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Under the ROS…Thiol network is the principal suspect for autophagy commitment

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Low molecular weight and protein sulphydryls undergo reactive oxygen species (ROS)-mediated oxidation. However, in contrast to the irreversible damages that oxidative conditions yield on biomolecules, the oxidation of reactive cysteines frequently results in reversible modifications, which represent the prototype of the molecular mechanisms underlying redox signaling. Many proteins involved in a wide range of cellular processes have been classified as "redox-sensitive," thereby modulating their function/activity dependent on the redox state of their critical thiols. Growing evidence from the past few years supports the idea that ROS production also correlates with the occurrence of autophagy. Nonetheless, the cysteine protease Atg4 remains the sole example of a protein whose redox regulation has been completely characterized and demonstrated to be necessary for the progression of autophagy. The principal aim of this commentary is to draw attention to the remarkable number of proteins that can fit the double role of: (i) being involved in autophagy, especially in autophagosome formation and (ii) sensing alterations of the cellular redox state by means of reactive cysteine residues. We will also attempt to provide a hypothetical model to explain the possible functional role of thiols in the occurrence of autophagy and outline a network of redox reactions likely concurring to allow the correct initiation and completion of autophagosomes.

Key words: redox signaling, sulphydryls, glutathione, reactive oxygen species, autophagy

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ROS Affect the Intracellular Redox State by Targeting Thiols

From a mere chemical viewpoint, the natural protein targets of reactive oxygen species (ROS) that have the capability to function as molecular switches in signal transduction are the sulfur-containing residues. Cysteine, better than methionine, fits this role since, in basic environments, the sulphydryl group can undergo deprotonation, thus enhancing its nucleophilicity and propensity to oxidation by ROS (mainly H_2O_2) to sulfenic acid. The sulfenate derivative is unstable and, unless there is the occurrence of over-oxidation (such as occurs under a massive oxidative burst), it usually converts into a disulfide bridge with another cysteine. This species can be easily reduced back by enzymatic systems (e.g., thioredoxin/thioredoxin reductase system) or nonenzymatic reactions (e.g., thiol/disulfide exchange), providing, in such a way, the character of *reversibility* essential for signaling (**Fig. 1**). Physiologically, the steady-state concentration of ROS and, hence, of the oxidized/reduced ratio of protein sulphydryls, is regulated by the synergism between the canonical antioxidant enzymes and the thiol-containing molecules, such as glutathione (GSH) and thioredoxin (Trx), whose reducing power relies upon NADPH availability. GSH, Trx and protein thiols constitute the intracellular thiol pool and contribute, together, to the maintenance of the cellular redox state.

