Review

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Thiol-based switching mechanisms of stress-sensing chaperones

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Abstract: Thiol-based redox switches evolved as efficient post-translational regulatory mechanisms that enable individual proteins to rapidly respond to sudden environmental changes. While some protein functions need to be switched off to save resources and avoid potentially error-prone processes, protective functions become essential and need to be switched on. In this review, we focus on thiol-based activation mechanisms of stress-sensing chaperones. Upon stress exposure, these chaperones convert into high affinity binding platforms for unfolding proteins and protect cells against the accumulation of potentially toxic protein aggregates. Their chaperone activity is independent of ATP, a feature that becomes especially important under oxidative stress conditions, where cellular ATP levels drop and canonical ATP-dependent chaperones no longer operate. *Vice versa*, reductive inactivation and substrate release require the restoration of ATP levels, which ensures refolding of client proteins by ATP-dependent foldases. We will give an overview over the different strategies that cells evolved to rapidly increase the pool of ATP-independent chaperones upon oxidative stress and provide mechanistic insights into how stress conditions are used to convert abundant cellular proteins into ATP-independent holding chaperones.

Keywords: disulfides; oxidative stress; protein unfolding; redox regulation.

Introduction

Reactive oxygen species, including superoxide anion and hydrogen peroxide are constantly generated in cells living under aerobic conditions. Effective antioxidant enzymes have evolved to protect the cellular redox homeostasis. Yet, organisms can encounter conditions in which oxidant levels exceed the threshold that can be balanced by cellular detoxification systems, causing a damaging and potentially lethal condition, termed oxidative stress (Imlay 2013; Sies 2015). Reactive oxygen and nitrogen species can modify and damage proteins, lipids, and DNA, thereby challenging the integrity of the entire cell. In proteins, the thiol group of cysteines belongs to the most reactive targets and can undergo different reversible and irreversible modifications. The formation of protein disulfide bonds and mixed disulfides with low-molecular weight (LMW) thiols, like glutathione (GSH), are reversed in most cells by the thioredoxin (Trx) and glutaredoxin (Grx) system. Overoxidation of cysteines to sulfinic or sulfonic acids in contrast causes irreversible damage, leading to protein unfolding and potential aggregation (for recent reviews see Goemans and Collet 2019; Reichmann et al. 2018; Ulrich and Jakob 2019). Oxidants significantly vary in their reactivity with protein thiols and their effects on protein integrity and stability. Hydrogen peroxide, for instance, is a rather slow-acting oxidant—except for proteins with exquisitely peroxide-sensitive thiols—and does not cause substantial protein unfolding or aggregation (Winter et al. 2008; Winterbourn 2008; Winterbourn and Peskin 2016). In comparison, hypochlorous acid, which is produced as a highly potent antimicrobial during mammalian host defense from H_2O_2 and chloride ions, rapidly modifies a wide range of amino acid side chains, causing widespread protein unfolding and aggregation (Sultana et al. 2020; Winter et al. 2008; Winterbourn et al. 2016).

Excessive levels of H_2O_2 or HOCl also induce a substantial drop of cellular ATP (Colussi et al. 2000; Kumsta et al. 2011; Winter et al. 2005). The decrease is attributed to the reversible oxidative inactivation of glycolytic enzymes like GAPDH, which results in the redirection of glucose from glycolysis to the pentose phosphate

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pathway (Hildebrandt et al. 2015; Ralser et al. 2007). Thus, ATP-generating pathways are inactivated in order to produce NADPH, which delivers the electrons for Trx reductase and glutathione reductase to ultimately restore the cellular redox homeostasis (Holmgren et al. 2005). Moreover, cellular ATP is actively converted into long chains of polyphosphate, which not only function as redox inert, high energy storage compounds, but stabilize proteins and have been shown to decrease aggregation in bacteria exposed to oxidative or heat stress (Gray et al. 2014; Yoo et al. 2018).

Depleting cellular ATP levels downregulates all ATP-dependent pathways, including gene expression which reduces the amount of nascent, aggregation-prone polypeptides (Morano et al. 2012; Shenton et al. 2006). Keeping cells in a dormant-like state appears to increase the organisms' chance of surviving toxic stress conditions. However, decreasing cellular ATP also comes with several downsides. One critical drawback is the inactivation of ATP-dependent canonical chaperones, such as heat shock protein (Hsp) 60, Hsp70 and Hsp90 as well as proteases, like the 26S proteasome (Hartl et al. 2011). How do cells then deal with protein aggregation under oxidative stress conditions? Research over the past two decades revealed that cells respond to oxidative protein unfolding stress by post-translationally activating a distinct set of ATP-independent chaperones, which act as protein holdases that bind unfolding protein intermediates and maintain them in a folding-competent state (Goemans et al. 2018; Jakob et al. 1999; Muller et al. 2014; Reichmann et al. 2012; Wyatt et al. 2014). Once non-stress conditions have been restored, these chaperones get inactivated again and transfer client proteins to ATP-dependent foldases.

Thiol-based redox switches evolved as efficient regulatory mechanisms that allow proteins to immediately respond to changes in the cellular redox state (Leichert and Dick 2015). Since the discovery of bacterial Hsp33 as first redox-regulated chaperone (Jakob et al. 1999), stress-induced activation of ATP-independent molecular chaperones has been shown to protect cellular proteins against irreversible oxidative damage across all kingdoms. In this review, we will focus on thiol-based switches of redox-regulated chaperones, explore the mechanisms by which these proteins sense oxidative stress, and discuss how redox-dependent conformational changes contribute to their function as molecular chaperones.

