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REVIEW

S-Glutathionylation and S-Nitrosylation as Modulators of Redox-Dependent Processes in Cancer Cell

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Abstract—Development of oxidative/nitrosative stress associated with the activation of oncogenic pathways results from the increase in the generation of reactive oxygen and nitrogen species (ROS/RNS) in tumor cells, where they can have a dual effect. At high concentrations, ROS/RNS cause cell death and limit tumor growth at certain phases of its development, while their low amounts promote oxidative/nitrosative modifications of key redox-dependent residues in regulatory proteins. The reversibility of such modifications as S-glutathionylation and S-nitrosylation that proceed through the electrophilic attack of ROS/RNS on nucleophilic Cys residues ensures the redox-dependent switch in the activity of signaling proteins, as well as the ability of these compounds to control cell proliferation and programmed cell death. The content of S-glutathionylated and S-nitrosylated proteins is controlled by the balance between S-glutathionylation/deglutathionylation and S-nitrosylation/denitrosylation, respectively, and depends on the cellular redox status. The extent of S-glutathionylation and S-nitrosylation of protein targets and their ratio largely determine the status and direction of signaling pathways in cancer cells. The review discusses the features of S-glutathionylation and S-nitrosylation reactions and systems that control them in cancer cells, as well as their relationship with redox-dependent processes and tumor growth.

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INTRODUCTION

It has been commonly recognized that cellular redox balance is determined by the ratio between the generated reactive oxygen species (ROS) and reactive nitrogen species (RNS), as well as by the activity of antioxidant system maintaining cellular redox homeostasis essential for various biological processes, such as cell proliferation/differentiation, metabolism, and immune response [1, 2]. ROS/RNS are continuously generated as byproducts in various cellular processes. An increase in the ROS/RNS content above a physiological range contributes to the development of oxidative/nitrosative stress [3, 4]. Chronic oxidative/nitrosative stress causes damage to the cells, tissues, and organs and is often associated with pathogenesis of various diseases including cancer [3].

Many tumor cell lines are characterized by the development of mild oxidative/nitrosative stress associated with the induction of oncogenic pathways due to the elevated ROS/RNS generation which can be partially compensated by the adaptive activation of antioxidant defense systems [2, 5]. ROS/RNS production by tumor cells results from the redox-dependent modulation of multiple signaling cascades affecting cell metabolism [6]. Oxidative/nitrosative stress can contribute to the tumor progression caused by genome instability and chromosomal

Abbreviations: ETC, electron transport chain; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; Grx, glutaredoxin; GSH/GSSG, reduced/oxidized glutathione; GSNO, nitrosoglutathione; GSNOR, S-nitrosoglutathione reductase; NF-κB, nuclear factor κB; NO, nitric oxide; NOS, NO synthase; Nrf2, nuclear factor erythroid 2-related factor 2; PDI, protein disulfide isomerase; PKC, protein kinase C; Pr-SH, protein thiol; Pr-SSG, S-glutathionylated protein; Prx, peroxiredoxin; Srx, sulfiredoxin; RNS, reactive nitrogen species; ROS, reactive oxygen species; Trx, thioredoxin; TrxR, thioredoxin reductase.

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abnormalities leading to the activation of oncogene levels and downregulation of oncosuppressors, as well as alterations in the metabolism of tumor cells [7]. DNA damage results in the hydrolysis of DNA bases with the formation of adducts that impair normal cell growth by inducing gene mutations and altering normal gene transcription.

Adaptation of tumor cells adapt to oxidative stress can be short- and long-term and occur via metabolic and genetic reprogramming, respectively [3]. An essential role in these adaptations belongs to post-translational modifications leading to the redox-dependent alterations in the activity of proteins [8]. Low ROS/RNS levels maintained by cellular antioxidant systems allow protein modifications via the ROS/RNS-triggered electrophilic attack on nucleophilic groups in amino acid residues of regulatory proteins. The reversibility of such reactions ensures redox switch in the activity of signaling proteins. The most common redox modifications in tumor cells are S-glutathionylation and S-nitrosylation of SH group of protein Cys residues [9-12]. The activity of these reactions is often related to the tumor progression.

Here, we discuss the features of S-glutathionylation and S-nitrosylation in tumor cells, the systems that control these reactions, as well as their involvement in the redox-dependent processes and tumor growth.

ROS GENERATION AND DEVELOPMENT OF OXIDATIVE STRESS IN CANCER CELLS

Tumor cells are characterized by an increased ROS generation largely initiated by physical factors and oncogene-induced malignant cell transformation [3, 13]. Mitochondria are the major producers of intracellular ROS; normally, 0.2-2% of electrons transferred by the electron transport chain (ETC) complexes I, II, and III are utilized for the formation of superoxide ($O^{\bullet}_2^-$) which rapidly dismutates into hydrogen peroxide (H_2O_2). Hydrogen peroxide acts as a secondary messenger in signal transduction due to its relatively long half-life and ability to diffuse through the aquaporin channels in the membrane followed by the Fenton reaction in the presence of transient metal ions (Fe^{2+}, Cu^{2+}, Co^{2+}) resulting in the production of highly reactive hydroxyl radicals. The reaction between superoxide and nitric oxide (NO) leads to the formation of peroxynitrite ($ONOO^-$) which controls signaling pathways via tyrosine nitration in target proteins [1].

Mutations in the ETC components promoting ROS formation have been found in numerous tumor types, thus proving an essential role of this mechanism in the modulation of tumor cell phenotype [14]. Mutations in mammalian NADH dehydrogenase [complex I containing 44 subunits, seven of which (ND1, ND2, ND3, ND4, ND4L, ND5, and ND6) are encoded by mitochondrial DNA and the rest – by the nuclear genome] can result in the elevated $O^{\bullet}_2^-$ production, this sustaining ROS-dependent oncogenesis pathways. In particular, mutations in the *ND2* gene contribute to oncogenesis and metastasis in breast, pancreatic, and oral cavity cancers and in head and neck carcinomas. Similar association were found for mutations in the *ND6* gene in lung cancer and in the *ND4* gene in acute myeloid leukemia and glioblastoma [14, 15]. Thus, mutations in *ND4* resulting in the decreased activity of complex I and elevated ROS generation promote proliferation of both hyper- and hypometastatic sublineages of Lewis lung carcinoma cells that can be suppressed by ROS scavengers [16]. Mutations resulting in the suppression of complex II in hereditary pheochromocytoma/paraganglioma and renal cell carcinoma elicit elevated ROS generation and reduce ATP synthesis by oxidative phosphorylation [17, 18].

Oncogenes can markedly stimulate ROS generation not only by the mitochondria, but also via activation of prooxidant enzymes. Thus, the K-RAS oncogene upregulates mitochondrial ROS level by triggering transformation of pancreatic acinar cells into pancreatic intraepithelial neoplasia, as well as activates NOX2 and NOX4 isoforms of NADPH oxidase, the main function of which is $O^{\bullet}_2^-$ generation [19, 20].

Similar to RAS, c-Myc induces metabolic rearrangement of tumor cells through the activation of glycolysis, mitochondrial biogenesis, and glutaminolysis [14]. It was shown that in glioma cell lines c-Myc controls a transcriptional program that promotes catabolism of glutamine as a carbon source to fuel the tricarboxylic acid cycle, thereby supporting ETC-coupled ROS production [21]. Upregulated *MYC* expression in the P493-6 B cells line results in the increased mitochondrial mass, oxygen consumption rate, and ETC activity, which promotes ROS production due to the increased electron flow through the ETC [22].

As noted above, NOX is an important source of ROS in tumor cells [23]. NOX isoforms belong to the NADPH oxidase family consisting of seven isoenzymes (NOX1-NOX5, DUOX1, DUOX2) that transfer electrons from $NADPH(H^+)$ through membranes to molecular oxygen with the generation of superoxide. Upregulated *NOX2* expression has been observed in breast, colon, stomach, and prostate cancers and in myelomonocytic leukemia [24]. Overexpression of *NOX4* gene was found in DU145, PC-3, and LNCaP prostate cancer cells [25]. Downregulation of *NOX2* expression results in a significant decrease in the activity of IKKε kinase (inhibitor of nuclear factor kappa-B kinase subunit epsilon), a key player in cell transformation, invasiveness, and development of resistance to chemotherapy [26]. NOX5 might have a dual role in tumor cells depending on its expression level and cellular context. It can either promote cell death via regulation of Ca^{2+} ion level and c-ABL kinase activity or activate tumor growth through the transcription factors STAT5A (signal transducer and activator

of transcription 5A) and CREB (cAMP response element-binding protein). Hence, NOX5 may account for a balance between proliferation and death of tumor cells in skin, breast, and lung cancers [27].

Cyclooxygenase (COX) and lipoxygenase (LOX) strongly contribute to the development of oxidative stress in tumor cells. Their activity results in the emergence of hydroperoxides of polyunsaturated fatty acids that are transformed into highly reactive bifunctional electrophiles hydroxynonenal and 4-oxo-nonenal, which form crosslinks in proteins and DNA [28]. It has been convincingly demonstrated that the LOX-catalyzed metabolism of arachidonic and linoleic acids is involved in the development of malignant neoplasms. The level of fatty acid hydroperoxides emerging due to the metabolism of arachidonic or linoleic acid affects regulation of cell growth and survival, angiogenesis, cell invasion, metastasis, and immunomodulation. Thus, 12-LOX promotes progression and metastasis of prostate cancer [29]. The activity of 5-LOX isoform plays an important role in the survival and proliferation of prostate cancer cells by maintaining high levels of the *MYC* gene expression [30].