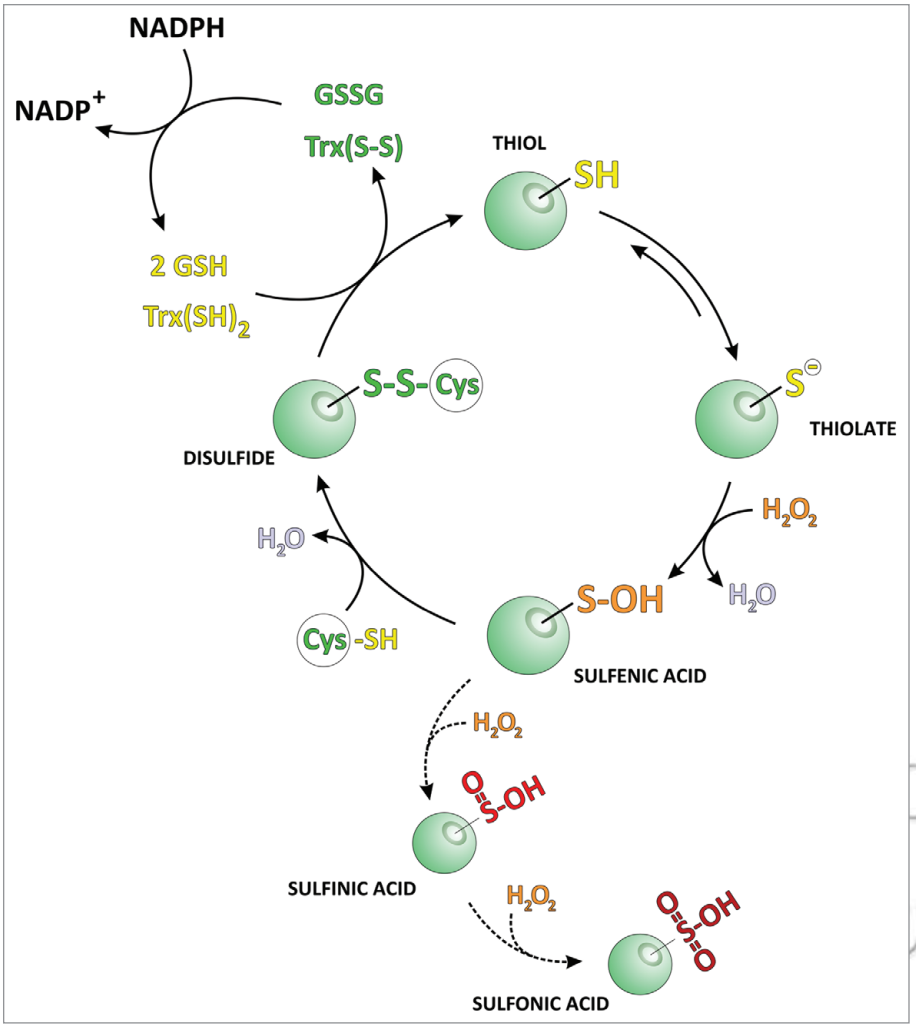

Figure 1. Oxidation/regeneration of a typical reactive cysteine residue of a redox-sensitive protein. In the presence of basic surrounding residues (dark green circle), cysteines located at the surface of redox-sensitive proteins can easily undergo ionization, also at physiological pH. Despite the low reactivity of the sulphydryl form, the thiolate anion is easily oxidized by H_2O_2 to a sulfenic derivative (*-SOH*). This is a very unstable species that reacts rapidly with another neighboring cysteine residue (*Cys-SH*) to generate a more stable inter- or intra-molecular disulfide bridge. The regeneration of the reduced cysteine is mediated by GSH, glutaredoxin (Grx) or Trx, whose reduction is ensured by the catalysis of the NADPH-dependent enzymes GSSG reductase, Grx reductase or Trx reductase, respectively. Under massive oxidative conditions (*dotted arrows*) sulfenic cysteine can undergo further irreversible oxidation reactions, thus leading to the formation of the hyperoxidized sulfinic (*-SO_2H*) or sulfonic (*-SO_3H*) cysteine. Colors used for the cysteine residues reflect the oxidation states of the sulfur atom: *yellow*, -2; *green*, -1; *bright orange*, 0; *red*, +2; *purple*, +4.

Indications for a Leading Role of Thiols in Autophagy

Many cellular processes are modulated by ROS and, in turn, by changes in the cellular redox state. Several pieces of evidence demonstrate that highly reducing environments stimulate cell proliferation, and a moderately oxidizing state initiates cell differentiation, whereas further shifts towards a more oxidizing setting lead to cell death.[1] Recently, it has emerged that the activation of autophagy correlates with an increase of the intracellular levels of ROS as well.[2] However, despite the plethora of observations regarding ROS and autophagy, whether and how changes in redox homeostasis or thiol/disulfide ratio, play a role in the signal transduction mechanisms regulating self-digestion processes remains elusive. In this regard, it has been recently demonstrated that the TP53-induced glycolysis and apoptosis regulator (TIGAR) inhibits autophagy by increasing the levels of NADPH through the redirection of glucose-6-phosphate to the oxidative branch of the pentose phosphate pathway.[3] Other evidence of the inverse correlation between reductant levels and autophagy rate comes from studies in yeast, which demonstrate that a decrease of intracellular GSH is indispensable for the occurrence of mitophagy.[4] On the basis of these observations, the question spontaneously arising is: "Are there proteins able to sense redox unbalance and regulate the autophagic process accordingly?" Despite the wide number of proteins functionally modulated by reversible oxidation of reactive cysteine(s), the protease Atg4 remains the sole example of a mammalian protein whose redox regulation has been demonstrated to be necessary for the progression of autophagy.[5] Nevertheless, if one inspects the proteins reported to have a role in such a process, it would become apparent that, in theory, many of them could be affected by changes in the cellular redox state.