Oxidative activation of the bacterial chaperone Hsp33

The stress-sensing 33 kDa heat shock protein Hsp33 is chaperone-inactive in its reduced state. Four strictly

conserved cysteines arranged in a CXC and CXXC motif coordinate a zinc ion in the C-terminal redox-sensing domain and maintain Hsp33 in a chaperone-inactive conformation (Ilbert et al. 2007; Janda et al. 2004; Vijayalakshmi et al. 2001). Upon exposure of Hsp33 to oxidizing conditions, the four cysteines form two disulfide bonds which cause the release of zinc and induce conformational changes that convert the monomeric chaperone-inactive Hsp33 into highly chaperone-active dimers (Graf et al. 2004; Ilbert et al. 2007; Winter et al. 2008). Importantly, the oxidative activation of Hsp33's chaperone function is fully reversible. Upon restoring non-stress conditions, Hsp33 dimers are reduced by the Grx and Trx system (Hoffmann et al. 2004). The release of client proteins requires in addition the restoration of cellular ATP levels in order to ensure refolding of clients by the *E. coli* DnaK (Hsp70) chaperone system (Hoffmann et al. 2004; Reichmann et al. 2012; Winter et al. 2005). Thus, client proteins are only released from Hsp33 when both the cellular redox balance and protein homeostasis have been restored. Recent single molecule studies revealed that activated Hsp33 not only prevents protein aggregation but guides also the folding process by promoting the formation of partially folded conformations in the client proteins (Moayed et al. 2020).

Mechanism of Hsp33 activation

Oxidative disulfide bond formation triggers the conformational change of fully folded to partially disordered, chaperone-active Hsp33 (Bardwell and Jakob 2012; Reichmann et al. 2012). Two intramolecular disulfide bonds are formed between the two cysteines of the CXXC- and CXC-motif, respectively (Figure 1). Since all four cysteines of the tetrahedral zinc center are arranged in an equal distance to each other (Janda et al. 2004; Vijayalakshmi et al. 2001), the question arises how the formation of other disulfide bonds is avoided? Structural and functional analyses revealed that the highly charged, metastable linker region (~50 aa), which connects the C-terminal redox-sensing domain with the compactly folded, mainly hydrophobic N-terminal domain, plays a crucial role in the oxidative activation of Hsp33. Exposure to H₂O₂ leads to the formation of only one disulfide bond, connecting the two cysteines of the distal CXXC-motif. Once this disulfide bond has formed, zinc is released and the C-terminal domain of Hsp33 partially unfolds. In this conformation, Hsp33 remains chaperone-inactive, but is primed to become activated upon unfolding conditions. The linker has turned into a

thermolabile protein folding sensor that readily unfolds when Hsp33 is exposed to increased temperatures or bile salts (Table 1) (Cremers et al. 2014; Ilbert et al. 2007). Only then, the proximal CXC motif can form the second disulfide bond, which locks the linker region in an unfolded state and constitutively activates Hsp33 as a molecular chaperone (Figure 1) (Cremers et al. 2010; Graf et al. 2004). Kinetically fast oxidants, like hypochlorous acid which cause widespread protein unfolding, induce the formation of both disulfide bonds and activate Hsp33's chaperone function within seconds at any temperature (Winter et al. 2008). Hsp33 from *Chlamydomonas reinhardtii*, which lacks the CXC-motif and thus only loosely coordinates zinc, has lost its redox-regulated activation mechanism and can be activated by elevated temperatures even in its reduced form (Segal and Shapira 2015).

The C-terminal redox-sensitive domain of Hsp33 mediates the stress-dependent switch from an inactivate to an active chaperone, whereas the highly charged linker region functions as a gatekeeper for Hsp33's oxidative activation. Reversing the order of the amino acids in the linker region or replacing it by a non-native sequence of similar length results in a constitutively active chaperone (Rimon et al. 2017). All mutant variants showed an increased sensitivity towards oxidation, suggesting that the linker region affects the reactivity of at least some of the cysteines involved. Replacement of the native linker region also affected the transfer of substrates to the DnaK/J system, emphasizing the function of the polar linker domain in client binding and release (Groitl et al. 2016; Rimon et al. 2017).

Hsp33 function in bacteria

Bacteria lacking Hsp33 are significantly more sensitive towards oxidative stress conditions that lead to protein unfolding (Table 1) (Cremers et al. 2014; Jakob et al. 1999; Sultana et al. 2020; Winter et al. 2008). The protective function of Hsp33 has been shown to serve as a first line of defense in *E. coli* upon oxidative stress and might explain the high abundancy of Hsp33 even under basal conditions (Dahl et al. 2015). Little, however, is known about the *in vivo* role of Hsp33 under non-stress conditions. Rimon et al. (2017) recently proposed that reduced Hsp33 might serve as a house-keeping factor preserving a “healthy proteome” since Hsp33 interacts with members of the proteostasis network under normal conditions in a linker-independent manner (Reichmann et al. 2018; Rimon et al. 2017). A similar role was suggested by an earlier study showing Hsp33-dependent sequestration of the elongation factor Tu (EF-Tu) for degradation by Lon protease in order to slow down *de novo* protein biosynthesis in strains lacking the canonical chaperones DnaK and trigger factor (Brüel et al. 2012). Although *in vitro* studies confirmed that reduced, chaperone-inactive Hsp33 binds to EF-Tu, induces EF-Tu aggregation and thus susceptibility to proteolytic degradation (Jo et al. 2019), *in vivo* evidence for the specific role of the reduced form of Hsp33 in preserving protein homeostasis under physiological conditions still needs to be provided.

