Protein Quality Control under Oxidative Stress Conditions

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http://dx.doi.org/10.1016/j.jmb.2015.02.014
Edited by J. Buchner

Abstract

Accumulation of reactive oxygen and chlorine species (RO/CS) is generally regarded to be a toxic and highly undesirable event, which serves as contributing factor in aging and many age-related diseases. However, it is also put to excellent use during host defense, when high levels of RO/CS are produced to kill invading microorganisms and regulate bacterial colonization. Biochemical and cell biological studies of how bacteria and other microorganisms deal with RO/CS have now provided important new insights into the physiological consequences of oxidative stress, the major targets that need protection, and the cellular strategies employed by organisms to mitigate the damage. This review examines the redox-regulated mechanisms by which cells maintain a functional proteome during oxidative stress. We will discuss the well-characterized redox-regulated chaperone Hsp33, and we will review recent discoveries demonstrating that oxidative stress-specific activation of chaperone function is a much more widespread phenomenon than previously anticipated. New members of this group include the cytosolic ATPase Get3 in yeast, the *Escherichia coli* protein RidA, and the mammalian protein α2-macroglobulin. We will conclude our review with recent evidence showing that inorganic polyphosphate (polyP), whose accumulation significantly increases bacterial oxidative stress resistance, works by a protein-like chaperone mechanism. Understanding the relationship between oxidative and proteotoxic stresses will improve our understanding of both host–microbe interactions and how mammalian cells combat the damaging side effects of uncontrolled RO/CS production, a hallmark of inflammation.

The Origin of Oxidative Stress

Reactive oxygen species (ROS) such as superoxide ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2), and hydroxyl radicals ($^{\cdot}OH$) are metabolic by-products, which occur naturally in all organisms that live an aerobic lifestyle. They are constantly produced during electron transfer in the respiratory chain [1], by enzymes such as NADPH oxidases [2,3], and in organelles such as the peroxisomes [4]. Non-physiologically low levels of ROS negatively affect cell growth, development, and differentiation [5–7], illustrating the importance of ROS as second messengers that control metabolic processes and signaling pathways (Fig. 1) [8]. Non-physiologically high levels of ROS, on the other hand, can cause irreversible oxidative modifications of virtually all cellular macromolecules, including lipids, DNA, and proteins (Fig. 1) [9]. Maintaining redox homeostasis requires a concerted cellular effort, involving expression of a variety of different ROS-detoxifying enzymes (e.g., superoxide dismutase, peroxiredoxin, and catalase), oxidoreductases of the thioredoxin (Trx) and glutaredoxin (Grx) systems, NADPH-regenerating systems such as the nicotinamide nucleotide transhydrogenase, and the production of the small redox-buffering tripeptide glutathione (GSH) [10]. Together, these systems keep cytosolic protein thiols reduced and protect cells against the accumulation of toxic oxidants (Fig. 1) [5,11].

However, even the best systems sometimes fail. When they do, such as during aging, age-related neurodegenerative diseases (e.g., Parkinson's disease), or metabolic diseases (e.g., diabetes) [12], ROS begin to accumulate in the cell, causing cells to experience a stress condition commonly known as oxidative stress. Oxidative stress can be caused by a variety of different mechanisms, including defects in specific antioxidant or redox-maintaining systems,

ROS production during host defense, UV light, gamma and X-rays, pollutants and smoke, or due to metal-catalyzed Fenton reactions [13]. All of these processes produce free radicals, capable of oxidizing cellular structures [14–16]. Apart from lipid peroxidation and DNA damage, ROS are especially known for their high reactivity toward sulfur-containing amino acids and metal-containing cofactor sites in proteins, causing reversible and irreversible inactivation of many different proteins and representing a major threat toward the cellular proteome [17]. It is thought that oxidative damage to a cell's proteome contributes to the etiology of a variety of different human protein-folding diseases, including Alzheimer's, Parkinson's, and prion disease [18].

The Benefit of Oxidative Stress—ROS and RCS as Physiological Antimicrobials

Oxidative stress is not always a bad thing; in fact, production of high levels of ROS and the related reactive chlorine species (RCS) [17] plays an important physiological role in the innate immune response, where it provides a powerful strategy for killing invading pathogens [19]. When bacteria are taken up by neutrophils, NADPH oxidases localized in the phagosomal membrane catalyze the reduction of molecular oxygen to superoxide ($O_2^{\cdot-}$). After being rapidly dismuted to H_2O_2 by superoxide dismutases, myeloperoxidases convert H_2O_2 and chloride ions into the RCS hypochlorous acid (HOCl). HOCl is extremely reactive and bactericidal even at low micromolar concentrations [20], making it one of the most powerful physiological oxidants known (for a comprehensive overview, see Refs. [17] and [19]). Not surprisingly, HOCl is the active ingredient of household bleach and is one of the most commonly used disinfectants in medical, industrial, and domestic settings [21]. Once released into the phagosome, HOCl evokes a very rapid toxic effect on bacteria, contributing to the effective neutrophil-mediated killing of invading microbes [22–24].

