Modulation of oxidative stress as an anticancer strategy

Chiara Gorrini[1], Isaac S. Harris[1,2*] and Tak W. Mak[1]*

Abstract | The regulation of oxidative stress is an important factor in both tumour development and responses to anticancer therapies. Many signalling pathways that are linked to tumorigenesis can also regulate the metabolism of reactive oxygen species (ROS) through direct or indirect mechanisms. High ROS levels are generally detrimental to cells, and the redox status of cancer cells usually differs from that of normal cells. Because of metabolic and signalling aberrations, cancer cells exhibit elevated ROS levels. The observation that this is balanced by an increased antioxidant capacity suggests that high ROS levels may constitute a barrier to tumorigenesis. However, ROS can also promote tumour formation by inducing DNA mutations and pro-oncogenic signalling pathways. These contradictory effects have important implications for potential anticancer strategies that aim to modulate levels of ROS. In this Review, we address the controversial role of ROS in tumour development and in responses to anticancer therapies, and elaborate on the idea that targeting the antioxidant capacity of tumour cells can have a positive therapeutic impact.

The regulation of redox homeostasis is fundamental to maintaining normal cellular functions and ensuring cell survival. Cancer cells are characterized by increased aerobic glycolysis (termed the Warburg effect) and high levels of oxidative stress[1]. This oxidative stress is exerted by reactive oxygen species (ROS) that accumulate as a result of an imbalance between ROS generation and elimination. The high ROS levels in cancer cells are a consequence of alterations in several signalling pathways that affect cellular metabolism. These ROS levels are counteracted by elevated antioxidant defence mechanisms in cancer cells[2].

For many years, researchers have theorized that cancer cells depend on the activation of an oncogene or the inactivation of a tumour suppressor gene for their survival — a hypothesis known as 'oncogene addiction'[3]. Based on the idea that oncogenes and tumour suppressor genes are a critical force in the malignant transformation of cells, pharmaceutical companies have focused on developing drugs that target these genes. However, recent studies have shed light on the vital mechanisms that ensure the survival of cancer cells, including the ability to escape from immune surveillance as well as the ability to cope with aneuploidy and to undergo metabolic adaptations that provide cancer cells with a secure energy supply and form a defence mechanism against various cellular stresses[4]. Thus, targeting the 'cart' (immune surveillance, aneuploidy and metabolism) rather than the 'horse' (oncogenes and tumour suppressor genes) may be a promising strategy for eliminating cancer cells while sparing normal cells.

In the context of cell metabolism, it is now apparent that cancer cells adapt to the imbalanced redox status created by their rapid growth and other conditions, such as oxygen and limited availability of nutrients, by developing alternative metabolic reactions that render them insensitive to further stress inducers such as chemotherapy and radiation[5]. Here, we discuss the cellular sensors and modulators of oxidative stress, and consider how a deep understanding of their function can inform the development of a new therapeutic strategy against cancer.

Defining the basis of oxidative stress

ROS are broadly defined as oxygen-containing chemical species with reactive properties. These include the superoxide ($O_2^{\bullet -}$) and hydroxyl ($HO^{\bullet}$) free radicals as well as non-radical molecules such hydrogen peroxide (H_2O_2). These molecules are principally derived from the oxygen that is consumed in various metabolic reactions occurring mainly in the mitochondria, peroxisomes and the endoplasmic reticulum (ER). It is estimated that about

[1]The Campbell Family Institute for Breast Cancer Research, University Health Network, 620 University Avenue, Toronto, Ontario M5G 2C1, Canada.
[2]Present address: Harvard Medical School, 240 Longwood Ave, Boston, Massachusetts 02115, USA.
*These authors contributed equally to this work.
Correspondence to T.W.M.
e-mail: tmak@uhnresearch.ca
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2% of the oxygen consumed by mitochondria is reduced to form superoxide; mitochondria are therefore considered to be a major source of ROS[6,7]. Peroxisomes are involved in both the scavenging of ROS (through catalase-mediated decomposition of H_2O_2) and in the production of ROS (through β-oxidation of fatty acids and flavin oxidase activity)[8]. The ER constitutes an oxidizing environment that favours disulphide bond formation and protein folding, and increases ROS levels through protein oxidation[9].

ROS are constantly produced by both enzymatic and non-enzymatic reactions. Enzyme-catalysed reactions that generate ROS include those involving NADPH oxidase, xanthine oxidase, uncoupled endothelial nitric oxide synthase (eNOS), arachidonic acid and metabolic enzymes such as the cytochrome P450 enzymes, lipoxygenase and cyclooxygenase. The mitochondrial respiratory chain is a non-enzymatic source of ROS.

The modulation of intracellular ROS levels is crucial for cellular homeostasis, as different ROS levels can induce different biological responses[1,10]. At low to moderate levels, ROS act as signalling molecules that sustain cellular proliferation and differentiation, and activate stress-responsive survival pathways[11]. For example, H_2O_2 can serve as a signal for proliferation, differentiation and migration[12]. ROS can also act as signal transduction molecules that induce pro-inflammatory cytokines[13] and the nuclear factor-κB (NF-κB) pathway[14]. However, excessive ROS production damages cellular components such as DNA, proteins and lipids. Tight regulation of both ROS-producing (inducer) pathways and ROS-detoxifying (scavenger) pathways is thus required (BOX 1, FIG. 1).

Role of ROS in cancer cells

A link between ROS and cellular transformation was first identified in 1981, when it was found that insulin elevated intracellular H_2O_2 levels and increased tumour cell proliferation[15]. More than three decades later, the role of ROS in cancer remains controversial. Cancer cells have a high demand for ATP because it provides the 'fuel' for aberrant proliferation. However, the dark side of this uncontrolled energy production is the accumulation of ROS, which needs to be counteracted by scavenging mechanisms to ensure cell survival. Numerous studies have evaluated ROS levels and production under various circumstances with the goal of delineating when ROS are oncogenic and when they are tumour suppressive[16].

At low to moderate levels, ROS may contribute to tumour formation either by acting as signalling molecules or by promoting the mutation of genomic DNA. For instance, ROS can stimulate the phosphorylation of mitogen-activated protein kinase (MAPK) and extracellular signal-regulated kinase (ERK), cyclin D1 expression and JUN N-terminal kinase (JNK) activation, all of which are linked to tumour cell growth and survival[17,18]. ROS have also been shown to reversibly inactivate tumour suppressors such as phosphatase and tensin homolog (PTEN) and protein tyrosine phosphatases (PTPs) owing to the presence of the redox-sensitive cysteine residues that reside in their catalytic centre[19,20]. Interestingly, PTPs can also regulate signalling events to increase antioxidant expression and reduce ROS levels[21].

ROS are also involved in normal stem cell renewal and differentiation[22]. Although cancer stem cells (also known as tumour-initiating cells; TICs) share similar phenotypes with normal stem cells, relatively little is known about their redox status. A recent study has shown that breast and liver cancer stem cells tend to have low ROS levels owing to the increased expression of ROS-scavenging systems[2,23]. If TIC expansion is crucial for the first stages of tumour formation, then maintaining low ROS levels in these cells may be crucial for the survival of pre-neoplastic foci. Thus, although treatments such as chemotherapy and radiotherapy — which induce the production of ROS — are useful for eliminating the bulk of cancer cells, such approaches may fail to cure the patient owing to the superior ability of TICs to survive in conditions of high ROS by upregulating levels of antioxidants. As ROS are critical mediators of the lethal effects of ionizing radiation and some anticancer drugs, TICs may be preferentially spared and actively selected by treatments that depend on high ROS levels. Moreover, the additional oxidative stress induced by these treatments may cause further DNA damage and mutations, leading to the development of drug-resistant tumour cells.

At high levels, ROS promote cell death and severe cellular damage. Cancer cells need to combat high levels of ROS, especially at early stages of tumour development. Recent research has revealed that conditions that induce oxidative stress (as described in BOX 1) also increase the selective pressure on pre-neoplastic cells to develop powerful antioxidant mechanisms[2]. High ROS levels are also induced by detachment from the cell matrix[24]. This aspect represents a challenge for metastatic cancer cells that need to survive during migration to distant organs.

Therefore, cancer cells characteristically have a high antioxidant capacity that regulates ROS to levels that are compatible with cellular biological functions but still higher than in normal cells. We believe that targeting these enhanced antioxidant defence mechanisms may represent a strategy that can specifically kill cancer cells, including TICs, while sparing normal cells.

ROS as sensors and modulators in cell signalling

There are numerous regulators, both positive and negative, that have a significant impact on the expression of antioxidant genes. Understanding the specific pathways that are affected by these controllers is essential before formulating therapeutic approaches towards the modulation of ROS levels. Below, we have listed the major regulators of antioxidant gene expression and the canonical antioxidant pathways that they direct.

NRF2. As noted in BOX 1, the transcription factor nuclear factor erythroid 2-related factor 2 (NRF2) is arguably the most important regulator of the expression of molecules that have antioxidant functions within the cell[25] (FIG. 2). Under resting conditions, NRF2 is constitutively degraded by the Kelch-like ECH-associated protein 1 (KEAP1)–Cullin 3 (CUL3) E3 ligase complex. Under

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Box 1 | ROS inducers and ROS scavengers

Under physiological conditions, redox homeostasis is achieved by the constant balancing of reactive oxygen species (ROS) generation with ROS elimination (FIG. 1).

ROS inducers

Hypoxia. Hypoxia arises from an imbalance between oxygen supply and consumption. Major causes of hypoxic stress include a reduced presence or structural abnormality of microvessels supplying nutrients in a tissue, increased distance between the tissue and its nutrition-supplying blood flow and a reduced oxygen-transporting capacity of the blood owing to anaemia. Hypoxia is known to stimulate the production of ROS by mitochondria[191], and these ROS in turn activate hypoxia-inducible transcription factor 1 (HIF1)[192]. HIF1 is a heterodimeric transcription factor that promotes angiogenesis, survival, glycolysis and tumour progression[193].

