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COMMENTARY

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Globin-based redox signaling

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ABSTRACT

In recent years, moderate levels of reactive oxygen species (ROS) have become recognized as signaling cues that participate at all levels of cellular organization. Globins, with their redox-active heme iron and ubiquitous presence, seem ideally suited to participate in ROS metabolism. Here we comment on our recent findings that show the participation of a globin, GLB-12, in a redox signaling pathway in *Caenorhabditis elegans*. We found that GLB-12 produces superoxide, a type of ROS, after which this is converted to what appears to be a hydrogen peroxide gradient over the plasma membrane by the activity of intracellular and extracellular superoxide dismutases. In the first part, we discuss in more detail the different regulatory mechanisms that increase the effectiveness of this redox signal. In the second part, we comment on how specific structural and biochemical properties allow this globin to perform redox reactions. Interestingly, these properties are also observed in 2 other *C. elegans* globins that appear to be involved in redox biology. We therefore hypothesize that globins involved in redox signaling display similar structural and biochemical characteristics and propose that a subgroup of globins can be added to the group of proteins that play a vital role in redox signaling.

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Introduction

Reactive oxygen species (ROS) are continuously produced, modified and again removed in all living organisms.[1-4] The traditional view of ROS and ROS-associated reactions is one of oxidative stress and damage, which leads to the decline of health in aging and disease. ROS levels are indeed increased in diseases ranging from cancer over neurodegenerative pathologies to diabetes. On the other hand, ROS can also operate as signaling molecules, a function that has been widely documented in recent years, but is sometimes received with reservation. This skepticism stems from the apparent paradox between the specificity that is required for signaling and the seemingly indiscriminate reactivity and transient nature of ROS. However, ROS are not one distinct chemical entity, and each ROS has unique chemical and biological properties. In addition, it appears that organisms have evolved a range of mechanisms to harness the reactivity of ROS and incorporate it in cell signaling. This controlled and regulated interaction of ROS with biological signaling is termed redox signaling, and it is now well appreciated that redox signaling cascades participate in a wide variety of essential physiological processes.[1-7]

There are currently 2 sources for ROS signaling known, namely mitochondria and a group of membrane-bound enzymes, NADPH-dependent oxidases and their dual oxidase relatives (Nox/Duox).[8-10] In *C. elegans*, mitochondrial redox signaling has been associated with e.g. longevity,[11] mitochondrial protein homeostasis[12] and actin-based wound healing,[13] while Duox are involved in tyrosine cross-linking to stabilize the extracellular matrix.[14] Both mitochondria and Nox/Duox produce $O_2^{\cdot -}$ (superoxide) to create a redox signal, after which $O_2^{\cdot -}$ is rapidly converted to the more stable H_2O_2 (hydrogen peroxide).

A major topic in this field is to understand the cellular mechanisms that define specificity in redox signaling, especially given the chemical simplicity of the redox signaling molecules $O_2^{\cdot -}$ and H_2O_2. One mechanism that appears to increase specificity is the spatiotemporally well-defined generation of H_2O_2. This model is mainly based on the properties of Nox/Duox:

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these proteins colocalize with their redox targets, such as phosphatases and kinases, at the plasma membrane within the cell or within cell organelles.[15,16]. Because of this, $O_2^{\cdot-}/H_2O_2$ produced by Nox/Duox will directly target the downstream target proteins and will not cause unwanted oxidation of other proteins. However, many questions remain, such as if these regulatory mechanisms are only relevant for Nox/Duox signaling, and how is redox specificity achieved in the many biological processes that are redox sensitive.

Recently we showed that a globin in *C. elegans*, named GLB-12, actively produces $O_2^{\cdot-}$ to create a redox signal that is essential for reproduction.[17] In this commentary, we discuss these findings in light of current understanding of how redox signaling is regulated. We propose that multiple molecular mechanisms are responsible for the specificity in this redox signaling pathway, and that these mechanisms are potentially a general feature of redox signaling pathways. In the second part, we argue that GLB-12 is most likely not the only globin that actively drives redox signaling, but instead represents a specific subgroup within the globin family that is functionally adapted to participate in redox biology.