In addition to the role of HOCl production in the antimicrobial action of neutrophils, HOCl might also be involved in controlling bacterial colonization of mucosal barrier epithelia, such as the airways and the intestine [25,26]. Epithelial cells express the enzyme dual oxidase (DUOX), a member of the nicotinamide adenine dinucleotide phosphate oxidase family. DUOX, like myeloperoxidase, has been suggested to convert peroxide into HOCl [27]. It is also possible, however, that the antimicrobial function of DUOX is primarily due to the production of H_2O_2, which is rapidly converted to the highly bactericidal hypothiocyanite by lactoperoxidases [154]. In either case, knockdown of the *duox* gene in the fly gut leads to increased bacterial colonization and a significantly increased rate of death caused by infections [27,28]. Duox$^{-/-}$ mice show a significant decrease of neutrophil invasion during the development of allergic asthma in a murine model and increased levels of pathogens that colonize the intestinal epithelial cells [29]. These results emphasize the physiological importance of oxidative stress in general and HOCl production, in particular, in
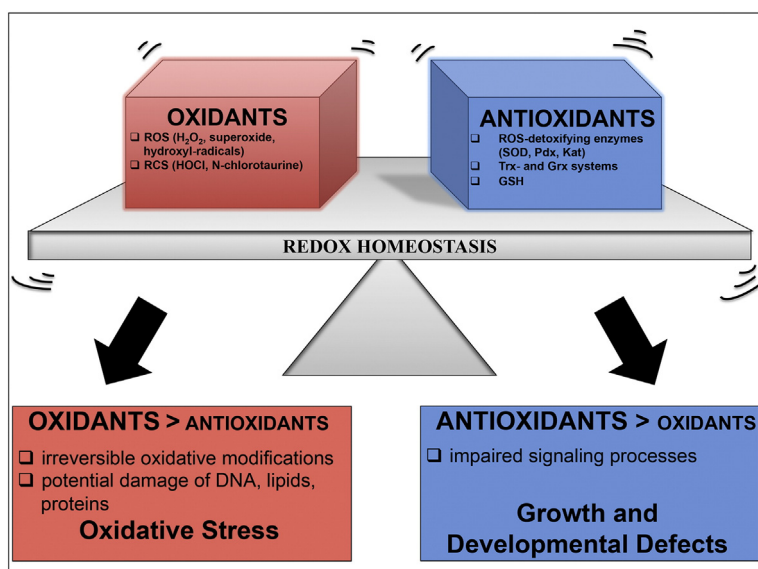
Fig. 1. Redox homeostasis—A balance between oxidants and antioxidants. ROS and RCS are constantly produced as by-products of cellular processes. They are involved as second messenger in signaling pathways, influencing a variety of different cellular processes. Antioxidant systems [including ROS-detoxifying enzymes, such as superoxide dismutase (SOD), peroxiredoxin (Prx), and catalase (Kat); oxidoreductases including the thioredoxin (Trx) and glutaredoxin (Grx) system; and the non-protein thiol glutathione (GSH)] work together to maintain a reducing environment and prevent accumulation of oxidants beyond physiological levels. However, defects in antioxidant systems or exposure to increased concentrations of RO/CS can shift this ratio. While accumulation of RO/CS causes widespread oxidative damage and is thought to be involved in aging and many age-related diseases, diminished levels of RO/CS affect growth, development, and differentiation.

combating microbial pathogens and controlling the bacterial population in the host [25,27,30]. On the downside, however, uncontrolled production of HOCl by neutrophils can cause a variety of diseases and is thought to be involved in the tissue damage at sites of chronic inflammation and in arteriosclerosis [31].

Proteins—The Primary *In Vivo* Targets of Oxidative Damage

Almost 70% of all oxidized molecules in oxidatively stressed cells are of proteinaceous nature [32], indicating that proteins are the most prominent *in vivo* targets of oxidants. Reactive oxygen and chlorine species (RO/CS) cause numerous post-translational protein modifications, including oxidation of sulfur-containing side chains, chlorination of side-chain amines, oxidation of histidines and tryptophans, dityrosine formation, and others (Fig. 2) [17,33]. These oxidative side-chain modifications can lead to oligomerization, fragmentation, destabilization, aggregation, and/or enhanced degradation of proteins [34–37]. While some RO/CS-mediated posttranslational modifications are intentional, reversible, and part of redox-regulated processes (see below), irreversible protein modifications are typically destabilizing and capable of a triggering a major secondary stress on the proteostasis network of the cell (Fig. 2).

The most vulnerable (i.e., reactive) targets in proteins are the sulfur-containing side chains of methionine and cysteine residues (Fig. 2) [17,38]. With reaction rates in the 10^6–10^7 M^{-1} s^{-1} range, HOCl rapidly chlorinates cysteine thiols (R-SH) forming R-SCl [39]. Chlorinated thiols either directly attack other thiols or amines, or exchange with H_2O to form sulfenic acid (R-SOH). The reaction of peroxide with cysteines, which also yields sulfenic acid intermediates, is up to 6 orders of magnitude slower except for proteins such as peroxiredoxin that contain unusually peroxide-reactive cysteines in their active site [40,41]. Due to its highly unstable nature, any sulfenic acid intermediate typically reacts very quickly with other protein thiols to form either intramolecular or intermolecular disulfide bonds (R_1-S-S-R_2) (Fig. 2). Reactions with non-protein thiol antioxidants, such as GSH or free cysteines, result in the formation of mixed disulfides known as S-glutathionylation (R-S-S-GSH) and S-cysteinylation (R-S-S-R_{Cys}), respectively. Sulfenic acids can also react with vicinal primary or secondary amino groups, thereby forming reversible sulfenamides or, upon further oxidation, irreversible sulfinamides or sulfonamides [17]. Alternatively, sulfenic acids can be further oxidized by RO/CS to sulfinic (R-SO_2H) or sulfonic (R-SO_3H) acid (Fig. 2): two typically irreversible thiol modifications that often lead to increased rates of protein degradation. The only known example of reversible sulfinic acid formation was found in select eukaryotic peroxiredoxins, which are reduced by the ATP-dependent sulfinic acid reductase sulfiredoxin [42,43].

Disulfide bonds, whether intramolecular, intermolecular, or mixed, are fully reversible thiol modifications, which can be reduced by members of the thioredoxin or glutaredoxin family (Fig. 2) [44]. Both types of oxidoreductases reduce oxidized protein thiols via direct thiol/disulfide exchange [45]. However, whereas Trx reduces predominantly disulfide bonds and sulfenic acid intermediates, Grx preferentially reduces S-glutathionylated proteins [46]. Both proteins enter the reaction in their reduced form and leave the reaction in their oxidized form. Trx is subsequently reduced by thioredoxin reductase, whereas reduction of oxidized Grx is achieved by GSH. Its oxidation product GSSG is subsequently reduced by glutathione oxidoreductase (Gor) [46,47]. Each of these reductases receives their reducing equivalents from NADPH, closely tying cellular redox homeostasis to the levels of NADPH [46,48].

Methionine residues are also very oxidation sensitive. Methionine can be oxidized by RO/CS to methionine sulfoxide at rates of up to 3.4×10^7 M^{-1} s^{-1} (for HOCl) [49,50]. Since the role of methionine oxidation and the methionine sulfoxide reductase (MSR) enzymes involved in repairing oxidized methionines in protein structure, function, and resistance to oxidative damage has been recently reviewed [51–53], we will touch only briefly on this topic here. It has become clear, however, that the repair of oxidized methionine is intimately connected to chaperone function and maintenance of proteostasis under redox stress conditions in many types of cells. In yeast, for example, MSR enzymes preferentially reduce oxidized methionines in unfolded protein substrates [54]. In humans, MSR-catalyzed methionine sulfoxide reduction restores chaperone function to oxidatively inactivated α-crystallin [55]. In the bacterial pathogen *Helicobacter pylori*, resistance to oxidative stress depends on MSR not only for repairing oxidized methionines in the ROS-degrading enzymes catalase and alkyl hydroperoxide reductase but also for restoring activity to oxidatively inactivated GroEL chaperones [56–58]. Like cysteine, under severe oxidizing conditions, methionine can also be further oxidized to methionine sulfone or methionine sulfoximine. These are irreversible and toxic end products, which likely lead to protein degradation [59].