Metabolic defects. Because ROS are by-products of metabolic reactions, altered metabolism can be a source of oxidative stress. Cancer cells have a high metabolic activity and require high levels of ATP to sustain their uncontrolled proliferation and growth. These two aspects result in sustained mitochondrial respiration, which leads to excessive ROS accumulation[5].

ER stress. The correct folding of proteins in the endoplasmic reticulum (ER) is essential for cell survival and normal physiological functions. The ER constitutes an oxidizing site where nascent proteins are engaged by the folding machinery to achieve the correct conformation and post-translational modifications. Misfolded proteins can elicit ER stress and the unfolded protein response (UPR), which eventually results in ROS accumulation[194]. Mitochondrial ROS production can also be stimulated by ER stress-induced calcium release and depolarization of the mitochondrial inner membrane[195].

Oncogenes. Previous studies have reported that oncogene activation increases ROS levels in cancer cells. For example, ectopic expression of *MYC* has been shown to lead to the upregulation of the expression of mitochondrial genes and increased ROS production[196]. Similarly, ectopic expression of *RAS* and *KRAS* increases ROS levels through the regulation of NADPH oxidase[197]. However more recent data have shown that the physiological expression of hyperactivated *RAS* (RAS^{G12D}) and *MYC* lowers ROS levels by triggering an antioxidant response[75]. These data suggest that the endogenous expression of the *MYC* and *RAS* oncogenes downregulates ROS, which contrasts with earlier data[198,199] that were obtained in less physiological conditions.

ROS scavengers

NRF2. Normal cells counteract ROS by producing enzymes with antioxidant functions. Examples of these are phase II detoxification enzymes such as haem oxygenase (HMOX1), NAD(P)H:quinone oxidoreductase 1 (NQO1), glutathione *S*-transferases (GSTs) and UDP-glucuronosyltransferases (UGTs). The transcription factor nuclear factor erythroid 2-related factor 2 (NRF2) controls the expression of these enzymes and is considered to be a master regulator of intracellular antioxidant responses[200].

Glutathione. Glutathione (GSH) is the most abundant non-enzymatic antioxidant molecule in the cell and is essential for cell survival and redox homeostasis[201]. GSH synthesis is catalysed by glutamate–cysteine ligase (GCL) and GSH synthetase (GSS). Modification of GSH metabolism has been observed in many tumour types.

NADPH. Reduced NADPH is an important metabolite that is essential for many cellular processes. In particular, NADPH is required for the regeneration of GSH and thioredoxin (TXN), which have an important role in the elimination of H_2O_2 (REF. 202). Perturbed NADPH production leads to impaired cell division and mitochondrial membrane permeability, thereby increasing cell sensitivity to oxidative stress and inducing apoptosis[175].

Tumour suppressors. Tumour suppressors such as forkhead box O (FOXO) transcription factors, retinoblastoma-associated protein RB and breast cancer susceptibility 1 (BRCA1) have been implicated in the control of oxidative stress[203]. The role of the tumour suppressor p53 is more complex, as p53 has both pro-oxidant and antioxidant capacities[64].

Dietary antioxidant compounds. Dietary antioxidants such as vitamin C, vitamin E, selenium and β-carotene are non-enzymatic systems that, although less specific than the enzymatic ones, appear to be equally important in cellular responses to oxidative stress[204]. For example, vitamin C (ascorbic acid), which is mostly present in the cell in its redox form, ascorbate, acts as a reductant and enzyme cofactor[205]. Vitamin E is considered a 'chain-breaking' antioxidant; it acts as a ROS scavenger by reacting with free radicals and converting these into tocopheryl radicals, thus lowering their radical damaging abilities[206]. Selenium is a non-metal element that forms part of antioxidant selenoproteins such as glutathione peroxidase and thioredoxin reductase[207]. β-carotene is the most abundant carotenoid in human diet. Its antioxidant property derives from its ability to quench singlet oxygen and trap peroxyl radicals[208].

conditions of oxidative stress, KEAP1 is oxidized and modified so that it cannot bind to NRF2, thereby leading to the stabilization of NRF2 and its translocation to the nucleus[26]. NRF2 is the sole controller of the enzymes that are responsible for producing glutathione (GSH), which is the most abundant antioxidant cofactor within the cell[26,27]. NRF2 drives the expression of the two subunits that comprise the glutamate–cysteine ligase (GCL) complex — namely, the modifier subunit (GCLM) and the catalytic subunit (GCLC). GCL catalyses the reaction of glutamate with cysteine, which is the rate-limiting step in GSH synthesis. NRF2 also controls the abundance of cysteine within cells, which is the rate-limiting substrate of GSH synthesis (and the synthesis of numerous other molecules). This occurs through NRF2-mediated promotion of the expression of solute carrier family 7 member 11

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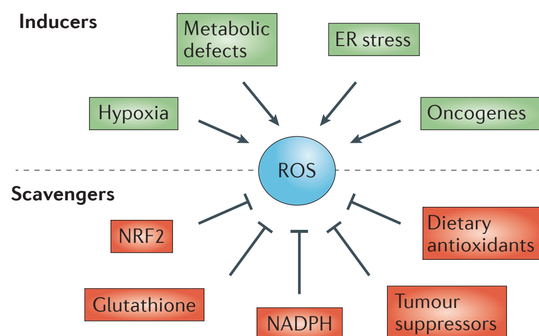

Figure 1 | **Determination of cellular redox status by a balance between levels of ROS inducers and ROS scavengers.** The production of reactive oxygen species (ROS) can be induced by hypoxia, metabolic defects, endoplasmic reticulum (ER) stress and oncogenes. Conversely, ROS are eliminated by the activation of the transcription factor nuclear factor erythroid 2-related factor 2 (NRF2), the production of glutathione and NADPH, the activity of tumour suppressors (such as breast cancer susceptibility 1 (BRCA1), p53, phosphatase and tensin homolog (PTEN) and ataxia telangiectasia mutated (ATM)) and the action of dietary antioxidants. ROS inducers and scavengers are described in detail in BOX 1.

(*SLC7A11*), which encodes the cystine/glutamate transporter XCT[28]. In exchange for glutamate, XCT imports cystine into the cell. The reduction of cystine to cysteine is catalysed by either GSH or thioredoxin reductase (TXNRD)[29]. Furthermore, cysteine can be found with selenium in the form of selenocysteine and incorporated into the active catalytic centres of antioxidant enzymes such as TXNRD and glutathione peroxidase (GPX)[30]. Finally, XCT has been implicated as a key enzyme supporting tumour development[31]. XCT is stabilized by CD44, a surface-expressed tumour-associated antigen that is also a marker for TICs[2,32].

NRF2 supports not only GSH synthesis but also GSH utilization, as it controls the expression of numerous ROS-detoxifying enzymes such as GPX2 and several glutathione *S*-transferases (GSTA1, GSTA2, GSTA3, GSTA5, GSTM1, GSTM2, GSTM3 and GSTP1)[33,34]. When GPXs or GSTs catalyse the reduction of ROS through GSH, the oxidized GSSG complex is formed. GSH is regenerated from GSSG through the activity of glutathione reductase (GSR) and the reducing agent NADPH. The production of NADPH is closely regulated by cell metabolism[1]. There are only three ways in which NADPH is generated within cells via glucose and glutamine metabolism: via the pentose phosphate pathway (PPP), which involves glucose-6-phosphate dehydrogenase (G6PD); through the conversion of pyruvate to malate by malic enzyme (ME) isoforms; and through the conversion of isocitrate to α-ketoglutarate by isocitrate dehydrogenase (IDH) isoforms. Intriguingly, mutations in IDH1 and IDH2 have been found to exist at a high frequency in glioma and acute myeloid leukaemia (AML)[35–39].

NRF2 is able to modulate glucose and glutamine metabolism by directly increasing the transcription of all NADPH-generating enzymes, which links NRF2 to NADPH production and subsequent GSH regeneration[40]. NADPH is also a reducing agent for the regeneration of thioredoxin via TXNRD[41]. Furthermore, peroxiredoxins utilize thioredoxin to reduce ROS levels. Thioredoxin 1, TXNRD1 and peroxiredoxin 1 are direct transcriptional targets of NRF2 (REFS 42,43).

In addition to its direct involvement in ROS detoxification via GSH metabolism, NRF2 indirectly helps to modulate ROS levels by regulating free Fe(II) homeostasis. Fe(II) catalyses the Fenton reaction, which describes the conversion of H_2O_2 to the highly reactive $OH^\bullet$ radical[44]. A major source of free Fe(II) is via the breakdown of haem molecules by haem oxygenase (HMOX1)[45], and NRF2 stabilization leads to the upregulation of HMOX1 transcription[46]. At first glance, upregulation of HMOX1 by NRF2 would seem to be counterintuitive, as uncontrolled release of Fe(II) into a cell would promote the Fenton reaction. However, in conjunction with HMOX1 upregulation, NRF2 boosts the transcription of genes encoding ferritin light chain (FTL) and ferritin heavy chain (FTH), which are components of the ferritin complex[42]. The ferritin complex first detoxifies Fe(II) by converting it into Fe(III) and then stores it within its own structure where it cannot be utilized for the Fenton reaction[47]. Interestingly, high serum levels of ferritin correlate with poor prognosis in multiple cancers[48]. Thus, NRF2 reduces the production of harmful $OH^\bullet$ radicals from ROS by promoting the release of Fe(II) from haem molecules and its subsequent sequestration.

FOXO and p53. Although primarily known as inducers of cell cycle arrest and cell death, the forkhead box O (FOXO) family of transcription factors and the tumour suppressor p53 have a major role in preventing oxidative stress by inducing antioxidant gene expression[1].