GLB-12 regulates reproduction by generating a redox signal

We initially selected GLB-12 based on its promising RNAi depletion phenotype, indicating its involvement in reproduction, vulval and general development. A more detailed analysis showed that *glb-12* RNAi depletion causes severely reduced fecundity and multiple defects during germline and oocyte development. Interestingly, we discovered that GLB-12, unlike the majority of globins, cannot bind O_2, but instead becomes oxidized when it is exposed to air. In addition, the crystal structure of GLB-12 showed that this globin possesses unique properties that support a role in electron transfer, while electrochemical analysis revealed that it has a reduction potential sufficiently low to favor electron transfer from its heme iron to O_2. Based on these results, we hypothesized that GLB-12 could interact with O_2 to create $O_2^{\cdot-}$, which in turn could lead to a redox signal (Fig. 1). We indeed observed that *in vitro* GLB-12 is capable of actively converting O_2 to $O_2^{\cdot-}$ by electron transfer. In line with this, GLB-12 interacts *in vivo* with the main intracellular superoxide dismutase (SOD) to generate

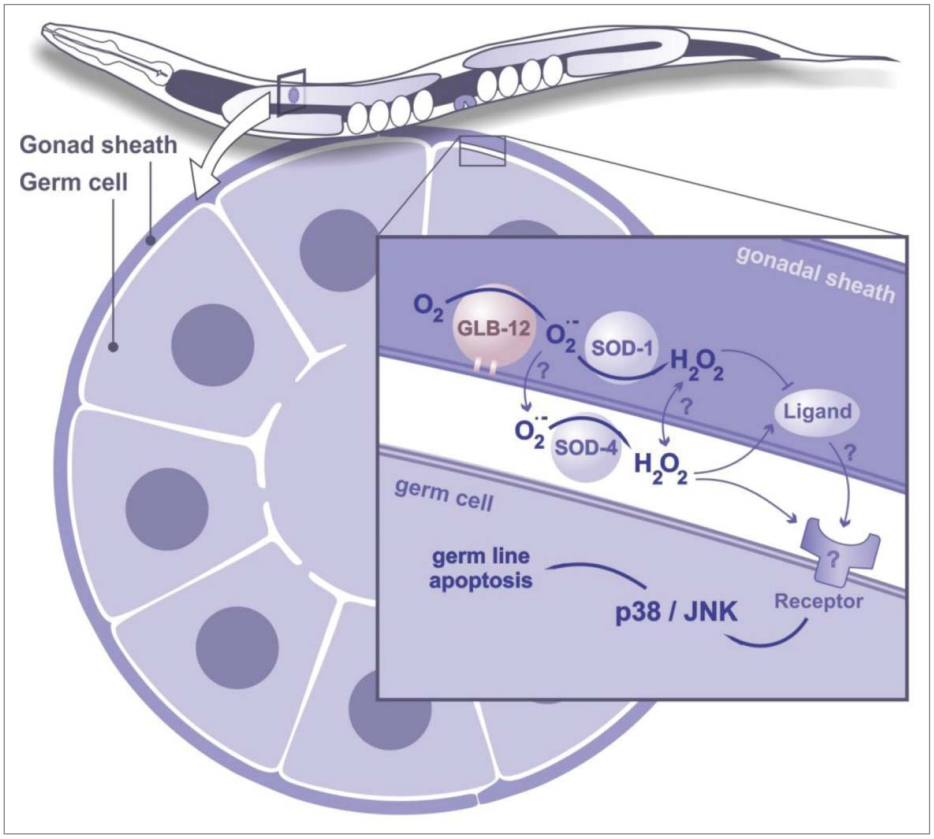

Figure 1. Outline of the redox signaling model for *Caenorhadbitis elegans* GLB-12.

the more stable H_2O_2. Surprisingly, the GLB-12 based redox signal is also modulated by the extracellular SOD. These results strongly supported the role of GLB-12 as a redox signaling protein and showed that this globin, together with 2 SODs, forms what appears to be a redox signaling module (Fig. 1). Interestingly, this signal is regulated at the subcellular level by multiple control layers, which is discussed in more detail below.