Apart from sulfur-containing residues, HOCl and H_2O_2, to a lesser extent, also react with histidine, tryptophan, lysine, tyrosine, and arginine side chains, as well as with the amino-terminus of proteins (Fig. 2) [60]. While the reaction rates are significantly lower than with cysteines [39], many of the reaction products are irreversible (Fig. 2), increasing the tendency of proteins to aggregate or to

Fig. 2. Reversible and irreversible protein modifications by RO/CS. RO/CS cause oxidative modification of a number of different residues in proteins. Oxidation of histidines and tryptophans and the formation of dityrosines, sulfinic/sulfonic acids, and methionine sulfone/sulfoximine intermediates are irreversible modifications and lead to protein unfolding, aggregation, and degradation. Disulfide bond formation, methionine sulfoxide formation, and N-chlorination are reversible protein modifications and often used to regulate protein function in response to oxidative stress. The systems responsible for reducing oxidative protein modifications *in vivo* are listed in brackets: Grx, glutaredoxin; Trx, thioredoxin; GSH, glutathione; MSR, methionine sulfoxide reductase; Gor, glutathione oxidoreductase.

be degraded. In addition, HOCl reacts with a protein's primary and secondary amines, resulting in chloramine formation. Chloramines themselves are effective oxidants, able to directly chlorinate, and oxidize other biomolecules. However, in comparison to HOCl, chloramines are 4 to 5 orders of magnitude less reactive but are more selective towards thiol groups [19,60].

The Danger of Oxidative Stress: Protein Unfolding and Aggregation

As outlined above, oxidative stress-derived protein modifications can lead to the loss of a protein's secondary or tertiary structure, which in turn impacts its activity, stability, and solubility. Moreover, the integrity of the cellular proteome is also highly dependent on regulated posttranslational protein modifications, such as phosphorylation, acetylation, ubiquitination, or methylation. These processes are catalyzed by enzymes, which can also fall victim to oxidative modifications [61,62]. *In vivo* studies agreed with this notion and revealed extensive oxidative protein aggregation in HOCl-treated bacteria [63]. This result was independently confirmed by the fact that HOCl treatment of bacteria triggers the heat shock response, a highly conserved transcriptional response that is known to be induced by the accumulation of protein-folding intermediates [64,65]. While H_2O_2 treatment in *Escherichia coli* yields in little to no protein aggregation and no significant heat shock response, exposure to disulfide stress (caused by either diamide or genetic depletion of the Trx/GSH systems) revealed a considerable overlap between heat shock and

oxidative stress response pathways in both Gram-positive and Gram-negative bacteria [66–68]. Eukaryotes appear to experience oxidative protein unfolding during peroxide treatment [69]. The reason for this discrepancy is unclear. One possibility is increased production of highly reactive hydroxyl radicals in eukaryotes, which could be fostered by the presence of a more significant pool of Fenton metals. Since hydroxyl radicals are extremely reactive and known to cause protein unfolding and aggregation, increased production of hydroxyl radicals would certainly lead to a massive stress on the proteome [70].

The Challenge of Oxidative Stress: Maintaining Proteostasis

Cells employ a large proteostasis network to maintain proteome stability and functionality during non-stress and stress conditions. This network consists of numerous different chaperones, folding catalysts, and proteolytic components [37,71]. Under stress conditions, the most important task is to sequester unfolding proteins, reducing the amount of aggregation-sensitive folding intermediates and preventing the accumulation of protein aggregates. This is the job of molecular chaperones, which harbor binding sites for unfolding proteins and prevent aggregate formation. Several different families of chaperones, including members of the HtpG (Hsp90), GroEL (Hsp60), DnaK (Hsp70), DnaJ (Hsp40), and IbpA/B (sHsps) family, contribute to maintaining proteostasis during stress conditions [72–74]. The majority of these proteins are under heat shock control and are among the first responders when

protein-unfolding intermediates accumulate. The Hsp90, Hsp70, and Hsp60 chaperones are "foldases": typically energy-dependent proteins that use ATP binding and hydrolysis to support refolding of damaged proteins once stress conditions subside [75]. The sHsp "holdases" are energy-independent chaperones that prevent aggregation without supporting refolding [76,77]. The second arm of the proteostasis network during stress consists of ATP-dependent proteases, which degrade proteins that are irreversibly damaged and/or unable to refold. The mechanisms by which proteins are triaged between these two fates are still subject of very active investigations [78,79].

Transcriptional and translational processes take time, involve numerous oxidation-sensitive proteins, and require ATP. However, sudden exposure to highly proteotoxic oxidants such as HOCl or hydroxyl radicals requires an immediate response, transiently inactivates transcription and translation processes, and substantially reduces cellular ATP levels [80,81]. That oxidative stress reduces cellular ATP levels has been observed in every organism studied. This phenomenon was originally thought to be due to the oxidative inactivation of glyceraldehyde dehydrogenase (GAPDH), a central enzyme of glycolysis. GAPDH has long been known for its exquisite sensitivity to RO/CS, which cause rapid modification of its active site cysteine (reviewed in Ref. [82]). This block in glycolysis together with the oxidative modification of other ATP-generating systems was thought to cause the very rapid decline in ATP levels [83,84]. While this might still be the case in eukaryotic cells, recent studies in *E. coli* revealed that the observed ATP decline in HOCl-stressed bacteria is due to the active re-routing of ATP into long chains of inorganic polyphosphates (see below) [85].

There is little doubt that decreasing cellular ATP levels is a clever strategy to protect the most oxidation-sensitive targets, such as newly synthesized proteins [75], against oxidative damage. However, loss of ATP equivalents also constitutes a major problem, particularly for maintaining proteostasis, since most chaperones and proteases rely on ATP for their function. Cells appear to deal with this dilemma by employing stress-specific, ATP-independent holdase chaperones. These chaperones, which are mostly inactive under non-stress conditions, are able to directly sense protein-unfolding oxidants and rapidly respond with an increase in their chaperone function.

