutathione peroxidase (GPx), peroxiredoxin (Prx), thioredoxin (Trx), glutathione disulfide reductase (GSR), thioredoxin reductase (TrxR) [60–66]. The antioxidant function of these proteins would be elaborated later within this section.

Superoxide produced via cellular processes is removed through a series of reactions involving dismutation of $O_2^{\bullet-}$ to H_2O_2 by the action of superoxide dismutase (SOD) enzymes (Fig. 1D). Three SOD isozymes are characterized to date, namely CuZnSOD (SOD1), MnSOD (SOD2) and ECSOD (SOD3) which exhibit differences in their structure, spatial distribution and metal-containing catalytic center. Catalytic activity of SOD1, found primarily in the cytosol and mitochondrial intermembrane space, is driven by copper (II) ion (Cu^{2+}) and zinc (II) ion (Zn^{2+}) [67]. In contrast, catalytic activity of SOD2 is driven by manganese (III) ion (Mn^{3+}) and is found primarily in the mitochondrial matrix. SOD3 has a similar structure to SOD1 with an additional secretory sequence that allows its secretion into extracellular space where it converts extracellular $O_2^{\bullet-}$ to H_2O_2 [68]. Like $O_2^{\bullet-}$, extracellular H_2O_2 can diffuse back across the plasma membrane via aquaporin channels to carry out its intracellular signaling functions [69].

Within the cell, H_2O_2 can be further detoxified via a number of antioxidant defense systems. Catalase catalyzes the decomposition of H_2O_2 to H_2O and O_2. Besides catalase, H_2O_2 can also be reduced to H_2O by GPx. The reduction of H_2O_2 to H_2O by GPx involves the oxidation of glutathione to glutathione disulfide (GSH to GSSH), which is subsequently recycled to form GSH via GSR [70,71]. In addition to GSH, another major antioxidant enzyme, Prx, scavenges H_2O_2, peroxides and peroxynitrite ($ONOO^-$) through oxidation of its own redox-active thiol groups. Oxidized Prx (Prx^{oxd}) then uses reduced Trx (Trx^{red}) as an electron donor to restore its reducing capacity. Trx^{oxd} is, in turn, regenerated through reduction by Trx reductase. Central to redox-cycling of the antioxidant defense system is cofactor NADPH which serve as an electron donor for the reduction of GSSG and Trx via GSR and Trx reductase, respectively [72].

As these antioxidants are crucial in maintenance of intracellular redox-homeostasis, compromised antioxidant function coupled with aberrant ROS generation results in an altered redox milieu (*pro-oxidant state*), associated with carcinogenesis and its progression.

4. Redox Paradox in cancer cell fate

Due to the ability to spontaneously induce oxidative damage to biological systems and involvement in the etiology of various pathological states, ROS have historically been regarded as noxious molecules. Despite these overstated deleterious effects, mounting evidence has challenged the dogmatic view of ROS as noxious molecules and revealed an essential, yet complex signaling role for ROS under both normal and pathophysiological states. Indeed, ROS have been implicated in myriad of cellular processes and signaling networks including those associated with the various phases of carcinogenesis:

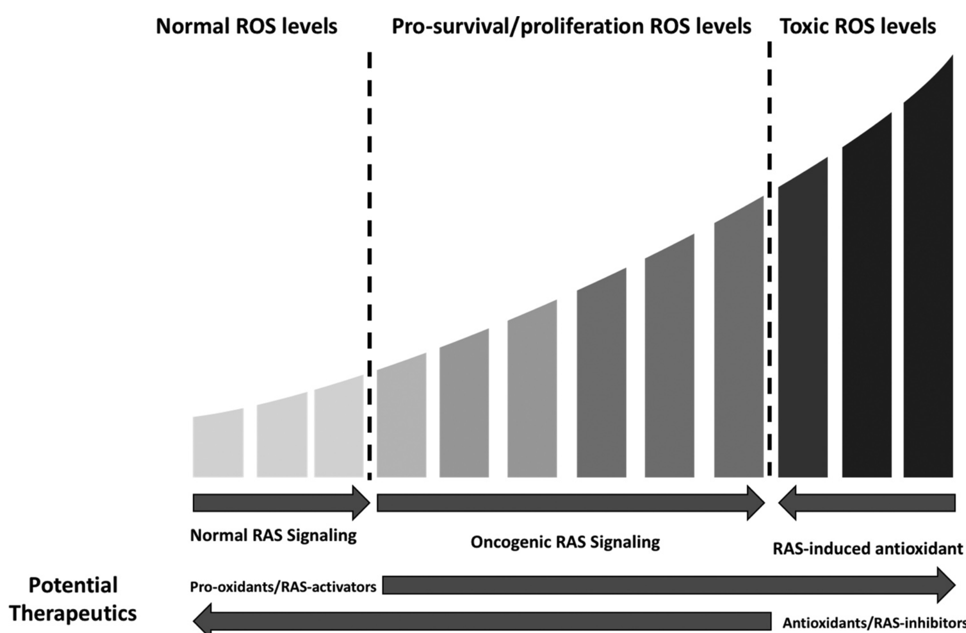

Fig. 2. Biphasic effects of ROS within RAS signaling. ROS evoke a variety of cell responses based on its concentration, spatial and temporal distribution, and cell type. Generally, moderate increase in ROS levels promotes cell proliferation and survival while extremely high levels of ROS promotes DNA damage, growth arrest, senescence and cell death. Oncogenic RAS signaling has been shown to promote cell proliferation and survival associated with ROS production. Accompanied by the increase in ROS production, RAS signaling also induces activation of antioxidant response to buffer ROS levels from reaching toxic levels. Potential therapeutics may consider leveraging on RAS signaling to augment ROS levels.

initiation, promotion and progression. These effects are a result of the redox-regulation of cell growth and survival mechanisms, such as inhibition of cell death (apoptosis resistance) and/or activation of proliferative pathways or conversely excessive DNA damage and cell execution. The latter effects of ROS are associated with excessive ROS accumulation that results in extensive macromolecule damage such as lipid peroxidation, protein oxidation and DNA damage, triggering cell death/growth arrest pathways that facilitate the removal of cells with irreparable lesions. An example is the p53 tumor suppressor, which is critically involved in the induction of apoptosis or growth arrest in response to oxidative stress-induced DNA damage [73,74]. Similarly, increase in intracellular ROS can also induce apoptosis via sustained activation of c-Jun N-terminal Kinase (JNK) [75,76], possibly stemming from the ROS-dependent inactivation of mitogen-activated protein kinase (MAPK) phosphatase (MKP) that dephosphorylates/inactivates JNK. The inactivation of MKP is a result of catalytic cysteine oxidation to sulfenic acid by H_2O_2 [77]. In another study, ROS has also been implicated in the activation of apoptosis signal-regulating kinase-1 (ASK1) which induces apoptosis through downstream activation of JNK/p38 MAPK [78].

In addition to apoptosis, ROS has also been demonstrated to be a robust activator of other forms of cell death. For instance, mutant RAS-selective small molecule compound, erastin, increases cytosolic and lipid ROS production via inhibition of cysteine uptake through cysteine/glutamate antiporter (system X_c^-) required for cysteine-dependent GSH synthesis, compromising antioxidant defense, ultimately resulting in an iron-dependent, oxidative cell death known as *ferrotoposis* [79]. A number of studies has also implicated ROS in mediating *necrotopsis*, a programmed form of necrotic cell death. Notably, combination treatment of Smac-mimetic BV6 and tumor necrosis factor-α (TNF-α) induces ROS-dependent assembly of receptor-interacting serine/threonine-kinase 1(RIPK1)/RIPK3 necrosome which drives necroptosis via phosphorylation and activation of mixed lineage kinase domain like pseudokinase (MLKL) [80]. Interestingly, deficiencies in critical TNF-α-induced necroptosis signaling proteins such as TNFR-associated factor (TRAF)2, Fas-associated death domain (FADD) and RIPK3 ameliorate ROS production and reduce cell death, indicating the importance of a ROS amplification loop in mediating this unique form of cell death [80,81].

Contrary to its death-inducing property, a substantial amount of evidence has also revealed that ROS, within physiological levels, function as secondary messengers. This signaling property of ROS is often mediated by the reversible oxidation of cysteine thiol groups within proteins, altering their function and activity, thereby regulating the respective signaling pathways the proteins are involved in [82–86]. Notably, H_2O_2 inactivates tumor suppressor phosphatase and tensin homolog (PTEN), a negative regulator of phospoinositide-3-kinase/protein kinase B (PI3K/AKT) signaling pathway via reversible oxidation of the cysteine residues, resulting in increased PI3K/AKT signaling. The inactivating oxidative modification can be reversed by Trx-mediated reduction of PTEN, thereby restoring its phosphatase activity and downregulation of PI3K/AKT signaling [84]. Similarly, $O_2^{\bullet-}$ is able to reversibly inactivate PTEN via oxidative modification, resulting in increased phosphoinositol-(3,4,5)-triphosphate levels, thereby increasing PI3K/AKT signaling [85]. Further supporting the role of oxidants in signal transduction, H_2O_2 has also been implicated in the reversible oxidation and inhibition of SH2 domain containing protein tyrosine phosphatase (PTP), resulting in burst of protein tyrosine kinases activity, a principle mode of mitogenic signal transduction [86].