Examples of Putative Redox-sensing Proteins Implicated in Autophagy

In this commentary, we will attempt to link lines of evidence obtained in the last few years and provide a rationale suggesting that the thiol redox state can profoundly influence autophagy, especially during the phagophore elongation steps and autophagosome completion. Nevertheless, this topic does not exclude the possibility that redox dysbalance might regulate autophagy at multiple levels. For instance, it has been recently indicated that both the cationic and the neutral amino acid transporter E16 (SLC7A5) and B (SLC6A19), respectively, undergo oxidation to a sulfenic derivative.[6] This could have consequences for mTORC1 signaling, as it profoundly depends on nutrients, especially amino acid availability. Indeed, amino acid starvation, in particular the absence of leucine or treatments with rapamycin, results in a rapid dephosphorylation of the mTORC1 and, subsequently, to Atg1-driven activation of autophagy.[7,8] Besides this role of ROS as "starvation mimicker," a direct oxidation

of mTOR has also been suggested to result in the inhibition of its activity.[9] However, whether ROS could hinder amino acid incorporation and, hence, give rise to a self-digestion process, has never been demonstrated.

Atg7, Atg10 and Atg3. Among the molecular factors playing a primary role in each step of autophagy, from nucleation, to expansion and completion of vesicles, about 30 different Atg proteins have been functionally identified as molecular adapters or enzymes regulating the autophagic machinery. In particular, it is now well established that two ubiquitin-like protein conjugation systems, sharing Atg7 as a common E1 ubiquitin activating enzyme homologue, operate during the formation of the autophagic vesicles by means of thiol-mediated reactions.[10] The first system catalyzes the addition of a molecule of phosphatildylethanolamine to Atg8/LC3 through the intermediate action of the E2-like enzyme, Atg10. The second one catalyzes the formation of an isopeptide bond between Atg12 and Atg5, by means of another E2-like enzyme, Atg3. Redox mechanisms are deeply involved in the regulation of ubiquitination processes.[11] In particular, both E1 and E2 are redox-sensitive enzymes, as the catalytic cysteines, that catalyze ubiquitin transfer via the formation of a thiol ester bond, can undergo reversible oxidation to disulfide upon ROS production or thiol/disulfide dysbalance, such as decrease of the GSH/GSSG ratio.[11] Since the oxidation of the catalytic cysteines restrains the formation of the thiol ester bond, both of these processes are inhibited by pro-oxidant conditions and favored by a reductant levels increase. Consistent with these notions, it can be speculated also that Atg7-Atg3 and Atg7-Atg10 systems sense alterations in intracellular redox state. However, oxidative conditions should cause the inhibition of Atg7-Atg3 and Atg7-Atg10 mediated catalysis and, in turn, abolish their capability to act as positive regulators of autophagy. Since this hypothesis is apparently conflicting with the above reported correlation between oxidative stress and autophagy, other redox-sensitive proteins must function as the early targets of ROS and, maybe, act as an "antioxidant" buffer against oxidative inhibition of autophagosome maturation.

Rab proteins. Rab proteins are small GTPases of the Ras super-family that often act together in a coordinated manner to regulate retrograde and anterograde Golgi trafficking. Rab GTPases are membrane-associated and active in effector recruitment in the GTP-bound state, and inactive cytosolic proteins in the GDP-bound state. Membrane insertion requires the irreversible modification of two carboxyl-terminal cysteines with isoprenyl lipid (geranylgeranyl) moieties. Among this huge class of proteins, Rab1a, Rab10 and Rab33b have been recently counted in the list of proteins undergoing sulfenylation.[6] Especially for what Rab33b concerns, this reversible modification could affect immature autophagic vesicle (AVi)/autophagosome formation. Indeed, it has been proposed that Rab33b is involved in the recognition/association of the phagophore with the Atg16L complex.[12] It has been proposed that this event allows the recruitment of the Atg3~Atg8/LC3 intermediate, thereby influencing the site of LC3 lipidation and phagophore membrane elongation.[12] Also in this case, oxidation reactions occurring on the Rab33b reactive cysteine(s), could result in the inhibition of phagophore elongation. Therefore, on the basis of what was previously discussed, protective mechanisms against oxidation of Rab33b and other Rab proteins should take place in order to avoid the unsuccessful completion of AVi/autophagosome and, in general, to allow vesicle traffic.