Another significant source of ROS is oxidoreductase ERO1 (ER oxidoreductin 1), which is often overexpressed in many types of tumor cells. Along with protein disulfide isomerase (PDI), ERO1 plays a major role in the oxidative reactions during endoplasmic reticulum (ER) stress [31]. In the oxidized state, PDI acts as a disulfide donor; in the reduced state, it is capable of disulfide bond isomerization in its ligands. Reduced PDI formed by the interaction with misfolded proteins can be re-oxidized by ERO1 further oxidation of which requires FAD (cofactor) and results in the emergence of H_2O_2 that is transported from the ER through aquaporin 8 [32].

The Warburg effect associated with the activation of anaerobic glycolysis, contributes to the elevation of intracellular ROS levels in most tumor cells due to the lowered antioxidant status resulting from the reduction in the pyruvate level and NADH(H^+) formation followed by a decrease in the activity of mitochondrial transhydrogenase and NADPH(H^+) content. It is accompanied by a decrease in the level of glutathione (GSH), one of the major low-molecular-weight antioxidants, in the mitochondrial matrix due to the suppressed activity of NADPH(H^+)-dependent glutathione reductase, which reduces GSH from its oxidized form (GSSG) [33]. To some extent, activation of glycolysis is restricted by overexpression of M2 pyruvate kinase, an enzyme with low catalytic activity, resulting in the accumulation of phosphoenolpyruvate and partial suppression of triose phosphate isomerase followed by the activation of pentose phosphate pathway (PPP) [34]. As a result, an increase in the NADPH(H^+) level causes by the increase in the ROS generation due to the activation of NADPH oxidase isoforms that use NADPH(H^+) as a coenzyme. ROS and M2 pyruvate kinase form a negative feedback loop that maintains ROS levels. In turn, ROS-regulated transcription factor HIF-1α (hypoxia-inducible factor 1-alpha) alters expression of genes, including those involved in the Warburg effect and related pathways. Moreover, it was also found that PKM2 acts as a coactivator of HIF-1α, which, in turn, regulates c-Myc proto-oncogene controlling expression of genes associated with cell growth and proliferation [32, 34]. Alterations in the energy metabolism can be linked to the expression of genes regulated by p53, such as *SCO2*, *TIGAR*, and *PIG3* [35].

Elevation of ROS content in tumor cells can also be caused by a lack of regulation by tumor suppressors of the antioxidant enzyme genes. For example, inactivation of the *TP53* tumor suppressor gene downregulates expression of genes for superoxide dismutase (*SOD2*), glutathione peroxidase (*GPX1*), and sestrin (*SESN1*, *SESN2*) [5]. Post-translational modifications, e.g., SOD2 acetylation [36], can occur that cause antioxidant enzymes to become prooxidant.

Development of chronic oxidative stress, which in tumor cells is mostly triggered by high ROS levels, results in the adaptation of cell signaling and emergence of the so-called aggressive tumor cell phenotype. The rise in the ROS levels alters the activity of PTP (protein tyrosine phosphatase), PTEN (phosphatase and tensin homolog) and MAPK (mitogen-activated protein kinase), thereby promoting the MAPK/ERK (extracellular signal-regulated kinase) and PI3K (phosphatidylinositol 3-kinase/protein kinase B)/Akt (RAC-alpha serine/threonine-protein kinase) and PKD (protein kinase D)/NF-κB (nuclear factor κB) intracellular signaling cascades [6]. The proliferative advantages of high ROS levels and reduced risk of apoptosis are maintained by the activation of redox-dependent transcription factors in tumor cells [3]. According to the rheostat model of adaptation to the oxidative stress, the first line of protection against moderate ROS concentrations is provided by the activated nuclear factor erythroid 2-related factor 2 (Nrf2), whereas at higher ROS levels, it "switches on" AP-1 and NF-κB transcription factors, so that the further increase in the ROS generation activates apoptosis [3].

Cell response to extremely high ROS concentrations involves transcription factor p53, which controls cell cycle, aging, and apoptosis. It should be noted that during severe oxidative stress, excessive nuclear Nrf2 binds to the regulatory site within the *Klf9* (Krüppel-like factor 9) gene promoter and activates its expression. Klf9 binding to specific repressive sites in the genes of antioxidant enzyme and downregulates expression these proteins, thus causing cell damage by ROS [37].

An increase in the ROS content followed by alterations in the activity of antioxidant systems and cell redox status can be an important regulator of cellular homeostasis in the redox-dependent adaptation to chronic oxidative stress related to the initiation and progression

of malignant neoplasms, which should be taken into account while developing chemotherapy regimens.

THE ROLE OF NITRIC OXIDE IN CANCER CELLS. DEVELOPMENT OF NITROSATIVE STRESS

In mammals and humans, NO is synthesized mainly by NO synthase (NOS). NO is represented by three isoforms: constitutive NOS1 (neuronal, nNOS) and NOS3 (endothelial, eNOS) isoforms and inducible NOS2 (iNOS). The homology between the isoforms is ~50% [38]. The activity of constitutive NOS1 and NOS3 is regulated mostly by phosphorylation, S-nitrosylation, protein–protein interactions, and alterations in the calcium levels, which ensures a steady-state level of NO activity accounting for the regulation of tissue homeostasis [39]. In contrast, inducible NOS2 synthesizes large amounts of NO in response to various stimuli [39]. Recently, it was found that mtNOS, a homologue of NOS1 found in the matrix and on the inner membrane of mitochondria, significantly impacts the functioning of these organelles [40].

All NOS isoforms function as homodimers consisting of two subunits linked via a zinc ion tetrahedral coordination complex with four Cys residues (two from each monomer) located within the CysXXXXCys motif in the enzyme oxidative domain. Due to presence of this motif, NOS binds L-arginine (substrate) and its coenzyme tetrahydrobiopterin (BH_4) which facilitates dimerization and substrate binding and is necessary for the enzymatic reaction to proceed [39]. Each monomer contains two domains with the reductase and oxygenase activities at C- and N-termini of the molecule, respectively. The reductase domain binds NADPH(H^+), FAD, and FMN involved in the electron transfer to the oxygenase domain of the neighboring monomer. The oxygenase domain provides dimerization and ensures coordination of the zinc ion, BH_4, heme, and L-arginine. NO is synthesized in the reaction of L-arginine with oxygen resulting in the formation of L-citrulline via the intermediate product N^{ω}-hydroxy-L-arginine (Fig. 1).

In addition to its radical form ($NO^{\bullet}$), NO can exist as nitrosonium (NO^+) and nitroxide (NO^-) ions depending on the microenvironment. Reactions involving NO can be divided into direct and indirect [41, 42]. In direct reactions typically occurring at low NO concentration, NO interacts directly with a target molecule. In indirect reactions, which take place at much higher NO concentrations, NO reacts with oxygen or superoxide with the formation of reactive nitrogen species (RNS) that then react with biological targets.

Indirect interactions can result in nitrosative and oxidative stress [42]. In the case of oxidative stress characterized by high ROS (primarily $O^{\bullet}_2^-$) levels, NO reaction with superoxide produces peroxynitrite ($ONOO^-$) and nitrous oxide (NO_2), both being strong oxidizing agents. On the contrary, in the case of nitrosative stress characterized by high NO levels, N_2O_3 formed in the reaction between NO and O_2 (autoxidation) and $NO/O^{\bullet}_2^-$ reaction, acts as a mild oxidizer that mainly acts on nitrosated nucleophiles such as amines and thiols [43]. Usually, nitrosative stress involves interactions between nitrosonium ion (NO^+) and thiols, secondary amines, and hydroxyl groups. The balance between oxidation and nitrosation largely depends on the NO level.