In this review, we will focus exclusively on the chaperone arm of the proteostasis network, since regulation of the proteasomal system and its role during oxidative stress has been expertly reviewed in the very recent past [78,79,86]. We will discuss several groups of redox-regulated chaperones— first, the prototypical prokaryotic 33-kDa heat shock protein (Hsp33), then a number of multifunctional cellular components that gain chaperone activity under oxidative stress conditions: (i) the eukaryotic ATPase Get3/TRC40, (ii) the bacterial enamine/ imine deaminase RidA, (iii) the mammalian protein α2-macroglobulin, and (iv) the prebiotic inorganic polymer polyphosphate (polyP). Whereas both Hsp33 and Get3 sense oxidants via highly sensitive cysteine residues whose oxidation status directly affects their ability to interact with unfolded client proteins, RidA and α2-macroglobulin appear to be activated by non-cysteine side-chain modifications. We will conclude with polyP, whose production seems to be largely responsible for the drop in cellular ATP levels upon HOCl stress and that acts similar to a holdase chaperone in bacteria, preventing protein aggregation.

Hsp33—The Inaugural Member of the Redox-Regulated Chaperone Family

Hsp33 (gene name *hslO*) was first identified in 1993, when Fred Blattner revisited the σ^{32}-controlled heat shock response in *E. coli* [87]. Transcriptional analyses confirmed that Hsp33 is expressed under non-stress conditions but that its expression is dramatically increased when bacteria experience stress conditions that induce protein-unfolding conditions [87,88]. What made the protein sequence of Hsp33 unique among known heat shock proteins at that time was the presence of four absolutely conserved cysteine residues, located in the C-terminus of the protein. These four cysteine residues were suggestive of metal binding and instigated our investigations into the function of this highly conserved protein [89].

Extensive studies over the past 16 years revealed that Hsp33 indeed uses these four cysteines as high-affinity zinc ligands. However, instead of serving a purely structural role, we found that the four cysteines function as posttranslational redox switch, effectively translating changes in ROS levels into structural and functional changes in Hsp33. While inactive as chaperone when reduced and zinc coordinated, Hsp33 converts into a highly effective chaperone holdase when disulfide bonded and zinc free [63,75]. Activators of Hsp33's chaperone function are RCS (e.g., HOCl) [63] or combinations of peroxide stress and unfolding conditions, induced by either elevated temperatures [89–91] or as recently noted by bile salts [92]. Bacteria might encounter any of one of these stress conditions during the innate immune response (see earlier sections), on their way through the gastrointestinal tract [92], or during inflammation and fever episodes [93]. All of these conditions cause oxidative protein unfolding and lead to the aggregation of many essential proteins in the absence of Hsp33 *in vivo* [63,92,94]. Not surprisingly, bacteria lacking Hsp33 are significantly more sensitive toward these stress conditions [92,94]. Since Hsp33 is highly conserved among prokaryotes and unicellular parasites such as *Leishmania* but absent from higher eukaryotes, it is a potentially promising drug target to

diminish the virulence of pathogenic bacteria such as *E. coli* or *Vibrio cholerae*. However, virulence studies have yet to be conducted to test this idea.

The activation and inactivation mechanism of Hsp33

Probably one of the most intriguing questions regarding Hsp33 concerned its mechanism of activation: how can a protein be activated under protein-unfolding conditions, which are detrimental for a host of different proteins, including several chaperones [63,75]? To our surprise, we found that, upon exposure to oxidative protein-unfolding conditions, Hsp33 also oxidatively unfolds. However, instead of losing activity and aggregating, Hsp33 uses these massive structural rearrangements to specifically activate its chaperone function [63,89,92]. This was a truly unexpected discovery, which immediately raised the next question as to how this would work from a mechanistic point of view. Reduced, chaperone-inactive Hsp33 consists of a compactly folded N-terminal domain, a highly flexible ~40-aa-long linker region, and a C-terminal redox-sensing domain, containing the Cys_{232}-X-Cys_{234}-X_{27-32}-Cys_{265}-XX-Cys_{268} redox switch motif [95]. The four highly conserved cysteines coordinate one zinc ion with high affinity in a tetrahedral arrangement [96]. The Zn^{2+} ion stabilizes the cysteine thiols in their highly reactive thiolate anion form [96–98], bringing the cysteines into close proximity for disulfide bond formation to occur and contributing significantly to the thermodynamic stability of the zinc-binding domain. Once exposed to the appropriate oxidative stress conditions, the four cysteines engage in two disulfide bonds, zinc is released, and the zinc-binding domain unfolds. What is truly crucial for the activation of Hsp33, however, is the unfolding of the central linker region [99], which connects Hsp33's N-terminus with the C-terminal redox switch domain. Not particularly conserved but highly charged, this linker region is stably folded under reducing, non-stress conditions yet rapidly unfolds upon the formation of both disulfide bonds. Destabilization of the linker region by site-specific mutagenesis successfully uncouples linker unfolding from disulfide bond formation and zinc release and yields a constitutively active yet fully reduced and zinc-coordinated Hsp33 [99]. These results provided strong evidence that the redox switch domain serves primarily as a rheostat, whose function is to control the stability of the linker region via its own thiol/disulfide status.

As previously mentioned, Hsp33 is only activated by slow-acting oxidants, such as H_2O_2, when combined under protein-unfolding conditions. This mechanism seems to assure that Hsp33 is only activated when proteins unfold under oxidative stress conditions. Peroxide treatment alone is capable of oxidizing the two distal cysteine residues (Cys232 and Cys234) in Hsp33, but not the two proximal cysteines (Cys265 and Cys268). This oxidation causes a loss in zinc binding, which in turn destabilizes the linker region. However, the destabilization is not sufficient to trigger linker unfolding under otherwise non-stress conditions [90,95]. Upon exposure to unfolding conditions, such as elevated temperatures or bile salts, however, the linker region unfolds, causing activation of chaperone function. In addition, formation of the second disulfide bond (Cys232–Cys234) is now facilitated, which locks the linker region in the unfolded state and maintains the chaperone activity of Hsp33 until reducing non-stress conditions are restored. Fast-acting oxidants such as HOCl gain access to all four cysteine residues and thus cause very rapid oxidative unfolding and the activation of Hsp33's chaperone function [63,92]. Subsequent dimerization and potentially even higher oligomer formation of oxidized Hsp33 monomers follows [91], potentially increasing the client binding interface [99,100].