The activation of FOXO transcription factors is controlled by their phosphorylation, which is mediated by AKT and by serum/glucocorticoid-regulated kinase (SGK)[49–51]. Phosphorylation of FOXO transcription factors results in their binding to the 14-3-3 protein (also known as YWHAQ), which triggers nuclear exclusion and cytoplasmic sequestration[52]. The response of FOXO transcription factors to oxidative stress is controversial, as reports have shown that they can be either activated (by JNK and macrophage stimulating protein 1 (MST1; also known as HGFL)) or inhibited (by AKT following PTEN inactivation) after ROS levels are increased[53–55].

Although FOXO transcription factors have roles in the regulation of GSH-mediated detoxification by inducing the transcription of GPX1 and GSTM1 (REFS 56,57), in Fe(II) homeostasis by inducing the transcription of HMOX1 (REF. 58) and in the regulation of mitochondrial function via PTEN-induced putative kinase (PINK1)[59,60], one could argue that FOXO transcription factors primarily exert their antioxidant effects through the regulation of superoxide dismutases (SODs), catalase and sestrin 3 (a cysteine sulphinic acid reductase)[61]. Similar to HMOX1, the fact that SODs catalyse the generation of H_2O_2 from $O_2^{\bullet-}$ appears to counteract antioxidant detoxification. Yet it is only in this form that the reactive $O_2^{\bullet-}$ can then be reduced to H_2O by catalase. SODs and catalase are transcriptionally regulated by FOXO

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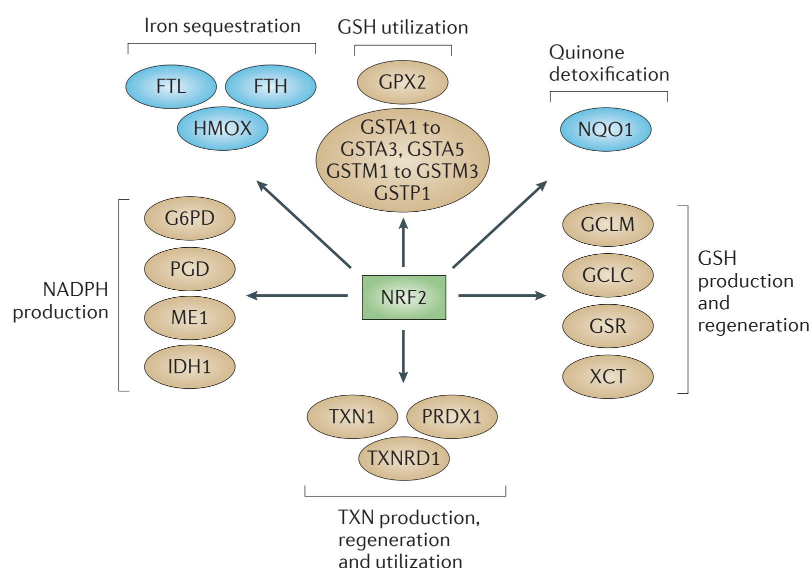

Figure 2 | **NRF2 as the master regulator of antioxidant responses.** Nuclear factor erythroid 2-related factor 2 (NRF2) controls several different antioxidants pathways. The first is glutathione (GSH) production and regeneration, which is regulated by the following antioxidants: the glutamate–cysteine ligase complex modifier subunit (GCLM), the GCL catalytic subunit (GCLC), the cystine/glutamate transporter XCT and glutathione reductase (GSR). The second is GSH utilization, which is regulated by the glutathione *S*-transferases (GSTA1, GSTA2, GSTA3, GSTA5, GSTM1, GSTM2, GSTM3 and GSTP1) and glutathione peroxidase 2 (GPX2). The third is thioredoxin (TXN) production, regeneration and ultilization, which is regulated by TXN1, thioredoxin reductase 1 (TXNRD1) and peroxiredoxin 1 (PRDX1). The fourth is NADPH production, which is controlled by glucose-6-phosphate dehydrogenase (G6PD), phosphoglycerate dehydrogenase (PHGDH), malic enzyme 1 (ME1) and isocitrate dehydrogenase 1 (IDH1). Both GSH and TXN utilize NADPH to regenerate themselves once they have reduced reactive oxygen species (ROS). These four groups of antioxidant genes — which are all upregulated by NRF2 — have both complementary and overlapping functions. Additional antioxidants that are controlled by NRF2 include NAD(P)H:quinone oxidoreductase 1 (NQO1) and enzymes regulating iron sequestration, such as haem oxygenase (HMOX1), ferritin heavy chain (FTH) and ferritin light chain (FTL). Notably, several NRF2 target genes have not been included in this figure because they do not pertain to antioxidant functions.

transcription factors, whereas the expression of ferritins and HMOX1 is regulated by NRF2 (REFS 55,62). Sestrin 3 (but not sestrin 1 or sestrin 2) is another important antioxidant target of FOXO transcription factors[61]. Although controversial, it has been shown that members of the sestrin family can reduce peroxiredoxins that have become overoxidized and thereby rendered inactive, thus regenerating their catalytic activity[63].

The final transcription factor that has a major role in controlling antioxidant gene expression is p53 (REF. 64). p53 has a controversial role in ROS regulation as it can promote both pro- and antioxidant responses[64] (the pro-oxidant role of p53, which is mediated mainly through cell death pathways, has been comprehensively reviewed in REF. 22). An important antioxidant target of p53 is TP53-induced glycolysis and apoptosis regulator (TIGAR)[65]. TIGAR encodes a protein that is similar to the glycolytic enzyme fructose-2,6-bisphosphatase, which degrades fructose-2,6-bisphosphate. A decrease in fructose-2,6-bisphosphate levels inhibits the activity of the rate-limiting enzyme phosphofructokinase 1 (PFK1), thereby blocking glycolysis and promoting the shuttling of metabolites to the PPP. By upregulating TIGAR, p53 amplifies PPP-mediated NADPH production. Another important antioxidant target of p53 is glutaminase 2 (GLS2)[66]. It is somewhat surprising that a tumour suppressor controls the transcription of GLS2, as the oncogene *MYC* controls GLS1 expression[67]. Tight control of GLS expression is essential to GSH synthesis, as GLS converts glutamine to glutamate, and glutamate is subsequently converted to GSH via GCLC and GCLM. Finally, like FOXO transcription factors, p53 can influence sestrin expression[63]. By regulating sestrin 1 and sestrin 2, p53 promotes the activity of peroxiredoxins.

FOXO transcription factors and p53 have complementary functions in the antioxidant response, in that p53 controls sestrin1 and sestrin 2, whereas FOXO transcription factors control sestrin 3. Similarly, p53 promotes GSH production through GLS2, whereas FOXO transcription factors promote GSH-mediated detoxification via GPX1 and GSTM1. Together, FOXO and p53 control both distinct and overlapping antioxidant genes that are not regulated by NRF2 (FIG. 3). For example, NRF2 promotes NADPH- and GSH-mediated detoxification, whereas FOXO transcription factors control SODs and catalase. Similarly, NRF2 controls thioredoxin-mediated peroxide detoxification and disulphide reduction (via thioredoxin 1, TXNRD1 and peroxiredoxin 1), whereas p53 and FOXO transcription factors control the expression of the cysteine sulphinic acid reductase family members sestrin 1, sestrin 2 and sestrin 3, which have a role in regenerating peroxiredoxins and increasing the activity of the thioredoxin antioxidant pathway. In addition, both FOXO transcription factors and p53 upregulate cyclin-dependent kinase inhibitor 1A (CDKN1A; also known as p21 or WAF1), which promotes NRF2 stabilization[68,69]. NRF2, FOXO and p53 also promote HMOX1 expression[46,58,70]. These transcription factors all control free Fe(II) sequestration via HMOX1 expression, which suggests that free Fe(II) sequestration has a central role in controlling ROS levels. It will be interesting to investigate the impact of these transcription factors on other components of iron regulation, such ferroportin and hepcidin, which have been shown to influence breast tumorigenesis[71].

The role of ROS modulators in cancer

Although previously thought to be simply a bystander effect, it has been shown that the modulation of ROS levels by oncogenes and tumour suppressor genes has a considerable impact on both the initiation and progression of tumorigenesis. Below, we examine in detail the cancer-associated pathways that have the biggest role in regulating ROS levels.

Role of oncogenes in oxidative stress. In the past, the roles of NRF2 in antioxidant gene expression and ROS detoxification were believed to be facets of a tumour suppressor function[72,73]. More recent research, however, has provided evidence to the contrary. Targets of NRF2 such as GCLM and HMOX1 have been implicated in promoting cancer cell survival because they neutralize

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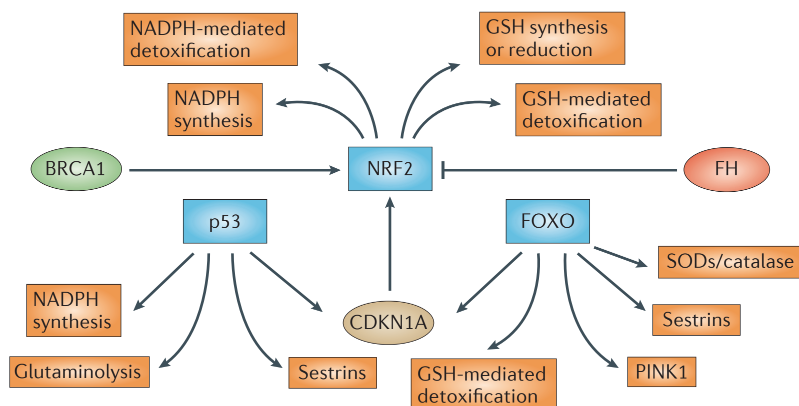

Figure 3 | **NRF2, p53 and FOXOs support complementary antioxidant pathways.** Whereas nuclear factor erythroid 2-related factor 2 (NRF2) mainly affects reduced glutathione (GSH)- and NADPH-related responses, forkead box O (FOXO) proteins and the tumour suppressor p53 regulate superoxide dismutases (SODs), catalase, PTEN-induced putative kinase 1 (PINK1) and sestrins. p53 promotes glutaminolysis via glutaminase 2 (GLS2), which produces the glutamate required for GSH synthesis. In addition, both FOXOs and p53 control NRF2 via the expression of cyclin-dependent kinase inhibitor 1A (CDKN1A). NRF2 activity is also controlled positively by breast cancer susceptibility 1 (BRCA1) and negatively by fumarate hydratase (FH).

the toxic effects of oxidative stress[2,74]. In addition, there is accumulating evidence that putative oncogenes can affect NRF2 regulation; for example, *KRAS* and *MYC* have been shown to stabilize NRF2 and promote the NRF2-mediated antioxidant response[75]. Mutations in PTEN that lead to hyperactive and oncogenic phosphoinositide 3-kinase (PI3K)–AKT signalling also activate NRF2 (REF. 40). The neuronal oncogene *DJ1* (also known as *PARK7*) promotes NRF2 stability by interfering with the binding of KEAP1 to NRF2 (REFS 76–79). Furthermore, the brain-specific carnitine palmitoyltransferase 1C (CPT1C) has a role in protecting cells from oxidative stress[80] and other conditions such as glucose deprivation and hypoxia, although its relationship with NRF2 has not yet been clarified[81,82].