The level of NOS expression and NO content in tumors vary significantly. NO might have either oncogenic or antitumor effect [44] depending on the its concentration, duration of cell exposure, and cell adaptation. At low concentrations (<200 nM), NO typically promotes cell survival and proliferation, while at higher concentrations (>400 nM), it facilitates cell cycle arrest, apoptosis, and senescence [45, 46]. High NO content results in the emergence of reactive NO derivatives (N_2O_3, $ONNO^-$, NO_2, NO_2^-) that cause DNA damage by suppressing the activity of DNA ligase, thus increasing the

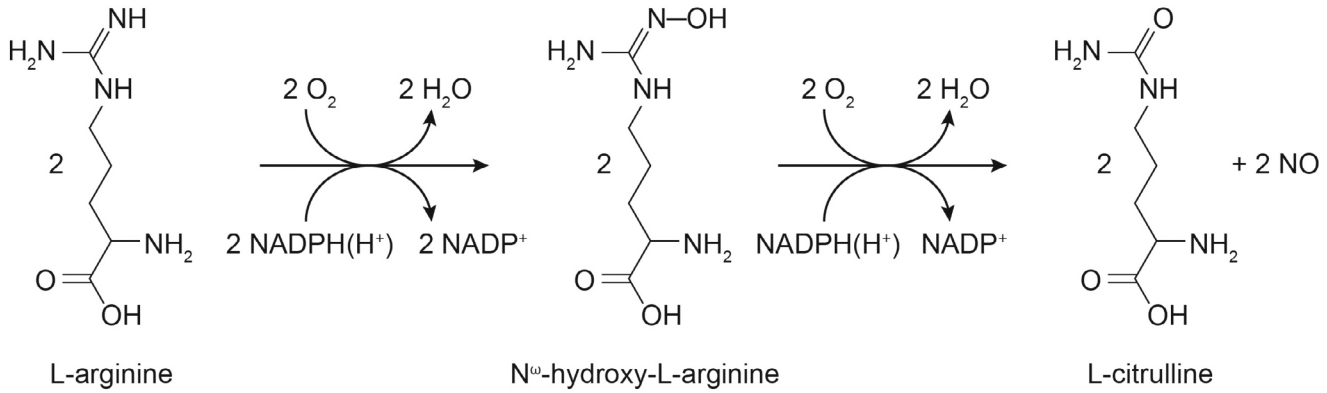

Fig. 1. NO synthesis catalyzed by NOS. At the first stage, L-arginine is hydroxylated into N^{ω}-hydroxy-L-arginine using a molecule of oxygen and two electrons transferred from NADPH(H^+). At the second stage, N^{ω}-hydroxy-L-arginine is converted into L-citrulline and NO using another oxygen molecule and an electron from NADPH(H^+).

number of DNA single-strand breaks. They can also inactivate DNA repair proteins and serve as a source of genotoxic nitrosamines [47, 48]. Moreover, NO-related cytotoxic effects are also associated with the NO ability to inhibit the activity of mitochondrial respiratory chain proteins by binding to FeS clusters [49].

Therefore, high NO concentrations, in particular, resulting from the NOS2 activity (which here acts as a potential cytostatic/cytotoxic factor of the immune system) can exert the antitumor effect, whereas chronically induced NOS2 promotes activation of tumor growth [50]. Moreover, low levels of NO synthesized by NOS1 and NOS3 accelerate tumor progression by modulating angiogenesis, apoptosis, cell cycle, invasion, and metastasis [51].

Aberrant *NOS1* expression was found in the brain tumors, lung tumors, and gliomas [46]. Low NO concentrations elicited by NOS1 trigger cell proliferation primarily via a cGMP-coupled mechanism due to the activity of cytosolic guanylate cyclase [52]. Ovarian carcinoma cell lines (OVCAR3, SKOV3, ES-2) are characterized by a high NOS1 expression resulting from the increased cell proliferation, invasion, and chemoresistance [46].

Recent studies have shown that NOS3 can serve as a negative prognosis marker in cancer, because its expression correlates with cell invasion, metastasis, angiogenesis, and resistance to therapy [53, 54]. *NOS3* is highly expressed in colorectal cancer cells with various phenotypes, including poorly differentiated adenocarcinomas, suggesting that NOS3 can be used as a novel biomarker for colorectal cancer with unfavorable prognosis [53].

It has been found in several clinical trials that *NOS2* expression is associated with a large variety of malignant neoplasms including breast, liver, cervical, ovarian, prostate, nasopharyngeal, lung, stomach, pancreatic, colon, and esophageal cancers, melanoma, and glioma [55]. High levels of NOS2 and inflammatory marker COX-2 have been observed in many tumor types, suggesting that upregulated *NOS2* expression correlates with the increase in the tumor metastatic potential and can be used as a negative prognostic marker in patients with breast, pancreatic, gastric, liver, and colon cancers, glioma, and melanoma. Transfection of *NOS2* into tumor cells resulted in more aggressive tumor phenotype *in vivo*, although *in vitro* proliferation of transfected cells was reduced [56]. *NOS2* expression is suppressed by p53, whereas high NO levels promote p53 stabilization due to its phosphorylation at Ser/Thr residues as well as intracellular p53 accumulation followed by the cell cycle arrest and apoptosis initiation [56, 57].

In oncogenesis, NO (at concentrations <200 nM) participates in several mechanisms involved in the tumor initiation and progression, including activation of DNA damage and inhibition of DNA repair, activation of oncogenes, suppression of apoptosis, promotion of gene mutations in chronic and malignant states (accumulation of mutant p53), and emergence of post-translational modifications (S-nitrosylation, Tyr nitration) that alter protein functions [54]. NO is involved in the signaling associated with malignant cell transformation (Wnt, Ras, ERK, Akt, cyclin D1, and mTOR pathways) and participates in angiogenesis, epithelial-mesenchymal transition, and metastasis [58]. Moreover, at low concentrations, NO suppresses apoptosis by inducing overexpression of the antiapoptotic proteins Bcl-2 and Bcl-xL, as well as via caspase inhibition [59]. On the contrary, long-term NO overproduction promotes apoptosis through the activation of caspases by release of the mitochondrial cytochrome *c* to the cytoplasm, which upregulates expression of *TP53* and *p38 MAPK* genes and suppresses expression of Bcl-2 family protein genes [60].

S-GLUTATHIONYLATION AND REGULATION OF REDOX-HOMEOSTASIS IN CANCER CELLS

Changes in the redox status of tumor cells are accompanied with the redox-dependent changes in the post-translational protein modifications largely due to the modification of Cys residues most sensitive to the ROS and RNS levels. Although the content of Cys residues in protein does not exceed 3% [9], they are among the amino acid residues most sensitive to oxidative modification. This provides redox-dependent regulation of protein functional activity, because Cys residues are involved in the formation of protein tertiary and quaternary structures and can be a part of the protein active site. In this regard, S-glutathionylation and S-nitrosylation at Cys residues are highly important redox-dependent post-translational proteins modifications.

The pK_a value of cysteine thiol group is determined by the structure of its microenvironment and can vary significantly (from 3.5 to >12). Usually, at physiological pH (7.0-7.4), the value of pKa is ~8.5. A decrease in pKa can be due to the stabilization of thiolate anion ($Pr\text{-}S^-$) by the electron-acceptor groups or a neighboring positive charge. On the contrary, the pKa value of thiolate increases in the presence of negatively charged groups or in the hydrophobic protein environment [10]. For instance, the pKa of the SH group decreases (usually to 5.0-7.0) in an immediate vicinity of basic amino acid residues (His, Lys, and Arg), whereas at physiological pH, the sulfhydryl groups dissociate. The formed thiolate anions are efficient nucleophiles, whose reactivity toward electrophilic targets increases dramatically [61].

S-glutathionylation (binding of GSH) is a reversible post-translational modification of SH groups in Cys residues that increases both protein molecular weight and negative charge (due to the addition of a Glu residue) and results not only in the protection of Cys residues from oxidative damage, but also in a redox-dependent alteration in the protein conformation and functional activity [10].

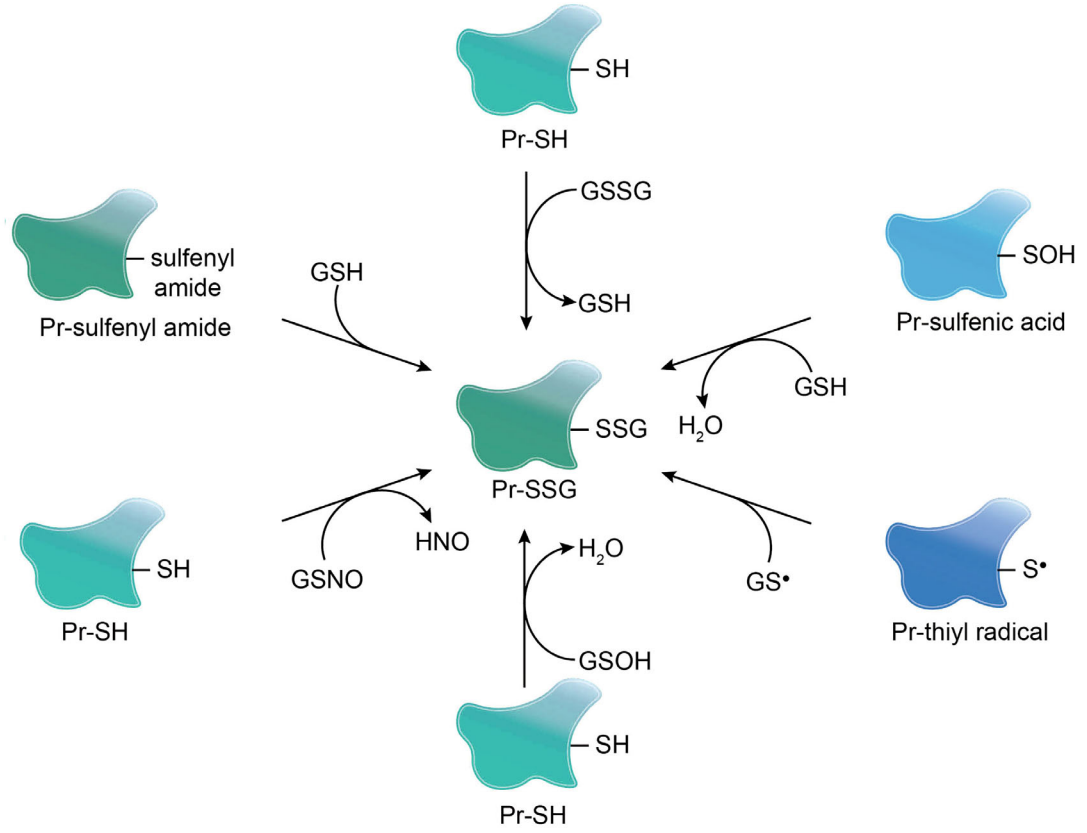

Fig. 2. Mechanisms of spontaneous S-glutathionylation.