PTEN. Phosphatase and tensin homolog deleted (PTEN) is a peculiar member of the huge class of protein tyrosine phosphatases (PTPs). It enters the lipid signal transduction pathways by catalyzing the dephosphorylation of phoshatidylinositol-3,4,5-trisphosphate [PtdIns(3,4,5)P] to PtdIns(4,5)P, thereby directly antagonizing signaling through the class I PtdIns3K. Like almost all PTPs, PTEN-mediated catalysis relies upon the formation of a cysteinyl-phosphate intermediate at the catalytic cysteine (Cys124), whose redox state is affected by changes in the surrounding redox environment.[13] In particular, it has been demonstrated that Cys124 undergoes reversible oxidation by means of the formation of a disulfide bridge with the backdoor Cys71, which results in PTEN inactivation.[14] Conditions in which PTEN is inactive, such as those occurring upon gene mutation, result in the constitutive activation of the Akt pathway, and therefore in autophagy inhibition.[15] Similarly, oxidative inactivation of PTEN could represent a repressive condition that reasonably should result in autophagy inhibition, unless reduction processes occur. However, apart from the above mentioned factors, proteins whose function was already proposed to antagonize the inhibitory effects of oxidative stress on PTEN—as well as on the other PTPs—have been identified.[16] They are a family of cysteine-based peroxidases, known as peroxiredoxins (Prxs), that function as a primary buffer against H_2O_2 and might be implicated in the redox regulation of autophagy.

Peroxiredoxin/Sulfiredoxin System: How to Sense H_2O_2 and Reduce Sulfinic Cysteines

Prxs were initially identified in yeast as a thiol specific antioxidant and were next found to be present in organisms from all kingdoms.[14] The mechanism of action is quite similar among all members of the Prx family; in particular, once oxidized by H_2O_2 to a sulfenic derivative, the conserved cysteine residue (Cys47), named *peroxidatic cysteine*, reacts with another sulphydryl (*resolving cysteine*) to form a disulfide bond. This sulphydryl can be provided by the GSH/glutathione transferase π (GST-π) system, such as in the case of Prx VI (*1-Cys* Prxs) or by a neighboring cysteine. In Prx V (*atypical 2-Cys* Prxs) this residue is part of the same protein resulting in the formation of an intramolecular disulfide bridge. In Prx I through Prx IV (*2-Cys* Prxs), the resolving cysteine (Cys170) comes from another Prx subunit, thus giving rise to two intermolecular disulfides within an obligate homodimer (**Fig. 2**), which are specifically reduced by the Trx/Trx reductase system at the expense of NADPH.[17] The main role of the most abundant Prx I and Prx II is to protect cellular components by removing the hydroperoxides and peroxinitrite physiologically produced. However, under

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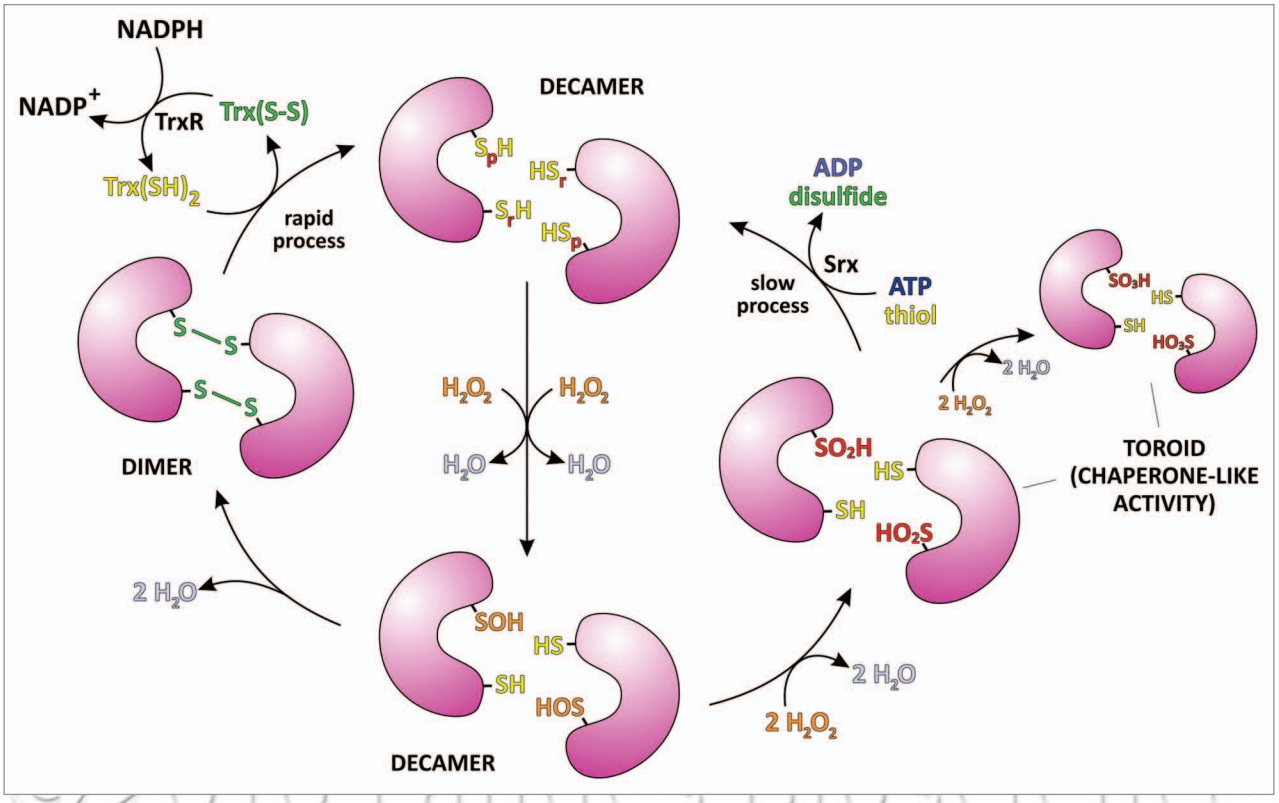