Moreover, mutations in NRF2 and in its regulator KEAP1 have been identified in tumour samples from patients with cancer, providing strong evidence for a role of NRF2 in tumorigenesis. For example, mutations in the KEAP1-binding domain of NRF2 prevent KEAP1-mediated modification of this transcription factor[83,84]. Inactivating mutations in KEAP1 itself have also been identified[85]. All of these mutations lead to the constitutive stabilization of the NRF2 protein in the nucleus. NRF2-related mutations have been found primarily in squamous cell carcinomas but also occur, albeit with lower frequency, in a broad spectrum of other tumour types, such as lung, skin, oesophageal, ovarian and breast cancer[83].

Like NRF2, FOXO transcription factors were originally considered to act exclusively as tumour suppressors. As these transcription factors have long been identified as promoters of organism longevity, it is not surprising that they have a role in supporting tumorigenesis[52]. Cancer cells in which the AKT signalling pathway is hyperactivated such that FOXO transcription factors are inhibited are particularly sensitive to changes in oxidative stress[61]. In addition, oncogenic factors such as β-catenin and transforming growth factor-β (TGFβ) can act synergistically to enhance the oncogenic functions of FOXO transcription factors[86,87]. For example, it has been shown that activated FOXO transcription factors — rather than the oncogene *AKT*, as previously thought — support the survival of AML cells[88]. Intriguingly, the FOXO genes are involved in chromosomal translocations that lead to alveolar rhabdomyosarcoma and acute lymphoblastic leukaemia (ALL)[89]. Specifically, the paired box 3 *(PAX3)–FOXO1* translocation is found in approximately 60% of alveolar rhabdomyosarcoma tumours[90]. The product of this fusion can no longer be inhibited by AKT signalling, which indicates that the FOXO-driven antioxidant pathway can cooperate with AKT to promote proliferation while also driving antioxidant expression by FOXO proteins[91].

Role of tumour suppressors in oxidative stress. Unlike oncogenes, tumour suppressors have a more variable role in the regulation of oxidative stress. Depending on the particular tumour suppressor, its inactivation can lead to the activation or suppression of antioxidant gene expression. For example, loss of PTEN leads to AKT hyperactivation, FOXO inactivation and an overall increase in sensitivity to oxidative stress[61]. Similarly, loss of the tumour suppressor retinoblastoma-associated protein RB leads to decreased FOXO activity and increased susceptibility to cell death in various cancer cell lines (for example, MDA-MB-458 (breast cancer), Saos-2 (osteosarcoma) and DU145 (prostate cancer)) that also fail to express the tumour suppressor tuberous sclerosis protein 2 (TSC2)[92]. Likewise, mutations in liver kinase B1 (LKB1; also known as STK11) that impair 5′ AMP-activated protein kinase (AMPK) activation and thus decrease NADPH production reduce the tumorigenic potential of various cancer cell lines, such as A549 (lung cancer), HeLa (cervical cancer) and MCF7 (breast cancer)[93]. In both instances, these conclusions were demonstrated using *in vitro* colony formation assays and orthotopic *in vivo* cell transplantations.

The breast cancer susceptibility 1 (*BRCA1*) gene has also been implicated in the control of oxidative stress. *In vitro* modulation of BRCA1 levels controls the expression of several NRF2-regulated antioxidant genes and reduces H_2O_2-induced DNA damage and apoptosis[94,95]. Our group has recently discovered that BRCA1 is a novel interacting partner and regulator of NRF2 that is required for an efficient antioxidant response[96] (FIG. 3). The ability of BRCA1 to positively regulate NRF2 has important consequences for its tissue-specific tumour suppression function, and BRCA1-deficient cells suffer from high ROS levels owing to an inefficient NRF2 response. Interestingly, oestrogen stimulation of BRCA1-deficient mammary epithelial cells rescued NRF2 protein expression to levels similar to those in BRCA1-proficient cells[96]. These findings suggest that oxidative stress blocks the transformation of *BRCA1*-null

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cells and that oestrogen stimulates the NRF2 pathway in *BRCA1*-null cells to a level that ensures their survival. Moreover, the connection between oestrogen and NRF2 explains why BRCA1 mutations mainly predispose individuals to breast and ovarian tumours.

The ataxia telangiectasia mutated gene, *ATM*, is another example of a tumour suppressor that regulates ROS levels. In fact, ATM-deficient cells from both patients with ataxia telangiectasia and from $Atm^{-/-}$ mice suffer from chronic oxidative stress[97]. High ROS levels are responsible for bone marrow failure in $Atm^{-/-}$ mice[98]. Interestingly, in the presence of oxidative stress, ATM can be directly activated by oxidation in the absence of DNA double-strand breaks[99]. It has been suggested that ATM acts as a ROS sensor that regulates autophagy through repression of mammalian target of rapamycin complex 1 (mTORC1)[100].

Fumarate hydratase is a tumour suppressor whose functional inactivation leads to the activation of an antioxidant programme[101,102]. Loss of fumarate hydratase leads to a build-up of fumarate, which subsequently succinylates KEAP1 and modifies it such that it cannot promote NRF2 degradation (FIG. 3). Interestingly, this mechanism of NRF2 stabilization — rather than the activation of the traditional oncogene hypoxia-inducible factor 1 alpha (*HIF1A*) — seems to be the driving force behind the formation of renal cysts and tumours in which fumarate hydratase mutations are found[101]. Furthermore, depletion of NRF2 in renal cell carcinoma cells increases ROS levels and decreases their proliferation potential[103].

Role of tumour supporters in oxidative stress. A tumour 'supporter' is a molecule that supports the proliferation of a cancer cell by managing stressors — such as oxidative, metabolic and hypoxic stress — that normally occur during the process of tumorigenesis. The M2 isoform of pyruvate kinase, muscle (PKM2) has been characterized as a cancer-specific isoform of pyruvate kinase[104,105]. PKM2 can be allosterically inactivated through receptor tyrosine kinase (RTK) signalling and is an isoform with minimal kinase activity[106,107]. As PKM2 is less efficient than PKM1 in converting phosphoenolpyruvate (PEP) to ATP and pyruvate, upstream glycolytic intermediates can flow into the PPP where NADPH-reducing equivalents can be synthesized. This constitutes a paradigm shift, as this tumour-specific metabolic enzyme actually works against the Warburg effect of aerobic glycolysis[1]. Further research has found that PKM2 is in fact redox-sensitive, which means that it is completely inactivated under conditions of high ROS, leading to the diversion of metabolites towards NADPH generation[108] (FIG. 4). PKM2 may be especially important during the initiation of solid tumours, as cells that detach from their matrix undergo reduced NADPH synthesis and increased oxidative stress[24].

Another metabolic pathway that is crucial for cancer cell survival and is indirectly related to antioxidant responses is serine biosynthesis, which is driven by phosphoglycerate dehydrogenase (PHGDH)[109,110]. Serine is a metabolic precursor of glycine, and glutathione synthetase (GSS) uses glycine for the second step of *de novo* GSH synthesis. Significantly, glycine is one of the metabolites that is most upregulated in cancer cells[111]. Serine biosynthesis is also connected to the PKM2 pathway as serine is an activator of PKM2 (REF. 112). Conditions of low serine lead to PKM2 inactivation, which drives metabolites away from glycolysis and towards PHGDH-regulated serine synthesis.

The exact role of many tumour-associated molecules is currently not well understood. For example, the transmembrane glycoprotein CD44, a tumour-associated surface protein, was mainly known for its role in controlling cell adhesion[113]. However, it has recently been shown that CD44 is a marker of TICs[114] and stabilizes the cystine/glutamate transporter XCT, thereby promoting GSH synthesis[32] — a process that is crucial for tumour cell survival.

Scavenging ROS as an anticancer therapy

If the oncogenic functions of ROS are mediated through the stimulation of mutagenesis and through their functions as second messengers in cell proliferation, the use of antioxidants should prevent tumorigenesis. Several studies of antioxidants, including vitamin E and selenium, have been conducted in this context. In 1993, the so-called Linxian trial was among the first large, randomized, double-blind, primary prevention studies to investigate the putative prevention of cancer through the consumption of a mixture of selenium, vitamin E and β-carotene supplements[115]. This cocktail was found to significantly reduce total mortality, total cancer mortality and mortality from gastric cancer. Interestingly, the protective effect of these antioxidants was still evident 10 years after the cessation of supplementation[116]. A large epidemiological study conducted in China showed that the intake of vitamin E (but not vitamin C), either from supplements or the diet, reduced the risk of liver cancer[117]. The opposite was found in two large studies involving supplementation with β-carotene and vitamin A or vitamin E, where no reduction — but instead an increase — in lung cancer incidence was observed[218,219].

