Functional Analysis of the *Caenorhabditis elegans* Globin Family

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Cover:

Front: A GFP reporter for GLB-12 reveals expression of this globin in the nervous system, the vulva and the reproductive system.

Back: Electrostatic surface of GLB-12, whereby the blue and red colors highlight positively and negatively charged surfaces, respectively.

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Summary

Globins are ubiquitous in nature: they can be found in the simplest of life forms, i.e. bacteria and Archaea, over the unicellular eukaryotes to multicellular and complex plants, fungi and animals[20-25]. Globins were among the first proteins to be studied, with the earliest report dating back to the 19th century[26]. Over the last century, the number of known globins was very limited and only a handful of globins were characterized in more detail, most notably hemoglobin in red blood cells and myoglobin in muscle tissue in vertebrates. As a result, globins were almost exclusively defined as respiratory proteins, facilitating O_2 uptake and transport throughout the body to support aerobic respiration. In the last three decades, our understanding of the function and distribution of these proteins has greatly evolved; the availability of genomic information from the three domains of life presented hundreds of new globin genes, which 1) revealed the ancient and ubiquitous nature of this superfamily, 2) presented new globin types and 3) indicated a high degree of functional adaptation to different environments[20-25]. Detailed analysis on selected globins has now allowed us to define a multitude of potential physiological roles for these proteins[12,27-30]. At the same time, many of these newly proposed functions, while supported by structural and biochemical work, still await strong validation from physiological studies.

The roundworm *Caenorhabditis elegans* is an established model organism for fundamental and biomedical research, offering a simple accessible anatomy and powerful molecular-genetic tools to study highly conserved aspects in development, behavior, disease and aging. *C. elegans* has thus far also been a very promising model system to study the functional diversity and physiological roles of the globin superfamily. It has the large number of thirty-three different globins, which are very diverse in gene and protein structure and are present in a variety of cell types[16,31,32]. It is assumed that this large globin family is the result of multiple evolutionary radiation and duplication events, together with the diversification and subfunctionalization of individual globin genes[31]. The variation in characteristics combined with the evolutionary history of this family indicates that its different members are involved in a range of physiological processes. More detailed analysis on a subset of these globins further supports this hypothesis, with globins functioning in O_2 sensing, participating in redox reactions and protecting against oxidative stress[33-37]. Within this present thesis, we aimed to further enhance our understanding on the potential roles that can be carried out by these *C. elegans* globins, by focusing on the molecular mechanisms used by several of them.

Summary

The first and main part of this research focused on the functional analysis of GLB-12. This globin was initially selected based on a promising RNAi depletion phenotype, which indicated the involvement of GLB-12 in reproduction, vulval development and general development. Following a more detailed analysis on the role of this globin in reproduction, we found that *glb-12* RNAi depletion causes severely reduced fecundity and multiple defects during germline and oocyte development. One of these defects was an abnormal increase in germline apoptosis levels and we discovered that this increase was mediated through the conserved JNK and P38 MAPK pathways. However, we also observed that several other germline defects associated with *glb-12* RNAi depletion occur independently of these two pathways, suggesting that GLB-12 influences other signaling cascades as well.

In collaboration with the group of Prof. Dr. L. Moens and Prof. Dr. Dewilde (Department of Biomedical Sciences, University of Antwerp), the group of Prof. Dr. K. De Wael (Chemistry Department, University of Antwerp), and the groups of Prof. Dr. M. Bolognesi and Prof. Dr. M. Nardini' (Physics Department, University of Genova, Italy) and Prof. Dr. A. Pesce (Biosciences Department, University of Milano, Italy), we were able to combine the phenotypical analysis of GLB-12 with a detailed biochemical and structural characterization. We found 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 superoxide, a molecule used in redox signaling. We indeed observed that *in vitro* GLB-12 is capable of actively converting O_2 to superoxide by electron transfer. Following this, we reasoned that, within the cell, the unstable superoxide produced by GLB-12 could potentially be converted by superoxide dismutases (SODs) to the more stable hydrogen peroxide, a second messenger in redox signaling. Also here we were able to confirm this hypothesis, showing that GLB-12 interacts with the main intracellular SOD, and surprisingly, also with the extracellular SOD. This strongly supported the role of GLB-12 as a redox signaling protein and showed that this globin, together with two SODs, forms what appears to be a redox signaling module. Finally, we were able to describe how the very specific membrane localization of GLB-12 increases the efficiency of its redox signal. In conclusion, this detailed analysis of GLB-12 revealed that a globin can act as an enzymatic source in redox signaling, thereby showing how this signal is regulated at the subcellular level by multiple control layers. From a more general perspective, these results add

the globin family to the limited group of proteins that can drive redox signaling and increase our understanding on how redox signaling proteins function.

In the second part, the role of the *C.elegans* globin family during periods of O_2 shortage was analyzed. In a wide range of organisms, globin genes are sensitive to O_2 deprivation[38-44]. Consequently, several functions related to O_2 metabolism have been proposed for these proteins, including facilitation of O_2 uptake and transport, protection against oxidative stress during and following hypoxia, and participation in stress signaling, for example by acting as O_2 or redox sensors[12]. In animals, the highly conserved transcription factor HIF-1 is responsible for most of the hypoxia-induced changes in gene expression[45,46], including that of globins in both vertebrates and invertebrates[39,40,47,48].

To identify *C. elegans* globin genes that are responsive to hypoxia and that are regulated by HIF-1, we analyzed the expression of the globin gene family following O_2 deprivation in wild-type worms, as well as in worms that lack HIF-1 and worms that have constitutively active HIF-1. We found that about one-third of the *C. elegans* globin genes is responsive to hypoxic stress, with both HIF-1-dependent and independent changes in expression. The majority of these globins is expressed in small subsets of neurons[31], with several of them clearly expressed in sensory neurons. These globins could act as O_2 sensors and be directly or indirectly sensitive to changes in O_2 concentration, inducing behavioral changes or metabolic and developmental adaptations when the environment becomes hypoxic. One globin, GLB-1, shows several biochemical and functional characteristics similar to vertebrate myoglobin[34]. Like myoglobin, GLB-1 might facilitate O_2 uptake, function as NO dioxygenase and protect against oxidative stress. Future work with knockout mutants for these globins will allow to further test these hypotheses.

The third part of this thesis was performed in collaboration with the group of Prof. Dr. Dewilde and Prof. Dr. L. Moens (Department of Biomedical Sciences, University of Antwerp) and describes a combined biochemical and functional analysis of GLB-26. A previous analysis showed that this globin, like GLB-12, has a low affinity for ligands, cannot form a stable oxygenated species and is oxidized instantaneously when exposed to air, suggesting a role involving redox reactions[34]. Expression analysis located it in muscle tissues that are involved in the defecation of the worm[31]. In the current study, we confirmed this very specific expression pattern, while on a subcellular level we showed that GLB-26 is localized to the cellular and nuclear membrane by the attachment of a myristoyl group. We further observed that GLB-26 is involved in the regulation of the defecation cycle when worms are exposed to conditions that

induce oxidative stress. In addition, GLB-26 shows a relatively low reduction potential, consistent with a role in electron transfer, and appears enzymatically active towards hydrogen peroxide. In conclusion, these results further support that GLB-26 participates in redox reactions, potentially as a redox sensor or as a redox enzyme, to regulate the worm's defecation cycle.

In addendum, I describe the results I obtained as a visiting scholar at the lab of Prof. Dr. Karen Oegema at the Ludwig Institute at the University of California, San Diego. Several observations, both from the literature and from our group, suggested that different forms of redox signaling are intimately associated with the regulation of *C. elegans* reproduction. In addition, the reproductive system of the worm has been put forward as a valuable tissue for the analysis of dynamic biological processes. As a visiting researcher, I was able to develop a first set of tools for the analysis of redox signaling within this tissue. The obtained results support the potential of the *C. elegans* reproductive system as a model to study redox signaling in a complex tissue.

Samenvatting

Globines zijn een fundamenteel onderdeel van het leven: ze kunnen worden teruggevonden in de meest eenvoudige levensvormen, de bacteria en Archaea, over unicellulaire eukaryoten tot de multicellulaire en meer complexe fungi, planten en dieren[20-25]. Globines behoren ook tot de oudste bestudeerde eiwitten, waarbij de eerste waarnemingen reeds dateren van de 19de eeuw[26]. Het aantal gekende soorten globines in de voorbije eeuw was echter erg beperkt en slechts enkele werden in meer detail bestudeerd, namelijk hemoglobine aanwezig in rode bloedcellen en myoglobine in spieren, beide uit gewervelde dieren. Dit had als gevolg dat globines bijna uitsluitend gedefinieerd werden als respiratorische eiwitten, zorgend voor een verbeterde O_2-opname en -transport doorheen het lichaam om zo aerobe ademhaling te ondersteunen. In de voorbije dertig jaar is onze kennis over de functie en verspreiding van deze eiwitten enorm toegenomen. Met de beschikbaarheid van genomische data vanuit de drie domeinen van het leven werden honderden nieuwe globinegenen ontdekt, wat 1) aantoonde dat globines behoren tot een oude en alomtegenwoordige eiwit-superfamilie, 2) nieuwe types globines opleverde en 3) suggereerde dat globines een hoge mate van functionele adaptatie aan verschillende omgevingen kunnen vertonen[20-25]. Meer gedetailleerde analyse van een beperkt aantal globines liet verder toe om een aantal mogelijke nieuwe functies voor deze eiwitten te definiëren[12,27-30]. De meeste van deze mogelijke functies zijn daarbij ondersteund door structurele en biochemische analyses, maar moeten nog gevalideerd worden met fysiologische resulaten.