Figure 2. Redox cycles of 2-Cys Prxs. 2-Cys Prxs are the principal intracellular buffer against H_2O_2-mediated oxidation. At physiological H_2O_2 concentration, the peroxidatic cysteine of each monomer (S_pH) undergoes oxidation to a sulfenic derivative, which is preparatory for disulfide bridge formation with the resolving cysteine of the other monomer (S_rH). Disulfide-bound 2-Cys Prxs are rapidly reduced back to the sulphydryl species by means of the Trx/Trx reductase system, which utilizes NADPH-provided electrons. Under H_2O_2 burst, hyperoxidation of peroxidatic cysteine to a sulfinic or sulfonic derivative can occur. Sulfinic 2-Cys Prxs can be slowly reduced by Srx, which requires ATP hydrolysis and thiol-containing molecules (e.g., GSH) as electron donors to generate the all-reduced forms of Prxs. Disulfide Prxs are largely present as dimers, whereas the sulphydryl and, mainly, hyperoxidized species have toroid structures, which have been suggested to confer additional functions to Prxs (e.g., chaperone-like activities, see text).

conditions of H_2O_2 overproduction, such as those occurring upon growth factors stimulation, hyperoxidation of the active site cysteine to a sulfinic acid derivative can take place. The reduced form of *2-Cys* Prxs is regenerated by sulfiredoxin (Srx), a 13-kDa enzyme containing a catalytic cysteine, which requires ATP hydrolysis and thiols as electron donors to operate the slow but complete reduction of sulfinylated Prxs (**Fig. 2**).[18,19] The discovery of Srx was the first demonstration of the ability of mammalian cells to reduce protein sulfinic acid, thus denying the general belief that oxidation to the sulfinic state is an irreversible cellular process. The slow rate of Prx reactivation by Srx (k_{cat} = 0.1–0.8 min^{-1})[18,19] has been suggested to be a functional adaptation to allow H_2O_2 to accumulate and propagate its signal. Moreover, it has been proposed that the redox state of the catalytic cysteine governs the quaternary structure of Prxs, with the reduced protein favoring decameric forms, and the disulfide being mainly in dimeric forms.[20]

Hypothesis on a Pro-autophagic Role of Prx and the Involvement of Sestrins

Recently, Prx I, Prx II, Prx VI and GST-π have been found to localize in the delimiting membrane of the autophagosome,[21] suggesting a role for them in the formation of this organelle. On the basis of the above-mentioned results, it could be speculated that this membrane-located distribution ensures a reducing environment where putative "redox-sensitive" proteins, implicated in vesicle maturation and traffic (e.g., Atg and Rab family members) can maintain their function and allow autophagy to proceed (**Fig. 3**). However, upon overproduction of ROS, the maintenance of reduced surroundings can increase the concentration of the sulfinylated or sulfonylated Prxs. Especially in regard to *2-Cys* Prxs members, it has been demonstrated that such hyperoxidized forms generate alternative structures similar to toroids that are structurally distinct from reduced Prx decamers. These hyperoxidized forms have been suggested to exhibit chaperone-like activity[19] and to protect mammalian cells from oxidative stress-induced apoptosis,[22] suggesting that the oxidized-to-hyperoxidized shift may have a role as a molecular switch that shuts down peroxidase activity and activates the chaperone-like function of Prxs. Also for the *1-Cys* Prxs member, Prx VI, a double function depending on the redox state of the catalytic cysteine has been proposed.[23] In particular, Prx VI catalyzes H_2O_2 and lipid peroxides reduction when in association with GST-π, which behaves as a GSH carrier for the regeneration of the reduced form of the protein. However, when the peroxidatic cysteine