In another study, a potentially protective effect of selenium against prostate cancer was described[118], but further investigation showed that the effect applied only to highly aggressive metastatic malignancies and was heavily dependent on particular genetic variants[119]. The very large 'Selenium and Vitamin E Cancer Prevention Trial' (SELECT; comprising 35,533 men from 427 study sites in the United States, Canada and Puerto Rico) found no initial reduction in the risk of prostate cancer in healthy individuals taking either selenium or vitamin E supplements. Indeed, long-term follow-up studies of these individuals showed that vitamin E supplementation significantly increased the risk of prostate cancer among healthy men[120]. These results show that the use of antioxidants in cancer prevention is complex and needs to be carefully evaluated.

The dietary intake of foods that are naturally rich in antioxidants has been suggested to the public as a way to protect against cancer. In previous years, natural products have been a more relevant area of drug

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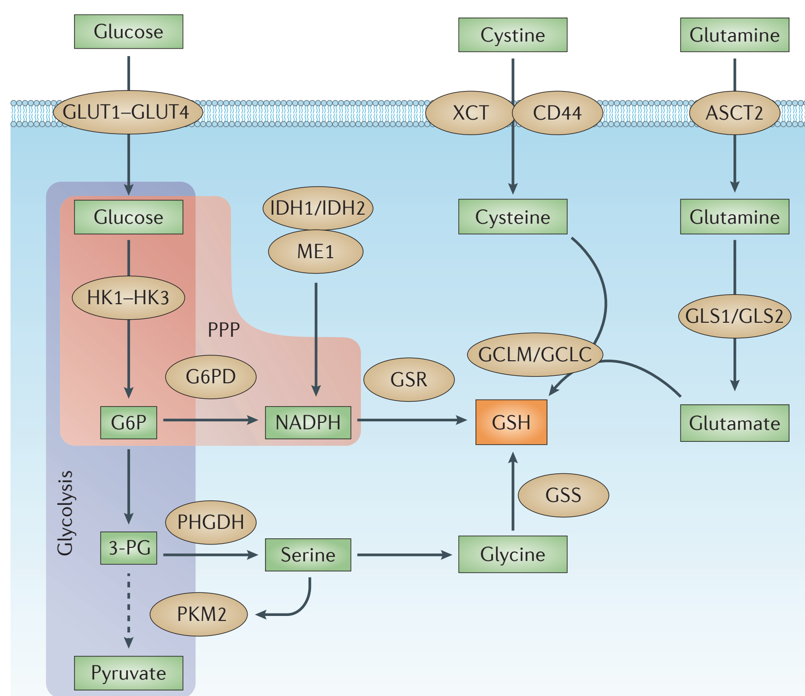

Figure 4 | **Multiple tumour supporter pathways promote GSH synthesis and regeneration.** The two main metabolites that drive cancer cell proliferation and survival are glucose and glutamine. After entering the cancer cell via glucose transporter 1 (GLUT1), GLUT2, GLUT3 or GLUT4 and being converted to glucose-6-phosphate (G6P) by hexokinases (HK1, HK2 or HK3), glucose can enter either the glycolysis pathway or the pentose phosphate pathway (PPP). The M2 isoform of pyruvate kinase, muscle (PKM2) slows glycolysis (dashed arrow) and allows for metabolite diversion into NADPH (via G6P dehydrogenase (G6PD) and the PPP), leading to the regeneration of the reduced form of glutathione (GSH) by glutathione reductase (GSR). NADPH can also be produced by isocitrate dehydrogenase 1 (IDH1) and IDH2 through the conversion of isocitrate to α-ketoglutarate, as well as malic enzyme 1 (ME1) through the conversion of malate to pyruvate. Metabolites diverted owing to PKM2 also promote serine biosynthesis via phosphoglycerate dehydrogenase (PHGDH), which leads to glycine production and, ultimately, *de novo* GSH production catalysed by glutathione synthetase (GSS). Glutamine, after being converted to glutamate through glutaminolysis via glutaminase 1 (GLS1) or GLS2, can either produce α-ketoglutarate (αKG) and enter the mitochondria (not shown), or react with cysteine to produce GSH via the glutamate–cysteine ligase complex modifier subunit (GCLM) or the GCL catalytic subunit (GCLC). Cysteine levels are controlled two ways: internal generation from methionine (not shown) or imported as cystine (via the cystine/glutamate transporter XCT) and reduced by GSH and/or thioredoxin antioxidant pathways (not shown). Cystine import is promoted by the tumour-associated antigen CD44 through its interaction with — and stabilization of — XCT. ASCT2, sodium-dependent neutral amino acid transporter type 2; 3-PG, 3-phosphoglyceric acid.

discovery research for novel and bioactive molecules[121]. Some nutrient components with antioxidant properties — such as vitamins A and D, genistein, (-)-epigallocatechin-3-gallate (EGCG), sulphoraphane, curcumin, piperine, theanine and choline — have been shown to control the aberrant expansion of cancer stem cells in breast, pancreatic and brain tumours[122]. As a result, the production of engineered foods containing increased amounts of these antioxidants and the consumption of these so-called 'superfoods' has become a highly profitable business that is sustained by industry claims on the links between antioxidant supplementation and cancer prevention. However, most of the data supporting these claims have been obtained *in vitro* or using animal models, and the number of population-based studies is still very small.

Increasing ROS as an anticancer therapy

The induction of oxidative stress can lead to the preferential killing of cancer cells. As summarized in TABLE 1, various drugs with direct or indirect effects on ROS have been used for effective cancer therapies. Drugs that directly affect ROS metabolism target two of the three major antioxidant pathways, such as those involving reduced glutathione and thioredoxin (FIG. 5). A more detailed investigation of their effects on cancer cell metabolism will help to define better-tailored therapies that have fewer side effects and a lower propensity for promoting the development of drug resistance.

Chemotherapy. As high ROS levels are harmful to cells, oxidative stress can have a tumour-suppressive effect. This imparts pressure on cancer cells to adapt by developing strong antioxidant mechanisms. But despite having an enhanced antioxidant system, cancer cells maintain higher ROS levels than normal cells[16]. This aspect offers an interesting therapeutic window because cancer cells might be more sensitive than normal cells to agents that cause further accumulation of ROS.

Several antineoplastic drugs that are currently used for cancer chemotherapy induce high levels of oxidative stress[123,124]. Patients who receive these drugs exhibit signs of ROS-induced lipid peroxidation in their plasma[124,125], have reduced blood levels of vitamin E, vitamin C and β-carotene[94,95], and decreased tissue GSH levels. For example, drugs such as taxanes (paclitaxel and docetaxel), vinca alkaloids (vincristine and vinblastine) and antimetabolites (anti-folates) promote the release of cytochrome *c* from the mitochondria — which induces cell death — and also interfere with the electron transport chain, resulting in the production of superoxide radicals[126].

Other drugs such as platinum coordination complexes (for example, cisplatin, carboplatin and oxaliplatin) and anthracyclines (for example, doxorubicin, epirubicin and daunorubicin) generate extremely high ROS levels[123]. For example, doxorubicin penetrates the inner membrane of cardiac mitochondria and competes with coenzyme Q_{10} in the electron transport chain to induce superoxide radical production, which is the basis of the cardiotoxicity of these drugs. Arsenic trioxide (As_2O_3) has been efficiently used for the treatment of acute promyelocytic leukaemia[127]. It induces apoptotic cell death in various cancer cells including leukaemia, myeloma and lung cancer via the induction of ROS[128]. 5-fluorouracil (5-FU) is a pyrimidine analogue that is used to treat several types of cancer, including colon cancer, rectal cancer as well as head and neck cancer[129]. 5-FU generates mitochondrial ROS via a p53-dependent pathway[130]. Tumour cells that adapt to oxidative stress by increasing the production of SOD2, peroxiredoxin 1 and B cell lymphoma 2 (BCL-2) are resistant to 5-FU[131].

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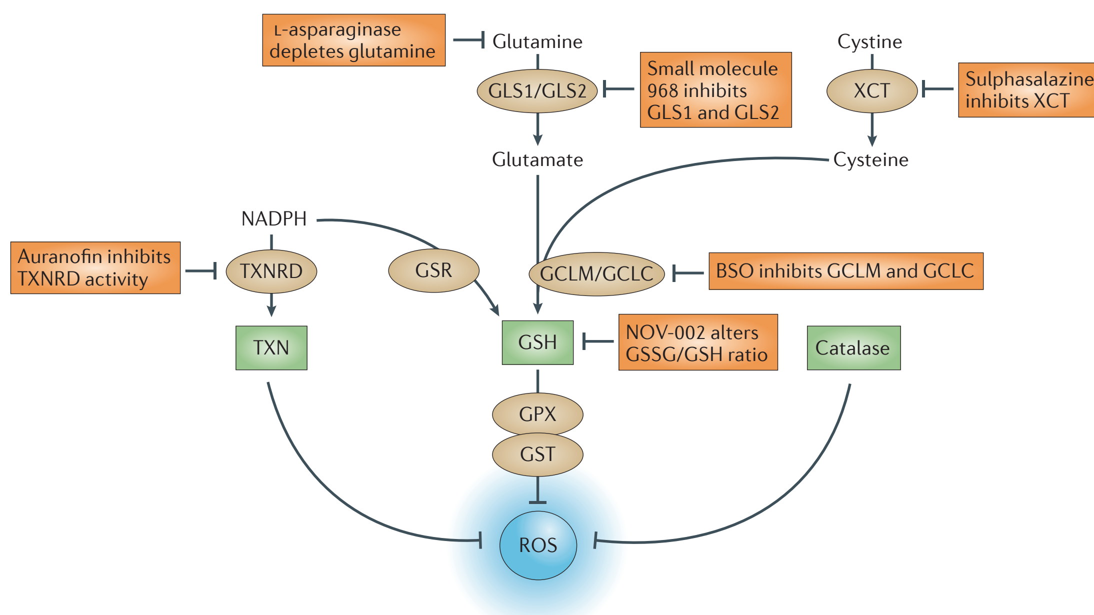

Figure 5 | **The antioxidant pathways that drive ROS detoxification.** There are three main pathways for the elimination of reactive oxygen species (ROS), and these involve reduced glutathione (GSH), thioredoxin (TXN) and catalase. TXN, a protein that reduces ROS levels, can be regenerated by thioredoxin reductase (TXNRD) using the metabolite NADPH. GSH, which can also be regenerated by NADPH via glutathione reductase (GSR), is derived from the metabolites glutamate and cysteine. GSH acts directly on eliminating ROS through the action of glutathione peroxidase (GPX) and glutathione *S*-transferase (GST). Catalase also acts directly on ROS and resides predominantly in peroxisomes. Glutaminase 1 (GLS1) and GLS2 produce glutamate, and the cystine/glutamate transporter XCT provides cysteine for the production of GSH through the action of the glutamate–cysteine ligase modifier subunit (GCLM) and the GCL catalytic subunit (GCLC). Whereas both TXN and GSH pathways rely heavily on NADPH production for sustaining their activity, catalase acts independently of NADPH. All three of these pathways contain key enzymes that could be specifically targeted to inhibit antioxidant responses within cancer cells. The figure also shows the anticancer drugs listed in TABLE 1 that exert a direct effect on glutathione metabolism. BSO, buthionine sulphoximine.