A pool of protein thiols exists in a dynamic equilibrium with the glutathione pool and can serve as a buffer for GSH regeneration. Protein S-glutathionylation occurs either spontaneously or enzymatically [11].

Non-enzymatic reactions of S-glutathionylation proceed by the thiol-disulfide exchange between protein thiol (Pr-SH) and oxidized glutathione (GSSG):

$$\text{Pr-SH} + \text{GSSG} \rightarrow \text{Pr-SSG} + \text{GSH}.$$

The reaction equilibrium constant K_{mix} is expressed as the [Pr-SSG]×[GSH]/[Pr-SH]×[GSSG] ratio, so that the proportion of glutathionylated proteins ([Pr-SSG] : [Pr-SH]) strongly depends on the local [GSH] : [GSSG] ratio [62]. Protein glutathionylation by about 50% (K_{mix} ~ 1; [Pr-SSG] : [Pr-SH] = 1) can occur upon a profound decrease in this ratio (from 100 : 1 to 1 : 1). Generation of large amounts of Pr-SSG requires a significant shift in the GSH/GSSG ratio towards GSSG, which is unlikely under normal physiological conditions (except in the case of severe oxidative stress). This means that the above mechanisms cannot serve as a dominating glutathionylation pathway [63]. An increase in the GSH/GSSG ratio has been found in many types of tumor cells, where it results from the induction of GSH synthesis and decrease in the GSSG content caused by the upregulation of NADPH(H^+) formation caused by the PPP activation [64] (adaptive response to oxidative stress). Also, low second-order rate constants for the reactions between various thiols and GSSG ($4.9\times10^2 - 1.2\times10^4$ $M^{-1}s^{-1}$) [65] suggest that thiol-disulfide exchange is an unlikely event.

Protein glutathionylation can be a result of protein interaction with sulfenic acid (GSOH), which is an oxidized form of GSH:

$$\text{Pr-SH} + \text{GSOH} \rightarrow \text{Pr-SSG} + H_2O.$$

Moreover, thiol-disulfide exchange can occur between Pr-SH and S-glutathionylated protein (Pr'-SSG):

$$\text{Pr-SH} + \text{Pr'-SSG} \rightarrow \text{Pr-SSG} + \text{Pr'-SH}.$$

S-glutathionylation can proceed via interaction between Pr-SH or GSH and oxidized derivative of protein cysteine residue, e.g., sulfenic acid (-SOH), thiyl radical (-S$^{\bullet}$), S-nitrosyl (-SNO), thiosulfinate (-S(O)SR), or sulfenyl amide (cyclic-S-N-CO-) (Fig. 2).

Oxidation of Pr-SH by ROS (e.g., H_2O_2) results in the production of sulfenic acid (Pr-SOH) that rapidly reacts with GSH with the formation of Pr-SSG:

$$\text{Pr-SOH} + \text{GSH} \rightarrow \text{Pr-SSG} + H_2O.$$

Oxidation of protein SH groups by micromolar concentrations of H_2O_2 proceeds rapidly. Sulfenic acid is unstable and can be further oxidized to sulfinic (Pr-SO_2H)

and finally to sulfonic (Pr-SO_3H) acid, which usually results in irreversible protein deactivation. Hence, S-glutathionylation of sulfenic acid can prevent further protein oxidation at cysteine residues [66]. Under physiological conditions, the intracellular level of H_2O_2 is maintained within a submicromolar range (10^{-9}-10^{-7} M) [67], therefore, spontaneous S-glutathionylation *in vivo* should proceed very slowly. However, oxidative stress developing in tumor cells is accompanied by the elevated H_2O_2 production. It should also be noted that the emergence of a sulfene group in proteins is a rare event due to the high activity of GPx1 and peroxiredoxin (Prx) that have higher second-order rate constants for the H_2O_2 reduction compared to the interaction between H_2O_2 and protein Cys residues [68]. At the same time, local inactivation of peroxidase leading to the high H_2O_2 levels can promote thiol sulfenylation.

One-electron oxidation of protein thiol or GSH, e.g., with hydroxyl radical ($^{\bullet}OH$), results in the formation of protein thiyl radical (Pr-S$^{\bullet}$) or glutathione thiyl radical (GS$^{\bullet}$) capable of generating glutathionylated radical intermediate (Pr-SSG$^{\bullet-}$) upon interaction with GSH or Pr-SH, respectively:

$$\text{Pr-S}^{\bullet} + \text{GSH} \rightarrow \text{Pr-SSG}^{\bullet-} + \text{H}^{+},$$
$$\text{Pr-SH} + \text{GS}^{\bullet} \rightarrow \text{Pr-SSG}^{\bullet-} + \text{H}^{+}.$$

Further interaction between Pr-SSG$^{\bullet-}$ and molecular oxygen results in the formation of superoxide and Pr-SSG. Thiyl radicals are generated under oxidative or nitrosative stress and represent the shortest-lived activated thiols [69]:

$$\text{Pr-S}^{\bullet} + \text{GS}^{\bullet} \rightarrow \text{Pr-SSG}.$$

An extremely rare mechanism for direct Pr-SSG generation is interaction of Pr-S$^{\bullet}$ and GS$^{\bullet}$ radicals:

$$\text{Pr-S}^{\bullet} + \text{GS}^{\bullet} \rightarrow \text{Pr-SSG}.$$

NO can elicit spontaneous S-glutathionylation. Although NO is a weak thiol oxidant, but both S-glutathionylation and S-nitrosylation (see below) can be promoted by secondary RNS formation. For instance, the anti tumor agent PABA/NO (O^2-[2,4-dinitro-5-(N-methyl-N-4-carboxyphenylamino)phenyl]1-(N,N-dimethylamino)diazen-1-ium-1, 2-diolate) triggered a dose-dependent increase in the intracellular NO levels in SKOV3 adenocarcinoma and HL60 promyelocytic leukemia cells followed by the development of nitrosative stress along with activation of S-glutathionylation of proteins, including β-lactate dehydrogenase, Rho GDP dissociation inhibitor beta, ATP synthase, elongation factor 2, PDI, nucleophosmin 1, chaperonin, actin, protein tyrosine phosphatase 1B, and glucosidase II [70]. It should be mentioned that interaction of nitrosoglutathione (GSNO) with thiols can also result in S-glutathionylation [71]:

$$\text{PrSH} + \text{GSNO} \rightarrow \text{PrSSG} + \text{HNO}.$$

It was shown that for tyrosine phosphatase 1B that generation of Pr-SSG as an intermediate can occur upon reduction of sulfenylamide, a cyclic structure with the -S-N-CO- fragment, that is formed with the involvement of the enzyme active site Cys215 residue under strong oxidative stress [72].

Reactions of non-enzymatic S-glutathionylation are non-specific and typically takes place under stress conditions. However, the rate and extent of this process increase significantly with the participation of enzymes. The leading role belongs to glutathione S-transferase P1-1 (GSTP1-1), whose expression is a highly prognostic marker in a wide range of tumors [73, 74]. High *GSTP1* expression is associated with the development of drug resistance of tumor cells that involves redox-dependent mechanism, inhibition of apoptosis, and suppression of cytotoxicity of anticancer drugs (doxorubicin, cisplatin) [75].

Peroxiredoxins (Prxs) is a family of thiol-specific peroxidase enzymes, whose expression is upregulated in various types of tumors [76]. Prxs are validated targets for the GSTP1-mediated reversible glutathionylation [77]. The catalytic Cys residue in Prx is prone to oxidation and loses its peroxidase activity upon substrate (H_2O_2) reduction. GSTP1 promotes glutathionylation of the oxidized Cys residue, thereby restoring Prx activity. The substrates for glutathionylation are two major Prx subclasses – 1-Cys Prxs (also known as Prx6) and 2-Cys Prxs [77]. Catalytically active Cys47 residue located inside the hydrophobic core of Prx6 and acts on both H_2O_2 and phospholipid hydroperoxides. After peroxide reduction, oxidized Cys47 acquires an access to the GSH-loaded GSTP1 for Prx6 reactivation [78]. Activation of Prx6 occurs upon assembly of a heterodimer with GSTP1-1, which promotes Prx6 glutathionylation at Cys47 resulting in the conformational changes within the heterodimer that ensure formation of a disulfide bond between Cys47 in GSTP1-1 and Cys47 in Prx6, followed by disulfide reduction with GSH and Prx6 Cys47 regeneration. There is strong evidence that different GSTP1 polymorphic forms can mediate Prx6 activation via other mechanisms, thereby affecting its response to the ROS levels. Compared to GSTP1-1B or GSTP11D, GSTP1-1A (the most common polymorphic GSTP1 isoform) has a higher affinity for Prx6 [79]. Moreover, *GSTP1A*-transfected breast cancer cells have a markedly higher peroxidase activity vs. *GSTP1B*-transfected cells [79], which can be related to changes in the distance between oxidized Cys47 and activated GSH bound to the GSTP1 molecule in different protein polymorphic forms.