In reverse of the activation of Hsp33, which requires both oxidizing and unfolding conditions, Hsp33's inactivation requires both reducing and refolding conditions [77]. Reducing conditions convert oxidized Hsp33 dimers into reduced Hsp33 dimers, which remain bound to their client proteins. This mechanism likely assures that Hsp33 does not release its client proteins until ATP levels are restored and fully functional ATP-dependent foldase systems such as the DnaK/DnaJ/GrpE chaperone system are available [77,100]. Once reducing and non-stress conditions are restored, Hsp33 transfers its client proteins to the DnaK/DnaJ/GrpE chaperone system, which supports their refolding to the native state. The precise mechanism of client release and the role of the DnaK//DnaJ/GrpE chaperone system during this release process still remain enigmatic.

Hsp33—A Conditionally Disordered Chaperone

Hsp33 undergoes substantial unfolding during its activation process. This mechanism made Hsp33 the founding member of a new category of so-called conditionally disordered chaperones [100–102]. Over the past few years, several other chaperones joined this category, protecting organisms against high heat (e.g., Hsp26) [103–105] or very low pH (e.g., HdeA) [106–108]. What these proteins have in common is that they are fully folded and chaperone inactive under non-stress conditions. During exposure to these rapidly proteotoxic stress conditions, they adopt a partially disordered conformation, which appears to be essential for their ability to bind protein-folding intermediates and protect them against aggregation [101] (Fig. 3). These results of course raised the intriguing question as to the role that the intrinsically disordered regions play in chaperone function. It appears that the disordered structure contributes to the increased plasticity of
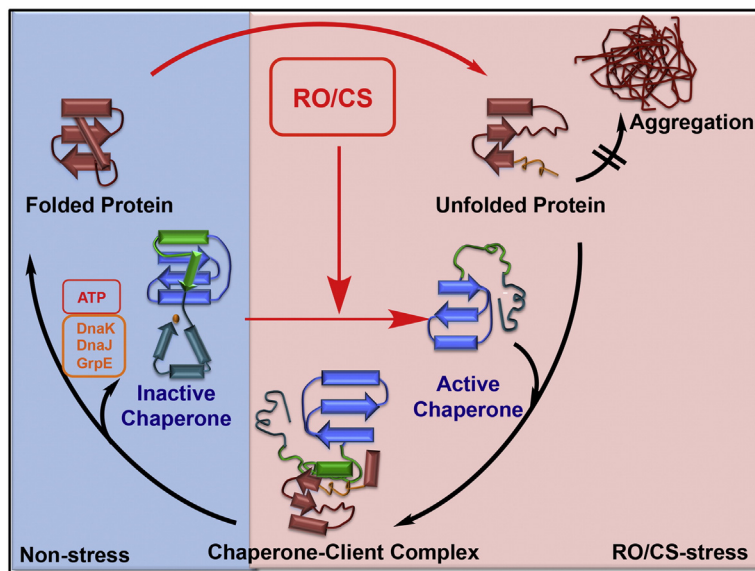
Fig. 3. Redox-mediated activation of conditionally disordered chaperones. RO/CS can cause substantial structural changes in proteins, leading to protein unfolding and aggregation. Bacteria employ the stress-specific molecular chaperone Hsp33 to sequester unfolding intermediates and prevent accumulation of toxic protein aggregates. Under non-stress conditions, Hsp33 is well folded and is chaperone inactive. Its four conserved cysteine residues are reduced and involved in high-affinity binding of zinc. Upon exposure to oxidative protein-unfolding conditions, Hsp33 undergoes oxidative disulfide bond formation, zinc release, and massive structural rearrangements, including significant protein unfolding. In this partially intrinsically disordered conformation, Hsp33 is chaperone active and is able to interact with partially unfolded protein intermediates. Upon return to non-stress conditions, the disulfide bonds are reduced and the client proteins are transferred to the DnaK/DnaJ/GrpE system, which uses ATP to refold the client proteins. Hsp33 is specific for prokaryotes and unicellular eukaryotes. Very recently, Get3 has been identified to serve as the likely functional analogue of Hsp33 in yeast.

client binding [100] and promotes promiscuity [101,104,107]. Moreover, hydrogen–deuterium exchange studies combined with mass spectrometry provided the first evidence that Hsp33's linker region might be in fact directly involved in client–chaperone interactions. The results of this study revealed that Hsp33 linker region regains stability upon client binding, suggesting that Hsp33 uses partially unfolding client proteins as scaffold to refold its linker region and thereby increase complex stability [100,101] (Fig. 3). Future structural studies are needed to uncover further details about the Hsp33–client interactions and define the extent to which intrinsic disorder is directly involved in the interaction with client proteins.

Get3—A Redox-Regulated Dual-Function Protein in Eukaryotes

Hsp33, while highly conserved in prokaryotes, is absent from higher eukaryotes. This raised the obvious question as to how eukaryotic cells defend themselves against oxidative protein unfolding. One group of proteins that might be involved in this process are 2-Cys peroxiredoxins, which have been shown to gain chaperone activity upon peroxide-mediated overoxidation of their active site cysteine (for a more detailed overview, see Refs. [109–111]). However, recent studies suggest that overoxidation

is not essential and that other triggers, including high temperature and low pH [112,113], might activate the chaperone function of peroxiredoxin as well. This makes peroxiredoxin less of a specialized and more of a general ATP-independent chaperone, which protects cells against a variety of different stress conditions, including oxidative stress. The other candidate, Get3, appears more Hsp33-like in its properties as a redox-regulated chaperone and has been recently shown to protect eukaryotic cells against oxidative protein unfolding [114]. On the surface, Get3 and Hsp33 have little in common. Reduced Hsp33 is a monomeric, non-ATP binding protein, which coordinates zinc using four highly conserved cysteines. Reduced Get3 is a dimeric ATPase, involved in binding and targeting tail-anchored proteins (TA) to the endoplasmic reticulum membrane. It too binds zinc, but zinc binding involves a Cys-XX-Cys pair from both monomers, stabilizing the reduced Get3 dimer [114]. Upon closer inspection, however, the two proteins do have some intriguing similarities; like Hsp33, the yeast protein Get3 (i) contains a Cys-X-Cys- X_{40-50}-Cys-X-X-Cys motif, (ii) protects unfolding proteins against aggregation *in vitro* when purified under aerobic conditions [115], (iii) shows oxidation sensitivity *in vivo* [114,115], and most importantly (iv) leads to an oxidative-stress-sensitive phenotype when deleted in yeast [114]. Growth studies of a *get3*-deficient yeast strain under various stress conditions identified two of the four conserved cysteine residues (Cys285/Cys288)

as essential for complementation of the growth defects [116].