undergoes hyperoxidation to sulfinic or sulfonic acid, Prx VI exhibits Ca^{2+}-independent phospholipase A_2 (iPLA2) activity, which mediates apoptosis upon oxidative stress conditions.[23] Interestingly, iPLA2 has been implicated in the formation of membrane tubules emanating from the trans-Golgi network,[24] in the dynamic maintenance of the Golgi architecture[24,25] and in various trafficking pathways, including the transport of cargo proteins in the anterograde pathway of secretion.[25] Given these additional enzyme activities that ROS overproduction endows to Prxs, it can be assumed that, besides the protection towards oxidative inactivation of the autophagic machinery, Prxs can positively regulate autophagosome maturation by means of other (accessory) mechanisms. For instance, the chaperone activity of Prx I and II can contribute to the autophagic sequestration of protein aggregates, whereas the iPLA2 activity of hyperoxidized Prx VI could contribute in correctly driving vesicle traffic. In addition, a massive hyperoxidation of Prxs (mainly I and II) can concomitantly leave H_2O_2 free to target other thiol-containing proteins, such as Atg4. If we consider the slow rate of Prxs reduction by Srx, this could explain in part why Atg4-mediated cleavage of lipidated Atg8/LC3 is a transient event, whose oxidative inhibition would allow AVi/autophagosome maturation. Finally, Prx I has been shown to bind to the GST-π/c-Jun N-terminal kinase (JNK) complex, thereby preventing the release of JNK from the complex and inhibiting its activation.[26] As JNK is a known positive regulator of autophagy by mediating Bcl-2 phosphorylation and disruption of the Bcl-2/Beclin 1 complex, this could be another way by which Prxs regulate autophagy during H_2O_2 signaling.

In this model of autophagy, whether to preserve the reduced state of a sulphydryl or to allow its oxidation has a deep impact on whether Srx-mediated catalysis occurs, and therefore the steady state levels of sulfinic cysteine represent certainly the rate-limiting event. However, oxidation of cysteine to sulfinic acid is not restricted to Prxs. Critical cysteine residues of many other proteins (e.g., glyceraldehyde 3-phosphate dehydrogenase) are also oxidized to sulfinic acid. It has been calculated that about 1.4% of the cysteine residues of soluble proteins in rat liver are present as sulfinic acid.[23] Therefore, a thiol/sulfinate shift, besides representing a putative novel redox switch for diverse proteins, could require the existence of sulfinic reductases of broad specificity. Consistent with this hypothesis, a family of Srx-like proteins, named sestrins, has been identified because the deletion of their genes causes decreased tolerance to H_2O_2.[28] Moreover, sestrin 2 has been recently demonstrated to be a positive regulator of autophagy and is induced upon starvation as a downstream gene target of p53.[29] Although sestrins are required for cellular regeneration of sulfinic-containing Prxs, other lines of evidence indicate that they are unable to catalyze this reaction in vitro,[30] thereby maintaining this as an active issue of debate. Nevertheless, the demonstration that sestrins are involved in H_2O_2 tolerance shows that they can at least contribute to the reduction of the sulfinylated Prxs (e.g., indirectly by means of still uncharacterized mediators) and/or can have a role in reducing sulfinic-derivatives of other proteins in vivo. What is certain is that the inactivation of sestrins, as well as of Prx I and II correlates with increased incidence of tumors in mice,[31] and that ablation of Prx I and Prx II results in hemolytic anemia,[32] as in the case of the unsuccessful autophagy observed in reticulocytes from Bnip3L knockout mice.[33]