Interestingly, GSTP1-1 is a redox-dependent enzyme. Its inhibition results in the glutathionylation at Cys47 and Cys101 residues, which prevents its binding to JNK1 (c-Jun N-terminal kinase 1) [73]. Direct protein–

protein interactions between GSTP1-1 and JNK1 inhibit the activity of this kinase and suppress JNK-induced stress response and apoptosis. Hence, by causing GSTP1-1 inactivation, oxidative stress activates JNK.

Upregulation of the *GSTP1* expression in tumor cells can promote oncogenesis via glutathionylation and inhibition of tumor suppressor p53 which plays a significant role in DNA repair, cell cycle control, cell differentiation, and suppression of tumor growth [80]. For instance, high content of glutathionylated p53 was found in prostate adenocarcinoma and melanoma cells [81], although such increase in p53gluthionylation could also be facilitated by oxidative stress and DNA damage.

S-glutathionylation promotes activation of the Nrf2 transcription factor that controls expression of genes encoding enzymes of the detoxification and antioxidant defense systems [82, 83]. In the case of pronounced oxidative stress, excessive Nrf2 accumulated in the nucleus binds to the regulatory region in the *Klf9* gene promoter and activates its expression, resulting in the Klf9-driven downregulation of expression of antioxidant enzyme and cell damage due to the elevated ROS levels [37]. In the norm, Nrf2 is bound to the cytosolic Keap-1 (Kelch-like ECH-associated protein 1), which limits the activity of Nrf2. Inhibition of Nrf2 is a plausible strategy of effective cancer treatment. However, recent studies showed that not only Nrf2 inhibitors, but also its activators can induce apoptosis in tumor cells [84, 85], thus suggesting a new approach to the antitumor therapy. Despite a large number of available Nrf2 activators, only few of them promote cell apoptosis. The search for drugs activating Nrf2 and facilitating cell apoptosis has shaped a number of novel concepts in the cancer therapy, e.g., activation of S-glutathionylation. For instance, immortalized HeLa cervical carcinoma cells were found to be depleted of GSH after exposure to 2-(7-(diethylamino)-2-oxo-2H-chromen-3-yl)cyclohexa-2,5-diene-1,4-dione. This resulted in the increased S-glutathionylation of Keap-1 followed by the activation of Nrf2 nuclear translocation, which resulted in the activation of *p53* transcription, decrease in the intracellular Bcl2 content and elevation in the Bax content, and promotion of tumor cell apoptosis [86].

An important role in tumor proliferation, invasion, oncogenesis, and metastasis is played by the isoforms of protein kinase C (PKC) [87]. Overexpression of the PKC gene is associated with tumor growth due to the synergistical activation of several signaling pathways controlling cell survival and proliferation, including NF-κB, Stat3 (signal transducer and activator of transcription 3), PI3K/Akt, and ERK pathways [88, 89]. PKC isoforms (α, β, γ, ε, ζ) are inactivated by oxidative S-glutathionylation, which was demonstrated using diamide and GSH [90].

S-glutathionylation targets an extremely broad range of proteins involved in all aspects of tumor cell activity. Thus, glutathionylation inhibits energy metabolism enzymes, including NADH dehydrogenase, cytochrome oxidase, ATPase, pyruvate dehydrogenase, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) [74]. By inhibiting pyruvate kinase M2 and 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase bifunctional enzyme that generates fructose 2,6-bisphosphate, S-glutathionylation promotes PPP and facilitates formation of NADPH(H^+), which increases the GSH level by increasing the activity of glutathione reductase, an enzyme that reduces GSSG using NADPH(H^+) as a coenzyme [91, 92]. S-glutathionylation modulates intracellular signaling pathways by altering the activity of proteins, in particular, Akt, MEKK1 (mitogen-activated protein kinase kinase 1), protein tyrosine phosphatase 1B, and Ras proteins. S-glutathionylation of H-Ras at Cys118 modulates its intrinsic GTPase activity, thus eliciting activation of the downstream p38 and Akt [93]. Both caspase-3 (an important mediator of apoptosis) and its precursor procaspase-3 undergo S-glutathionylation. Moreover, S-glutathionylation of procaspase-3 inhibits its capacity for proteolytic activation. The p17 subunit of caspase-3 is S-glutathionylated at Cys135 located in the active site, which affects its access to the substrate and suppresses enzyme activity [94].

S-glutathionylation is a reversible post-translational modification, whereas deglutathionylation, as a rule, is catalyzed by the enzymes and proceeds under much tighter control. Glutaredoxin isoforms (Grx1 and Grx2) are among the most effective Pr-SSG-reducing enzymes. Depending on the GSH/GSSG ratio, Grx either facilitates deglutathionylation or, instead, promotes S-glutathionylation (Fig. 3) [95]. When the GSH/GSSG ratio decreases and the H_2O_2 content rises, Grx2 functions as a glutathionylation enzyme (e.g., toward respiratory complex I), whereas at a high GSH/GSSG ratio and low H_2O_2 concentration, it exhibits deglutathionylation activity. The interplay between Grx-catalyzed S-glutathionylation/deglutathionylation and cellular redox status may represent an adaptation ensuring that S-glutathionylation reactions will not be reversed as long as oxidative stress persists [96, 97].

Grx-catalyzed S-glutathionylation proceeds in several stages. First, the disulfide bond in GSSG undergoes a nucleophilic attack by the Grx-S^- thiolate anion resulting in the generation of glutathionylated intermediate Grx-SSG. Activated cationic radical $[GS^{\cdot}]^+$ is transferred from Grx-SSG to the target protein with the formation of Pr-SSG, while Grx is returned to the catalytically active state. This process can also result in the reversible generation of Grx-S_2 from Grx-SSG [96]. During deglutathionylation, the protein (Pr-SSG) is attacked by enzyme's thiolate anion (Grx-S^-) with the generation of the covalent intermediate (Grx-SSG) and release of the reduced protein (Pr-SH). Next, Grx-SSG is reduced by GSH resulting in GSSG formation (this stage determines the rate of deglutathionylation).

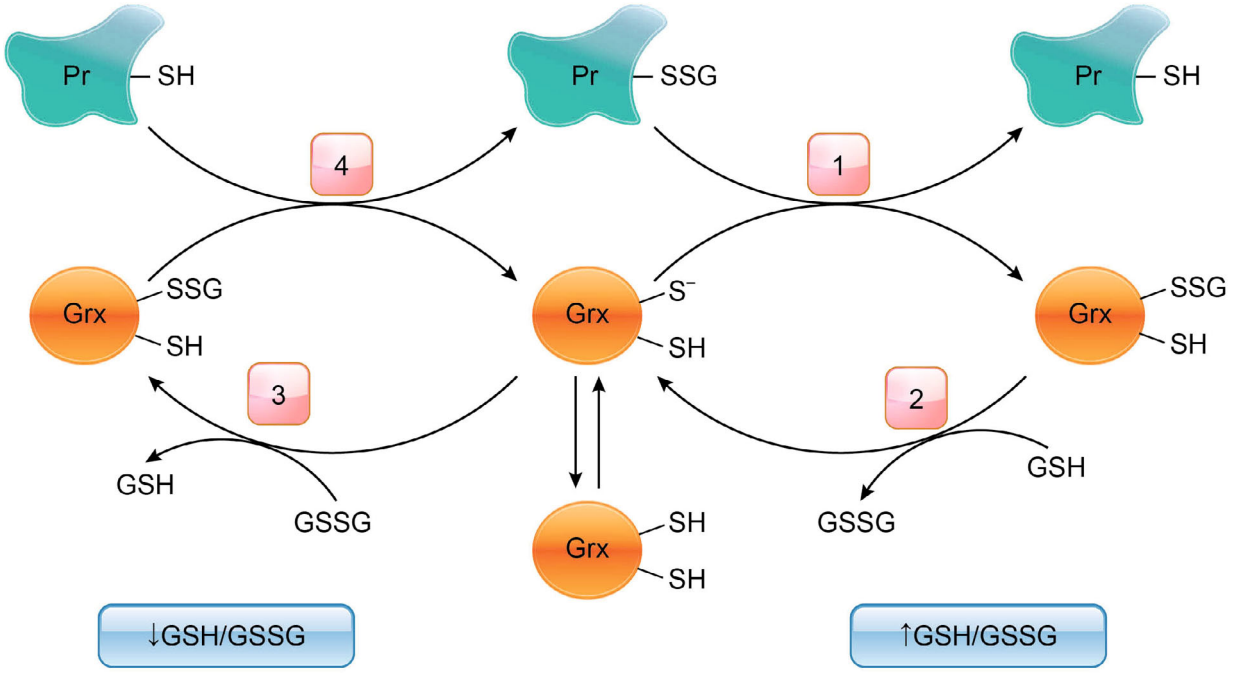

Fig. 3. GSH/GSSH ratio-driven catalysis by Grx. At a high GSH/GSSG ratio, Grx catalyzes protein deglutathionylation: (1) glutathionylated protein (Pr-SSG) is attacked by the enzyme's thiolate anion (Grx-S-) with the formation of reduced protein (Pr-SH) and intermediate (Grx-SSG) that is (2) reduced by GSH with the generation of active Grx-SH and GSSG. At a low GSH/GSSG ratio, Grx catalyzes S-glutathionylation: S-glutathionylated Grx (Grx-SSG) formed in the reaction with GSSG (3) reacts with the protein with the formation of S-glutathionylated protein (Pr-SSG) (4).