We therefore decided to investigate the potential chaperone activity of Get3 in more detail. Indeed, we found that, upon exposure to hydroxyl radicals, whose *in vitro* production can be elicited via the Fenton reaction using a combination of Cu^{2+} and peroxide [114], Get3 forms two disulfide bonds and releases Zn^{2+} [114]. These redox changes trigger massive structural rearrangements, causing the inactivation of Get3's ATPase activity and the formation of tetrameric and higher oligomeric species with high, ATP-independent chaperone holdase activity [114]. These results are fully consistent with previously reported co-localization studies of Get3, which showed that Get3 associates with both unfolding proteins and other chaperones under ATP-depleted conditions *in vivo* [115]. Importantly, all of the conformational and functional changes in Get3 are fully reversible, requiring both reducing conditions and the presence of ATP [114].

What makes the Get3 case particularly fascinating is the fact that Get3 has an apparent completely different function in its reduced state. In fact, reduced Get3 was originally identified as the central member of the GET (*g*uided *e*ntry of *t*ail-anchored proteins) pathway. In this role, Get3 shuttles tail-anchored proteins (TA) from the ribosome to the endoplasmic reticulum membrane using ATP binding and hydrolysis to modulate client binding and release [117,118]. Site-directed mutagenesis studies revealed that the chaperone function of oxidized Get3 is independent of, and potentially mutually exclusive with, the membrane targeting function of reduced Get3 [114]. Phenotypic studies using a mutant Get3 variant, which was no longer capable of sorting TA proteins but had wild-type-like redox-regulated chaperone activity, revealed that the chaperone function and not the sorting function of Get3 is responsible for the growth deficit that is observed in *get3*-deficient cells during oxidative stress [114]. These findings are consistent with previous reports about a potential dual function in the Get3 homologue TRC40 in *Caenorhabditis elegans*. Deletion of TRC40 was found to cause severe growth deficits and increased sensitivity toward cisplatin [119], an anticancer drug thought to cause oxidative stress *in vivo* [120]. Importantly, complementation studies using either wild-type TRC40 or a mutant variant lacking two of the four cysteines revealed that both proteins rescued the growth defect of a TRC40 deletion strain. However, only wild-type TRC40 was able to complement for the observed cisplatin sensitivity. These results strongly suggest that Get3's redox-regulated chaperone functions and plays a crucial role in oxidative stress protection in higher eukaryotes as well [119]. These are exciting findings but many questions remain open: how does Get3 recognize its client proteins? What fates do client proteins have after the stress conditions subside? What is the role of the other GET pathway components and might they possibly be involved in client release, refolding, and/or restoration of proteostasis after oxidative stress? Time will tell how Get3 (TRC40) balances its two important functions in the cell.

Chaperone Activation by Non-Cysteine Oxidation

HOCl causes a number of other oxidative modifications in proteins, including N-chlorination, methionine oxidation, and dityrosine formation (Fig. 2). These mechanisms have now also been found to play a regulatory role in the activation of chaperone proteins during oxidative stress.

E. coli RidA—Activation by reversible N-chlorination

N-Chlorination of proteins occurs during severe HOCl stress. It most likely affects the ε-amino groups of lysine residues, the guanidinium groups of arginines, and the terminal amino groups of polypeptides. Chloramine formation is known to have detrimental effects on molecular level, including protein inactivation, unfolding, and aggregation. In addition, protein fragmentation due to halogen transfer and cross-linking events have been reported in response to treatment with chloramines [35,121–123]. Intriguingly, the *E. coli* protein RidA was recently identified to specifically sense RCS via reversible N-chlorination, using these modifications to turn into a chaperone-active state [124].

RidA is member of the functionally diverse YjgF/ER057c/DUK114 family of proteins, which is highly conserved in all domains of life [124]. In *Enterobacteriaceae*, such as *E. coli* and *Salmonella enterica*, RidA is an enamine/imine deaminase, which speeds up the IlvA-catalyzed deamination of threonine into 2-ketobutyrate [124,125]. Redox proteomic studies under nitrosative stress revealed that the only cysteine residue of RidA is oxidatively modified during peroxynitrite treatment, resulting in the loss of the stimulatory effect of RidA on the activity of IlvA [126].

Very surprisingly, however, in the presence of HOCl or other RCS such as monochloramine, RidA not only failed to stimulate but also in fact strongly inhibited the activity of IlvA [124], indicating that peroxynitrite and HOCl treatment cause a markedly different outcome for RidA's activity. The results furthermore suggested that HOCl-treated RidA might form a tight complex with IlvA, thereby inhibiting its catalytic activity. This led to the hypothesis that HOCl-treated RidA might function as a protein-binding holdase chaperone. *In vitro* aggregation studies using a variety of different client proteins, including unfolded IlvA, agreed with this conclusion and demonstrated that HOCl-treated RidA but not untreated RidA prevents protein aggregation under stress conditions [124]. Notably, RidA

appeared to specifically sense RCS, since no other tested oxidants, including peroxide or diamide, had any effect on its chaperone function. Even more astounding was the finding that HOCl treatment of the cysteine-free RidA variant had precisely the same chaperone-activating effect, excluding the possibility that modification of RidA's sole cysteine is involved in the activation process. Instead, HOCl-treated chaperone-active RidA trimers were found to associate into higher oligomers with substantially decreased levels of free amino groups and, based on mass spectrometry analyses, up to seven different N-chlorination sites [124]. These results led to the conclusion that chlorination of several lysine and/or arginine residues might contribute to the observed increase in hydrophobicity, a hallmark of binding sites in chaperones [127]. Deletion of RidA increased *E. coli*'s sensitivity to HOCl and caused the accumulation of aggregated proteins, suggesting that RidA is a true member of the proteostasis network during HOCl-mediated oxidative stress. N-Chlorination of RidA is fully reversible, and treatment with DTT, with ascorbic acid, or with the physiological redox systems Trx or GSH abolished RidA's chaperone activity *in vitro*. RidA's chaperone function appears to be not specific for *E. coli* since HOCl treatment of the *Drosophila melanogaster* homologue DUK114 also activates the chaperone function *in vitro* (albeit irreversibly) [124]. What remains to be done is identify the residues whose chlorination contributes to the chaperone function of RidA, to determine the lifetime of chloramine within the reducing environment of the cytosol, and to define the precise place that RidA holds in the cellular proteostasis network.