From the standpoint of carcinogenesis, the biphasic functions of ROS can be difficult to grasp given its conflicting role in cancer. On the one hand, cancer cells often display higher levels of oxidative stress compared to normal cells due to oncogenic stimulation and altered metabolism, which are important for cancer initiation and progression [87–90]. On the other hand, ROS can also induce multiple tumor suppressive mechanisms such as activation of p53 [73,77,91]. In fact, augmentation of oxidative stress is the basis of many chemotherapeutic and radiotherapeutic agents' mechanism of action [92–94]. *How then, do cancer cells reconcile with the conflicting effects of ROS on cell fate determination?* Underpinning these contradictory responses seem to be a concentration-dependent pathology, wherein a threshold exists to distinguish between the signaling functions and toxic effects of ROS (Fig. 2). Hence, while leveraging on the beneficial effects of ROS to promote its own survival and proliferation, a cancer cell must also actively keep its ROS levels in check by enhancing its own antioxidative capacity to prevent cell death. Herein, we will focus on how RAS, a potent oncogene, promote and maintain the cancer phenotype in a redox-regulated manner.

5. Rising ROS levels float RAS signaling

RAS are 21 kDa small GTPase that regulate diverse cellular

processes such as cell proliferation, survival and metabolic reprogramming. In human, three subfamily RAS genes – *NRAS, HRAS and KRAS* - encode for 4 highly homologous RAS isoforms, NRAS, HRAS and splice variants KRAS4a and KRAS4b. Localized on the cell plasma membrane, RAS proteins serve as critical signaling nodes which transduce extracellular signals from membrane receptors to a complex network of intracellular effector pathways. This is achieved by transitions between its active guanosine triphosphate (GTP)- bound conformation upon GDP loading and inactive guanosine diphosphate (GDP)-bound conformation upon GTP hydrolysis. Under physiological conditions, tandem switching between "active" and "inactive" conformations is modulated by GTP exchange factors (GEFs) and GTPase-activating proteins (GAPs), ensuring signal transduction to be transient and efficiently controlled. Hence, RAS are known to be effective "molecular-switches". Molecular analysis of RAS-driven tumors has identified RAS mutational hot spots in codons 12, 13 and 61 at varying frequencies [95]. These mutations disrupt GTP hydrolytic capacity of RAS GTPase resulting in incessant proliferative and survival signaling through stimulation of downstream effectors such as RAF/MEK/MAPK, PI3K/AKT/mTOR and RalGEF/Ral pathways.

Apart from receptor-mediated and mutational activation, increased ROS levels have also been linked to the activation of RAS. Notably, ROS-induced activation of RAS was first observed in human T cells where increased •NO levels increased RAS-GDP dissociation, allowing for subsequent GTP binding and activation [96]. The molecular target of nitric oxide-mediated RAS activation was later identified to be the highly conserved NKXD (asparagine-lysine-X-aspartate) nucleotide-binding motif of RAS superfamily where Cys^{118} is the variable X residue in HRAS, NRAS and KRAS [97,98]. Presumed to be mediated by S-nitrosylation of Cys^{118}, the guanine nucleotide exchange (GNE) of RAS is facilitated via the formation of RAS-thiyl intermediate (RAS-S•) during the process of S-nitrosylation of the thiol group of Cys^{118} by nitrogen dioxide ($•NO_2$), a reaction product of •NO and O_2, rather than the S-nitrosylated end-product [99–101]. Similarly, $O_2^{•-}$ and H_2O_2 in the presence of transition metals such as copper (Cu^{2+}) and iron (Fe^{2+}), and peroxynitrite ($ONOO^-$) have also been reported to facilitate RAS-GDP dissociation and GTP exchange [102,103]. RAS-S• intermediate may also be formed during S-glutathiolation of RAS Cys^{118} residue, stimulating RAS activation through GNE [104]. However, this modification prevents Cys^{118} from engaging in further radical-based reactions [104]. Site-directed mutagenesis of Cys^{118} to serine impedes carcinogen-induced lung carcinogenesis in mice. Interestingly, duo-site mutated $KRAS^{C118S,\ G13D}$ displayed decreased sensitivity towards epidermal growth factor (EGF) stimulation compared to single-site mutated $KRAS^{G13D}$, suggesting synergism between ROS and oncogenic RAS mutations in promoting carcinogenesis. Taken together, these studies provide evidence of ROS-dependent activation of RAS signaling in promoting carcinogenesis.