Mycobacterium tuberculosis lacks any Hsp33 homologs. However, very recently the protein Rv0991c has been identified to function analogously to Hsp33 (Becker et al. 2020). The redox-regulated protein with unstructured

Figure 1: Activation of *E. coli* Hsp33 chaperone function. (A) Domain structure of Hsp33. (B) The four highly conserved cysteine residues shown in (A) coordinate a zinc ion (red circle) in the C-terminal redox-sensing domain (yellow). Oxidative disulfide bond formation of the CXXC-motif results in zinc release and partial unfolding of the C-terminus. Under additional unfolding conditions, the thermolabile linker domain (red) unfolds and the CXC-motif forms the second disulfide bond, which keeps the linker region in the unfolded state and leads to the exposure of hydrophobic surfaces and Hsp33 dimerization. Once unfolded, Hsp33 acts as a highly efficient, ATP-independent chaperone.

Table 1: Thiol-based functional and structural switches of stress-sensing chaperones.

Protein family	Protein	Subcellular localization	Function under non-stress conditions	Activating conditions <i>in vitro</i>	Thiol-based switching mechanism	Chaperone-active conformation	Phenotypes linked to ATP-independent chaperone function	References
Hsp33	<i>E. coli</i> Hsp33	Cytoplasm	Unfoldase, potential role in proteostasis	H ₂ O ₂ + Temp >43 °C, H ₂ O ₂ + bile salt, HOCl	Disulfide bond formation, zinc release	Partially unfolded, non-covalent dimers	Resistance towards oxidative stress and heat, bile salts or bleach	Cremers et al. 2014; Ilbert et al. 2007; Jo et al. 2019; Rimon et al. 2017; Winter et al. 2008
Ruc	<i>M. tuberculosis</i> Rv0991c	Cytoplasm	–	H ₂ O ₂ and Cu ²⁺	Disulfide bond formation	–	–	Becker et al. 2020
Get3/TRC40	Yeast Get3	Cytosol	ATP-dependent TA protein targeting factor	H ₂ O ₂ and Cu ²⁺	Disulfide bond formation, zinc release	Partially unfolded, tetramers and oligomers	Resistance towards oxidative stress, heat and metal stress	Powis et al. 2012; Shen et al. 2003; Voth et al. 2014
2-Cys Prx	Yeast Tsa1 and 2 (Prx1 and 2)	Cytosol	Peroxidase	H ₂ O ₂ and/or Temp >43 °C	Hyperoxidation of the peroxidatic Cys	Non-covalent HMW complexes	Resistance towards heat stress, growing under zinc depletion	Jang et al. 2004 Cell; MacDiarmid et al. 2013
	Human Prx2						Resistance towards H ₂ O ₂ stress in HeLa cells	Moon et al. 2005
	<i>A. thaliana</i> Prx	Chloroplast stroma		H ₂ O ₂ , CHP		Decamer and various oligomeric forms	–	Koenig et al. 2013
	<i>H. pylori</i> AhpC	Cytoplasm		–		Non-covalent HMW complexes	–	Chuang et al. 2006
	<i>S. mansoni</i> Prx1	Cytosol		H ₂ O ₂ and/or pH 4.2		Two stacked decamers	–	Saccoccia et al. 2012
	<i>Anabaena</i> Atr4641	Cytoplasm		H ₂ O ₂	–	Non-covalent HMW complexes	–	Banerjee et al. 2015
	<i>P. aeruginosa</i> Prx	Cytoplasm		H ₂ O ₂	–	HMW complexes	–	An et al. 2010
	<i>L. infantum</i> mTXNPx	Mitochondrial matrix		Temp >40 °C	Disulfide bond formation (inactivation)	Decamer	Heat tolerance, crucial for parasite infectivity	Castro et al. 2011; Morais et al. 2017; Teixeira et al. 2015
Trx	<i>T. brucei</i> Trx2	Mitochondrial matrix	Potential role in mitochondrial protein import	Temp >40 °C	Disulfide bond formation (inactivation)	Monomer	Heat tolerance, increased parasite infectivity	Currier et al. 2019
Hsp70	Yeast BiP	ER	ATP-dependent foldase	GSSG, GSH + diamide or HOCl or CHP	Sulfenylation and S-glutathionylation of the single Cys	Monomer	–	Wang et al. 2014; Wang et al. 2016
	Human BiP (Grp78)	ER		H ₂ O ₂	Disulfide bond formation		Resistance towards oxidative ER stress	Wei et al. 2012
	Mammalian Hsc70	Cytosol		GSSG, Temp >43 °C	S-glutathionylation		–	Hoppe et al. 2004

AhpC, alkylhydroperoxide reductase; BiP, Binding immunoglobulin protein; CHP, cumene hydroperoxide; GET, Guided entry of tail-anchored proteins; Hsc, heat shock cognate protein; Hsp, heat shock protein; Prx, peroxiredoxin; TA, tail-anchored; Trx, thioredoxin.