α₂-Macroglobulin—Activation by methionine oxidation/dityrosine formation

α₂-Macroglobulin is a very abundant glycoprotein in mammals, present within the highly oxidizing milieu of the blood plasma and extracellular space. The functions of α₂-macroglobulin are quite diverse but it is probably best known for its ability to trap and inhibit a variety of different extracellular proteinases, irrespective of their mechanism or specificity [128]. In addition, α₂-macroglobulin interacts with a number of other biomolecules, including different hormones and cytokines. α₂-Macroglobulin has also long been known for its sensitivity toward HOCl [129]. Earlier studies showed that HOCl treatment causes the homo-tetrameric protein to dissociate into stable dimers, which no longer interact with proteinases but show increased affinity for other binding partners, including the LDL receptor protein LRP [130]. These results suggested that HOCl-mediated dissociation into dimers is not simply a random, non-specific inactivation process but might be part of a regulatory mechanism. Indeed, very recently, Wyatt *et al.* demonstrated that HOCl-treated α₂-macroglobulin dimers show substantially increased surface hydrophobicity and are highly active in preventing the aggregation of a range of different model clients *in vitro* [131]. More specifically, HOCl-treated α₂-macroglobulin was able to prevent the HOCl-mediated aggregation of several disease-associated proteins, including fibrinogen and LDL, and showed increased activity toward preventing Aβ₁₋₄₂ fibril formation [131]. This interaction was even more effective when Aβ₁₋₄₂ was treated with HOCl as well. Neurotoxicity assays suggested that these interactions are indeed physiologically relevant; compared to non-treated α₂-macroglobulin, the researchers found that the efficacy of HOCl-treated α₂-macroglobulin to protect neuroblastoma cells against Aβ₁₋₄₂ toxicity was significantly improved. These results strongly suggest that α₂-macroglobulin switches into a physiologically relevant chaperone upon exposure to HOCl stress.

The mechanisms by which α₂-macroglobulin gets activated as a chaperone remain unclear so far. Since it is present in a very oxidizing environment, the cysteines are constitutively oxidized. It has been shown that HOCl-activated α₂-macroglobulin undergoes methionine oxidation, tryptophan oxidation, and dityrosine formation [129,131]. However, which combination of oxidation events is necessary or sufficient to activate the chaperone function remains to be investigated. The observed oxidative modifications are thought to be irreversible [131]. However, more controlled *in vitro* oxidation and careful analysis of the oxidation and activity state of α₂-macroglobulin *in vivo* might be necessary to ultimately answer this question.

Polyphosphate: A Protein-Like Inorganic Chaperone

Polyphosphates (polyP) are prebiotic polymers [132]: highly conserved, universal, and structurally extremely simple. They exist as long, typically unbranched chains of phosphoanhydride-bond-linked phosphates, which can reach lengths of up to 1000 P_i units [133]. More than 20 years ago, the late Arthur Kornberg *et al.* showed that polyP-deficient bacterial cells suffer from a number of different phenotypes, including increased sensitivity toward multiple stressors such as heat shock, heavy metal exposure, peroxide, and starvation [134]. The molecular reason for this effect was unknown, but speculations considered the fact that PolyP's energy status is equivalent to ATP and thus might serve as a suitable storage compound for phosphate and energy during stress conditions [133]. Alternatively, it was proposed that polyP might be involved in the regulation of the σ^{38}-dependent general stress response system of *E. coli* [135,136], which controls the transcription of various genes related to ROS resistance, including *katE* (encoding catalase), *sodC* (encoding superoxide dismutase), and the polyphosphate kinase (PPK)

encoding gene *ppk* itself [137]. In addition to increased sensitivity toward ROS, polyP-deficient prokaryotic cells display also defects in biofilm formation, virulence, and motility [133,138]. In higher eukaryotes, polyP affects blood clotting and is involved in apoptosis, mTOR activation, and neuronal signaling [139–141]. It remained fascinating how a molecule that is so structurally simple can be involved in all these seemingly unrelated functions in diverse organisms.