6. RAS: A master redox-rheostat in cell fate determination

6.1. Pro-oxidant activity of RAS

Besides the amenability of RAS structure/activity towards oxidative modifications, RAS activation is also a strong inducer of cellular ROS production associated with tumor-promoting effects of RAS. In that regard, many critical intracellular pro-oncogenic networks that are modulated by mutant RAS signaling such as autophagy, NF-κB and MAPK activation, PI3K/AKT/mTOR and hypoxia signaling appear to be highly intertwined with cellular redox metabolism. Mutant KRAS was shown to promote oxidative stress in pancreatic cancer by induction of miR-155 through NF-κB and MAPK pathway, resulting in downregulation of transcription factor, FOXO3A, which transcribes for antioxidant genes, SOD2 and catalase [105]. Besides transcriptional downregulation of ROS-scavenging enzymes, RAS has also been shown to induce mitochondrial $O_2^{•-}$ production from complex I and III of the ETC required for activation of proliferative and survival signaling, although a consensus has not been reached as to whether this is due to an increase in mitochondrial dysfunction or mitochondrial metabolism [106–108]. The conflicting reports may be due to differences in cell models used [106–108]. It is also worth mentioning that AKT, a downstream effector of RAS, can further increase oxidative stress by increasing respiratory capacity and/or suppressing FOXO3A activity [109–111], conceivably resulting in a ROS amplification loop to favor a pro-oxidant milieu for carcinogenesis. Supporting the role of ROS in promoting cancer cell proliferation, null-mutation of Prx1 greatly enhanced proliferation of non-small cell lung cancer (NSCLC) via ROS-dependent stimulation of ERK/CyclinD1 pathway in KRAS-driven lung carcinogenesis, suggesting a tumor suppressor role of the antioxidant activity of Prx1 [112]. Similarly, oncogenic RAS was observed to inhibit transcription of sestrin family genes *SESN1* and *SESN3*, resulting in increased intracellular ROS levels [91].

Perhaps the most convincing evidence linking ROS to RAS-induced transformation and cancer progression is its ability to directly induce $O_2^{•-}$ production via the intermediary activation of Rac1 GTPase [113], a critical component of NOX complexes [25,114]. $O_2^{•-}$ production via Rac1 has been shown to be an important mediator of mitogenic signaling and is required for malignant transformation and cancer progression in various RAS-driven cancer models [108,115–119]. Moreover, RAS activation in colorectal carcinoma strongly correlates with NOX1 expression and its expression has been linked to activation of MAPK [30,89,120]. Apart from NOX, our group has demonstrated that Rac1 localized to the mitochondria where it interacts with Bcl-2 and increases mitochondrial $O_2^{•-}$ production, promoting survival [121].

Besides the aforementioned studies demonstrating RAS as an inducer of ROS production which subsequently promotes mitogenic and survival signaling, there exists a preponderance of evidence attesting ROS production as an integral component of RAS-induced transformation. In pancreatic cancer, where frequency of KRAS mutations are up to 90%, constitutive RAS activation was shown to induce cellular ROS production via NOX4 to promote cancer progression in a Rac1-dependent manner [122]. RAS-induced ROS production via NOX4 also promotes accumulation of DNA damage which may result in the acquisition of additional oncogenic hallmarks or oncogene-induced senescence if DNA-damage response is activated [122,123]. Previously thought to be a tumor suppressive mechanism to arrest cells at risk of malignant transformation, mounting evidence suggests that senescent cells can evoke pro-tumorigenic changes in tumor microenvironment through acquisition of senescence-associated secretory phenotype (SASP) (reviewed by Coppé) [124].

Although oncogenic RAS promotes carcinogenesis through incessant proliferative and survival signaling, it has become clear over the years that oncogenic RAS also requires other mechanisms to sustain tumor progression. One of these critical mechanisms tied to RAS-driven transformation and redox-signaling is autophagy, a "self-eating" mechanism through which organelles, part of cytoplasm or proteins are digested to sustain metabolic needs or prevent accumulation of damaged/unwanted proteins and organelles [87,125–128]. Indeed, autophagy manifests in KRAS-driven malignant transformation of MCF10A breast epithelial cells through ROS-dependent activation of stress kinase, JNK, inducing upregulation of critical autophagy-related genes Atg5 and Atg7. Suppression of autophagy via genetic knockdown of autophagy-related (*Atg*) genes and ROS scavenging in this model, attenuated malignant transformation [126]. In agreement with this study, our group has shown that a small molecule compound, 1,3-dibutyl-2-thiooxo-imidazolidine-4,5-dione (C1), was able to trigger beclin-1 independent autophagy in various cancer cell lines and primary cells from lymphoma patients through ROS-dependent activation of MAPK, ERK and JNK [12]. It has also been reported that autophagy is an important mechanism for sustaining glycolysis during RAS-driven tumorigenesis and deficiencies in autophagy resulted in lower glycolytic flux and reduction in cell proliferation, indicating that autophagy