Specificity in redox signaling is regulated by multiple molecular mechanisms

Spatial and temporal regulation of redox signaling

As stated above, cells can potentially regulate physiological redox signaling by controlling the production of $O_2^{\cdot -}$ and H_2O_2 in a spatial and temporal manner at the subcellular level. This is based on the fact that $O_2^{\cdot -}$ and H_2O_2 are unable to migrate far from their source of production because of their inherent reactivity, combined with the redox-buffering and antioxidant capacity of the cell. Given this, the effectiveness of redox signaling can thus be regulated by the colocalization of enzymatic sources of these ROS and their downstream targets.[18] Colocalization also improves selectivity, preventing pathological oxidation of targets such as proteins and lipids. This model of redox signaling regulation is based on the tissue- and subcellular-specific expression of Nox/Duox proteins, and a similar property is seen for GLB-12. At the tissue level, this globin is expressed in clearly distinct parts of the gonadal sheath. At the subcellular level, GLB-12 is membrane-bound by myristoylation and palmitoylation, thereby targeting it to specific subdomains within the cell membrane. As such, any $O_2^{\cdot -}$ produced by GLB-12 will be restricted to a very specific region within the gonadal sheath, allowing for a spatially defined redox signal. We indeed found that this membrane-association of GLB-12 is important to deliver its redox signal. Similar to Nox/Duox, GLB-12 is thus restricted to very specific subcellular regions to deliver its redox signal.

Modulation of local redox buffer capacity

On top of localizing the enzymatic source of a redox signal with its potential downstream target, an additional layer of regulation can be achieved by alterations in the local redox buffer capacity.[1] For example, the conversion of $O_2^{\cdot -}$ to H_2O_2 occurs spontaneously in an aqueous environment, but can be strongly enhanced by the presence of SODs. The presence and interaction of SODs with a enzymatic source of $O_2^{\cdot -}$ would thus enhance the conversion to H_2O_2, which is a more potent redox signaling molecule, and thus increase the effectiveness of the redox signal. This mechanism has been observed for all SOD types: 1) for the main cytoplasmic SOD-1, by inhibiting MPK-1 phosphorylation in the oocyte preceding fertilization, most likely by the conversion of mitochondrial $O_2^{\cdot -}$ to H_2O_2;[19] 2) for the mitochondrial SOD-3; by its conversion of mitochondrial $O_2^{\cdot -}$ to H_2O_2 to generate a pro-longevity signal;[20] and 3) for the extracellular SOD, by its localization at membrane microdomains to promote VEGF receptor type2 signaling[21] (reviewed in [22]).

Also GLB-12 interacts with a SOD to increase the effectiveness of its redox signal: reduction of GLB-12 levels leads to a more severe phenotype in a knockout background for the main cytoplasmic SOD-1, and this can be reverted by introducing a SOD-1 translational reporter. Given that these 2 proteins are expressed in the gonadal sheath and are both intracellular, these results indicate that SOD-1 converts $O_2^{\cdot -}$ produced by GLB-12 to H_2O_2. GLB-12 also interacts with the extracellular SOD-4, and this in an opposite manner compared to SOD-1: loss of SOD-4 will alleviate the severity of the *glb-12* RNAi phenotype, and also here this effect is reverted by introducing a SOD-4 translational reporter. In this case however, it is unclear if the redox signal by GLB-12 is directly or indirectly modulated by SOD-4. If SOD-4 directly modulates the GLB-12 redox signal, this implies that $O_2^{\cdot -}$ produced by GLB-12 needs to pass the cell membrane before it can be converted to H_2O_2 by SOD-4. Alternatively, SOD-4 may participate in an independent, extracellular redox signal that converges on the same downstream target as the GLB-12 redox signal. Overall, these observations further support the role of SODs in modulating the strength of redox signals, this by enhancing the conversion of $O_2^{\cdot -}$ to H_2O_2 and so increasing local concentrations of the latter.