C-terminus (Ruc) acts as a molecular chaperone upon oxidation, and enables refolding of client proteins by the *M. tuberculosis* Hsp70 system. Ruc is required for full virulence of *M. tuberculosis* in mice. Ruc homologs are found throughout *Actinobacteria* which, except for a single species, lack Hsp33. Thus, it might be reasonably assumed that Ruc fulfills the role of Hsp33 in *Actinobacteria* in order to prevent irreversible aggregation of unfolding protein intermediates during oxidative stress (Becker et al. 2020).

Dual function proteins with thiol-dependent chaperone activity

Get3: tail-anchored protein targeting factor and molecular chaperone

Hsp33 is highly conserved in bacteria but absent in eukaryotes except for some unicellular flagellates like *Chlamydomonas* and members of the *Trypanosomatidae* family. In yeast, Get3 has been identified to function as a Hsp33-like, redox-regulated chaperone activated under ATP-depleting oxidative stress conditions (Powis et al. 2013; Voth et al. 2014). Although structurally unrelated, Get3 shares several features of Hsp33, including the conserved CXC- and CXXC-motif and the ability to coordinate zinc. However, reduced Get3 forms non-covalent homodimers and the cysteines of the respective CXXC-motifs coordinating the zinc ion in the dimer-dimer interface (Stefer et al. 2011) (Figure 2). Like for Hsp33, oxidative disulfide bond formation results in the release of zinc accompanied by partial unfolding of Get3 (Voth et al. 2014). The conformational changes induce the formation of chaperone-active, higher oligomeric species, which bind unfolding protein intermediates and prevent their aggregation. The activation of Get3's chaperone function is fully reversible and depends also on the restoration of ATP levels (Voth et al. 2014).

In contrast to Hsp33, Get3 has a well-known function under physiological conditions (Table 1). Reduced Get3 acts as an ATPase in the “guided entry of tail-anchored (TA) proteins” (GET) pathway, where it mediates the post-translational insertion of TA proteins into the membrane of the endoplasmic reticulum (ER) (Mateja et al. 2009; Schuldiner et al. 2008; Stefanovic and Hegde 2007). Get3 receives TA proteins from the cochaperone Sgt2, which is bound to the pre-targeting complex (Get4/5) (Figure 2) (for a recent review see Borgese et al. 2019). Sgt2 takes over proteins from cytosolic Hsp70 (Ssa1), which efficiently binds nascent TA proteins. This TA protein relay appears to

Figure 2: The dual function of Get3. Under non-stress conditions (left), reduced, homodimeric Get3 which coordinates a zinc in the dimer-dimer interface is part of the GET complex mediating the post-translational targeting of tail-anchored (TA) membrane proteins to the endoplasmic reticulum (ER). Get3 takes over clients from the co-chaperone Sgt2 at the pre-targeting complex (Get4/5), shuttles them to the ER membrane and hands them off to the GET receptor (Get1/2) upon ATP hydrolysis. Exposure to ATP-depleting, oxidative stress conditions (right) triggers Get3 oxidation and zinc release which causes major conformational changes. The oxidized Get3 tetramer is ATPase-inactive and acts as an efficient, ATP-independent chaperone. Restoration of non-stress conditions will convert Get3 back to its reduced, dimeric form which is chaperone-inactive and ATPase-active.

ensure the gradual loading of Get3 while maintaining the solubility and targeting competence of TA proteins (Cho and Shan 2018). A flexible α -helix lining the substrate-binding groove of Get3 facilitates substrate loading and has been proposed to function as a lid that prevents the interaction of TA proteins with other chaperones (Chio et al. 2019; Mateja et al. 2015). Cargo-loaded Get3 dissociates from the pre-targeting complex and shuttles TA proteins to the GET receptor complex (Get1/2) at the ER (Borgese et al. 2019). ATP hydrolysis seems to be required for Get3 to interact with the receptors. The interaction with Get1 induces the release of ADP and the insertion of the TA proteins into the ER membrane. ATP binding and the interaction with Get4/5 mediate the dissociation of Get3 from the ER and allows Get3 to enter another cycle (Borgese et al. 2019; Hu et al. 2009; Mateja et al., 2015, 2009).

Mechanism of Get3's chaperone activation

Exposure of Get3 to Cu^{2+} , H_2O_2 or $\text{H}_2\text{O}_2/\text{Cu}^{2+}$ results in oxidative disulfide bond formation and the release of the zinc ion (Voth et al. 2014). Get3 undergoes major conformational changes leading to its partial unfolding and the

exposure of hydrophobic surfaces, which allow binding of unfolding protein intermediates. Size exclusion chromatography connected to multi-angle light scattering (SEC-MALS) identified homo-tetramers as the minimal chaperone-active form and subsequent transmission electron microscopy provided a low-resolution structure of the oxidized Get3 tetramer. Importantly, as Get3's chaperone function is activated, its ATPase activity is switched off. Moreover, the TA binding site appears to be buried, thereby preventing the interaction with TA proteins. These structural changes are fully reversible upon Get3 reduction in the presence of Zn^{2+} and ATP (Voth et al. 2014). The precise mode of activation and the kinetics of disulfide bond formation remain under investigation. Disulfide mapping revealed the formation of two disulfide bonds in activated Get3, connecting the next adjacent cysteines of the CXC and CXXC motif, respectively. Whereas *in vitro* studies also revealed the formation of inter-subunit disulfide bonds, only intramolecular disulfide bonds were observed *in vivo* (Voth et al. 2014).