The production of ROS is also part of the mechanism of action of 2-methoxyestradiol, a metabolite of estradiol-17 beta. 2-methoxyestradiol was shown to inhibit the proliferation of, and induce apoptosis in, human neuroblastoma cells *in vitro* via an increase in ROS levels and through the loss of the mitochondrial membrane potential[132]. Oxidative stress induced by 2-methoxyestradiol is not due to the inhibition of SOD, as initially postulated[133]. *N*-(4-hydroxyphenyl) retinamide (4-HPR) is a synthetic retinoid that can induce apoptosis in many cancer cell lines via ROS production[134]. Interestingly, ascorbic acid, trolox and vitamin E reverse the effects of 4-HPR on cell death in human leukaemia cells[135].

17-allylamino-17-demethoxygeldanamycin (17-AAG) binds to and inhibits heat shock protein 90 (HSP90), which is a member of the heat shock protein family[136]. HSP90 is upregulated in response to stress and has important cytoprotective functions[137]. Drug combinations that affect the metabolism of GSH and thioredoxin potentiate the sensitivity of cancer cells to 17-AAG treatment[138]. HSP90 inhibition combined with the mTOR inhibitor rapamycin can cause ER stress and mitochondrial damage, enhancing oxidative stress and reducing tumour growth in a mouse model of RAS-driven tumour growth[139]. Therefore, inhibition of the anti-apoptotic functions of HSP90 combined with agents that induce oxidative stress represents an efficient strategy for killing cancer cells.

Inhibitors of the enzyme poly(ADP-ribose) polymerase (PARP) have shown promising effects against breast cancer, in particular for advanced triple-negative breast cancers associated with BRCA deficiency[140]. PARP is involved in the maintenance of DNA integrity as part of the base excision repair pathway[141]. PARP enzymes are therefore crucial in the response to different genotoxic effects, including oxidative stress[142].

Platinum-based compounds induce high ROS levels that cause apoptosis[143]. Therefore, the combination of PARP inhibitors with platinum coordination complexes would negatively affect the capacity of tumour cells to respond to oxidative stress. Indeed, the administration of PARP inhibitors such as veliparib or olaparib with carboplatin has proven to be highly promising in treating different types of cancers[144]. The combination of PARP inhibitors with platinum-based drugs has been shown to delay tumour growth in *Brca1*- and *Brca2*-deficient mouse models[145,146].

Promising results have also been obtained from ongoing clinical trials, although data are still limited. A combination of a PARP inhibitor (veliparib or olaparib) with

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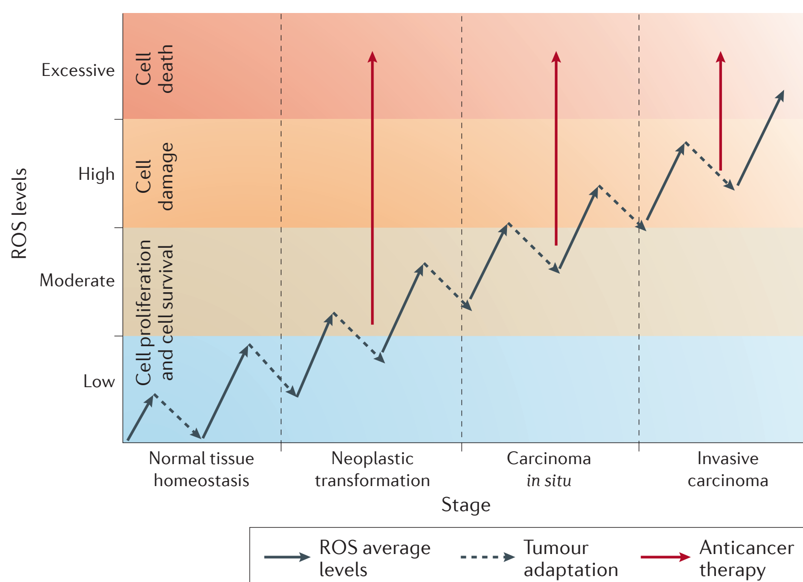

Figure 6 | **Interplay between ROS regulation and tumorigenesis at different stages.** During the transition phases from normal tissue to invasive carcinoma, cells experience a progressive increase in reactive oxygen species (ROS) levels owing to metabolic aberrations acquired following transformation (represented by the solid arrows)[209]. Cancer cells escape cell death and damage induced by high ROS levels by increasing their antioxidant defences that lower ROS levels (dashed arrows). We propose to target the antioxidant mechanisms of tumour adaptation by an anticancer therapy that forces the accumulation of excessive ROS and the induction of cell death (solid red arrows). This can be achieved by treating cancer cells either with ROS-inducing therapies or with antioxidant-inhibiting therapies.

carboplatin is currently being tested in different Phase I and II clinical trials organized by the US National Institutes of Health (see the ClinicalTrials.gov website). The combination has been investigated in the following types of cancer: BRCA1- or BRCA2-associated stage III/IV breast or ovarian cancer (Phase II; ClinicalTrials.gov identifier: NCT01149083), sporadic invasive breast cancer (Phase II; ClinicalTrials.gov identifier: NCT01042379), HER2-negative breast cancer (Phase I; ClinicalTrials.gov identifier: NCT01251874) and refractory gynaecological cancers such as ovarian, fallopian, primary peritoneal, uterine and cervical cancer, as well as malignant mixed mullerian tumours (Phase I; ClinicalTrials.gov identifier: NCT01237067). Olaparib is also being tested in combination with cisplatin or carboplatin and other drugs (for example, paclitaxel, gemcitabine, vinorelbine, topotecan, bevacizumab or radiation) in the following Phase I/II clinical trials: advanced biliary, pancreatic, urothelial or non-small-cell lung cancer (NSCLC) (Phase I; ClinicalTrials.gov identifier: NCT01282333); advanced, persistent or recurrent cervical cancer (Phase I/II; ClinicalTrials.gov identifier: NCT01281852); triple-negative breast cancer and BRCA-associated breast cancer (Phase I; ClinicalTrials.gov identifier: NCT01104259); relapsed or refractory acute leukaemia, high-risk myelodysplasia or aggressive myeloproliferative disorders (Phase I; ClinicalTrials.gov identifier: NCT00588991); stage II, III or IV ovarian cancer (Phase I; ClinicalTrials.gov identifier: NCT00989651); and stage III NSCLC (Phase I/II; ClinicalTrials.gov identifier: NCT01386385).

Although a deeper investigation is required, it is conceivable that lack of DNA damage repair owing to PARP inhibition may sensitize tumour cells to carboplatin- or cisplatin-induced oxidative stress. Indeed, in NSCLC, PARP inhibitors synergize with cisplatin to induce DNA damage foci and mitochondrial membrane permeabilization and reverse resistance to cisplatin[147,148]. In conclusion, the capacity of some chemotherapeutic agents to cause an imbalance in ROS levels can offer a therapeutic opportunity for treating cancer.

Ionizing radiation. Ionizing radiation is widely used to treat many types of cancer. Two main types of ionizing radiation exist: the first is based on photons, which are generated from cobalt, cesium or a linear accelerator; and the second is based on particles such as electrons, protons, neutrons, α-particles and-β particles. Ionizing radiation induces a substantial increase in ROS levels[149]. NADPH oxidase, another important source of ROS, is also activated by radiation exposure, leading to persistent oxidative stress[150]. Mechanisms of cellular resistance to radiation are associated with higher antioxidant levels that scavenge ROS[2].

Drugs that inhibit the ubiquitin–proteasome pathway. The ubiquitin–proteasome pathway is responsible for the degradation of misfolded and mutated proteins and other proteins that are important in differentiation, development, proliferation, cell signalling and cell death. Therefore, a tight regulation of the ubiquitin–proteasome system is required for normal cell physiology. Proteasome-dependent proteolysis is involved in cellular events that are linked to tumorigenesis[151]. Cancer cells may have aberrant proteasome regulation. For these reasons, targeting the proteasome has been postulated as a form of antineoplastic therapy[152]. Proteasome inhibition leads to the accumulation of apoptotic proteins, oxidative damage and mitochondrial dysfunction. For example, ROS production mediates the cytotoxicity of the proteasome inhibitor *N*-benzyloxycarbonyl-Ile-Glu(O-t-butyl)-Ala-leucinal (PSI) in neuronal cells[153]. In the same cells, depletion of GSH exacerbates PSI-induced cell death. Bortezomib (Velcade; Millennium Pharmaceuticals) is another example of a proteasome inhibitor that causes oxidative stress[154]. Bortezomib is used to treat patients with relapsed mantle cell lymphoma (MCL) despite having varying clinical outcomes[155].