De rondworm *Caenorhabditis elegans* is een gewaardeerd modelorganisme voor fundamenteel en biomedisch onderzoek. Door zijn eenvoudig te bestuderen anatomie en de beschikbaarheid van krachtige moleculaire en genetische technieken kunnen geconserveerde processen in ontwikkeling, gedrag, ziektes en veroudering worden bestudeerd. *C. elegans* is tot nu toe ook een veelbelovend model voor de analyse van de functionele diversiteit binnen de globine superfamilie. Het bezit een groot aantal globines, 33 in totaal, die een grote heterogeniteit vertonen in gen- en eiwitstructuur en die in verschillende celsoorten kunnen worden teruggevonden[16,31,32]. Er wordt aangenomen dat deze grote en diverse globinefamilie het resultaat is van meerdere evolutionaire periodes van radiatie en duplicaties, gevolgd door een diversificatie en subfunctionalisatie van individuele globinegenen[31].
De variatie in eigenschappen samen met de evolutionaire geschiedenis van deze familie suggereert dat de verschillende leden betrokken zijn bij een diverse reeks fysiologische processen.

Meer gedetailleerde analyses van enkele van deze globines ondersteunen deze hypothese verder en wijzen erop dat globines kunnen werken als O_2-sensors, betrokken zijn bij redoxreacties en deelnemen aan bescherming tegen oxidatieve stress[33-37]. Binnen dit doctoraatsonderzoek werd getracht om de kennis over de mogelijke functies van de globines bij *C. elegans* uit te breiden, waarbij de nadruk werd gelegd op de moleculaire werking van enkele van deze eiwitten.

Het eerste en tevens grootste deel van dit onderzoek was gericht op de functionele analyse van GLB-12. Dit globine was initieel geselecteerd omdat het een veelbelovend fenotype vertoonde na RNAi-depletie, en suggereerde dat GLB-12 is betrokken bij voortplanting, ontwikkeling van de vulva en algemene ontwikkeling. Bij een meer gedetailleerde analyse bleek dat *glb-12* RNAi leidde tot een sterk verminderde reproductie en tot meerdere defecten bij de werking van het voortplantingsstelsel en eicelvorming. Hierbij hoorde ook een abnormale toename in celdood van kiemcellen, wat via de geconserveerde signaalcascades JNK and P38 MAPK wordt gereguleerd. We zagen echter ook dat andere defecten in het voortplantingsstelsel onafhankelijk van deze signaalcascades worden gereguleerd, wat suggereert dat GLB-12 meerdere signaalcascades beïnvloedt.
In samenwerking met de groepen van Prof. Dr. L. Moens en Prof. Dr. Dewilde (Department of Biomedical Sciences, University of Antwerp), de groep van Prof. Dr. K. De Wael (Chemistry Department, University of Antwerp), en de groepen van Prof. Dr. M. Bolognesi en Prof. Dr. M. Nardini (Physics Department, University of Genova, Italy) en Prof. Dr. A. Pesce (Biosciences Department, University of Milano, Italy) konden we de bovengenoemde fenotypische analyse van GLB-12 combineren met een gedetailleerde biochemische en structurele karakterisatie. Dit toonde aan dat GLB-12, in tegenstelling tot de meeste globines, niet in staat is om O_2 te binden, maar daarentegen geoxideerd wordt wanneer het wordt blootgesteld aan lucht. De kristalstructuur van GLB-12 liet zien dat dit globine unieke eigenschappen bezit die een rol in electronentransfer ondersteunen. Electrochemische analyse toonde verder aan dat GLB-12 een redoxpotentiaal heeft die voldoende laag is om electronentransfer van het haem-ijzer naar O_2 toe te laten. Op basis van deze resultaten konden we de hypothese naar voor brengen dat GLB-12 kan interageren met O_2 om superoxide te vormen, waarbij superoxide een molecule is die gebruikt wordt in redoxsignalisatie. GLB-12 bleek *in vitro* inderdaad in staat om actief O_2 om te zetten naar superoxide via electronentransfer. Hierop steunend konden we verder aannemen dat, binnenin de cel, superoxide geproduceerd door GLB-12 zou kunnen worden omgezet door superoxide dismutases (SOD) naar het stabielere waterstofperoxide, een tweede type molecule gebruikt in redoxsignalisatie. Ook hier bleek deze hypothese correct te zijn: GLB-12 interageert

met de voornaamste cytoplasmatische SOD, en verrassend genoeg ook met een extracellulaire SOD. Deze interacties zijn een verdere ondersteuning dat GLB-12 functioneert als een eiwit in redoxsignalisatie en toonden aan dat dit globine, samen met twee SODs, een redoxsignalisatie-module lijkt te vormen. Tenslotte zagen we ook dat de specifieke membraanlocalisatie van GLB-12 zorgt voor een toename in efficiëntie van zijn redoxsignaal. Tot besluit, deze gedetailleerde analyse van GLB-12 toonde aan dat een globine kan functioneren als een enzymatische bron in redoxsignalisatie, waarbij bleek dat dit signaal op subcellulair niveau door meerdere controlelagen wordt gereguleerd. Meer algemeen toont dit onderzoek aan dat de globinefamilie kan toegevoegd worden aan de erg beperkte groep van eiwitten die redoxsignalisatie aansturen en vergroot het de kennis over de werking van redox-signaliserende eiwitten.

In het tweede deel werd de rol van de *C.elegans* globinefamilie tijdens periodes van O_2-tekort geanalyseerd. In verschillende organismen blijken globinegenen gevoelig te zijn voor hypoxie[38-44]. Meerdere functies die gerelateerd zijn aan O_2-metabolisme zijn voorgesteld voor deze eiwitten, waaronder een versnelling van O_2-opname en –transport, bescherming tegen oxidatieve stress tijdens en volgend op hypoxische periodes, en deelname in stresssignalisatie, bijvoorbeeld door als O_2- of redoxsensor te werken[12]. In dieren is de sterk geconserveerde transcriptiefactor HIF-1 verantwoordelijk voor de meeste veranderingen in genexpressie na hypoxie [45,46], waaronder ook deze van globinegenen in zowel gewervelde als ongewervelde dieren [39,40,47,48].
Om globinegenen van *C. elegans* te identificeren die responsief zijn op hypoxische stress en die gereguleerd worden door HIF-1 werd de expressie van de volledige globinegenfamilie geanalyseerd na blootstelling aan hypoxie in wild-type wormen en in wormen die HIF-1 ontbreken, en verder in wormen die constitutief actief HIF-1 bezitten. Hieruit bleek dat ongeveer een derde van de *C. elegans* globinegenen gevoelig is aan hypoxische stress, met daarbij zowel HIF-1-afhankelijk als -onafhankelijke veranderingen in genexpressie. Een eerdere studie toonde aan dat de meerderheid van deze globinegenen geëxpresseerd is in een beperkt aantal zenuwcellen[31], waarbij duidelijk is dat enkele van deze genen specifiek in sensorische neuronen tot expressie komen. Deze globines zouden kunnen werken als O_2-sensoren, waarbij ze rechtstreeks of onrechtstreeks gevoelig zijn aan veranderingen in O_2-concentratie en veranderingen in gedrag, metabolisme of ontwikkeling aansturen wanneer de omgeving hypoxisch wordt. Eén globine, GLB-1, vertoont een reeks biochemische en functionele kenmerken die gelijkaardig zijn aan deze van myoglobine in gewervelde dieren[34]. GLB-1 zou daarom een vergelijkbare rol kunnen vervullen als myoglobine, door O_2-opname te versnellen, als NO-dioxygenase te functioneren en te beschermen tegen oxidatieve stress. Verder onderzoek

op basis van mutante wormen die specifiek deze globines ontbreken zal toelaten om de hier voorgestelde hypotheses te testen.

Het derde deel van deze thesis is uitgevoerd in samenwerking met de groep van Prof. Dr. Dewilde en Prof. Dr. L. Moens (Department of Biomedical Sciences, University of Antwerp), en beschrijft een gecombineerde biochemische en functionele analyse van GLB-26. Een eerdere studie toonde aan dat dit globine, net zoals GLB-12, een lage affiniteit vertoont voor liganden, O_2 niet stabiel kan binden en direct wordt geoxideerd wanneer het wordt blootgesteld aan lucht. Ook voor dit globine werd daarom een functie die gerelateerd is aan redoxreacties voorgesteld[34]. Verdere expressieanalyse localiseerde GLB-26 in spierweefsel dat geassocieerd is met de defecatie-cyclus van de worm[31]. In het huidig onderzoek werd dit erg specifiek expressiepatroon bevestigd, waarbij ook duidelijk werd dat GLB-26 op subcellulair niveau gebonden is aan de cel- en kernmembraan door middel van een myristoylgroep. Daarnaast blijkt dat GLB-26 betrokken is bij de regulatie van de defecatiecylcus wanneer wormen worden blootgesteld aan oxidatieve stress. Bovendien vertoont GLB-26 een relatief lage redoxpotentiaal, consistent met een functie in electronentransfer, en lijkt dit eiwit enzymatisch actief te zijn naar waterstofperoxide toe. Deze resultaten zijn een verdere ondersteuning dat GLB-26 deelneemt aan redoxreacties, potentieel als redoxsensor of als redoxenzyme, om zo de defecatiecyclus van de worm te reguleren.

In addendum zijn de resultaten beschreven die ik heb behaald tijdens mijn verblijf bij de groep van Prof. Dr. Karen Oegema (Ludwig Institute aan de Universiteit van Californie, San Diego). Meerdere waarnemingen, zowel vanuit de literatuur als vanuit onze eigen groep, suggereerden dat verschillende vormen van redoxsignalisatie een rol spelen in de regulatie van de voortplanting bij *C. elegans*. Bovendien is het voortplantingsstelsel van de worm een waardevol modelweefsel voor de studie van dynamische biologische processen. Tijdens mijn verblijf bij de groep van Prof. Dr. Oegema kon ik een eerste reeks technieken ontwikkelen voor de analyse van redoxsignalisatie in dit weefsel. De behaalde resultaten ondersteunen daarbij het potentieel van het voortplantingsstelsel van de worm als een waardevol model voor de studie van redoxsignalisatie in een complex weefsel.

PART I: INTRODUCTION

Chapter I:
General introduction and outline of the thesis

1. The globin superfamily

1.1. Introduction

Globins are an ancient and very large superfamily of proteins, characterized by a typical tertiary structure and the presence of a heme group[20,22-25]. 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. In recent years, a large number of new globins and globin-like proteins have been discovered and it is now appreciated that these proteins seem to be ubiquitous throughout nature. Biochemical, structural and functional analyzes of several of these newly discovered globins showed that a role in O_2 binding is not necessarily the main function carried out by globins[12,20,25,49]. However, even though we now know that globins can potentially fulfill a wide range of functions, our understanding of these is still largely incomplete.