Get3 function in yeast

Get3 and any of the other components of the GET pathway can be deleted in yeast without causing any obvious growth defects under non-stress conditions. This finding is in stark contrast to the fact that some TA proteins like Sed5 are essential in yeast and suggests that alternative targeting pathways for TA proteins must exist (Powis et al. 2013; Schuldiner et al. 2008). In comparison to non-stress conditions, deletion of *get3* leads to growth defects under a number of different stress conditions, including copper and heat stress (Metz et al. 2006; Powis et al. 2013; Shen et al. 2003; Voth et al. 2014). The observation that a Get3 mutant variant, which is deficient in TA protein targeting rescues the copper-sensitive phenotype of a *get3* deletion strain (Mateja et al. 2009) was an early indication for another, targeting-independent role of Get3. Since the targeting-deficient mutant was found to have a wild type-like, redox-regulated chaperone function *in vitro*, we concluded that the copper sensitive phenotype of a *get3* deletion strain is likely caused by loss of the Get3 chaperone activity and not by impaired TA protein targeting (Voth et al. 2014). A recent phylogenetic analysis suggested that handling hydrophobic substrates is a fundamental and likely the more ancient property of Get3, irrespective of its role in TA protein targeting. The chaperone function appears to be a common feature among Get3 homologues across phyla and has been later on adapted to serve in TA protein targeting in eukaryotes (Farkas et al. 2019).

TRC40 function in higher eukaryotes

TRC40 is the mammalian homolog of Get3, which post-translationally binds to hydrophobic C-terminal domains of TA proteins and shuttles them to the GET receptors CAML and WRD in the ER membrane (Schuldiner et al. 2008; Stefanovic and Hegde 2007). The knockout of TRC40 is embryonically lethal in mice (Mukhopadhyay et al. 2006). Yet, the conditional, tissue-specific deletion of the WRB receptor subunit affected only a small number of TA proteins (Rivera-Monroy et al. 2016) which is in line with the notion that losing TRC40 may affect the cell independently from TA targeting. Studies in mammalian cell culture systems revealed that even in the absence of a functional TRC40 pathway, most known TRC40 clients are properly targeted to membranes (Coy-Vergara et al. 2019). The finding agrees with previous *in vivo* studies showing that TA protein targeting is facilitated by several pathways, including the signal recognition particle (SRP) system (Abell et al. 2004; Casson et al. 2017) and the SRP-independent targeting (SND) pathway (Aviram et al. 2016; Hassdenteufel et al. 2017). Therefore, the TRC40 pathway is not essential for TA protein targeting. Considering the function of Get3 as a redox-sensitive molecular chaperone in yeast, it is tempting to speculate that TRC40 also serves as stress-specific chaperone in mammalian cells. Indeed, conditional TRC40 knockout studies in mice suggested that the dependence of syntaxin 5 on TRC40 is not conferred by its transmembrane domain but depends on interactions of TRC40 with its N-terminal cytosolic domain (Rivera-Monroy et al. 2016). Future studies will investigate whether TRC40 indeed functions as a molecular chaperone like its yeast homolog. These studies are, however, not trivial, particularly since purification of TRC40 is very challenging. Nonetheless, obtaining detailed mechanistic knowledge about the two functions of TRC40 is necessary in order to dissect the roles they play *in vivo*.

2-Cys peroxiredoxins: peroxidase and molecular chaperone

Proteins containing a thioredoxin (Trx)-fold motif are particularly sensitive to changes in the redox milieu and several members of the Trx superfamily have been shown to function as redox-regulated chaperones, such as *E. coli* Trx reductase and Trx (Kern et al. 2003). Human protein disulfide isomerase (PDI) also operates as oxidoreductase and molecular chaperone in the ER. Thiol-disulfide exchange reactions promote the switching between a

“closed” (reduced) and an “open” (oxidized) conformation, which facilitates client binding and release (Kar-amzadeh et al. 2017; Wang et al. 2012a). Among Trx-fold proteins, peroxiredoxins (Prxs) represent a large and highly conserved family of thiol-based peroxidases showing an exquisitely high reactivity towards H_2O_2 with catalytic rates of 10^5 – 10^8 $M^{-1}s^{-1}$ (Chae et al. 1994; Winterbourn and Peskin 2016). Within this family, 2-Cys Prxs belong to the most abundant cellular proteins, which primarily function as efficient scavenger of low levels of H_2O_2 and catalyze its reduction to water (Nelson et al. 2011). Given their high reactivity with H_2O_2 , 2-Cys Prxs have been shown to act in addition as receptor and transducer of the H_2O_2 signal (Sobotta et al. 2015; Stocker et al. 2018). In the nucleus, binding of Prx1 to telomeric DNA has been shown to regulate telomerase activity (Aeby et al. 2016; Lu et al. 2013) and Prx-dependent regulations play a crucial role in disease development such as cancer (Forshaw et al. 2019). Under specific stress conditions, 2-Cys-Prxs from various organisms have been shown to become peroxidase-inactive and function as potent ATP-independent chaperone (Table 1) (Jang et al. 2004; König et al. 2013; Moon et al. 2005; Rhee and Woo 2020; Toledano and Huang 2016).