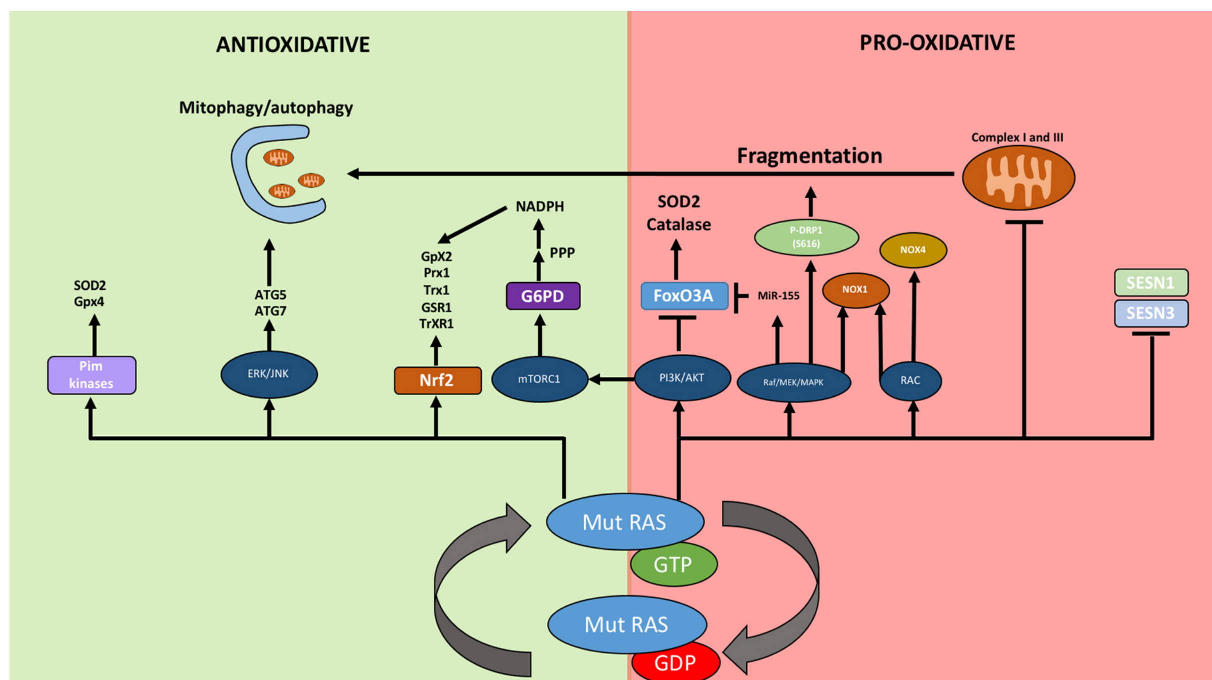

Fig. 3. Duality of RAS in redox-regulation. Mutant RAS has both oxidative and antioxidative functions to maintain ROS levels below lethal threshold. Mutant RAS drives production of ROS to promote cell proliferation and survival. Mutant RAS, through its downstream effectors have been shown to increase ROS production by stimulating NOX complex through Rac and also disrupting complex I and III activity of the mitochondrial. Mutant RAS also downregulates antioxidant expression through inhibition of FoxO3A transcription factor. Mutant RAS maintains sublethal levels of ROS by activating Nrf2 transcription of antioxidant genes. Mutant RAS also induces autophagy to remove dysfunctional mitochondria that contributes to ROS production. Also, mTORC1, a downstream effector of RAS/PI3K/AKT signaling has also been shown to upregulate G6PD expression required for synthesis of NADPH, an important cofactor in the reduction of redox-cycling enzymes such as GSH and Trx.

may be required to maintain ATP homeostasis during nutrient deprivation [128]. Interestingly, suppression of autophagy in RAS-driven NSCLC with p53 loss disrupts lipid metabolism and changes the fate of adenoma and carcinoma to benign oncocytoma [129]. Collectively, these studies testify the importance of autophagy in supporting metabolic reprograming during RAS-driven transformation and cancer progression, and changes in cellular-redox status mediated by oncoprotein RAS is an important mediator of these changes.

6.2. Anti-oxidant activity of RAS

While RAS promotes cellular transformation and tumor progression through induction of ROS, persistent ROS production as a result of RAS signaling can trigger growth arrest or cell death mechanisms. To overcome the deleterious effects of ROS, RAS must rely on failsafe mechanisms to neutralize and/or reduce ROS production. Indeed, a number of studies have demonstrated that RAS-driven transformation is associated with upregulation of antioxidant proteins to neutralize ROS production. For instance, upregulation of proteins involved in redox metabolism such as Prx3,4 and selenophosphate synthetase, a rate-limiting enzyme for synthesis of ROS scavenging selenoproteins such as GPx and TrxR, has been observed during RAS-driven transformation of ovarian epithelial cells [130]. More importantly, KRAS has been shown to directly enhance expression of master antioxidant transcription Nrf2, that positively regulates expression of critical ROS scavenging enzymes [131,132]. The increase in antioxidative capacity is also crucial to mediate RAS-driven transformation and malignant progression. Notably, Nrf2 has been shown to confer increased survival and tumor growth in later stages of malignancy and also chemoresistance in RAS-driven lung cancer [131,133]. In addition, inhibition of Nrf2 ubiquitination and degradation via deletion of Sag promotes KRAS-driven skin papillomagenesis [134]. Numerous reports also observed increased Nrf2 activity in diverse cancer types, lending credence to its importance in carcinogenesis [135–137]. Apart from compromising major cellular antioxidant systems, another study showed that loss of Prx1 in $HRAS^{V12}$-driven hepatocellular carcinoma was sufficient to induce apoptosis as a result of decreased ERK activation and increased ROS-induced DNA damage [138]. In another study, deletion of Pim kinase was shown to reduce SOD2 and GPx4 expression in $KRAS^{G12V}$-transformed MEFs and sensitize towards RAS-induced ROS-dependent cell death [139].