1.2. The globin fold

Globins are small heme proteins, typically 145-155 amino acids, that are characterized by a highly conserved tertiary structure, the **globin fold**[13,50] (**Figure 1**). This 3-over-3 (3/3) sandwich structure is created by six to eight α-helices, named A to H, that are separated by small loops. This globin fold generates a central pocket, which is occupied by the prosthetic heme group. The pocket is lined with hydrophobic amino acids and delineated sideways by the E- and F-helices (distal and proximal to the heme, respectively). For all globins, the amino acids within the protein are labelled in the following way: the first residue of helix A is termed residue A1. Nonhelical residues are named with both letters of the α-helices they connect, e.g. EF refers to the coiled region between the E- and F-helices. The N- and C-terminal regions are named NA and HC, respectively.

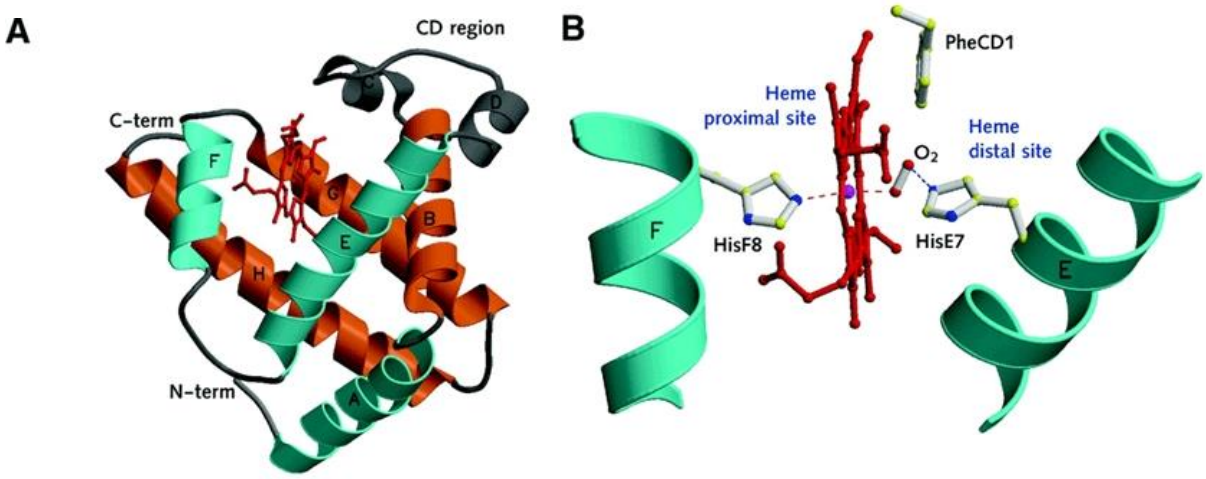


Figure 1: The classical globin fold[10]. A) Ribbon view of a typical 3-over-3 globin, highlighted in two colours, with the heme group in red and centrally located. The helices are labelled according to conventional globin nomenclature. **B) Detailed view of the heme pocket.** The proximal and distal side are defined by the F and E helix, respectively. HisF8 and PheCD1 contribute in positioning the heme group, HisE7 is an important regulator of ligand binding. The coordination bonds of heme iron with HisF8 and with O_2 are represented by a dashed red line, the hydrogen bridge between O_2 and the distal HisE7 is present as a blue line.

1.3. The heme group

The **heme** is a Fe-porphyrin molecule, consisting of four pyrrolic groups joined together by methine bridges, creating a planar ring structure (**Figure 2**). The central iron displays a bipyrimidal d2sp3 hybridization, therefore it can receive 6 electron pairs in its empty orbitals, thus allowing 6 binding partners. Four of these are delivered by the pyrrolic N-atoms from the heme group. The imidazole N-atom of the invariant His at position F8 in globins will bind as the fifth ligand at the proximal side of the heme and will, together with approximately 100 van der Waals interactions from surrounding amino acid side chains of the globin, localize the heme within the protein. This **pentacoordinated** heme iron can be found in the majority of characterized globins, leaving the sixth iron orbital free for the reversible binding of diatomic gasses, such as O_2, NO and CO.

A second and highly conserved His (His E7) is often located above the distal ligand site, but is not connected with the iron in pentacoordinated globins. This distal His however can provide an important stabilizing interaction with bound O_2 through hydrogen bonding and may modulate access/exit of exogenous ligands to/from the heme distal pocket ("E7 gate")[51-53]. Additional residues located at the distal and proximal side of the heme will affect heme:ligand association

and dissociation kinetics to different degrees, thereby defining the affinity of a globin for a given diatomic ligand[53,54]. For example, Phe CD1 is an important but not strictly conserved residue that helps to wedge the heme in the protein, while the residue at the distal position B10 often plays a crucial role in stabilizing the bound O_2.

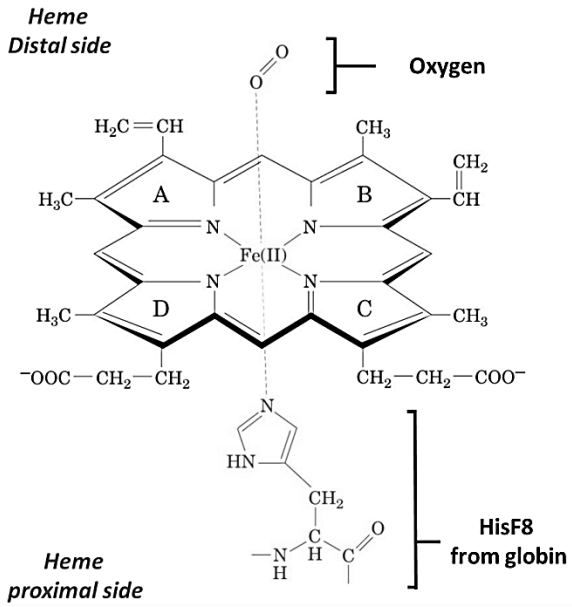


Figure 2: 2-dimensional representation of the heme group, together with the globin F8 histidine residue and O_2 as ligand. A-D are the 4 pyrrolic groups that create a planar ring structure. The Fe^{2+} is centrally located and is bound with 4 pyrrolic N-atoms, 1 histidine imidazole N-atom and is bound to O_2 via its sixth orbital. Modified from[7].

In addition to a pentacoordinated heme Fe, several more recently discovered globins display a **hexacoordinated** Fe, in which the distal HisE7 occupies the sixth binding site of the iron. Binding of exogenous ligands in hexacoordinated globines therefore has an additional level of regulation, characterized by 1) removal of HisE7 from the distal heme iron binding site, 2) formation of a transient reactive pentacoordinated heme, and 3) binding of the exogenous ligand, e.g O_2, to the distal heme iron binding site[55,56]. In addition, the hexacoordinated state of the iron seems to favor electron transfer[27,35,57], indicating that redox reactions involving the heme iron could be an important element in the physiological function of these globins. Since their initial discovery two decades ago, these hexacoordinated globins are now recognized to be broadly present in plants and animals[30]. The physiological significance of this coordination state is not yet understood, however.

Overall, heme reactivity exhibits an impressive dynamic range in both penta- and hexacoordinated globins, and it can be tuned by modulating the ligand access, the stability of the bound ligand by specific interactions with heme pocket side chains and the chemical reactivity of the heme.

1.4. Globin occurrence in living organisms

The rapid accumulation of genomic information over the last decades allowed the identification of globins in numerous organisms. Subsequent analysis of this information showed that these proteins form an ancient and broadly distributed superfamily[20,22-25]. They originated very early in the evolution of life, i.e. more than 3000 mya, and are now present from bacteria and Archaea over unicellular eukaryotes and fungi to the multicellular plants and animals.

The globin superfamily can be categorized in three families which combined display two types of globin folds[20,24] (**Figure 3**); the M family (for Myoglobin-like) and S family (for Sensor) exhibit the canonical 3/3 α-helical sandwich, while the T family (for Truncated) displays a structurally distinct 2/2 globin fold. Within each of these families, globins can occur as chimeric or as single-domain proteins.

For the M family this results in two subfamilies, the chimeric flavohemoglobins and the related single domain globins. The flavohemoglobins are composed of an N-terminal globin domain and a C-terminal FAD-reductase domain. Because of the presence of the reductase domain in flavohemoglobins, the globin iron can efficiently function as an electron donor ($Fe^{2+} \rightarrow Fe^{3+}$), after which the reductase domain will use the reducing power of NAD(P)H to regenerate ferrous heme ($Fe^{3+} \rightarrow Fe^{2+}$). This makes these chimeric proteins efficient NO-detoxifying enzymes and allow inducible protection following NO-stress[58]. In the related single domain globins, such as mammalian hemoglobin, the heme iron can also donate electrons, but subsequent reduction to ferrous heme is dependent on a cellular reductans and is therefore in general much slower. The best known function for these single domain globins is thus far in O_2 transport and storage, but other functions are increasingly being recognized, ranging from the catalysis of redox reactions to sensing, transport or scavenging of ligands such as O_2, NO and CO, and of other small molecules[12].

The S family encompasses the chimeric globin-coupled sensors and the related single domain protoglobins and sensor single domain globins. The chimeric globin-coupled sensors have an additional C-terminal functional domain, which can function as a transmembrane receptor or have various catalytic functions, such as diguanylate cyclase activity, phosphodiesterase activity toward cyclic diGMP and histidine kinase activity (reviewed by Martínková *et al.*[59]). Structural changes in the globin domain, for example induced by binding O_2, will be transduced to this second functional domain, switching on/off the latter. This can result in an aerotactic or gene regulatory response, but other, yet unknown roles, are also possible[60]. The globin fold in this family lacks the entire D-helix and part of the E-helix, whereby these structural changes are

predicted to be beneficial for O_2 sensing[12,61]. The functions of the related protoglobins and sensor single domain globins are still largely unknown.