2-Cys Prxs form non-covalent head-to-tail dimers (Figure 3). The N-terminal peroxidatic cysteine of one subunit reduces H_2O_2 under the release of water and formation of a cysteine sulfenic acid. The sulfenic acid intermediate condenses with the C-terminal resolving cysteine of the second subunit, thereby forming an inter-subunit disulfide under the release of a second H_2O molecule (Figure 3). The disulfide is subsequently reduced by Trx, which completes the peroxidatic cycle. Eukaryotic 2-Cys Prxs have been shown to be highly sensitive towards oxidative inactivation at elevated H_2O_2 levels. Oxidation of the cysteine sulfenic acid to a sulfinic acid by another H_2O_2 molecule results in the loss of peroxidase activity (Wood et al. 2003; Yang et al. 2002). In contrast to most cysteine residues, sulfinylation of the peroxidatic cysteine is reversible. Sulfiredoxin (Srx) catalyzes the ATP-dependent reduction and allows Prx to reenter the peroxidatic cycle (Biteau et al. 2003; Woo et al. 2003). Further oxidation of the sulfinic acid to a sulfonic acid is, however, irreversible.

Oxidation and reduction of typical 2-Cys Prxs are accompanied by large conformational rearrangements (Figure 3). Reduced 2-Cys Prxs favor the formation of (do) decameric, ring-like structures, which dissociate into dimers upon disulfide formation. For Prx1 and Prx2, it has been shown that hyperoxidation induces the formation of high molecular weight (HMW) multi-stacked rings which

confer ATP-independent chaperone activity and prevent protein aggregation during oxidative stress (Jang et al. 2004; Moon et al. 2005; Noichri et al. 2015; Wood et al. 2002). Although in Prx1 from *Saccharomyces cerevisiae* (Tsa1) some chaperone activity can be thiol-independently induced upon heat shock (Jang et al. 2004), the peroxidase-to-chaperone switch is primarily guided by hyperoxidation of the peroxidatic cysteine. A similar functional switch has been described for several other 2-Cys Prxs in different organisms and subcellular localizations as summarized in Table 1.

Mechanism of Prx chaperone activation

In human Prx2, hyperoxidation of the peroxidatic cysteine and switching from a peroxidase to a molecular chaperone has been shown to require the presence of a YF (Tyr-Phe) motif in the C-terminal domain (Moon et al. 2005). In a C-terminally truncated Prx2 mutant, H_2O_2 exposure failed to trigger the functional and conformational switch (Moon et al. 2005). It appears that the peroxidatic cysteine acts as efficient H_2O_2 sensor while the YF motif promotes the stress-induced structural rearrangements. Comparing 3D crystal structures of the chaperone-inactive LMW decamer and the chaperone-active HMW double-decamer of *Schistosoma mansoni* Prx1 provided further insights into the mechanism (Saccoccia et al. 2012). Hyperoxidation of the peroxidatic cysteine which is located at the beginning of the $\alpha 2$ helix results in unwinding of the first turn of the helix concerted by unfolding of the C-terminal tail. Unwinding of the first turn of helix $\alpha 2$ has been previously demonstrated for human Prx1 bound to Srx (Jonsson et al. 2008) and represents most likely a general structural change upon Prx hyperoxidation. Unfolding of the C-terminus changes the surface of the decameric ring and allows interacting with another Prx decamer to form a double decamer which is chaperone-active and binds unfolded proteins (Saccoccia et al. 2012).

The formation of hyperoxidized HMW complexes is reversible in the presence of Srx, which catalyzes the ATP-dependent reduction of the sulfinylated peroxidatic cysteine and restores the peroxidase-active conformation (Jang et al. 2004; Moon et al. 2013). Structural analyses showed that the interaction of hyperoxidized Prx with Srx also relies on the unfolding of its C-terminus (Jonsson et al. 2008; Saccoccia et al. 2012). In some organisms, like *H. pylori* and trypanosomads, the mechanism for reducing hyperoxidized 2-Cys Prxs remains to be uncovered since these cells lack known Srx homologs (Toledano and Huang 2016).

Figure 3: Thiol-dependent and -independent activation of 2-Cys Prx chaperone function. 2-Cys Prxs form head-to-tail homodimers arranged often in (do)decameric, ring-like complexes. In the peroxidatic cycle (left), H_2O_2 oxidizes the peroxidatic cysteine of one Prx subunit to a sulfenic acid, which forms an intermolecular disulfide with the resolving cysteine of the other subunit. Disulfide bond formation causes the dissociation of dimers from the oligomeric complex. The disulfide bond is reduced by thioredoxin (Trx). In the sulfenic acid cycle (right), the peroxidatic cysteine becomes hyperoxidized to a sulfenic acid, which stabilizes the decameric structure and triggers the formation of chaperone-active HMW complexes. Sulfenylated Prx can be reduced by sulfiredoxin (Srx) under ATP consumption. Aside of hyperoxidation, elevated temperatures have been shown to induce conformational changes and stacking of Prx decamers resulting in chaperone-active HMW complexes.