Sulfiredoxin (Srx) plays a pivotal role in deglutathionylation of some proteins (PRX1, actin, PTP1B), presumably, due to its higher affinity for these proteins compared to Grx. Unlike Grx, Srx is not inactivated by oxidative stress, as follows from its capacity to lower the level of S-glutathionylation upon the increase in the ROS content [98]. The mechanism of Srx-catalyzed deglutathionylation resembles that of Grx-mediated catalysis and includes generation of the Srx-SSG intermediate glutathionylated at the conserved Cys99 residue [99]. Srx-catalyzed deglutathionylation appears to have a broad substrate specificity. HEK293 cells transfected with *Srx* demonstrated a decreased total content of S-glutathionylated proteins generated under nitrosative stress after exposure to the NO donor PABA/NO [98].

It was shown that in T47D breast cancer epithelial cells, deglutathionylation can be catalyzed by the glutathione transferase isoform GSTO1-1 [100, 101]. GSTO1 is structurally similar to Grx, as it contains the Trx (thioredoxin)-like fold and GSH-binding site that can form a disulfide bond with GSH through the conserved Cys32 residue of the active site. In contrast, other GST isoforms (GSTA, GSTM, GSTP, GSTT, GSTS and GSTZ) have Tyr or Ser as catalytic residues. Similar to Grx, GSTO1-1 catalyzes deglutathionylation in two steps: Cys32 of the GSTO1-1 active site interacts with Pr-SSG resulting in the generation of reduced Pr-SH and mixed disulfide GSTO1-1-Cys32-SG that is further deglutathionylated with GSH with the formation of GSSG and functionally active GSTO1-1.

In addition, proteins can be deglutathionylated by PDIs, which are activated in various types of cancer cells (renal, lung and prostate cancers, melanoma). Overexpression of the PDI gene often correlates with metastasis, tumor invasiveness, chemoresistance, and lower survival of cancer patients [102, 103]. However, the importance of PDIs in cancer has not been fully elucidated, because PDIs mainly function by exchanging disulfide bonds with the target proteins. PDIs reside in the ER, where they catalyze oxidation of *de novo* synthesized proteins and take part in isomerization of proteins with improperly formed disulfide bonds to restore their native folding. Moreover, PDIs can be secreted or translocated to the cell surface to maintain proteins in a reduced state.

S-NITROSYLATION. REGULATION OF REDOX HOMEOSTASIS IN CANCER CELLS

S-nitrosylation is another reversible post-translational modification of protein thiol groups [4].

Typically, S-nitrosylation of cysteine thiol groups proceeds via one-electron oxidation that involves O_2 or a transition metal ion (e.g., iron or copper) [104], while direct NO addition involving a thiyl radical occurs rather rarely:

$$NO + Pr\text{-}S^{\cdot} \rightarrow Pr\text{-}SNO.$$

Interaction between NO and O_2 generates a set of oxides with a higher nitrogen oxidation state (the so-called

autoxidation), among which N_2O_3 is considered as the major nitrosylating agent contributing to the formation of protein nitrosothiols and nitrite ion. The rate of this reaction increases dramatically in a hydrophobic environment, e.g., in the membranes, where NOS3 resides [104]:

$$2NO + O_2 \rightarrow 2NO_2,$$
$$NO_2 + NO \rightarrow N_2O_3,$$
$$N_2O_3 + Pr\text{-}SH \rightarrow Pr\text{-}SNO + H^+ + NO_2^-.$$

One of the potential mechanisms of GSNO synthesis is interaction between GSH and N_2O_3:

$$N_2O_3 + GSH \rightarrow GSNO + H^+ + NO_2^-.$$

Moreover, NO_2 can interact with a thiol group with the formation of thiyl radical which further reacts with NO:

$$NO_2 + Pr\text{-}SH \rightarrow Pr\text{-}S^{\cdot} + H^+ + NO_2^-,$$
$$NO + Pr\text{-}S^{\cdot} \rightarrow Pr\text{-}SNO.$$

Both mechanisms are limited by the NO_2 formation rate, as well as by the NO availability.

S-nitrosylation can be catalyzed by transition metal ions (Fe^{3+} or Cu^{2+}) through the one-electron oxidation of NO and formation of nitrosonium (NO^+) that can nitrosylate a neighboring thiol:

$$Me^{(n+1)} + NO \rightarrow Me^n\text{-}NO^+,$$
$$Me^n\text{-}NO^+ + Pr\text{-}SH \rightarrow Pr\text{-}SNO + Me^n + H^+.$$

This mechanism was found for hemoglobin autonitrosylation and GSNO formation involving ceruloplasmin and cytochrome *c* [104].

It should be mentioned that S-nitrosylation can occur solely at specific Cys residues. The target Cys must be located: (i) in an immediate vicinity to the NO source, (ii) within the protein I/L-X-C-X_2-D/E motif specifically recognized by NOS, (iii) in a highly hydrophobic region created by the tertiary protein structure or membranes, (iv) in a proper microenvironment allowing thiolate anion formation [105].

According to these requirements, NO-generating NOS isoforms represent the primary targets for S-nitrosylation [106]. For instance, S-nitrosylation of NOS3 inhibits dimerization of this enzyme and, therefore, its activation. The latter suggests that S-nitrosylation of constitutive NOS isoforms may serve as a "self-shutdown" mechanism [106]. The I/L-X-C-X_2-D/E motif should be located within an α-helix and to create a large surface area to increase NO availability [107]. Moreover, the target Cys thiol group within the S-nitrosylation motif should electrostatically interact with neighboring charged residues (<6 Å), which increases its nucleophilicity and promotes formation of thiolate anion. Protein S-nitrosylation preferentially occurs at Cys residues embedded in the hydrophobic regions, to which NO and molecular oxygen can easily penetrate and where the reaction rate is accelerated 30-300 times [108]. The target Cys residue should be surrounded within a distance of 8 Å by a small number of bulky, sterically hindered amino acid residues (Phe, Tyr, Arg, Leu) that would prevent its interaction with NO [109].

Although S-nitrosylation targets only certain Cys residues, this selectivity can be partially abolished by transnitrosylation, during which a low-molecular-weight nitrosothiol (e.g., GSNO) or a protein nitrosylated at Cys residue or bearing a nitrosylated metal ion (e.g., in the heme) can interact with a protein undergoing S-nitrosylation and transfer nitroso group (ON-) to the Cys residue of this protein:

$$PrS^- + Pr'\text{-}SNO \leftrightarrow PrSNO + Pr'S^-,$$
$$PrS^- + Me\text{-}SNO \leftrightarrow PrSNO + Me.$$

This reaction facilitates sequential NO transfer from the site of its synthesis, including the transfer between different subcellular compartments [110]. Transnitrosylation takes place when two proteins interact directly which each other and possess appropriate redox potentials to ensure electron transfer followed by the NO transfer [110]. It is assumed that physical interaction between the two proteins induces conformational change allowing a recipient thiol to form thiolate anion that would attack the donor's nitrosyl group [110].

GSNO is the most common S-nitrosothiol and the main endogenous NO donor. It is formed in the mitochondria when the nitrosyl group is transferred from the iron of the cytochrome *c* heme to GSH resulting in transnitrosylation of various proteins playing essential role in cell signaling, such as NF-κB, STAT3, AKT, and EGFR [111, 112]. GSNO-mediated transnitrosylation of IκB kinase and NF-κB p65 and p50 subunits inhibits its NF-κB activation associated with the tumor development [113]. Another protein transnitrosylated with GSNO is STAT3 (protein promoting cell survival and proliferation), resulting in the suppression of STAT3 phosphorylation necessary for its activation [114]. GSNO induces apoptosis in lung cancer cells by nitrosylating Prx2 at Cys51 and Cys172, thus impairing its dimerization and reducing its antioxidant activity followed by accumulation of endogenous H_2O_2 and AMPK activation. Activated AMPK phosphorylates SIRT1, inhibiting its deacetylation activity toward p53 and FOXO1, as was demonstrated in A549 lung adenocarcinoma cells and NCI-H1299 non-small cell lung cancer cells, respectively [115]. The antitumor effect of GSNO observed in preclinical studies resulted in the suppression of tumor growth and potentiated the efficacy of radiotherapy [112]. Comparison of the antitumor activity of GSNO vs. bi- and mononuclear dinitrosyl iron complexes with thiol-containing ligands in solid tumors transplanted

in mice demonstrated that dinitrosyl iron complexes with GSH displayed a peak activity (inhibition of tumor cell growth by 90%) due to their ability to act as nitrosonium donors [116].