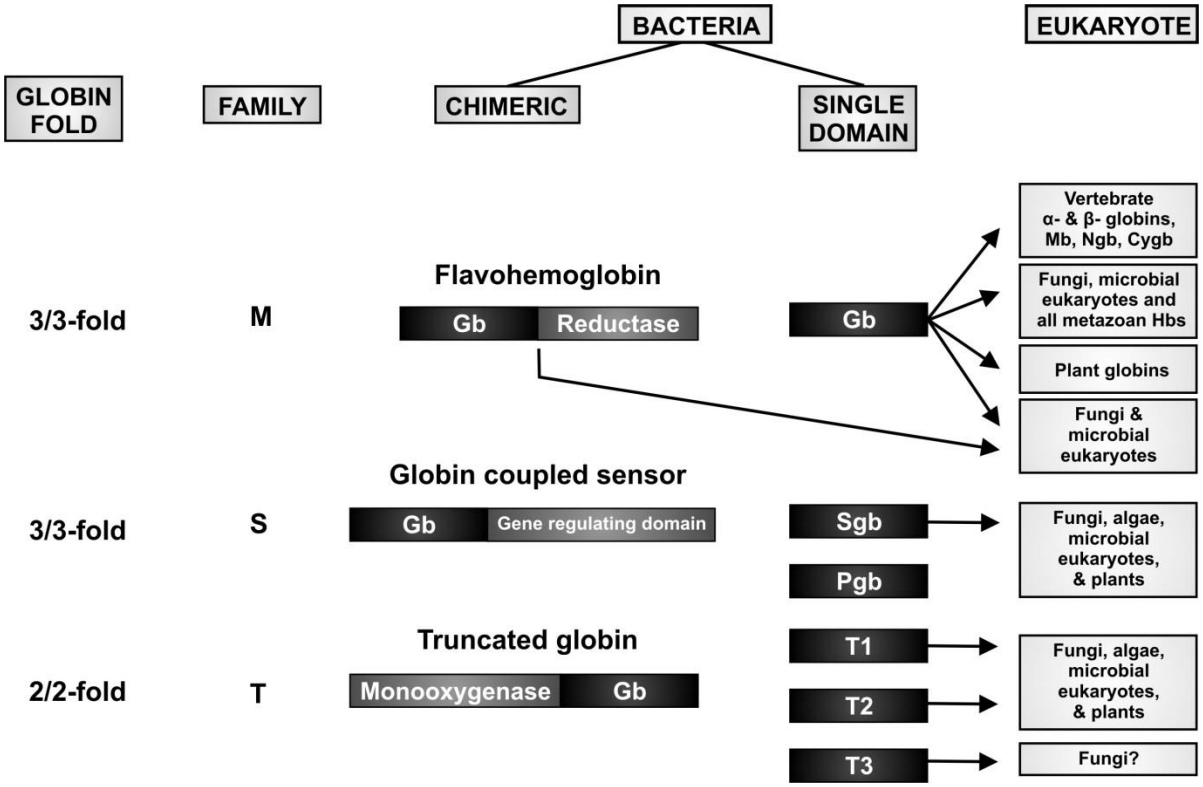


Figure 3: Diagrammatic representation of the known chimeric and single domain globin families, showing the relationships between bacterial and eukaryote globins. Gb: globin, Sgb: S family single domain globin, Pgb: protoglobin, T1-T3: truncated globin, Mb: myoglobin, Ngb: neuroglobin, Cygb: cytoglobin, Hb: hemoglobin. Modified from Vinogradov and Moens[12].

Finally, the T family displays a 2/2 instead of the typical 3/3 sandwich structure (**Figure 4**). This 2/2 fold originates from extensive modifications of the typical 3/3 fold, which are distributed throughout the entire protein. This alternative fold is thus not the result of a simple truncation event. Because of these modifications, the overall length of the majority of these proteins is 20 to 40 residues shorter compared to classical globins. Structurally, the anti-parallel helix pairs B/E and G/H are responsible for the 2/2 fold, while the A helix is reduced or absent, the CD-D region is drastically shortened and the F helix is substituted by a loop[62]. These globins are only distantly related to 3/3 globins and can be further organised in three subfamilies that differ in the residues involved in heme binding and in ligand access paths to the heme distal side. Chimeric proteins can also be found in this globin family; they consist of a truncated globin domain fused to an N-terminal monooxygenase domain. However, these chimeric globins have thus far only

been reported in two species[63]. The function of 2/2 globins remains largely unknown, but they are hypothesized to participate in redox reactions, O_2/NO sensing and O_2 metabolism[64-66].

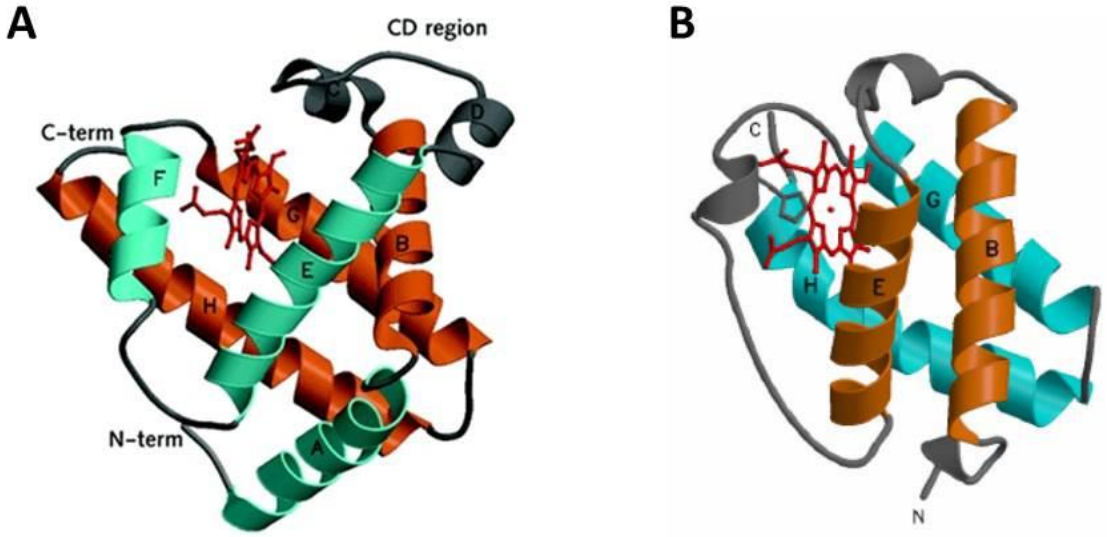


Figure 4: Secundary and tertiary structure of a typical 3/3 globin and a 2/2 globin[10]. A) 3/3 globin, B) 2/2 globin. The 3-over-3 sandwich of full-length globins and the 2-over-2 sandwich of truncated globins are highlighted in two colors, the helices are labeld according to conventional globin nomenclature (A, B, C.....H, starting from the N-terminus)[13,14]. The prosthetic heme group is in red.

The M, S and T families are not evenly distributed over the domains of life; only bacteria have representatives of all three families in both the chimeric and single-domain form, while Archaea and eukaryotes completely lack the flavohemoglobin and globin coupled sensors, respectively. Interestingly, all animal and plant globins and many globins in microbial eukaryotes belong to the M family. It is assumed that bacterial single domain globins belonging to the M family have been transferred to the last eukaryotic common ancestor and subsequently underwent a highly successful adaptive evolution parallel to the advent of multicellularity[67]. In higher vertebrates, this has resulted in globins that have become essential as O_2 transporters. However, as mentioned before, this is only one of several functions that can be carried out by globins belonging to this group. In addition, there are rare examples of globins in eukaryotes that are clearly related to single domain globins, but have become linked with other protein domains to form unusual chimeric proteins. This is for example observed for the recently discovered androglobin[68] and for one of the globins in *C. elegans*, GLB-33[32].

1.5. Properties and functions of metazoan globins

As stated above, globins originated more than 3000 mya, well before the presence of atmospheric O_2 on Earth. This makes it clear that the best-studied function of globins, O_2

transport by vertebrate hemoglobin, must be a relatively recent adaptation. It is hypothesized that the earliest functions of globins involved NO and O_2 -reactions, -sensing and -scavenging[12]. Members of the M and T family indeed function as enzymes in unicellular prokaryotes and eukaryotes, whereas in Archaea and bacteria, the globin-coupled sensors function as aerotactic O_2 sensors and gene regulators. Also the group of globins present in higher eukaryotes, all belonging to the M family, seems to have evolved to fulfill a wide range of functions. In the following sections, the known and potential functions of these single domain globins, belonging to the M family and present in higher eukaryotic animals, will be discussed in more detail.

1.5.1. Oxygen transport

The heme group in globins potentially allows for the reversible binding of diatomic ligands, such as O_2, NO and CO. This permits globins to fulfill their familiar role of supporting aerobic respiration. This role has been well studied for hemoglobin and myoglobin found in vertebrates and has led to a thorough understanding of the relationship between structure and function of these two globins. A similar function is proposed for many related globins found in vertebrates, invertebrates and plants.

In red blood cells of vertebrates, hemoglobin occurs as heterotetramers, comprised of two α- and two β-globin subunits. It binds O_2 in the pulmonary capillaries and releases it when it passes through the rest of the body. The occurrence of hemoglobin as a tetrameric complex allows for the cooperative binding of O_2, mediated by allosteric changes in the different subunits. This results in a stepwise increase in O_2 affinity upon additive O_2 binding and a sigmoidal O_2 dissociation curve, with high O_2 affinity when hemoglobin is present in the O_2 rich lung capillaries and low O_2 affinity to facilitate release in the O_2 demanding tissue capillaries (**Figure 5**). The acquisition of regulatory control over ligand binding, with interactions between globin subunits to create an allosteric molecule, appears to be a fundamental prerequisite for circulating globins whose primary function is O_2 transport. Accordingly, also many invertebrate globins involved in O_2 transport are present in the form of homodimers or multimers, showing allosteric control of O_2 binding[69].

Vertebrate myoglobin on the other hand is present as monomers in the cytoplasm of heart and skeletal muscles and does not display cooperative O_2 binding. The resulting O_2 binding curve is hyperbolic, with myoglobin displaying a higher O_2 affinity compared to hemoglobin in the O_2 poor tissue capillaries (**Figure 5**). O_2 released by hemoglobin can thus be efficiently captured by myoglobin, allowing myoglobin to act as a temporary O_2 storage.