Concluding Remarks

The molecular model coming out from this commentary suggests that, similarly to the above-described regulation of PTPs activity, a *two-module* thiol system is needed to allow autophagy being stimulated by pro-oxidant conditions, in which a first group of sulphydryl-containing proteins (e.g., PTEN, Atg and Rab proteins) must be maintained in the reduced state at the expense of a second one (e.g., Prxs) that represents the "sacrificial lamb" and that, once oxidized, positively contributes to the occurrence of the process. On the basis of the current knowledge in redox biology, we believe that this fine regulation does not rely upon the "quality" of oxidative stress applied to the cell—for instance superoxide radical ($O_2^{\cdot-}$) versus H_2O_2, as recently reported by Chen and coworkers[35]—since thiol oxidation is a *radical-free* rather than a *free radical*-dependent reaction, and therefore it is mainly mediated by H_2O_2 or directly responsive to a decrease in the GSH/GSSG ratio. Conversely, we are confident that the "extent" of oxidative stress is essential to modulate the Prxs-mediated protection of pro-autophagic factors and, hence, to determine the number protein targets undergoing oxidation. This scenario could also underlie the anti-apoptotic role of autophagy, for which a huge or constant redox dysbalance would overcome the capacity of the protective thiols and allow the oxidation of pro-autophagic proteins. This condition would affect the function of autophagosome machinery and hinder the elimination of damaged proteins or organelles, thereby resulting in shifts of the cell response towards a programmed demise. The perspective from which the data reported in this commentary have been looked at, although still speculative, attempts to provide interesting clues for the potential role of a thiol network in the occurrence of autophagy. Since GSH is the most abundant low molecular weight thiol within the cell, it represents one of the principal players in the preservation of this network. The maintenance of GSH intracellular levels depends on: (i) amino acid and ATP availability, necessary for its neosynthesis; (ii) NADPH-provided electrons, required for its reduction, thus electing GSH as a very early sensor of nutrient supply from the extracellular environment. On the basis of the tight relationship between energy and electron availability, as well as the general arising concept of cellular response to stress, as a combined outcome towards a wide range of stimuli (from oxidation to heat shock and starvation), we can speculate that redox signaling could be profoundly implicated in the mechanisms governing these processes and, hence, in the regulation of autophagy as well. This assumption is also corroborated by the similar phenotypes yielded by mutations of proteins entering autophagy, rather than DNA repair or antioxidant pathways. Besides the above-mentioned proteins,

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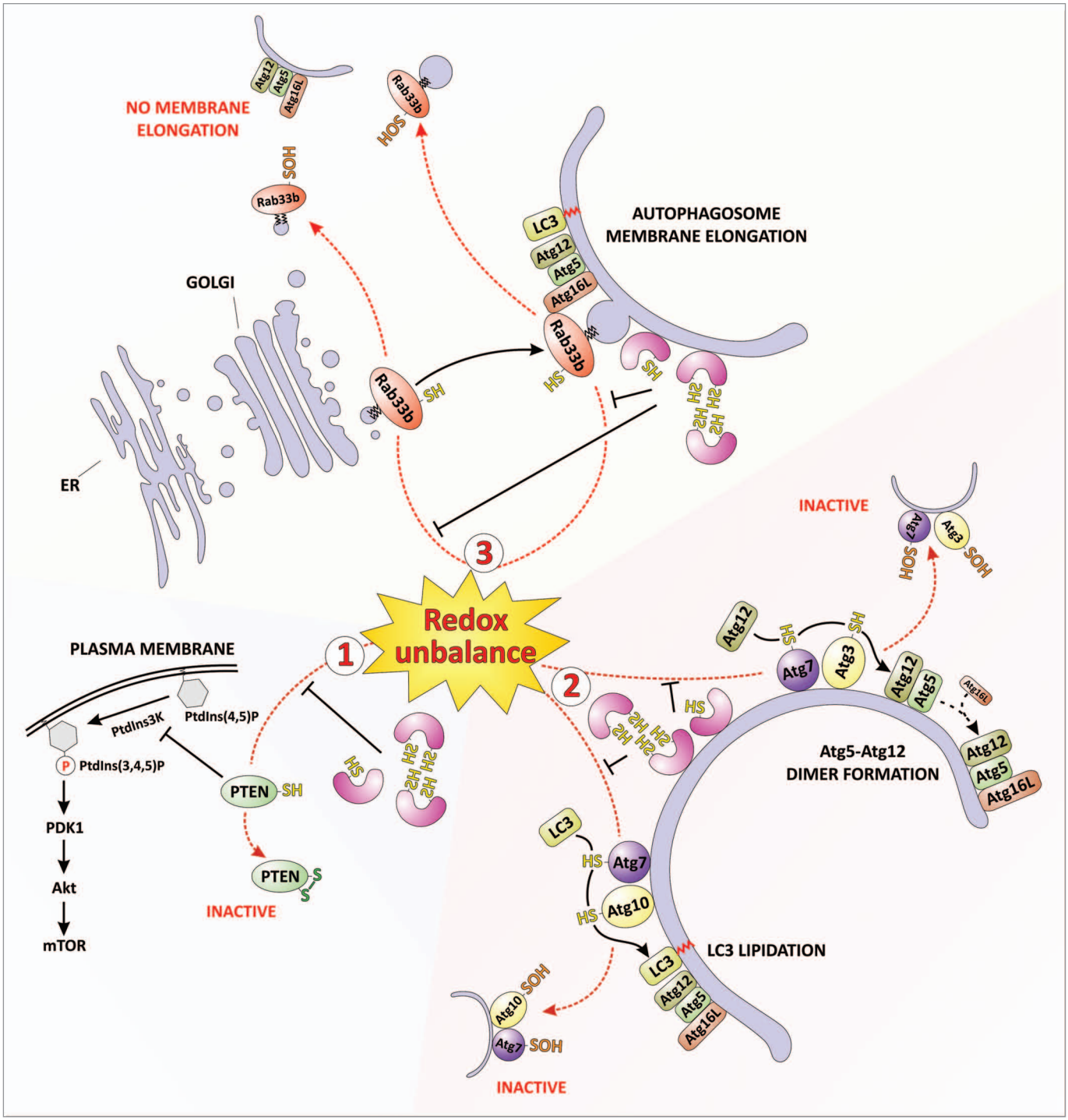