Nevertheless, as mentioned, a key to maintaining antioxidant function of ROS scavenging enzymes is the dependence on NADPH, which is mainly produced by the Pentose Phosphate Pathway (PPP) and the key rate-limiting enzyme for this anabolic pathway is glucose-6-phosphate dehydrogenase (G6PD). Alluding to its role in NADPH synthesis, G6PD has an important role in protecting cells against oxidative stress via NADPH-mediated restoration of GSH activity [140,141]. Interestingly, mammalian Target of Rapamycin complex 1 (mTORC1), a downstream effector of RAS/PI3K/AKT pathway, was shown to directly induce expression of G6PD, that is blocked by treatment with the mTOR inhibitor, rapamycin [142]. The direct connection between RAS signaling and PPP showcases an important antioxidant property of RAS, potentially during carcinogenesis.

Another feature in RAS-driven transformation is the accumulation of dysfunctional mitochondria which is a major source of ROS production [107,143]. In this regard, removal of dysfunctional mitochondria through autophagy (mitophagy) could be an important mechanism for ameliorating oxidative stress. Supporting this concept, mitophagy was shown to be an important mechanism for maintaining efficient oxidative metabolism in KRAS-driven lung cancer through maintenance of healthy pool of mitochondria, and genetic knockdown of *Atg5* and *Atg7* was shown to decrease cell survival associated with increased number of defective mitochondria and decreased respiration [87]. Corroborating these studies, many RAS-driven cancers often exhibit high basal levels of autophagy, demonstrating the importance of an

intact autophagic mechanism during RAS-driven carcinogenesis [129,144,145]. Interestingly, oncogenic KRAS signaling also promotes mitochondrial fragmentation, a pre-requisite to mitophagy initiation, through ERK2-mediated phosphorylation of dynamin-related protein 1 (DRP1) at serine 616 and deletion of DRP1 has been associated with impaired proliferative capacity of KRAS-driven tumors [146,147]. It is plausible that KRAS-induced mitochondrial morphology changes could also play an important role in facilitating the removal of damaged mitochondria and prevent excessive accumulation of ROS during transformation. Hence, the involvement of mitochondrial dynamics in RAS-driven carcinogenesis and its interplay with ROS warrants further investigation.

Taken together, these studies demonstrate strongly the duality of *RAS* oncogene in redox regulation through concurrent oncogenic stimulation of ROS production and increasing antioxidant capacity to ensure that ROS levels are kept within pro-carcinogenic levels while avoiding the lethal threshold (Figs. 2 and 3). However, in light of the conflicting reports, it would be interesting to investigate the circumstances and cellular context wherein RAS adopts a pro or anti-oxidant role. Understanding the plasticity of RAS in redox-regulation will help us develop more effective therapeutic strategies against RAS-driven tumors.

6.3. Redox-based strategies against RAS-driven cancers

Given the incidence of *RAS* mutations in cancer and its involvement in mediating acquisition of oncogenic hallmarks, it is unsurprising that direct inhibition of RAS is an important and attractive therapeutic strategy against RAS-driven tumors. However, a therapeutic grip on RAS oncogene remains challenging. Nevertheless, there has been some success in understanding the structure of RAS protein and how it can be drugged [148–150]. However, the therapeutic efficacy remains to be established. As a result, the bulk of research efforts are aimed at designing therapeutic strategies targeting upstream and/or downstream effectors of RAS signaling [151–154]. Despite the initial successes of these therapies, their durability in the clinic is ultimately compromised by multiple cross-talk, negative feedback and redundancies that exists within the complex RAS signaling network, leaving an open area for development of novel therapies [155–159].