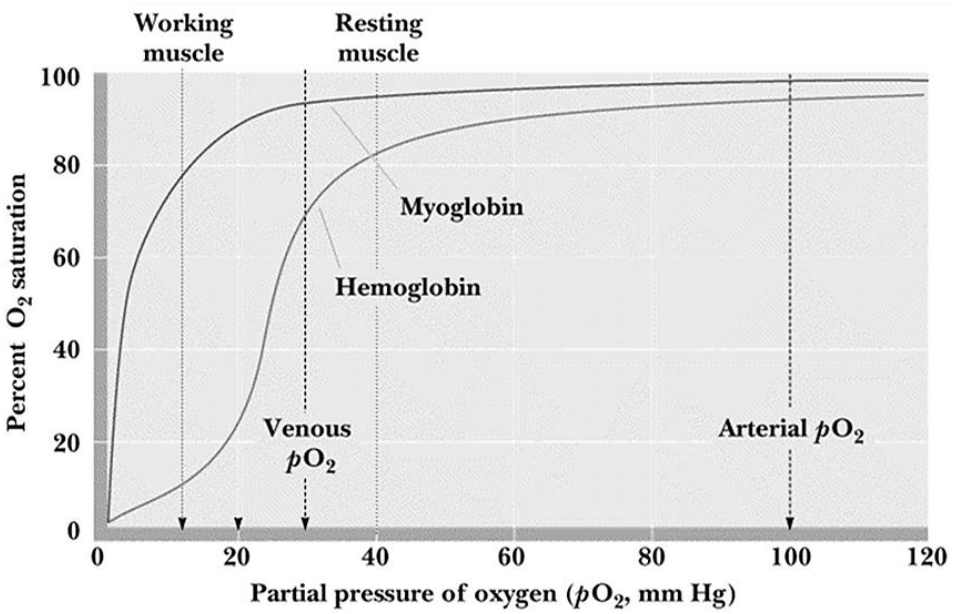


Figure 5: O_2 affinity of hemoglobin and myoglobin. Hemoglobin consists of four globin subunits, allowing it to bind O_2 in a positive cooperative manner: when O_2 binds to one of the subunits, the O_2 affinity of the remaining subunits will increase, and vice versa. This results in a sigmoidal O_2 binding curve. Myoglobin is present as a monomeric protein, resulting in a hyperbolic O_2 binding curve. Combined, this results in myoglobin being capable of binding O_2 released by hemoglobin at low P_{O2} and to deliver it to cells of highly active tissues.

A large fraction of the cytosolic pool of myoglobin releases O_2 within 20-40 seconds upon the onset of muscle contraction in the case of skeletal muscles[70] or when blood supply is briefly interrupted during normal systole in heart muscle[71,72], suggesting that O_2 is released for mitochondrial consumption during this period. Consequently, myoglobin shows the highest expression in muscles sustaining prolonged contractile activity, such as heart and skeletal muscles with high cytochrome c oxidase content and high oxidative capacity[73]. In addition, it often shows increased expression in skeletal muscles of hypoxia-tolerant mammals, such as burrowing and diving mammals and birds, potentially to allow increased body O_2 stores[74-76]. However, this correlation is not always as clear and the regulation of myoglobin expression seems to be complex and varies between species and tissues (reviewed by Helbo and colleagues[73]). In addition, while muscle globins are usually monomeric, oligomeric muscle globins have been observed in several invertebrate species. This oligomerization is sometimes associated with cooperative O_2 binding[69].

In addition to its role as a transient O_2 storage, myoglobin has been proposed to facilitate O_2 diffusion within the cell[77]. In such a role, the high O_2 affinity of myoglobin would allow it to compete with the ability of O_2 to dissolve in solution, resulting in a steeper O_2 gradient from the

cell membrane to within the cell and consequently in an increased O_2 flux within the cell[78]. It is somewhat controversial whether or not mitochondrial respiration is completely dependent on this function under normal conditions. There is however general consensus that in muscle tissue under hypoxic conditions, during prolonged physiological exercise or in diving mammals, myoglobin is essential for O_2 supply to mitochondria to support oxidative phosphorylation[38,79,80]. Interestingly, the reversible binding of O_2 in hemoglobins and myoglobins induces no chemical or redox changes to the heme iron group, despite the strong tendency of molecular oxygen to oxidize the heme iron. This oxidation is inhibited by the environment created by the hydrophobic pocket surrounding the heme group. For example, only 1-3% of hemoglobin within red blood cells is oxidized daily to methemoglobin. When hemoglobin and myoglobin become oxidized, the ferric heme (Fe^{3+}) is incapable of binding O_2 and needs to be reduced back to ferrous heme (Fe^{2+}). This reduction can occur enzymatically by the NADH-dependent methemoglobine reductase and metmyoglobine reductase[81-83], or non-enzymatically by intracellular reductans such as NAD(P)H and other antioxidants.

1.5.2. NO scavenger

The ability of NO to react rapidly with heme centers has long been recognized and is common to all heme proteins. It likely represents one of the earliest and most important functional properties of globins[12]. Also here has the detailed study of hemoglobin and myoglobin advanced our understanding of the physiological role of these reactions. When O_2 is present, both oxygenated hemoglobin and myoglobin will act as NO scavengers via their NO dioxygenase activity. This reaction is rapid and irreversible and results in the two-electron oxidation of NO to nitrate, converting the ferrous heme to the ferric form[84-86].

$$Gb(Fe^{2+})O_2 + NO \rightarrow Gb(Fe^{3+}) + NO_3^-$$

NO is a gaseous signaling molecule that can be produced endogenously by NO synthases[87], the latter being present in various cell types. NO production in endothelial cells can diffuse into the adjacent musculature to activate vasodilation and increase local bloodflow[88]. NO can also reversibly inhibit mitochondrial respiration by competing with O_2 for binding to cytochrome c oxidase in the respiratory chain[89]. Thus, the dioxygenase activity of hemoglobin allows to inhibit the activity of NO as a powerful vasodilator, while the removal of NO by myoglobin will protect mitochondrial respiration against NO inhibition.

1.5.3. NO synthase/ nitrite reductase

Remarkably, both hemoglobin and myoglobin have the capacity to switch from an NO scavenger to an NO synthase when O_2 becomes limiting[90,91]. Under this condition, the deoxygenated globins can function as a nitrite reductase that converts endogenous nitrite to NO. In this process, ferrous deoxyGb becomes oxidized to ferric metGb.

$$deoxyGb(Fe^{2+}) + NO_2^- + H^+ \rightarrow MetGb(Fe^{3+}) + NO + OH^-$$

The produced NO can bind to cytochrome c oxidase and will so inhibit respiration[90,92]. This prolongs O_2 availability, extends the O_2 gradient in cells during hypoxia and reduces the production of reactive oxygen species upon reoxygenation. In addition, NO production could also be important to regulate vasodilatation, which may enhance blood flow and increase O_2 supply to hypoxic tissues[93]. Mechanistically, because the K_m for O_2 of cytochrome c oxidase (0.1-0.5μM) is significantly lower than the P_{50} of myoglobin (3.1μM), myoglobin will become desaturated and will shift to nitrite reduction to generate NO during hypoxia before respiration rates becomes O_2 limited[90]. In this context, it is relevant to note that O_2 is also a substrate for nitric oxide synthase, but the latter requires relatively high O_2 concentrations (K_m ~10-20μM)[94]. In hypoxic conditions, nitrite reduction by myoglobin or hemoglobin thus potentially represents the predominant mechanism of NO generation, in which nitrite functions as a NO storage pool that may be utilized when O_2 levels drop. In line with this, applying nitrite indeed has beneficial effects in treating various human diseases like hypertension and tissue injury following hypoxia-reoxygenation[95-97].

The relationship of NO with hemoglobin and myoglobin is thus bound by O_2, with a shift from NO scavenging at normal O_2 concentrations to NO generation under hypoxia. This relationship may allow these two globins to act as O_2 sensors, modulating the signaling capacity of NO in response to O_2 tension[12,83].

1.5.4. Peroxidase activity & reactions with free radicals

Hemoglobin and myoglobin can also display peroxidase activity[98,99], and this function has been associated with protection against oxidative stress. Ferrous oxyGb (Fe^{2+}) and ferric Gb (Fe^{3+}) can react with H_2O_2, yielding ferryl Gb (Fe^{4+}), which is however also a potent oxidant[100]. Ferryl myoglobin itself can react with ferrous Gb (Fe^{2+}), yielding 2 ferric Gbs (Fe^{3+}), which can then be

reduced to ferrous Gb (Fe^{2+}) by MetGb reductases within the cell[101,102]. Although ferrous myoglobin has a much lower reaction rate with H_2O_2 compared with other H_2O_2 scavenging enzymes such as catalase, the high intracellular concentration of this globin may result in a considerable contribution to protection against oxidative stress[73].

$$Gb(Fe^{2+})O_2 + H_2O_2 \rightarrow Gb(Fe^{4+}) + H_2O + ½\ O_2 + O_2$$
$$Gb(Fe^{3+}) + H_2O_2 \rightarrow Gb(Fe^{4+}) + H_2O + ½\ O_2$$

$$Gb(Fe^{4+}) + Gb(Fe^{3+}) \rightarrow 2\ Gb(Fe^{3+})$$

However, because ferryl Gb is a powerful oxidant, its formation following the oxidation of ferrous Gb by H_2O_2 has also been associated with the initiation and exacerbation of many cardiovascular diseases[83]. In this scenario, ferryl Gb will oxidize proteins and lipids, forming cross-linked species and initiate lipid peroxidation. Both reactions will produce active radicals, which on their turn can react with ferric Gb and form additional ferryl species. Hence, a cascade of oxidations is initiated.

Finally, the formation of ferryl Gb has also been associated with the oxidation of polyunsaturated fatty acids, generating prostaglandin-like molecules with potent vasoconstrictor activity[99].