Another protein playing a prominent role in transnitrosylation is GAPDH, a glycolytic enzyme that catalyzes conversion of glyceraldehyde 3-phosphate to D-glycerate 1,3-bisphosphate. As a transnitrosylase, GAPDH is involved in the regulation of gene transcription and apoptosis [117, 118]. When S-nitrosylated at the active site Cys150 residue, it is translocated from the cytosol to the nucleus with a help of S100A8/A9 protein, which, in turn, undergoes S-nitrosylation due to the increase in the NOS2 activity during the stress response [107, 118]. S-nitrosylation of GAPDH enables its interaction with E3 Siah1 ubiquitin ligase resulting in the translocation of the GAPDH–Siah1 complex to the nucleus, where Siah1 mediates ubiquitination and degradation of nuclear proteins followed by apoptosis activation. GAPDH can also transnitrosylate proteins involved in DNA transcription and repair, including deacetylases sirtuin 1 (SIRT1) and histone deacetylase 2 (HDAC2), which are inhibited by S-nitrosylation. On the contrary, DNA-dependent protein kinase involved in DNA repair is activated by S-nitrosylation [118]. Moreover, by forming a complex with p53, GAPDH participates in the activation of p53-mediated apoptosis [117]. During the stress response, GAPDH is translocated to the mitochondria, where it transnitrosylates mitochondrial proteins such as Hsp60, acetyl-CoA acetyltransferase (ACAT1), and voltage-dependent anion channel 1 (VDAC1) [119]. This transnitrosylation regulates mitochondrial membrane permeability and functional activity, as well as cell death [120]. In tumor cells, GAPDH-mediated transnitrosylation of nuclear proteins is disturbed resulting in the impairment of stress-dependent apoptosis [118], e.g., because of Siah1 suppression, which facilitates GAPDH nuclear translocation [114]. Siah1 expression is directly controlled by the tumor suppressor p53 downregulated in many cancer types [121].

Calcium- and zinc-binding proteins S100A8 and S100A9 can also act as transnitrosylases. These proteins are involved in the regulation of inflammation and immune response and form the calprotectin heterodimer (S100A8/A9) [122]. When S-nitrosylated S100A8/A9 acquire the anti-inflammatory properties and inhibit mast cell activation and interaction between leukocytes and endothelium [123]. Moreover, in the case of NOS2 induction by S-nitrosylated inflammatory stimuli, S100A8/A9 transnitrosylate other proteins by facilitating the transfer of nitrosyl group from NOS2 to target proteins [107]. Currently, more than 100 proteins transnitrosylated by S100A8/A9 have been identified in cells and microvasculature [107], including GAPDH (see above), hemoglobin, and cytoskeletal proteins that bind cortical actin to the plasma membrane (ezrin, moesin) and vimentin (major intermediate filament protein in mesenchymal cells and metastatic tumor cells) [107]. It is believed that S-nitrosylation elicits conformational changes affecting protein stability and protein–protein interactions [124].

The content of S-nitrosylated cellular proteins is determined by the cell redox status and denitrosylation activity. Activation of antioxidant systems elevating cell reduction potential can prevent S-nitrosylation, whereas their suppression promotes S-nitrosylation [125]. The level of protein S-nitrosylation in cells is also controlled by the balance between S-nitrosylation and denitrosylation. Unlike S-nitrosylation, which is usually a non-enzymatic reaction (except in prokaryotes), denitrosylation can be both non-enzymatic or catalyzed reaction [12]. The cleavage of S-nitrosyl group can occur spontaneously in the case of exposure to reducing agents (ascorbate, GSH), metal ions (Cu^{2+}), UV radiation, ROS, or nucleophiles [12] or can be catalyzed by denitrosylases that remove the nitrosyl group from S-nitrosothiols.

S-nitrosoglutathione reductase (GSNOR) is one of the main denitrosylases that decomposes GSNO in the irreversible NADH-dependent reaction in the presence of GSH with the generation of products that are ultimately determined by the GSH/GSSG ratio. Thus, high GSH content favors production of GSSG and hydroxylamine, whereas low GSH/GSSG ratio is associated with the generation of glutathione sulfinic acid and ammonia. Therefore, cellular redox potential determined by the levels of NADH(H^+) and GSH is a critical parameter regulating formation of S-denitrosylation products [126]. The content of intracellular S-nitrosylated proteins is governed by GSNOR that eliminates GSNO (the most active player in transnitrosylation), thus controlling cellular Pr-SNO pool. Excessive *GSNOR* expression or defects in the *GSNOR* gene can disturb this balance, which would affect NO-dependent signaling and contributing to the emergence of some pathologies including tumor formation.

Downregulation of *GSNOR* expression is associated with the onset and progression of hepatocellular carcinoma and breast cancer [127, 128]. Thus, the lack of *GSNOR* expression presumably resulting from *GSNOR* chromosomal deletion was found in ~50% cases of hepatocellular carcinoma [127]. *De novo* hepatocarcinogenesis after tumor resection and poor prognosis in patients with hepatocellular carcinoma are often associated with reduced *GSNOR* expression and *NOS2* overexpression [129]. Intraperitoneal administration of diethylnitrosamine or lipopolysaccharide in mice with ablated *GSNOR* gene resulted in S-nitrosylation, ubiquitination, and proteasomal degradation of angiotensinogen, a protein essential for protection against diethylnitrosamine-induced hepatocellular carcinoma [127]. Moreover, some cases of breast cancer are also characterized by downregulation of *GSNOR* expression. In particular, HER2 (human

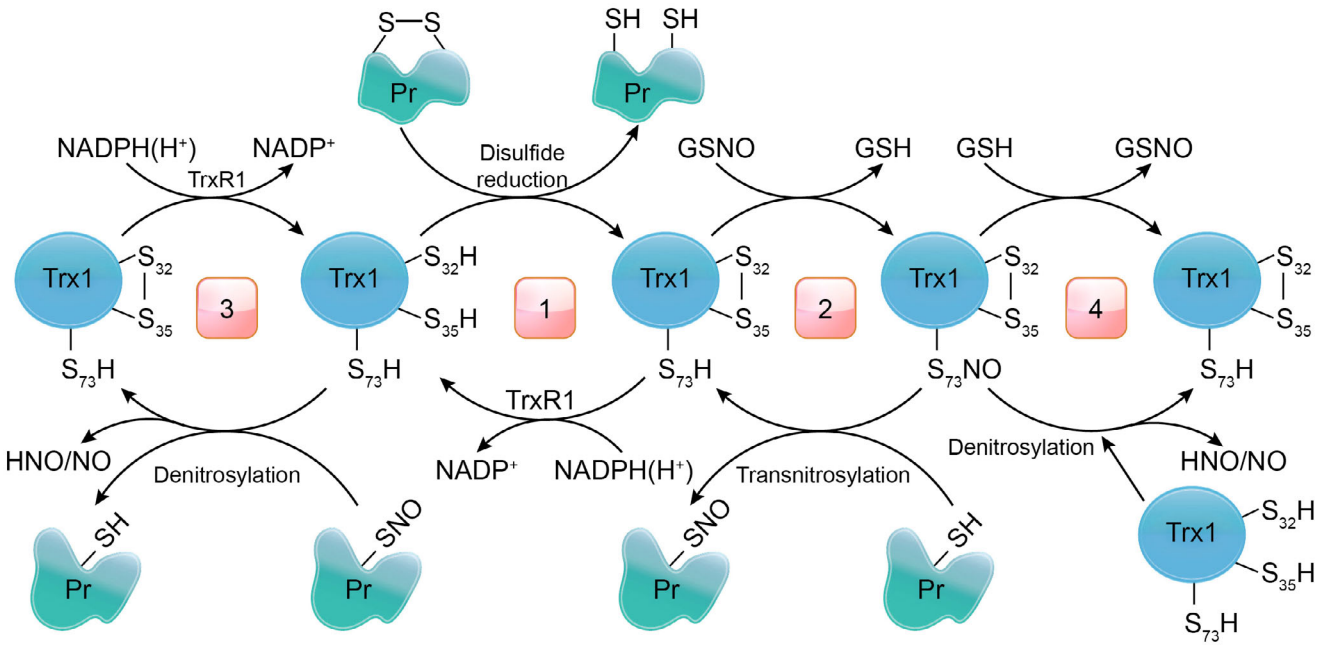

Fig. 4. Trx1/TrxR1-mediated transnitrosylation and denitrosylation. 1) Trx1-$(SH)_2$ reduces disulfide bonds in target proteins resulting in its oxidation into Trx1-S_2, which is then reduced by TrxR and NADPH(H^+). 2) At high ROS level, Trx1-S_2 is nitrosylated at Cys73 outside its active site into SNO-Trx1-S_2, which transnitrosylates target proteins. 3) Trx1-$(SH)_2$ denitrosylates target proteins with the formation of HNO or NO. 4) Denitrosylation of SNO-Trx1-S_2 can occur in the presence of Trx1-$(SH)_2$ or GSH.

epidermal growth factor receptor 2)-positive breast cancer, an aggressive type of cancer with unfavorable prognosis, is characterized by low *GSNOR* expression and increase in the pool of S-nitrosylated proteins involved in apoptosis, while upregulated *GSNOR* expression correlates with a higher patient survival rate [128].