Drugs that induce ER stress. ER stress is induced when the ER capacity of the folding protein is impaired, and it results in the accumulation of misfolded proteins. The cell responds to ER stress with a defence mechanism known as the unfolded protein response (UPR), but apoptosis and cell death are triggered if the level of stress is excessive. Recently, ER stress inducers have attracted attention from pharmaceutical companies as potential anticancer drugs. Pharmacologically

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aggravated ER stress can be useful for killing cancer cells via the induction of oxidative stress. Some examples of ER stress aggravators that also increase ROS levels are bortezomib, celecoxib, nelfinavir and the sesquiterpene lactone thapsigargin. The clinical use of thapsigargin is unfeasible owing to its high toxicity. A new promising drug, G202, has recently been developed through the chemical modification of this molecule and is currently being studied in the United States as part of a Phase II clinical trial[156] (ClinicalTrials.gov identifier: NCT01777594). Bortezomib has been developed as a proteasome inhibitor but it also aggravates ER stress[157,158]. Celecoxib is a non-steroidal anti-inflammatory drug that has been developed as a selective inhibitor of cyclooxygenase 2. It aggravates ER stress in a similar manner as thapsigargin[159]. Nelfinavir has been developed as an inhibitor of HIV protease. However, it induces potent ER stress and shows promising anticancer activity[160]. Nelfinavir is currently part of a Phase I clinical trial for the treatment of cervical cancer (ClinicalTrials.gov identifier: NCT01485731).

Drugs that affect GSH metabolism. GSH metabolism appears to be actively involved in protecting cancer cells from apoptosis and in mechanisms of multidrug and radiation resistance[161]. In particular, increased levels of GSH within tumour cells have been associated with resistance to platinum-containing anticancer compounds, anthracyclines and alkylating agents. For example, enhanced utilization of cysteine for GSH synthesis underlies the resistance of breast cancer cells to tamoxifen[162]. Thus, a therapeutic approach that modulates GSH levels within cancer cells could affect the efficacy of other anticancer therapies. Compared to normal cells, cancer cells with high GSH content seem to be more sensitive to drugs that affect GSH metabolism. Therefore, several approaches for blocking GSH synthesis in cancer cells have been attempted.

As noted above, GCL is the rate-limiting enzyme in GSH synthesis. GCL has therefore been an anticancer drug target for over 30 years. The classical drug used to inhibit GCL activity is buthionine sulphoximine (BSO)[163], which is currently the only known inhibitor of *de novo* GSH synthesis that is in clinical use. Phenylethyl isothiocyanate (PEITC)[164], which inhibits GPX and depletes GSH levels[165], has shown promise in a preclinical mouse model of ovarian cancer[164]. Piperlongumine, a naturally occurring alkaloid present in the long pepper (*Piper longum*), has been implicated in the regulation of GSTP1 activity[166]. *In vitro* treatment of cancer cells with piperlongumine increases ROS levels and induces cell death. *In vivo* administration of the same drug suppresses tumour growth in established tumour xenografts in mice (human bladder, breast and lung tumours in nude mice and mouse melanoma in B6 mice)[166].

GSH synthesis may also be modulated through the regulation of intracellular cysteine levels. Different concentrations of cysteine in the culture media affect GSH levels, which in turn regulate survival and protection from the drug-induced toxicity of chronic lymphocytic leukaemia (CLL) cells[31]. Sulphasalazine is an anti-inflammatory drug that is used for the treatment of rheumatoid arthritis, psoriatic arthritis and many other types of arthritis. It has also been found to specifically inhibit XCT activity[167,168]. Sulphasalazine treatment markedly decreases GSH levels and reduces the growth and viability of human pancreatic cancer cells both *in vitro* and *in vivo*[169]. Inhibition of XCT by sulphasalazine also appears to be useful in treating small-cell lung cancer (SCLC). In fact, cysteine depletion by sulphasalazine administration has been shown to inhibit SCLC growth *in vitro* and *in vivo* in a xenograft model[170]. However, like BSO, sulphasalazine is a drug that has never been improved upon and no other inhibitors of XCT are currently available.

Another promising anticancer drug that affects GSH metabolism is NOV-002. NOV-002 is a glutathione disulphide mimetic that alters the intracellular GSSG/GSH ratio and increases oxidative stress, decreasing tumour cell invasion, proliferation and survival[171,172]. In patients with HER2-negative breast cancers, administration of NOV-002 in combination with adjuvant chemotherapy (doxorubicin-cyclophosphamide followed by docetaxel) resulted in a favourable response rate and mitigation of side effects compared to adjuvant chemotherapy alone[171].

Drugs that affect thioredoxin metabolism. Similar to GSH, thioredoxin is central to one of the major redox systems in animal cells. The reduction of thioredoxin by NADPH is catalysed by TXNRD and supplies electrons for DNA synthesis, antioxidant defence, redox regulation of cellular signal transduction, cell growth and cell death. In tumour cells, changes to thioredoxin metabolism are implicated in resistance to chemotherapy. Auranofin (Ridaura; Prometheus) is a gold compound that is clinically used as an antirheumatic agent and functions as a thioredoxin inhibitor; interestingly, administering it in combination with BSO has been shown to result in increased sensitivity of head and neck squamous cell carcinoma cell lines to epidermal growth factor receptor (EGFR) inhibitors. This effect is reversed by pre-treatment with the antioxidant *N*-acetylcysteine, thus confirming the role of oxidative stress in mediating cell death[173]. Another study shows that the treatment of ovarian cancer cells with auranofin induces cytochrome *c*-mediated cell death, which confirms the importance of thioredoxin metabolism in tumour cell survival[174].

Drugs that affect glucose metabolism. The regulation of glucose metabolism is fundamental for the control of ROS generation. In both healthy neurons and cancer cells, regulation of glucose flux is fundamental for preventing oxidative stress and the activation of cytochrome *c*-mediated apoptosis[175]. High metabolism of glucose via the PPP produces intracellular GSH, which reduces cytochrome *c* and renders it inactive and unable to trigger cell death. Indeed, the inhibition of G6PD by 6-anicotinamide diminishes multidrug resistance in a doxorubicin-resistant human colon cancer cell line[176]. The coordination of the pro-apoptotic activity

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Table 1 | **Classification of anticancer treatments according to their direct or indirect role in regulating ROS levels**

Name	Mechanism of action; effects on ROS	Cancer types	Stage of development	Refs
Drugs with a direct role on ROS metabolism				
NOV-002	Glutathione disulphide mimetic; alters intracellular GSSG/GSH ratio	Lung, breast and ovarian cancer	Approved	171
Sulphasalazine	Inhibitor of cystine/glutamate transporter XCT; reduces intracellular transport of cysteine required for GSH synthesis	Pancreatic and lung cancer	Approved	169, 170
6-anicotinamide (6-AN)	Inhibitor of glucose-6-phosphate dehydrogenase; reduces GSH	Colon cancer	Approved	210
L-asparaginase	Depletes glutamine; reduces GSH	Leukaemia, pancreatic cancer	Approved	211, 212
Small molecule 968 (dibenzophenanthridine)	Glutaminase inhibitor; reduces GSH	Lymphoma, breast cancer	Approved	178, 179
Buthionine sulphoximine (BSO)	Glutamate–cysteine ligase complex inhibitor; inhibits *de novo* GSH synthesis	Ovarian and breast cancer, melanoma	Approved	213, 214
Drugs or treatments with an indirect role on ROS metabolism				
Ionizing radiation	Photons (from cobalt, cesium or a linear accelerator) or particles (such as electrons, protons, neutrons, α-particles and β-particles) affect chemical bonds and produce highly reactive free radicals and ROS, which cause damage to DNA and other cellular components	Different types of cancer	FDA-approved	149
G202	Binds to and blocks the sarcoplasmic/endoplasmic reticulum calcium ATPase (SERCA) pump, thereby increasing the concentration of cytosolic calcium that causes apoptosis; induction of ROS owing to ER stress	Hepatocellular carcinoma, prostate cancer	Phase II	156
Celecoxib	Inhibits cyclooxygenase 2 (COX2) activity but it also induces ER stress by causing leakage of calcium from the ER into the cytosol; induction of ROS owing to ER stress	Colorectal cancer, myeloma, Burkitt's lymphoma and prostate cancer	FDA-approved	215
Nelfinavir	Originally developed as HIV protease inhibitor but it also induces ER stress by an unknown mechanism; induction of ROS owing to ER stress	HPV-transformed cervical carcinoma, head and neck cancer, pancreatic cancer, melanoma and glioma	FDA-approved	216
N-benzyloxycarbonyl-Ile-Glu (*O*-t-butyl)-Ala-leucinal (PSI)	Proteasome inhibitor; induces ROS that lead to mitochondrial dysfunction	Leukaemia	FDA-approved	153
Bortezomib	Proteasome inhibitor; induces ROS owing to ER stress	Mantle cell lymphoma, multiple myeloma	FDA-approved	154, 155
Anthracyclines (doxorubicin, daunorubicin or epirubicin)	Insert into the DNA of replicating cells and inhibit topoisomerase II, which prevents DNA and RNA synthesis. Induce the generation of oxygen-derived free radicals through two main pathways: a non-enzymatic pathway that utilizes iron, and an enzymatic mechanism that involves the mitochondrial respiratory chain	Different types of cancer	FDA-approved	217
17-allylaminogeldana-mycin (17-AAG)	HSP90 inhibitor; affects protein homeostasis during oxidative stress by disrupting HSP90–client protein complexes and promoting the degradation of the client proteins	Breast cancer, non-small-cell lung cancer	Phase I/II	136
5-fluorouracil (5-FU)	Inhibits thymidylate synthetase and/or incorporates into RNA and DNA; induces intracellular increase in $O_2^{\bullet-}$ levels	Colon cancer, rectum cancer, and head and neck cancer	FDA-approved	129
Arsenic trioxide (As_2O_3)	Reacts with cysteine residues on crucial proteins; inhibits mitochondrial respiratory function, thereby increasing free radical generation	Leukaemia, myeloma	FDA-approved	127
2-methoxyestradiol (2-ME)	Metabolite of estradiol-17β; induces free radicals and loss of mitochondrial membrane potential	Prostate cancer, leukaemia	FDA-approved	132, 133
N-(4 hydroxyphenyl) retinamide (4-HPR)	Synthetic retinoid derivative; induces apoptosis through the production of ROS and mitochondrial disruption	Prostate cancer, breast cancer, neuroblastoma	FDA-approved	134