These different outcomes suggest that the role of hemoglobin and myoglobin, when present in an H_2O_2 rich environment, likely entails a fine balance between protection against oxidative stress and functioning in redox biology, or contributing to exaggerating oxidative stress[103].

1.5.5. Binding and transport of sulfide

Bivalves and annelids living in H_2S-rich environments, e.g. at sea level, in hydrothermal vents on the ocean floor and in mud of mangrove swamps, can symbiose with sulphur-oxidizing bacteria. These bacteria will supply metabolic byproducts when they have access to both O_2 and H_2S[29,104-107]. H_2S is normally a respiratory poison by binding the heme iron of globins, thereby preventing O_2 transport. However, these bivalves and annelids can survive in H_2S-rich environments and support the symbiosis by expressing two structurally distinct globins, one of which will transport H_2S, while the other will carry out O_2 transport whilst being remarkably resistant to H_2S binding. H_2S transport by globins has been observed or hypothesized to occur by multiple mechanisms: H_2S can bind to the heme iron forming ferric sulphide, it can bind to cysteine residues within the

globin[107], or it can bind to interior zinc ions within the globin fold[106]. At the same time, the globin that is responsible for O_2 transport will need to prevent oxidation of its heme iron by H_2S. One study showed that this is achieved by the presence of a hydrogen bonding network at the distal heme side, effectively reducing heme accessibility and so heme iron oxidation[104]. The simultaneous transport of O_2 and H_2S by structurally distinct globins highlights the flexibility and the modular nature of the structure-function relationship of globins, and provides a good example of how globins can allow their carriers to occupy new ecological niches.

1.5.6. Novel functions

Our understanding of the above described functions has mainly resulted from the extensive analysis of vertebrate hemoglobin and myoglobin. Over the last two decades, a large number of new globins have been discovered in a wide range of animals. In vertebrates, this includes neuroglobin[57,108,109], mainly present in nerve cells, cytoglobin[110,111], expressed in a variety of tissue types, globin E[112], an eye specific globin found in birds, globin X[113], present in fish and amphibians, globin Y, present in lower vertebrates[114] and androglobin[68], a testes-specific chimeric globin (reviewed by Burmester and Hankeln[115]). Neuroglobin and cytoglobin have received the most attention thus far and seem capable of fulfilling multiple functions, similar to hemoglobin and myoglobin[83]. In addition, they both display specific features that seem associated with their *in vivo* role. Both globins are hexacoordinated, allowing increased regulation of ligand binding, and have redox-sensitive thiols that seem to modulate their function depending on the local redox state[116]. In addition, cytoglobin is present as a dimer and displays cooperative O_2 binding, potentially permitting greater O_2 loading and unloading within a narrow range of O_2 tensions[117]. The roles of these and other newly discovered vertebrate globins are however not or only partially understood.

In invertebrates, globins and globin-like proteins display an even greater functional and structural variety compared to their vertebrate counterparts[12,29]. They can occur as cytoplasmic proteins in a range of cell types, including circulating cells of the coelomic fluid. As cytoplasmic proteins, they are often present as monomers or as complexes made from a small number of subunits. Invertebrate globins can also occur as extracellular proteins, freely dissolved in vascular, coelomic or perienteric fluids. Most remarkably, in annelids these extracellular globins occur as giant polymers, with a molecular mass of 3-4MDa[118,119]. Many invertebrate globins are hypothesized to function in O_2 transport and metabolism, similar to vertebrate hemoglobin and myoglobin. For example, the water flea *Daphnia pulex* contains several two-domain globin

isoforms, all with moderate O_2 affinity and present in the hemolymph. Interestingly, their relative concentrations and subunit composition changes depending on the O_2 tension of the environment, thereby leading to an adjustment of the overall O_2 affinity[120]. Also the giant extracellular globin complexes present in annelids seem to function as O_2 transport vehicles; the organization in large, multisubunit complexes appears to allow a large capacity for binding O_2, together with an increased functional regulation and an extensive modular cooperativity in O_2 binding.

However, many globins display characteristics that do not support a traditional role in O_2 metabolism; they can display very high O_2 affinities, making it difficult or even impossible to release bound O_2 at physiological O_2 levels, as is observed for the extracellular globin in the nematode *Ascaris*[121]. This globin can potentially act as an O_2 scavenger or as an O_2 storage in case of extreme anaerobic conditions, but these functions remain speculative. Other globins are even incapable of binding ligands, which is observed for the *C. elegans* globin GLB-6. This globin is hypothesized to participate in redox reactions, but its mode of action is currently unknown. A recent bioinformatics analysis also indicated that several animal globins are most likely membrane bound, indicative of a sensing, signaling or regulatory function[122]. Although the physiological role for many of the newly discovered globins is not known yet, it is clear that these will include novel functions.

2. The model organism *Caenorhabditis elegans*

2.1. Introduction

C. elegans is a small (adult length approximately 1.2 mm) free-living nematode or roundworm that can be found in many parts of the world. It is a filter-feeder, surviving on a variety of bacteria that develop in decaying organic matter such as rotting fruit. However, its ecology is largely unknown and most laboratory strains were taken from anthropogenic environments such as gardens and compost piles[123]. It was adopted in 1963 by Sydney Brenner as a relatively simple metazoan model organism for the study of developmental biology[124]. Since then it has become an established model system for biological research in many fields, including genomics, cell biology, neuroscience and aging. Notable research using *C. elegans* includes the discovery and analysis of controlled cell death, RNA interference and microRNAs, and the use of GFP as a reporter gene.

2.2. Morphology, anatomy & life cycle

C. elegans occurs as two sexes: self-fertilizing hermaphrodites with two sex chromosomes (XX) and males with only one sex chromosome (XO) (**Figure 6**). Males arise infrequently by spontaneous meiotic non-disjunction of the sex chromosome in the hermaphrodite germ line, but can also be kept at higher frequency (up to 50%) through repetitive mating. A single hermaphrodite lays approximately 300 eggs. When inseminated by a male, the number of progeny can exceed 1,000. The majority of research is done on hermaphrodites.

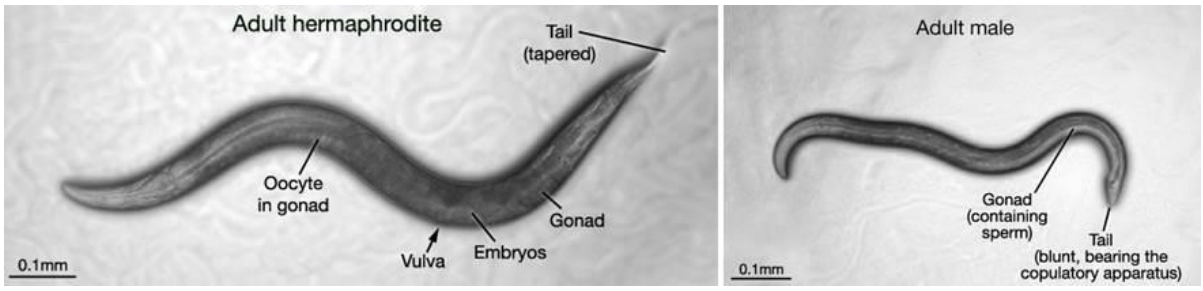


Figure 6: *C. elegans* occurs as two sexes, hermaphrodites and males[6].

The *C. elegans* life cycle consists of the embryonic stage, four larval stages (L1-L4) and adulthood (**Figure 7**). The larval stages are separated by a molting period, during which a new, stage-specific cuticle is synthesized and the old one is shed.

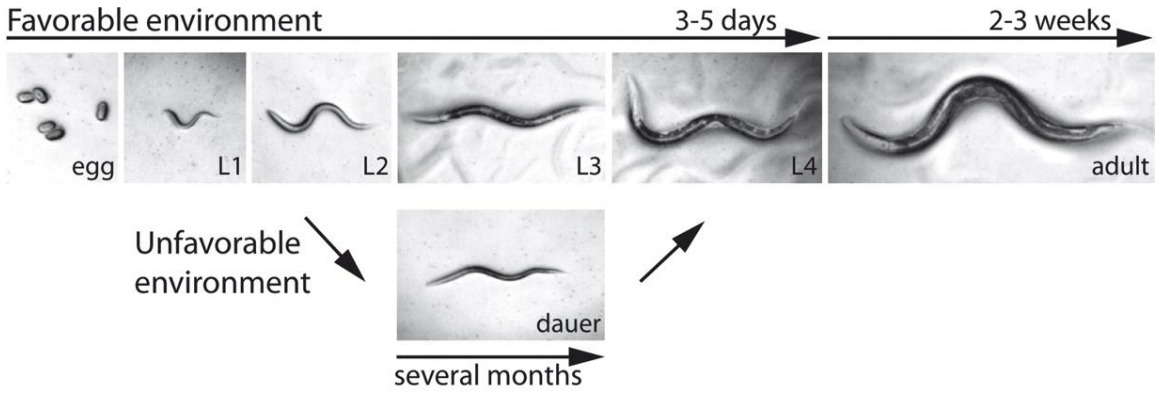


Figure 7: *C. elegans* life cycle[8]. In favorable environments, *C. elegans* undergoes reproductive development and progresses rapidly from embryo through four larval stages (L1–L4) to the adult in 3–5 days, depending on the temperature. Adults then live another 2–3 weeks. In unfavorable conditions, *C. elegans* undergoes development to a specialized third larval stage, the dauer diapause, which can survive for several months without feeding. Upon return to favorable environments, dauer larvae recover to reproductive adults with normal life spans.

Under adverse conditions, such as overcrowding, food scarcity or high temperatures, the L2 larvae can develop to an alternative third larval stage, the dauer. This stage is non-feeding, non-aging and capable of surviving harsh conditions for up to 4 months[125]. When conditions improve again, it can regain development and become a normal, reproductive adult.

C. elegans has the typical nematode body plan, which is an unsegmented, cylindrical body shape that is tapered at both ends (**Figure 8**). Simplified, this consists of an outer tube (body wall) and an inner tube (alimentary canal), which are separated by a fluid-filled body cavity, the pseudocoel. The hydrostatic pressure caused by the pseudocoel is responsible for maintaining the body shape. The body wall consists of cuticle, hypodermis, excretory system, neurons and muscles, and the inner tube comprises the pharynx, intestine and the rectum (**Figure 9**). In the adult hermaphrodite and male, the gonad will fill most of the pseudocoelomic space. The presence of these different tissues makes that the adult worm contains all major differentiated cell types, including muscle cells, neurons, intestinal cells, germ cells and epidermis.