In HepG2 hepatocellular carcinoma, 769P renal carcinoma, RD embryonic rhabdomyosarcoma, and MCF7 breast adenocarcinoma cells, GSNOR deficiency promoted S-nitrosylation of FAK1 (focal adhesion kinase 1) at Cys658, which potentiated its autophosphorylation activity and sustained tumor cell motility and growth [130]. FAK1, also known as protein tyrosine kinase 2 (PTK2), plays an important role in the metastatic transformation of tumor cells, which allows to consider GSNOR as an oncosuppressor.

Thioredoxin (Trx) significantly contributes to the denitrosylation reactions. An interest in Trx is also due the fact that it represents a universal enzyme that displays denitrosylase and transnitrosylase activities in addition to the disulfide reductase activity, which makes it one of the key players in cellular redox homeostasis [131]. The disulfide reductase and denitrosylase activities of Trx are mediated by the Cys32 and Cys35 residues in its active site. These residues form a disulfide bond that is reduced by thioredoxin reductase (TrxR) in the presence of NADPH(H^+) (Fig. 4) [131, 132]. Denitrosylation is a multistage process that can proceed by different mechanisms, but ultimately results in the release of nitroxide (HNO) or NO.

Trx1 can function as a transnitrosylase when Cys32 and Cys35 in its active site form a disulfide bond, which takes place in the case of high ROS levels and/or low TrxR activity. Oxidized Trx1 can be S-nitrosylated at Cys73 located outside the active site, allowing it to act as a transnitrosylase toward Cys residues in target molecules. Interestingly, the stability of Trx-SNO is regulated by reducing agents. For example, reduced Trx1-$(SH)_2$ and GSH promote denitrosylation of cellular Trx-SNO thus affecting the balance between Trx-mediated nitrosylation and denitrosylation [133, 134].

Overexpression of the *TXN1*(*C32S/C35S*) gene (mutant Trx1 lacking the disulfide reductase activity because of Cys32 and Cys35 substitution with serine) in HeLa cells promoted nitrosylation of specific target proteins. Proteomics analysis revealed 47 new candidate protein targets for the Trx1-mediated transnitrosylation [133], the most well-characterized of which was effector caspase-3. Based on the Trx1 redox status, this modification can lead to either activation or inactivation of caspase-3 [133, 135]. Trx1-$(SH)_2$ catalyzes denitrosylation of caspase-3 nitrosylated at Cys163, whereas Trx1-SNO prevents apoptosis by transnitrosylating caspase-3 at the same cysteine residue and induces oncogenic cell transformation [134, 136-138].

Although it was demonstrated *in vitro* and *in vivo* that caspases-8 and -9 initiating signaling cascade resulting in apoptosis, also have catalytic Cys residues that can be S-nitrosylated [139], it still remains unclear whether Trx1 transnitrosylates these enzymes. Experimental data indicate that the redox-dependent Trx1/Trx1R circuit is involved in denitrosylation of SNO-caspase-9 and reductive reactivation of caspase-8 [140, 141]. Undoubtedly,

Trx1-related regulation of caspase activity plays an important role in the apoptotic signaling pathways.

Beside caspases, other targets may also be involved in NO-dependent regulation of tumor apoptosis. One of the mechanisms of oncogenesis is alteration in cell signaling pathways, especially those involved in apoptosis activation. Cells of the tumor microenvironment secrete TNF superfamily cytokines (FasL, TRAIL, and TNFα) with the pro- or anti-oncogenic properties. The exact effect of these cytokines is determined by multiple factors including post-translational modifications, including S-nitrosylation and denitrosylation, of signaling cascade proteins [142, 143]. In particular, the DR4 receptor that binds TRAIL is considered as a tumor suppressor protein involved in the induction of extrinsic apoptotic pathway. The NO donor nitrosylcobalamin (vitamin B_{12} analog) exhibited a pronounced antiproliferative activity in melanoma and renal and ovarian carcinoma cells by eliciting S-nitrosylation of DR4 at Cys336 located in the cytoplasmic domain, thus contributing to the DR4 activation. The C336A point mutation resulted in the loss of nitrosylcobalamin ability to activate apoptosis in target cells [144].

As shown in in colorectal cancer cells using nitroglycerin as an NO donor, the Fas receptor can be S-nitrosylated at Cys199 and Cys304 [145]. However, only S-nitrosylation at Cys304 results in the stimulation of its proapoptotic activity by ensuring Fas recruitment to lipid rafts, which increased cell sensitivity to FasL and apoptosis induction [145]. S-nitrosylation of the transcription factor YY1 (Ying Yang 1), which represses Fas receptor, led to the YY1 inactivation resulting in the upregulation of *FAS* gene expression and tumor cell sensitization to Fas agonists [146].

S-Nitrosylation affects signal transduction not only by altering the functioning of cell receptors, but also by modulating the downstream transcription factors and kinases involved in signaling cascades affecting cell viability. In particular, S-nitrosylation/denitrosylation regulate the activity of STAT3 and NF-κB, which are among important transcription factors involved in the tumor progression, chemoresistance, and metastasis [147, 148]. For example, STAT3 governs expression of genes encoding cell survival proteins (Bcl-xL, cIAP, survivin, and Mcl-1), cell cycle proteins (e.g., c-Myc, CDK2, cyclin E, CDK1, and cyclin B), and proteins participating in tumor angiogenesis (e.g., VEGF). NF-κB regulates expression of proteins necessary for cell survival (cIAP, Bcl-2, Bcl-xL, and XIAP), cell cycle (c-Myc and cyclin D), and multidrug resistance (MDR1) [114]. In HNSCC head and neck squamous carcinoma cells, STAT3 activation by phosphorylation is reversibly suppressed by the GSNO-mediated S-nitrosylation at Cys259 [114]. GSNO also contributes to the decrease in the baseline and cytokine-stimulated NF-κB activation. Reduction in the STAT3 and NF-κB activity is associated with the cell cycle arrest in the S and G2 phases, decreased proliferation, and apoptosis activation, as well as decrease in the expression of genes encoding cell cycle regulators, such as c-Myc and anti-apoptotic proteins Bcl-xL and cIAP (cellular inhibitor of apoptosis protein 1). Systematic administration of GSNO in mice with HNSCC xenografts inhibited tumor growth that was further suppressed by a combination of GSNO with cisplatin and radiation therapy. It is believed that GSNO, which blocks NF-κB and STAT3 proteins controlling cell survival and proliferation, has a potential to enhance the effect of routine therapies [114].

S-nitrosylation also modulates activity of cell signaling kinases. For instance, activation of the ERK1/2 cascades determining tumor cell proliferation and survival occurs via phosphorylation at Thr202 and Tyr204 residues of ERK1 and ERK2 kinases (extracellular signal-regulated kinases 1 and 2) displaying 85% homology. GSNO used an NO donor in U251 glioma cells [149] induced S-nitrosylation of ERK1 at Cys183 located near Thr202 and Tyr204 residues, resulting in the decreased ERK1/2 phosphorylation and cell growth inhibition. Cys183 replacement with Ala prevented ERK1 S-nitrosylation, restored ERK1/2 phosphorylation, and suppressed GSNO-induced apoptosis. Human glioma tissues demonstrate elevated ERK1/2 phosphorylation along with the decreased ERK1/2 S-nitrosylation. Taking into account the relationship between S-nitrosylation and phosphorylation of ERK1/2 demonstrated in *in vitro* and *in vivo* studies, these processes might be involved in the redox-dependent development of drug resistance in gliomas [149].

In conclusion, ROS/RNS exert a dual effect on tumor cells. On one hand, ROS/RNS promote malignant cell transformation by activating signaling pathways resulting in the enhanced cell proliferation, survival, and stress resistance. On the other hand, high ROS/RNS levels can slow down tumor progression due to the development of oxidative/nitrosative stress that causes cell death and limit tumor growth at specific stages of cancer development. Low ROS/RNS concentrations maintained by endogenous antioxidant systems enable oxidative/nitrosative modifications of key redox-dependent residues in regulatory proteins. The reversibility of S-glutathionylation/S-nitrosylation proceeding by electrophilic attack by ROS/RNS on nucleophilic Cys residues provides a redox "switch" that controls the activity of signaling proteins, as well as regulation of tumor cell proliferation and programmed death. Deeper understanding of the redox-driven control of cellular homeostasis, in particular, via S-glutathionylation/S-nitrosylation, will allow to improve the antitumor therapy regimens by taking into account the regulation of key enzymes controlling these processes and a balance between them.

Contributions. E.V.K. conceived the study, wrote the manuscript, and supervised the study; M.D.N. wrote the

"S-Glutathionylation in tumor cells. Regulation of redox homeostasis" and "S-Nitrosylation. Regulation of redox homeostasis in tumor cells" sections of the manuscript and edited the text.

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