Membrane transport and sequestration

Plasma membranes form a physical barrier for ROS participating in redox signaling. This also implies that ROS transport across membranes could be actively

regulated by the presence of selective membrane channels, allowing an additional level of control. Indeed, this has been observed for both $O_2^{\cdot -}$ and H_2O_2, in which the permeability of membranes to these molecule can be modified by the presence of certain classes of anion channels[23] and aquaporins,[1,24,25] respectively. In line with this, it has recently been reported that Nox proteins can work together with aquaporins to regulate transport of extracellularly produced H_2O_2 across the plasma membrane to influence intracellular signaling cascades.[18] These results suggest that different tissues, cells or cell organelles can be tuned for their sensitivity to H_2O_2 mediated signaling, depending on the type of aquaporins or similar channels that are present in their plasma membranes. In this context, it is fascinating that the GLB-12 based redox signal appears to be modulated by both an intracellular and an extracellular SOD. This indicates that in this redox signaling pathway the amount of $O_2^{\cdot -}$ and/or H_2O_2 on both sides of the cell membrane is important for the regulation of downstream signaling. It therefore appears reasonable that also in this redox pathway cell membrane permeability for $O_2^{\cdot -}$ and H_2O_2 is regulated by the presence of membrane channels. A major question is how exactly $O_2^{\cdot -}$ and/or H_2O_2 on either side of the membrane influence downstream signaling, especially because loss of the intracellular and an extracellular SOD have opposite effects on knockdown of GLB-12. We hypothesize that the separation of these 2 ROS by the cell membrane provides an additional level of regulation in the GLB-12 mediated redox signaling pathway. Also this property might form a more general principle to increase redox signaling specificity.

Globins can actively drive redox signaling

Globins form an ancient and ubiquitous superfamily of heme-associated proteins.[26] In vertebrates, the role of hemoglobin and myoglobin as O_2 carriers has been extensively studied and globins in a wide range of organisms appear to fulfill such a function. At the same time, our understanding of the function and distribution of these proteins has greatly evolved over the last decades, and detailed analysis on selected globins has now allowed us to define a multitude of potential physiological roles for these proteins.[27-31] Enzymatic redox reactions appear to be an inherent part of many globins, and several redox-related functions have consequently been proposed for these proteins.[32] *C. elegans* possesses the remarkably high number of 33 globins,[33] and 6 of these have thus far been examined in more detail. Besides GLB-12, 2 other globins appear to play a role in redox signaling, namely GLB-6 and GLB-26.[34,35]

Interestingly, these 3 globins show largely comparable biochemical characteristics; they display strong hexacoordination, are spontaneously oxidized when exposed to ambient air and show reduced or absent ligand binding. In hexacoordinated globins, the heme group is coordinated by 2 histidine side chains. Initial analyses of hexacoordinated globins showed that their distal histidine side chain is capable of reversible dissociation to allow the stable binding of gaseous ligands, like CO, NO and O_2.[31] However, GLB-6 is almost incapable of binding ligands, while GLB-12 and GLB-26 bind CO only with reduced affinities. This indicates that the heme iron in these globins is very tightly hexacoordinated and that these proteins are not involved in a role that requires reversible ligand binding. Furthermore, the spontaneous heme iron oxidation under air rules out a function in O_2 storage. Instead, the fast oxidation rate of the heme iron indicates a function in redox reactions for all 3 globins. The absence of ligand binding most likely helps to keep the reduction potential of the heme iron unaffected.

The crystal structure for the GLB-6[35] and GLB-12[17] globin domain has been solved, while the GLB-26 3-dimensional structure has been modeled using GLB-6 as template.[36] These results show that the 3 globins also have surprisingly similar structural characteristics. The proximal side of the heme group in these globins is exposed to the hydrophilic environment, which results in a lower redox potential and high autoxidation rate. A missing D-helix in GLB-6 and GLB-26 and a stabilization of the E-helix through hydrogen bonds in GLB-12 results in a restriction of the helices at the distal side of the heme, which is expected to hamper ligand binding and thus corresponds to the observed ligand binding characteristics.