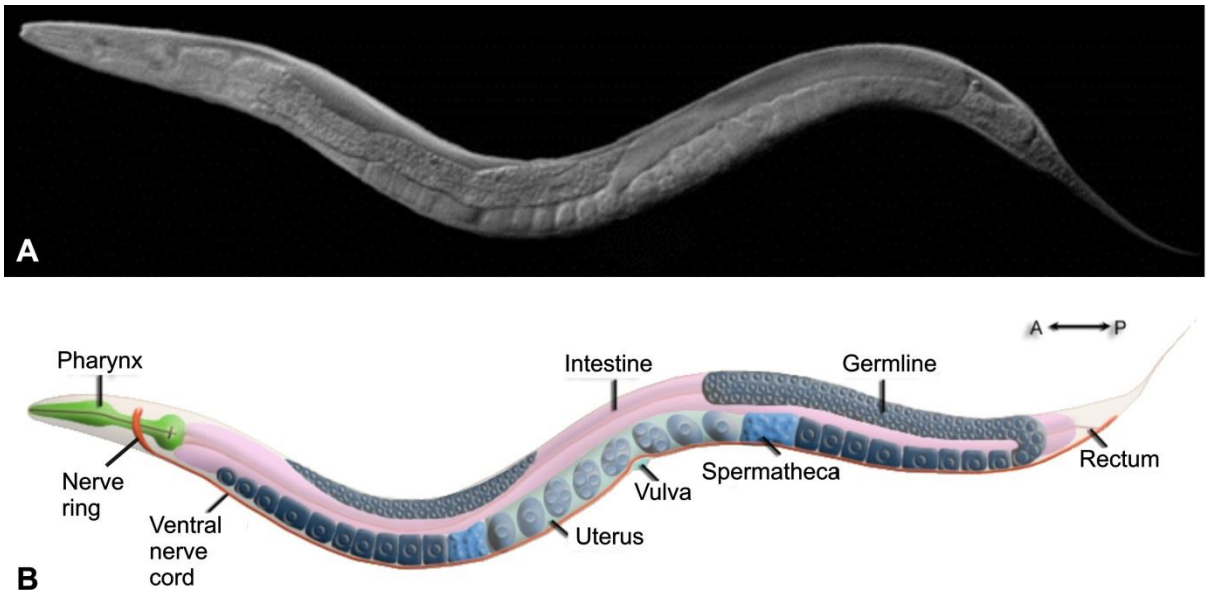


Figure 8: (A) Nomarski image of an adult-stage hermaphrodite *C. elegans*[2]. (B) A lateral (left side) anatomical diagram of an adult-stage hermaphrodite *C. elegans* with emphasis on the digestive and reproductive systems[17].

A collagenous **cuticle** surrounds the worm on the outside and lines the pharynx and rectum. Various tissues, such as the alimentary, reproductive, sensory and excretory system, open to the outside through this cuticle. The **muscle system** consists of striated body wall muscles, arranged into strips in four quadrants, two dorsal and two ventral, along the whole length of the animal. Smaller, nonstriated muscles are found in the pharynx and around the vulva, intestine and

rectum. The **alimentary system** runs straight from head to tail and consists of a pharynx, intestine and rectum. The two-lobed pharynx pumps a suspension of microorganisms into the pharyngeal lumen, after which this is grinded and moved to the intestine. The pharynx is a nearly autonomous organ with its own neuronal system, muscles and epithelium, while the intestine is formed by twenty epithelial cells with extensive apical microvilli, resembling the intestinal cells of higher organisms.

Figure 9: Schematic representation of the body with cross sections from head to tail[5].

A) Posterior body region. Body wall (outer tube) is separated from the alimentary system (inner tube) and gonad by a pseudocoelom,

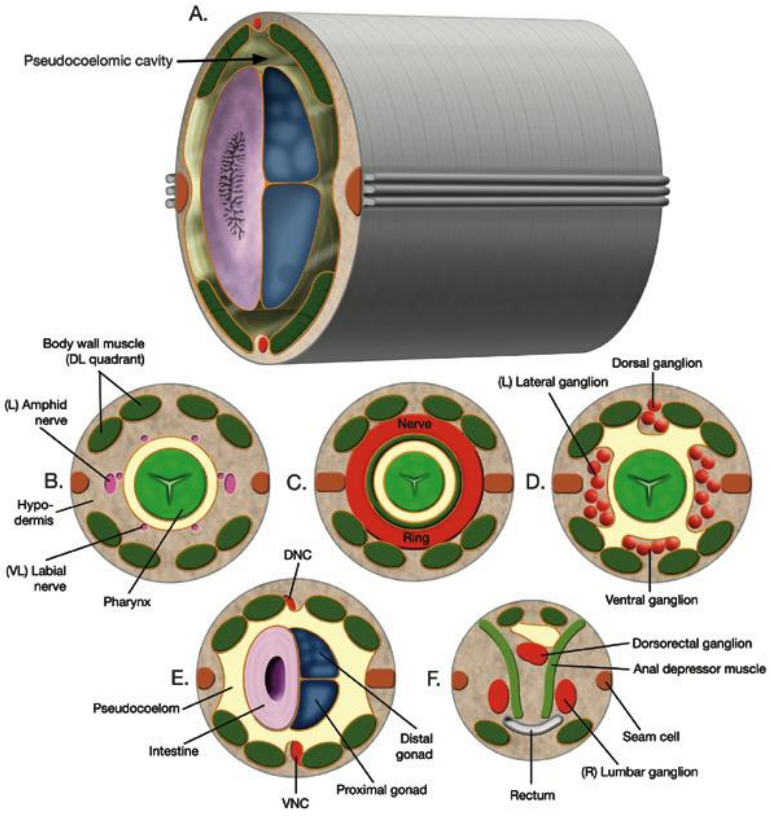
B) anterior head region,

C) central region of the head,

D) posterior region of the head,

E) posterior body, with DNC: dorsal nerve cord; VNC: ventral nerve cord,

F) Section through tail, rectum area.



The **nervous system** is the most complex organ of *C. elegans*, with 302 neuronal cells and 56 glial cells out of the total 959 somatic cells. The majority of neurons are located in the head, in between the two lobes of the pharynx. This region also harbors many neuronal processes that form a circumpharyngeal nerve ring, with ventral and dorsal nerve cords extending posteriorly, running almost to the tail. In the body, neurons are mainly found along the ventral midline and in the tail. Despite its overall small number of cells, the *C. elegans* nervous system is capable of regulating a wide variety of behaviors and responses to environmental stimuli.

Finally, the **reproductive system** consists of two bilaterally symmetric, U-shaped gonad arms that are connected to a central uterus through the spermatheca (**Figure 10**).

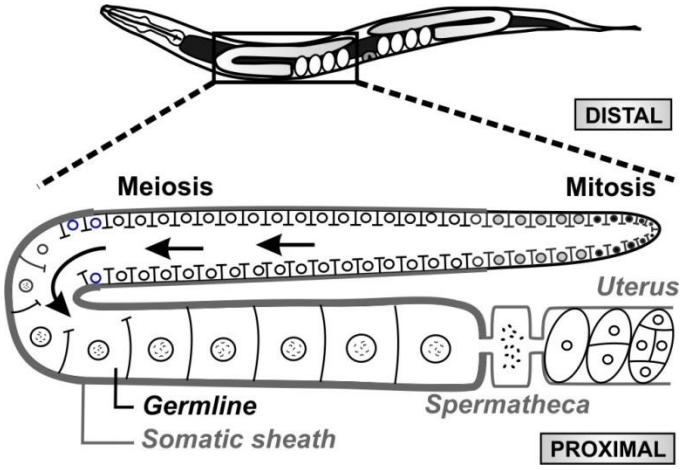


Figure 10: Schematic representation of the anatomy of the *C. elegans*' reproductive system.

The U-shaped gonad arms consist of a central germ line covered with thin somatic sheath cells. These somatic sheath cells are intimately associated with the germ line and have a critical role in its development, organization, and function[126,127]. The distal part of the germline is a syncytium, consisting of 1000 mitotic and meiotic nuclei in cup-shaped compartments open to a common cytoplasmic core. As they progress from the distal tip to the proximal region of the germline, compartments mature, become meiotic, and, as they pass through the bend of the gonad arm, enlarge and further mature to oocytes. The oocytes are fertilized by sperm present in the spermatheca. The resulting diploid zygotes are stored in the uterus and laid off through the vulva, which protrudes at the ventral midline. Hermaphrodites first produce all their sperm in the L4 stage (approximately 150 sperm cells per gonadal arm) and then produce only oocytes as adult organisms.

2.3. The advantages of *C. elegans* as a model organism

Following its selection in 1964 as a potential model organism by Sydney Brenner, *C. elegans* is now being used by a global and still growing research community, due to its many advantages for research. It has a short life cycle, small size and is easy to propagate and maintain. It is a multicellular eukaryotic organism that is still simple enough to be studied in great detail, and displays a stereotypical and largely invariant development, whereby the developmental fate of every single somatic cell has been mapped[128]. Also the complete connectivity of the nervous system in the adult animal has been outlined[129]. The transparency of *C. elegans* forms another advantage, allowing the analysis of cellular differentiation and other developmental processes in the intact organism. These features have led to the extensive use of *C. elegans* in research fields such as development, behavior and signal transduction.

Notably, *C. elegans* was the first multicellular organism of which the genome was sequenced[130]. The genome sequence data have emphasized the substantial conservation of biological processes across the animal kingdom and many cell biological mechanisms in the worm can directly be translated to those in humans. In addition, the genome projects of *C. elegans* and other organisms has led to a wealth of information and to the development of high-throughput and genome-wide analyzes. This lead to the rise of –omics research (e.g. transcriptomics, proteomics, metabolomics, …). For many of these screening techniques, a small transparent worm is ideally suited and also in these post-genomics fields, *C. elegans* has become one of the major model organisms[131].