Based on the studies presented, the apparent reliance of RAS-driven tumors on ROS to sustain its tumorigenic needs presents itself as a targetable vulnerability to be exploited for therapeutic management. One possible strategy to target RAS-driven tumors will be to reduce its pro-proliferative and survival capacity through reducing intracellular ROS levels (Fig. 2). In support of this concept, one study showed that treatment of KRAS-driven pancreatic cancer cells with antioxidant, vitamin E evokes an apoptotic response through transcription factor EGR-1-dependent upregulation of pro-apoptotic *BAX* [160]. However, whether or not ROS downregulation was involved in *BAX* upregulation was not reported in the study. A more direct strategy proving the viability of this strategy was demonstrated in another study where inhibition of NOX4 by flavoprotein inhibitor, diphenyleneiodonium (DPI), inhibited $O_2^{\cdot-}$ production and triggered apoptosis in KRAS-driven PANC-1 pancreatic cancer cells [161]. More specifically, reduction of $O_2^{\cdot-}$ downregulated phosphorylation/activation of AKT and relieved its inactivating activity on apoptosis signal-regulating kinase 1 (ASK1), triggering cell death [161]. Similarly, anti-diabetic drug, metformin, was also shown to selectively target KRAS-driven pancreatic cancer cells, PANC-1 and Mia PaCa, by downregulating NOX2 and NOX4 levels while upregulating SOD2 levels, resulting in overall decreased cell survival associated with decreased intracellular ROS levels [28].

The reliance of RAS-driven tumors on increased intracellular ROS production, by the same token, also makes them inherently vulnerable to further increases in oxidative stress. Therefore, a viable strategy to target RAS-driven cancers by inducing massive ROS production to reach lethal levels in these cells [162,163] (Fig. 2). To that end, Shaw et al. has screened over 50,000 compounds and discovered a class of compounds which selectively targets KRAS mutant cells [164]. Among which, the most potent member, lanperisone, induces ferrotopsis through an increase in intracellular ROS levels, that was dependent on MAPK activation [164]. In another chemical library screen aimed at identifying new compounds targeting EGFR and KRAS-driven lung cancer, identified novel compound LCS-1 showed promising activity against KRAS and EGFR-driven lung cancer cell lines through decreases in PI3K/AKT and MAPK signaling [165]. Interestingly, a follow-up study identified SOD1 as a likely target of LCS-1 and silencing of SOD1 conferred increased sensitivity of lung cancer cells towards LCS-1, possibly owing to further increases in ROS levels due to additive SOD1 loss [166].

Another dietary supplement, ascorbate (vitamin C), was also shown to selectively target colorectal cancer cells expressing BRAF or KRAS mutations in which GLUT1 expression is increased [167]. Interestingly, the observed effect involved the uptake of dehydroascorbate (DHA), oxidized vitamin C, via glucose transporter GLUT1, which subsequently exhaust antioxidant GSH levels via its own reduction to ascorbate [167]. Due to depletion of GSH levels, intracellular ROS increases and subsequently inactivates glyceraldehyde-3-phosphate dehydrogenase (GAPDH)-in KRAS or BRAF-driven glycolytic addicted cells, resulting in energetic crisis and cell death [167]. Consistent with this finding, an earlier study also demonstrated the anti-tumor effects of ascorbate in KRAS-driven pancreatic cancer cells was due to an increase in ROS production [168]. Interestingly, overexpression of GLUT1 transporter in wild-type cells alone did not increase its susceptibility towards ascorbate treatment, suggesting that the toxic effects of ascorbate towards mutant KRAS-expressing cells requires coupling to additional factors unique to KRAS-driven tumors. One metabolic liability that may render KRAS-mutant expressing cells susceptible towards ascorbate is its reliance on ROS to promote cell proliferation and tumorigenesis [106]. Thus, the toxic effect of ascorbate is likely due to the synthetic lethal combination created by high rates of ROS production and increased GLUT1 expression in mutant KRAS-expressing cells.

Along similar lines, we recently unraveled a novel mechanism of drug-induced mutant KRAS-mediated increase in oxidative stress with potential therapeutic implications in RAS-driven cancers. The small molecule (C1) triggered hyperactivation of KRAS in cells expressing mutant KRAS resulting in AKT-dependent ROS production and execution of cell death [76]. In addition, cell death induced by C1 displayed features of apoptosis and autophagy mediated by ROS-dependent activation of ERK and JNK [12]. Remarkably, similar increase in RAS signaling as a result of mutant and wild-type KRAS heterodimer disruption also increased sensitivity of lung cancer cells towards MEK inhibition [169]. It would be interesting to investigate if this increase in sensitivity is also due to the pro-oxidant effects of RAS signaling which could open up new avenues for redox-based therapies in treating RAS-driven cancers. Taken together, these studies provide a proof- of-concept for developing ROS-inducing strategies in targeting RAS-driven cancers.