Previous results of our group indicated that GLB-26 has a role in redox signaling.[34] GLB-26 is exclusively expressed in the stomato-intestinal and anal depressor muscle cell and in the head mesodermal cell, all of which appear to be associated with the regulation of the defecation cycle. We observed that the defecation cycle is normal in a *glb-26* knockout strain, but is

differentially affected in WT and *glb-26* knockout worms when they are exposed to high concentrations of a ROS producing compound. High ROS levels might influence redox reactions carried out by GLB-26, explaining these observations. It is however unclear what the exact nature is of these reactions.

GLB-6 is not yet functionally analyzed. However, in addition to their detailed physicochemical and structural analysis of GLB-6, Yoon and colleagues reported that a GLB-6 overexpressing worm suppresses worm aggregation, a behavior related to O_2 concentration.[35] Because GLB-6 is expressed in several sensory neurons,[37] they hypothesize that this globin functions as a sensory protein. Because of the large number of biochemical and structural similarities between GLB-6 and GLB-12, it is conceivable that these 2 globins have a comparable function in the nervous system. In addition, it should be noted that, while GLB-6 seems exclusively expressed in neurons, these results are based on a transcriptional reporter, containing only the gene's promoter. We observed in several cases that including a gene's introns and 3′UTR region in a reporter can reveal expression in additional tissues. An obvious question is thus if GLB-6, like GLB-12, is expressed in additional tissues and thus also functions in multiple processes.

GLB-6, GLB-12 and GLB-26 also appear to share a similar subcellular location. Both GLB-12 and GLB-26 are membrane-bound by protein acylation, and, while not functionally tested for GLB-6, several acylation sites for this globin are predicted with varying confidence (personal observation). This restriction in subcellular location for these 3 globins supports a spatially confined function, such as cell signaling. As stated before, we observed that the membrane-localization of GLB-12 is associated with its role in redox signaling, whereby we hypothesize that the tightly defined localization mediated by acylation increases the specificity of the GLB-12-redox signal. Interestingly, a membrane localization dependent on the presence of fatty acids has also been identified for several non-*C. elegans* globins.[38-40] More recently, a bioinformatical screening starting from 7697 globin sequences identified 90 globins with potential myristoylation sites, of which 65 also appear to carry one or more palmitoylation sites.[41] The authors of this screen propose a function related to lipid protection or signaling for these globins. These results could potentially also be an indication that multiple globins, spread over different organisms, are involved in redox signaling.

In conclusion, GLB-6, GLB-12 and GLB-26 are 3 hexacoordinated globins which share several biochemical and structural characteristics that associates them with a role in redox biology. Additional functional analysis of GLB-12 shows that this globin acts as a redox signaling protein. A first set of functional results for GLB-26 also support a role in redox biology for this protein, while further research should show if GLB-26 and GLB-6 participate in redox processes comparable to those of GLB-12. From a broader perspective, the group of hexacoordinated globins shows considerable variation in ligand binding kinetics, reduction potential and structural properties. The characterization of these 3 *C. elegans* globins suggests that hexacoordinated globins that participate in redox biology are likely to show several properties that support stable electron transfer of the heme iron; 1) a tightly coordinated heme, 2) reduced ligand binding properties, 3) a polarized heme cavity, 4) a relatively low redox potential, and 5) a restricted subcellular location. While it is very likely that additional hexacoordinated globins will function in redox biology, future research should show if these properties are indeed essential for such a role.

Conclusion

The regulatory mechanisms acting in the GLB-12 redox signal are also operating in Nox/Duox signaling, and could therefore be general principles in this field. Furthermore, globins involved in redox biology appear to have evolved specific structural and biochemical adaptations.

Abbreviations

Duox	Dual oxidases
GLB	globin
H_2O_2	Hydrogen peroxide
Nox	NADPH-dependent oxidases
$O_2^{\cdot -}$	Superoxide
ROS	Reactive oxygen species
SOD	superoxide dismutase

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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