In contrast to most known Prxs, the *Leishmania infantum* mitochondrial 2-Cys Prx (mTXNPx) works as an effective chaperone only in its fully reduced form (Teixeira et al. 2015). Activation of the chaperone function is induced by temperature-mediated restructuring of the decamer, which exposes a previously buried hydrophobic patch and allows binding of unfolded protein clients in the center of the decameric ring (Teixeira et al. 2015; Teixeira et al. 2019). Upon restoration of non-stress conditions, client proteins are released and transferred to ATP-dependent chaperones for refolding. Replacing the oxidation-sensitive peroxidatic cysteine by a serine residue maintains the protein in a decameric and/or higher oligomeric conformation even under non-reducing conditions and results in a constitutively chaperone-active mutant variant (Teixeira et al. 2015). *Vice versa*, mutant variants that are unable to form decamers no longer protect parasite proteins against temperature-induced aggregation (Moraes et al. 2017). *L. infantum* mTXNPx is essential for host virulence and thermotolerance in the insect form of the parasite (Castro et al. 2011; Teixeira et al. 2015). The fact that these phenotypes can be rescued by a mutant variant that lacks the peroxidatic cysteine indicates that not the peroxidase function is essential under these conditions, but the ability of mTXNPx to protect clients from temperature-induced aggregation.

We recently described a similar switch for mitochondrial Trx2 in *Trypanosoma brucei* (Table 1). Like *Leishmania*

mTXPNx, the chaperone function of reduced Trx2 is activated by temperature-mediated structural changes and protects other proteins against thermal aggregation by maintaining their folding-competent state. Oxidative disulfide bond formation inhibits activation of the chaperone function. Replacing all five cysteines by serine residues generates a constitutively chaperone-active mutant variant, which complements for the essential function of the wild type protein and confers parasite infectivity in the mouse model (Currier et al. 2019). Both examples show that structural remodeling appears to be necessary for chaperone activation, which can be induced by elevated temperatures as well as possibly by other protein-unfolding stresses (Teixeira et al. 2015). What remains to be investigated is whether this mode of activation only applies to mitochondrial proteins or extends also to cytosolic members of the Trx family.

Thiol-based switching of ATP-dependent foldases to ATP-independent chaperones

Hsp70 proteins are well known for their crucial roles in maintaining cellular proteostasis (Hartl et al. 2011; Rosenzweig et al. 2019). In addition to their ATP-dependent function as foldases, several members of

this family have been demonstrated to act as ATP-independent holding chaperones in response to oxidative stress. In yeast, the ER-localized Hsp70 chaperone BiP is sensitive towards changes in the redox milieu of the ER. The single cysteine of BiP becomes oxidized to a sulfenic acid as peroxide levels rise in the ER (Wang et al. 2014). This intermediate can further react with GSH under the formation of a mixed disulfide (Wang and Sevier 2016). Treating recombinant BiP with glutathione disulfide (GSSG) also resulted in the S-glutathionylation, a mechanism that might be of physiological relevance considering the much lower GSH:GSSG ratio in the ER compared to the cytosol (Birk et al. 2013; Schwarzlander et al. 2016). Both, sulfenylation and S-glutathionylation result in inactivation of the ATPase activity and activation of the chaperone holding function *in vitro* (Wang et al. 2014; Wang and Sevier 2016). The functional switch is fully reversible and the BiP nucleotide exchange factor (NEF) Sil1 has been recently identified to catalyze the reduction of BiP, which restores its ATPase activity and reverses the enhanced holdase activity (Siegenthaler et al. 2017). A Sil1 mutant that lacks its catalytic cysteines is unable to reverse BiP oxidation and restore its ATPase activity. Interestingly, upon reduction, the ATPase activity is further stimulated by Sil1 acting as NEF. Lhs1, another ER-resident NEF of BiP, was unable to restore ATPase activity of oxidized BiP, presumably because Lhs1 does not act as a reductant. However, in the presence of both, Sil1 and Lhs1, an enhanced steady-state ATPase activity of BiP was measured. This effect is likely the result of the Sil1-mediated reduction of BiP followed by an enhanced rate of ATP-turnover promoted by Lhs1 and might explain the advantage of having both NEFs in the ER (Siegenthaler et al. 2017).

The functional switch of BiP appears to not only help preventing protein aggregation, but avoids also ATP-consuming and potentially futile cycles of substrate binding and release under oxidative stress conditions. A similar mechanism has been proposed for the mammalian homolog Grp78, where disulfide bond formation was found to increase its ATP-independent chaperone activity (Table 1) (Wei et al. 2012). Interestingly, the ER-localized non-selenocysteine containing glutathione peroxidase NPGPx has been shown to transfer H₂O₂-derived oxidative equivalents to Grp78 via the formation of a transient intermolecular disulfide bond.

The redox regulation of Hsp70's chaperone function appears not to be restricted to ER-resident homologs. Mammalian cytosolic heat shock cognate protein Hsc70 is also susceptible to protein S-glutathionylation, which

induces an ATP-independent chaperone function and can be reversed by Grx1 (Hoppe et al. 2004). For other cytosolic members of the Hsp70 family, like bacterial DnaK and yeast Ssa1, S-glutathionylation and disulfide bond formation, respectively, have been shown to impair ATP-dependent chaperone function, causing the release from their respective heat shock factors and initiation of the heat shock response (Wang et al. 2012b; Winter et al. 2005; Zhang et al. 2016). The finding emphasizes once more that thiol switches not only act to initiate a specific function, but can also serve to inactivate functions that might be futile or even harmful under stress conditions. In this respect, it is also of particular interest to unravel the role of interaction partners such as J-proteins, which potentially play a crucial role in regulating the functional switch.