Despite the promising laboratory findings for redox-based approach towards treating oncogenic KRAS-driven cancers, clinical trials involving use of redox-active compounds are met with limited success. One limitation of the use of redox-active dietary supplements such as vitamins in treatment of cancer is its oral bioavailability. Early double-blind placebo-controlled clinical studies of vitamin C in patients with advanced cancer showed no significant therapeutic benefits when administered orally at 10 g per day [170,171]. Another study showed that despite high dosage of vitamin C, plasma concentration rarely exceeds 200 μM when administered orally due to limited absorption and drug clearance. As a result, the maximum dose achievable through oral administration is well below the effective dose used in in vitro studies [167]. By contrast, plasma concentration of vitamin C can reach as high as 15 mM when administered intravenously [172]. A recent pilot phase 1/2a clinical studies using combination therapy of intravenous vitamin C with chemotherapeutics carboplatin and paclitaxel in patients with

Table 1
Novel RAS-targeting approaches using redox-altering compounds.

DRUG	MECHANISM	CANCER TYPE	REFERENCE
Vitamin E (Antioxidant)	EGR-1-dependent upregulation of BAX	Pancreatic cancer	[160]
DPI	Reduces AKT-dependent inhibition of ASK1 by downregulating $O_2^{\cdot-}$	Pancreatic cancer	[161]
Metformin	Downregulates NOX2 and 4 levels, Upregulates SOD2 levels	Pancreatic cancer	[28]
Lanperisone	Iron and MAPK-dependent ROS signaling; ferroptopsis	$KRAS^{G12D}$-transformed MEFs and mouse sarcoma cells	[164]
LCS-1	SOD1 inhibitor	Lung adenocarcinoma	[165,166]
Vitamic C	Depletion of intracellular GSH leading to oxidation of GAPDH, resulting in energetic crisis	KRAS and BRAF-driven colorectal cancer cells	[167]
C1	Hyperactivation of KRAS leading to AKT-dependent ROS production leading to cell death	Colorectal cancer, pancreatic cancer and lung cancer	[12,76]

ovarian cancer showed an improved overall survival and median time of 8.75 months longer disease progression/relapse in vitamin C/carboplatin/paclitaxel-treated patients compared to patients receiving only standard carboplatin/paclitaxel therapy [173]. However, the difference between two groups of patients was not statically significant and the efficacy of vitamin C in cancer therapy still warrants further clinical investigation. Intravenous administration of vitamin E in combination with chemotherapeutics was also studied clinically but no therapeutic benefits has been observed [174]. Another limitation and drawback of vitamin C and E in cancer therapy may also be attributed to its short biological half-life [175,176]. Hence, use of vitamin in cancer therapy would be impractical as patients would need to be dosed with high concentrations of vitamins very frequently. Moreover, several studies revealed pro-oxidant activity in vitamins, contrary to their presumed antioxidative roles [167,177]. Thus, it would be important to understand which role would vitamins adopt in vivo and at the tumor site.

As mentioned, NOX enzymes are also potential targets for ROS-modulating strategies and inhibition of NOX enzymes has proven effective against several cancer cell lines [28,119]. Among which, NOX1 and NOX4 has been implicated in RAS-driven carcinogenesis [30,119,123,161]. NOX1 is abundantly expressed in colon epithelial cells and appears to play an important role in mucosal wound repair after inflammation and loss of NOX1 function has been associated with inflammatory bowel disease [178–180]. Expression of NOX4, on the other hand, is far more ubiquitous than NOX1, and its diverse function has recently been reviewed in [181]. Given the importance NOX enzymes in physiological functions, inhibition of NOX during cancer therapy in patients may have severe implications and calls for cautious optimism.

Lastly, given the complexity of RAS oncogene in redox-regulation, it is conceivable that RAS-driven tumors may exhibit redox-heterogeneity across different cell types and stages of carcinogenesis, warranting for personalized redox-profiling of tumors in any given patient to identify potential hormetic thresholds. Only then, we would be able to effectively target RAS-driven tumors through manipulation of ROS levels using drug or combinational therapy (Table 1).

7. Concluding remarks

The treatment of RAS-driven cancers has been therapeutically challenging due to the complexity and diversity of RAS signaling readouts. However, an interesting aspect with potential therapeutic implications is the intricate interplay between RAS networks and cellular redox status. Observations supporting bi-directional signaling (RAS-ROS-RAS) argue in favor of an amplification loop that on the one hand provides a permissive environment for transformation, survival and carcinogenesis, yet on the other hand presents as a vulnerability worth exploiting for the therapeutic management of RAS driven malignancies. These observations support a *Redox Rheostat* function for RAS. It is worth noting that the contribution of ROS towards cell fate may be governed by cell-specificity and stages of carcinogenesis, and therefore for redox-based therapies to be effective, the functional biology of RAS from the standpoint of redox status in specific cancer cell types and stages have to be clearly established. Furthermore, is there a propensity for RAS to switch from an anti-oxidant role to a pro-oxidant role in response to extrinsic or intrinsic stimuli? If so, what mechanisms confer the plasticity of RAS in redox-regulation? Understanding these phenomena would be critical for the design and development of novel redox-based approaches for the efficient execution of RAS driven cancers.

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