Figure 3. For figure legend, see page 1005.

many others could be comprised as being among the putative redox-sensitive members of the autophagic machinery. For instance, one of the last to be identified and that could meet these expectations is Rubicon, for which a cysteine-rich containing domain has been pointed out to be indispensible for the interaction with Beclin 1 and the resulting inhibition of class III PtdIns3K.[34] Although no evidence has been provided yet about the redox sensitivity of Rubicon, it cannot be excluded that oxidative insults could affect protein/protein interaction and, thereby, Rubicon activity by means of changes in cysteine(s) redox state.

Overall, the above reported examples of sulphydryl oxidation on proteins located on the membrane surface of the autophagosome and, generally, on proteins involved in the regulation of a self-eating process remain only a theoretical indication of what might occur during AVi/autophagosome maturation. The role

Figure 3 (See opposite page). Hypothesis on the possible redox implications in autophagy. Many proteins involved in autophagy have been demonstrated to sense redox alterations by means of reactive cysteines. In this scheme we have summarized some of the possible processes in which redox reactions could influence autophagosome formation and, more generally, the occurrence of autophagy. Under redox unbalance, such as ROS overproduction or a decrease in the GSH/GSSG ratio, the catalytic cysteine of PTEN can be oxidized to an intramolecular disulfide with the so-called backdoor cysteine, thereby compromising its phosphatase activity on PtdIns(3,4,5)P. (1) This represents a repressive condition similar to those occurring upon mutation of the protein, in which autophagy is inhibited. (2) Redox alterations can also affect the ubiquitin-like systems Atg7-Atg10 and Atg7-Atg3, whose function relies upon the cysteine-based transfer of Atg12 and Atg8/LC3 respectively, resulting, in such a way, in the oxidative inactivation of the proteins and in a general impairment of autophagosome membrane elongation. (3) Similar circumstances could also occur if Rab proteins, especially Rab33b, undergo sulfenilation. Prxs-mediated reductions can allow autophagy to proceed, as they counteract the oxidative inhibition of the autophagic machinery. Besides their cytosolic localization, Prxs have been also found on the phagophore surface, thus reinforcing the hypothesis of a role for them in the maintenance of a reducing environment for proteins directly involved in the maturation of autophagosomes. Moreover, in the hyperoxidized forms resulting from ROS scavenging, Prxs change their structure and can further act as pro-autophagic molecules. In particular, it has been demonstrated that sulfi(o)nylated Prx I, II and Prx VI show chaperone-like and Ca^{2+}-independent phospholipase A_2 activities, respectively, thus contributing to the autophagic sequestration of protein aggregates, and to correctly regulate vesicle traffic. In the figure, oxidation reactions (*dotted red arrows*) of the pro-autophagic proteins PTEN, Atg7, Atg3, Atg10 and Rab33b are shown as possible events, whose inhibition by Prxs-mediated catalysis could underlie the correct progression of autophagy.

of the antioxidant activity of GSH, Prxs and the reduction systems of sulfinic cysteines, as well as their spatial distribution and the redox forms under which they are present are only some issues that deserve to be investigated in the future to comprehend these and many other aspects of the possible crosstalk between redox reactions and modulation of autophagy.

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