Concluding remarks

The stress-specific activation of ATP-independent chaperones serves as first line of defense to protect cells from the formation and accumulation of potentially toxic protein aggregates under severe stress conditions. As high-affinity binding platforms for unfolding proteins, these chaperones do not require energy, which is particularly important under oxidative stress conditions, where cellular ATP levels drop (Colussi et al. 2000; Winter et al. 2005). In turn, reductive inactivation generally relies on the restoration of ATP levels to ensure that client proteins are only released when ATP-dependent foldases are again functional. It remains to be seen which additional cellular factors contribute to the (in)activation of respective chaperones. In the case of Get3/TRC40, the interaction with the GET receptors triggers nucleotide release, whereas ATP-binding of Get3 is necessary to interact with Get4/5 in order to enter another cycle of TA protein targeting (Figure 2) (Borgese et al. 2019). Since the inactivation of Get3's chaperone function depends *in vitro* on the presence of ATP (Voth et al. 2014), it is tempting to speculate that in the cell, GET pathway components contribute to fine-tuning Get3's functional switch.

Many stress-inducible chaperones are dual function proteins. Whereas in some cases the functions are mutually exclusive and proteins have to lose their non-stress function to turn into chaperones, it is possible that the chaperone function of some stress-inducible proteins also plays a role for the interaction with specific proteins under physiological conditions. However, investigating those aspects is not trivial and reveals one major caveat when

studying dual function proteins. In order to assign *in vivo* phenotypes to a specific protein function, one has to find a way to manipulate one of the activities without affecting the other. Therefore, structural features need to be identified first that allow designing mutations which target only one of the two functions.

The fact that stress-sensing chaperones often are dual function proteins also suggests that more cellular proteins might have the ability to switch to effective chaperones under stress conditions. Systemic approaches are required to globally identify redox-regulated chaperones and investigate their functions under both reducing and oxidizing conditions. The Reichmann lab recently presented a toolbox of diverse techniques for studying redox-regulated chaperone activity, including guidelines for preparing fully reduced and oxidized proteins and mapping of conformational changes via hydrogen-deuterium exchange mass spectrometry (Fassler et al. 2018). In addition to thiol-based switching mechanisms discussed here, more and more thiol-independent mechanisms have been identified to convert proteins into active chaperones during stress as recently outlined in Goemans and Collet (2019), Reichmann et al. (2018) and Sultana et al. (2020).

Equally intriguing is the potential role of redox-regulated chaperones in signaling pathways. It is well-established that oxidants like nitric oxide, peroxynitrite and H_2O_2 act as second messengers and oxidative thiol modifications serve to transduce molecular signals (D'Autreaux and Toledano 2007; Forman et al. 2014; Sies 2017). 2-Cys Prxs have been shown to transduce H_2O_2 -derived oxidizing equivalents to specific target proteins (Bersweiler et al. 2017; Rhee et al. 2018; Sobotta et al. 2015; Stocker et al. 2018). It will be interesting to see in how far oxidation-induced conformational changes and the ability of 2-Cys Prx to form high-affinity binding platforms affect interactions with specific target proteins and thus, potentially promote the redox relay.

Thiol-based switching mechanisms allow the immediate activation of stress-sensitive chaperones in response to cellular changes. Given the effort of organisms to maintain a healthy proteome, the role of stress-inducible chaperones in protecting cells during disease- or aging-related changes of the redox homeostasis will be of overall interest. With our increasing understanding of tissue-specific redox protein networks (Xiao et al. 2020) that underlie distinct physiologic and metabolic changes, it will be exciting to learn more about tissue-specific functions of redox-regulated chaperones and their role in redox-modified disease signatures.

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Bionotes

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Kathrin Ulrich studied Molecular Bioscience at Heidelberg University and obtained her Ph.D. from Heidelberg University Biochemistry Center in 2017 where she worked on the unique thiol redox metabolism of African trypanosomes. Led by her interest in redox biochemistry, she has joined the lab of Ursula Jakob as postdoctoral fellow to study thiol switches of stress-sensing chaperones at the University of Michigan. In a joint collaboration with Blanche Schwappach at Göttingen University, she is investigating the redox-regulated switching mechanism of Get3 which turns into an efficient molecular chaperone upon thiol oxidation. Her main goal is to better understand how the oxidation status of specific cysteine residues allows such proteins to sense and respond to cellular stresses.

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Ursula Jakob received her Ph.D. in the Department of Physical Biochemistry from the University of Regensburg in 1995 for her work on the identification and characterization of Hsp90 and small heat shock proteins as molecular chaperones. She then moved to Michigan where she conducted a 5-year postdoctoral fellowship in the lab of Dr. James Bardwell. There, she discovered the heat shock protein Hsp33 as the first known redox-regulated chaperone. This work helped establish the field of redox-regulation in biology. In 2000, she received the Burroughs Wellcome Fund Career Award and started her independent research career at the University of Michigan one year later. She is now the Patricia S. Yaeger Collegiate Professor in the Department of Molecular, Cellular and Developmental Biology and Professor in the Department of Biological Chemistry at the University of Michigan Medical School. Her main interest concerns the role of oxidative stress and redox regulation in aging and disease.