Bacterial polyphosphate kinases (PPK) reversibly catalyze the generation of polyP directly from ATP, whereas exopolyphosphatases (PPX) can degrade polyP into P_i molecules (Fig. 4) [85]. Numerous independent studies have reported that deletion of the *ppk* gene in many species of bacteria leads to increased sensitivity toward ROS [85,133,142,143], and we very recently reviewed the mechanistic details of several pathways by which polyP protects bacteria from oxidative stress in a direct or in an indirect fashion [144]. A role for polyP in protein homeostasis under severe oxidative stress conditions, however, has only very recently been deciphered [85]. Measurement of ATP and polyP levels in *E. coli* cells lacking either PPK or PPX in comparison to wild-type cells revealed that 50% of the cellular ATP is converted into long polyP chains under severe HOCl stress (Fig. 4). Investigation of *ppk*-deficient strains of *E. coli* and *V. cholerae* revealed that the absence of polyP results in much higher sensitivity toward HOCl, suggesting that polyP has an important function in bacterial HOCl resistance [85]. However, the question arose of how polyP protects cells against HOCl. We found the answer in our observation that *ppk*-deficient strains accumulate large amounts of protein aggregates *in vivo* [85]. Consistently, the expression of heat shock response genes was upregulated under HOCl stress in the absence of polyP, indicating the need for molecular chaperones to combat protein unfolding and suggesting that polyP might work as a physiologically relevant chaperone that can functionally replace proteinaceous molecular chaperones when present in sufficient amounts [85]. *In vitro* and *in vivo* studies revealed that polyP prevents aggregation of a variety of denatured client proteins in a manner dependent on the concentration and polyP chain length and thus acts as a highly effective chemical chaperone (Fig. 4). PolyP binds unfolded proteins, prevents their aggregation, and finally releases them to ATP-dependent foldase chaperones such as the DnaK/DnaJ/GrpE system once non-stress conditions are restored [85]. The demand for polyP under severe HOCl stress is directly regulated by the oxidant itself via transient oxidative inactivation of PPX (Fig. 4) [85]. PolyP synthesis is an elegant way for cells to prevent protein aggregation during severe oxidative stress and an intriguing new component of the proteostasis machinery for the following reasons: polyP (i) does not require ATP for its chaperone function, (ii) is impervious to oxidative damage, (iii) works during ATP depletion, (iv) does not require time-consuming transcription/translation processes, and (v) can be reconverted into ATP by PPK, which can then be used by ATP-dependent foldases to promote protein refolding. However, several open questions remain, particularly concerning the exact
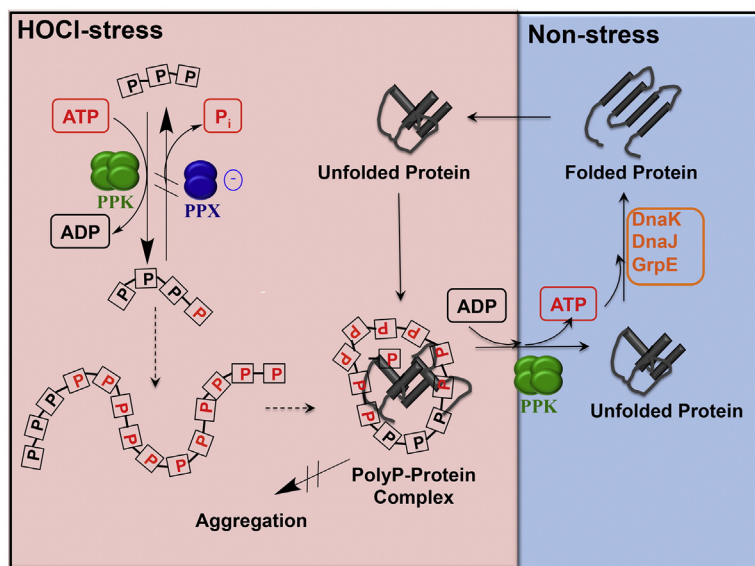
Fig. 4. Model of polyP's chaperone function. Treatment of bacteria with HOCl leads to the conversion of cellular ATP into long chains of polyphosphate (polyP) catalyzed by the conserved enzyme polyphosphate kinase (PPK). PolyP accumulation in the cell is a consequence of the reversible oxidative inactivation of the polyP-degrading enzyme polyP phosphatase (PPX), which contains an oxidation-sensitive cysteine in its polyP-binding site. PolyP accumulation results in a significant depletion of the cellular ATP level, affecting most ATP-dependent cellular processes, including ATP-depending chaperones, such as the DnaK/DnaJ/GrpE system. PolyP is able to compensate for the lack of ATP-dependent chaperones by serving as a scaffold that binds to unfolding proteins, preventing them from aggregation and keeping them soluble and refolding competent. Once reducing conditions are restored, polyP is reconverted to ATP and client proteins are released. Reactivated ATP-dependent foldases then support the refolding of these client proteins.

mechanism by which polyP binds unfolding client proteins to prevent their aggregation. Since the chain length of polyP molecules strongly influenced the chaperone effect of polyP [85], it is likely that polyP acts as a stabilizing scaffold. However, how polyP distinguishes between native and unfolded proteins is still not understood.

In addition to the newly discovered chaperone function of polyP, it has been known for some time that polyP plays a role in regulating proteolysis in bacteria. PolyP retargets the Lon protease of *E. coli* to degrade distinct substrate proteins, including ribosomal proteins [145] and the antitoxin modules of toxin–antitoxin systems [146]. This is thought to be important for the survival of amino acid starvation and accumulation of stress-tolerant persister cells, respectively [147,148]. What remains unknown, however, is how cells balance polyP's chaperone activity with its role in protein degradation, particularly under protein-damaging stress conditions. This question is an interesting future direction for studies of the role of polyP in proteostasis in bacteria.

Although polyP also exists in animals, where it—among other functions—stimulates proliferation [149], influences cytokinesis [150], and affects blood coagulation by enhancing fibrin polymerization [139,151], the gene encoding the polyP-generating polyphosphate kinase has not yet been identified. Identification of the polyP-generating enzymes will help fill the gaps in our current understanding of polyP's role in mammals. Given the fact that many neurodegenerative diseases including Alzheimer's diseases and Parkinson's disease have been associated with oxidative stress and are caused by the accumulation of protein aggregates, understanding the synthesis and role of polyP in mammalian cells might lead to improved therapeutic strategies for a variety of diseases.

Summary and Outlook

Controlled production of RO/CS has been shown to play an important role during the mammalian host defense [26,27,152]. Uncontrolled accumulation of RO/CS, on the other hand, has been associated with a number of pathophysiological processes and diseases [15,153]. One common effect that most RO/CS exert on cells is their damage to the proteome. Over the recent years, a number of protein and non-protein chaperones have been identified, which are specifically activated during oxidative stress conditions to reduce redox stress-induced protein aggregation. Their stress-specific, posttranslational activation together with their ATP-independent chaperone function make them ideally suited to prevent protein aggregation under stress conditions that are known to deplete the energy status of the cell. The chaperones differ primarily in their modes of activa-

tion. Hsp33 in bacteria and Get3 in eukaryotes use the oxidation status of some of their cysteine residues to become activated. RidA/DUK114, in contrast, require N-chlorination of their lysine and/or arginine residues to turn into active chaperone-holdases, and α2-macroglobulin, an extracellular chaperone, most likely uses methionine oxidation as trigger for its functional activation. Together with polyP, which is generated from ATP and in part responsible for the ATP depletion at least in bacteria, these chaperones are capable of binding a variety of different unfolding client proteins and prevent their irreversible aggregation. Once non-stress conditions are restored, the chaperone-activating modifications are reduced by members of the Trx/Grx/GSH systems. At the same time, polyP is reconverted into ATP, which releases the client proteins while fueling ATP-dependent chaperone systems to promote their refolding [85]. Obtaining a detailed view of the precise mechanisms of molecular chaperones under oxidative stress conditions and the processes they are affecting will shed light on our understanding of how molecular chaperones and the pathways they are affecting can be manipulated to alter their oxidative stress resistance. This will help us to design new specific drugs that target these players and potentially attenuate the resistance of pathogenic bacteria toward oxidative stress or help combat the oxidative protein damage associated with human diseases. It is also particularly intriguing that so many of the cellular components with redox-activated chaperone activity are multifunctional. It remains to be seen, however, what the significance of this pattern might be.

species; Trx, thioredoxin; Grx, glutaredoxin; Gor, glutathione oxidoreductase; GSH, glutathione; HOCl, hypochlorous acid; DUOX, dual oxidase; TA, tail-anchored; RO/CS, reactive oxygen and chlorine species.

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