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Table 1 (cont.) | **Classification of anticancer treatments according to their direct or indirect role in regulating ROS levels**

Name	Effects on ROS	Mechanism of action	Cancer types	Refs
PARP inhibitors	Inhibit the action of the enzyme PARP; reduce the capacity to repair ROS-induced DNA damage	Breast cancer	Phase III	142
Erastin	Downregulates mitochondrial VDACs and cysteine redox shuttle; alters the mitochondrial membrane permeability and blocks GSH regeneration	*RAS*^{V12}-expressing tumour cells	Phase I/II	182, 183
Lanperasone	Downregulates mitochondrial VDACs; alters the mitochondrial membrane permeability	*KRAS*^{G12D}-expressing tumour cells	FDA-approved	184
AGX-891	Inhibits mutant IDH1 isoform; alters the $NADP^+$/NADPH ratio	Glioma, leukaemia	Preclinical	188
AG-221	Inhibits mutant IDH2 isoform; alters the $NADP^+$/NADPH ratio	Advanced haematological malignancies	Phase II	220

ER, endoplasmic reticulum; FDA, US Food and Drug Administration; GSH, reduced glutathione; GSSG, oxidized glutathione; HPV, human papilloma virus; HSP90, heat shock protein 90; IDH, isocitrate dehydrogenase; PARP, poly(ADP-ribose) polymerase; ROS, reactive oxygen species; VDAC, voltage-dependent anion channel.

of cytochrome *c* with the PPP renders cancer cells with high intracellular glucose concentrations susceptible to cytochrome *c*-mediated apoptosis. Because normal cells do not exhibit the same dependence on intracellular glucose, this characteristic provides an opportunity to target the aberrant metabolism of tumour cells. Further investigations are underway to translate these mechanistic findings into druggable targets.

Drugs that affect glutamine metabolism. Glutamine is the precursor to glutamate, which is required for *de novo* GSH synthesis. In addition, glutamine metabolism has been shown to be crucial for cancer cell survival[177]. *In vitro* and *in vivo* depletion of glutamine by the small molecule 968, a dibenzophenanthridine that targets GLS activity, can inhibit the growth of transformed fibroblasts and human cancer cells[178]. Inhibition of GLS efficiently kills hypoxic cancer cells *in vitro* and delays tumour xenograft growth *in vivo*[179].

Glutamine depletion also underlies the mode of action of L-asparaginase, an agent that is clinically used to treat haematological cancers such as ALL in combination with chemotherapy. Besides hydrolysing L-asparagine, L-asparaginase also possesses GLS activity, which can drastically reduce glutamine levels[180]. It was thought that the mode of action of this drug was dependent on the reduction of serum asparagine levels. In fact, leukaemic cells do not produce L-asparagine because they are deficient in asparagine synthetase, and they therefore rely on free asparagine in the blood to maintain cell viability[180]. However, recent data have shown that the cytotoxic activity of L-asparaginase is ascribed to its modulation of glutamine levels[180]. This finding is supported by the fact that a form of asparaginase that does not affect glutaminase activity (isolated from *Wolinella succinogenes*) has failed in the clinic, which suggests that glutamine depletion mediated the antitumour activity of L-asparaginase.

Genotype-selective antitumour drugs that induce oxidative stress. Recent high-throughput drug screens have identified chemical compounds that selectively kill tumorigenic cells carrying specific gene alterations and not their isogenic counterparts. These compounds have been defined as genotype-selective drugs. For example, in a high-throughput screen for synthetic lethal interactions between genetic alterations and small-molecule compounds, Dolma and colleagues discovered a novel drug called erastin[181]. Efficient erastin-induced cell death requires the presence of the oncogenic allele of *RAS* (*RAS*^{V12}) and small T oncoprotein. Erastin-induced cell death was found to be induced through a novel mechanism that is morphologically, biochemically and genetically distinct from other forms of cell death. As this new form of cell death depends on intracellular iron levels, it has been called 'ferroptosis'[182]. Erastin-induced ferroptosis involves oxidative stress as erastin alters the permeability of the outer mitochondrial membrane[183] and inhibits cysteine-dependent GSH regeneration[182].

Another genotype-selective antitumour drug that has been discovered in a similar screen as erastin is lanperisone[184]. Lanperisone is a modified form of tolperisone, a piperidine derivative, which is used as a muscle relaxant to treat painful muscle spasms. Similarly to erastin, lanperisone induces a non-apoptotic form of cell death in *KRAS*^{G12D}-expressing tumour cells, and this form of cell death does not depend on the cell-cycle phase or protein translation and occurs through the induction of oxidative stress. The exact mechanism responsible for lanperisone-induced ROS generation is not known but preliminary results suggest that it occurs through the perturbation of voltage-gated ion channels[184].

As described above, IDH1 and IDH2 enzymes are frequently mutated in glioma and in AML. These mutations in IDH1 and IDH2 lead to the production of a novel oncometabolite called 2-hydroxyglutarate[185]. Although wild-type IDH1 and IDH2 are key to the production of the reducing agent NADPH, mutated IDH1 and IDH2 consume NADPH during the synthesis of 2-hydroxyglutarate. These findings have recently been confirmed in a mouse model with a point mutation in IDH1 (REFS 186,187). Importantly, this mouse model provides a tool for testing novel inhibitors of mutated IDH1 within a physiological environment. The IDH1 inhibitor AGX-891 was developed and found to specifically inhibit the enzymatic activity of IDH-mutant isoforms[188]. The compound AGI-6780 was also generated as an allosteric inhibitor of the

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IDH2-mutant form[220]. A recent derivative of this inhibitor, AG-221, is currently under investigation in a Phase II clinical trial (see the ClinicalTrials.gov website).

One would hypothesize that mutated IDH1 and IDH2 result in higher ROS levels owing to the increased consumption of NADPH, and inhibition with AGX-891 or AG-221 would actually increase NADPH levels and lower ROS levels. Surprisingly, however, results obtained from mice with mutated IDH1 show that cells have low levels of ROS regardless of an imbalance in the $NADP^+$/NADPH ratio[186]. Therefore, it can be speculated that inhibitors that are specific for mutant IDH1 may in fact induce high ROS levels, increase oxidative stress and ultimately lead to cell death. These inhibitors may represent another example of a genotype-specific antitumour drug that can specifically kill tumour cells with metabolic aberrations.

Conclusion

Over the past several years, substantial research has shown that altered cell metabolism has a crucial role in the development of different types of cancer. The exact nature of the impact of oxidative stress on cancer initiation, progression and/or response to therapy requires further investigation. Newly developed technologies such as deep DNA sequencing and metabolomics are important tools that will help to define how the metabolism of cancer cells adapts and provides a buffer against increased oxidative stress.

It has been estimated that approximately 45–80% of patients with breast cancer use antioxidant supplements after diagnosis or during breast cancer treatment[189]. There is still considerable controversy as to whether ROS modulation by either antioxidant supplementation or inhibition is clinically beneficial or detrimental for cancer treatment. In fact, some investigators have hypothesized that antioxidant supplements can be used both for cancer prevention and to potentiate chemotherapy and radiation therapy by providing protection against toxic side effects. However, none of these theories is supported by solid clinical and experimental data. Instead, as described above, numerous recent studies are suggesting an opposite scenario: that is, antioxidants provide crucial survival and proliferation signals to cancer cells; cancer cells depend on an increased antioxidant capacity to counteract elevated ROS levels; and antioxidant inhibitors represent a promising therapeutic strategy in anticancer therapy (FIG. 6). GSH metabolism seems to be the main target of currently used anticancer drugs. However, other enzymes with antioxidant capacity can be crucial for cancer cell survival and should therefore be considered as targets for new anticancer approaches. Further mechanistic insight into the role of cellular antioxidant molecules in different types of cancer is required, as these data have the potential to provide better-tailored anticancer treatments.

As discussed above, GSH metabolism, which is essential for maintaining a correct redox balance, has an essential role in the protection of tumour cells from stress and ensures survival in the extreme environments — such as hypoxia and nutrient deprivation — that are present in solid malignancies. For these reasons, combinations of GSH inhibitors (or other antioxidant inhibitors) with radiotherapy or chemotherapeutic drugs that cause cell death induced by oxidative stress may prove to be useful for killing cancer cells. This therapeutic approach stands in sharp contrast to the conventional strategy of targeting oncogenes and tumour suppressor genes — an approach that has turned out to be largely ineffective owing to the numerous oncogenes and tumour suppressors and their ability to trigger compensatory mechanisms (for example, mutations in *KRAS* that lead to resistance to anti-EGFR therapy)[190].

Now, with the advent of new technologies and numerous ongoing research studies, we are beginning to recognize the mediators of resistance to immune surveillance, aneuploidy and metabolic aberrations. In our view, it is likely that all oncogenes induce a common set of stress adaptations in cancer cells and that the pathways underlying these adaptations may represent the most critical weak point in most tumours. Therefore, molecules that mediate such adaptations, rather than oncogenes and tumour suppressors, could be the next important targets for future anticancer drug discovery studies.

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Competing interests statement
The authors declare competing interests: see Web version for details.

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