2.4. The globin family of *C. elegans*

An *in silico* screening of the complete genome of *C. elegans* showed the presence of 33 different globin genes[32], a surprisingly large number. All 33 globins have orthologs in closely related *Caenorhabditis* species as well as in more distinct nematode taxa, including both parasitic and free-living species (unpublished observations and [31]). The functionality and active transcription of these genes has first been confirmed by Reverse Transcription-PCR[32] and later on by the use of transcriptional reporters[31]. The *C. elegans* globin family is very diverse, displaying extensive variation in their DNA and amino acid sequence, as well as in their gene and protein structure and overall protein length, which suggests a long evolutionary history. The globin genes are spread over all six chromosomes, and except for *glb-5* and *glb-6*, they are not organized in gene clusters. Further, while vertebrate and plant globin genes have two and three introns at conserved positions, respectively, the *C. elegans* globin family lacks this conserved pattern and their introns are very diverse in number, size and position. This finding was particularly interesting at its time, because it was speculated that the four exons/three introns arrangement in globins of higher organisms was an ancestral and highly conserved feature. At the protein level, all but one of the *C. elegans* globins are composed of a single domain globin and all *C. elegans* globins show the invariant proximal histidine at position F8. However, other determinants of the globin fold are slightly relaxed and a large number of amino acid substitutions within the globin fold generate much structural diversity among this globin family. Despite this remarkable variety some globins still display significant sequence similarity to vertebrate myoglobin, neuroglobin and cytoglobin[16,32] (**Table 1**). In addition to this sequence variability, many *C. elegans* globins contain additional N-terminal, C-terminal and interhelical extensions. Most remarkably is GLB-33, which has an N-terminal G-coupled receptor-like domain with seven transmembrane helices

in addition to the globin domain. The additional regions of the other globins do not resemble any known protein domains. Because of the additional extensions, the total globin length ranges from 159 aa, which is approximately the standard globin length, to 542 aa.

Protein	Motif/similarity	Total length	Pre A helix	Post H helix	GH Inter-helix
GLB-1	globin	159	-	5	-
GLB-2	globin-like	230	26	26	21
GLB-3	globin	209	32	25	-
GLB-4	globin-like	344	-	120	22
GLB-5	globin	358	34	162	-
GLB-6	globin	389	195	40	-
GLB-7	ngb (Xenopus) 5.4 e-07	278	5	115	-
GLB-8	cygb (human) 3 e-05	404	193	22	-
GLB-9	globin	194	10	42	-
GLB-10	globin	198	26	16	-
GLB-11	globin-like	254	41	71	-
GLB-12	globin	266	30	56	21
GLB-13	ngb (human) 2.8 e-09	231	48	31	-
GLB-14	cygb (human) 8.2 e-12	236	83	-	-
GLB-15	globin-like	234	34	48	-
GLB-16	globin-like	322	59	99	-
GLB-17	cygb (Danio) 7.9 e-06	216	12	16	40
GLB-18	cygb (human) 0.00014	238	68	11	-
GLB-19	ngb (Xenopus) 4.1e-05	214	-	47	-
GLB-20	globin-like	311	94	75	-
GLB-21	globin	196	15	-	32
GLB-22	cygb (human) 0.06	387	132	87	-
GLB-23	globin	342	159	21	-
GLB-24	ngb (Xenopus) 0.006	322	141	24	-
GLB-25	globin-like	189	31	-	-
GLB-26	globin	183	25	6	-
GLB-27	globin	224	22	51	-
GLB-28	globin-like	217	55	23	-
GLB-29	cygb (human) 0.01	216	36	20	-
GLB-30	ngb (human) 0.0001	272	6	103	17
GLB-31	globin-like	169	8	6	-
GLB-32	globin	230	88	-	-
GLB-33	globin	542	372	8	-

Table 1: Overview of the structural characteristics of the *C. elegans* globin family. Adapted from [16]

The remarkable diversity of the *C. elegans* globin family indicates a very dynamic evolution; it is hypothesized that multiple gene duplications gave rise to the expansion of this family, followed by subfunctionalization and consequently the retention of the duplicated genes[31]. Therefore, it is expected that the *C. elegans* globin family carries out a wide range of functions.

Since their discovery, several family-wide screenings have been conducted to better understand what these functions could be. All *C. elegans* globins are expressed *in vivo*, but except for GLB-1

and GLB-26, they seem to be present at low to very low levels. Transcriptional reporters for the 33 globins and translational reporters for a subset of these showed that they are expressed in very specific tissues in the worm[31]. Most globins are present in a limited number of non-overlapping neurons in the head and tail region and in the nerve cord. A minority of the globin reporters could be found in non-neuronal tissues, including body wall, head and vulval muscles, and in the pharynx. RT-PCR expression analysis in all developmental stages of *C. elegans* showed that several globin genes show stage-specific changes in expression, including during the alternative dauer stage[16]. In addition, expression analysis in young adult worms exposed to anoxic conditions for a non-lethal time period showed that several globin genes are particularly responsive to O_2 deprivation. This could point to a role in O_2 metabolism or in protection against oxidative stress that is associated with reoxygenation of cells.

In addition to these family-wide screenings, five globins have been studied in more detail. Their characterization indicates that they carry out functionally distinct roles and thus further supports the diversity present in the *C. elegans* globin family. The first of these globins, GLB-1, has been mainly biochemically characterized and appears to function as a myoglobin-like protein[34]: it is the smallest *C. elegans* globin, having the average globin size, is mainly expressed in muscle tissue, is pentacoordinated and can reversibly bind O_2. Even though GLB-1 will bind O_2 with high affinity, this is still within a physiological relevant range and could support a role in O_2 supply. Expression of this globin is induced following hypoxia, further supporting a myoglobin-like role.

GLB-5 is functionally very well characterized[36,37]. Two groups have independently shown that this globin plays an important role in O_2 dependent behavior. GLB-5 is expressed in a limited number of sensory neurons in the head and tail of the worm, where it functions as an O_2 sensor, modulating neuronal responses and regulating feeding behavior under different O_2 levels. GLB-5 works together with a second O_2 binding protein, the soluble guanylate cyclase GCY-35; GLB-5 is responsible for an inhibitory input when O_2 decreases below 21%, while GCY-35 will activate neurons when O_2 rises towards 21%. Together, these proteins are responsible for fine-tuning the dynamic range of the O_2 sensing neurons they are expressed in, to a range close to atmospheric (21%) concentrations. Biochemical analysis showed that GLB-5 is a hexacoordinated globin[36] and oxidizes rapidly through reversible O_2 binding, which could be important for its function. While it is currently not known how GLB-5 transmits information, de Bono and colleagues[36] hypothesize that changes in O_2 levels trigger conformational changes in this globin that directly modulate downstream signaling molecules.

GLB-6 has received detailed structural and biochemical characterisation[35]. This globin is hexacoordinated and, remarkably, cannot bind any gaseous ligands, which indicates a very tight

coordination of the distal histidine with the heme iron. A role in ligand binding is thus excluded. Instead, GLB-6 seems ideally suited to participate in electron transfer reactions; it shows a low redox potential and is spontaneously oxidized when exposed to air. These unusual biochemical properties are supported by several unique features in the globin fold; a more exposed heme supports oxidation of the heme iron, while a restriction in distal helix movement can be correlated with reduced ligand binding. Similar to GLB-5, it is expressed in head neurons and has been hypothesized to function in aggregation behavior, possibly also working as an O_2 sensor. Interestingly, GLB-6 has a long (186 aa) N-terminal region preceding the globin domain, which could be important in facilitating signal transduction. However, this region does not harbor a recognizable protein domain.

A fourth globin, GLB-26, can bind some gaseous ligands, but its affinity for them is very low[132]. Like GLB-6, it is strongly hexacoordinated and oxidizes instantaneously to the ferric form when exposed to O_2. Also for this globin a role in electron transfer has been proposed. Furthermore, expression analysis showed that *glb-26* is upregulated following anoxia[16], while a transcriptional reporter for GLB-26 has located it in muscle cells that are associated with defecation[31]. It is therefore hypothesized that GLB-26 participates in redox reaction involving O_2 at normal conditions and in other redox reaction when O_2 is absent, potentially to regulate the defecation cycle at different conditions.

Most recently, GLB-13 was studied in more detail[33]. This globin, which is exclusively expressed in head and tail neurons[31], appears to be involved in protection against oxidative stress[33]. Low doses of paraquat, a ROS (reactive oxygen species) generator, caused increased levels of developmental arrest in a loss-of-function mutant for GLB-13, as well as increased expression of the *glb-13* gene in WT worms. GLB-13 is one of the *C. elegans* globins with homology to vertebrate neuroglobin[32], and the authors found that expressing neuroglobin in the *C. elegans* nervous system could rescue the sensitivity of the *glb-13* mutant to oxidative stress[33]. They conclude that GLB-13 and neuroglobin are functionally conserved proteins that are involved in the detoxification of ROS.

In conclusion, the *C. elegans* globin family has shown a remarkable diversity at all levels of analysis, including at a functional level. This makes *C. elegans* a very attractive model to study the potential roles that can be carried out by the globin superfamily and to increase our understanding on the *in vivo* function of these proteins.

2.5. The *C. elegans* globin family as potential model for globins in humans

In vertebrates, eight different globin types have currently been identified, of which five are present in most vertebrate taxa, including humans: hemoglobin in red blood cells, myoglobin in striated muscle cells, neuroglobin in nerve cells, cytoglobin in a variety of tissues, including fibroblast-related cell lineages, and androglobin in testis. The three remaining globins, globin E, X and Y, are restricted to certain taxa and are not found in mammals (reviewed by Burmester and Hankeln[115]). Apart from hemoglobin and myoglobin, these globins have only relatively recently been discovered. Since then, various novel functions have been proposed, including novel roles for hemoglobin and myoglobin. However, many of these potential functions have only been demonstrated *in vitro* and await rigorous proof *in vivo*[115].

C. elegans lacks a circulatory system and hence does not have hemoglobin-like proteins, while at this moment androglobin appears to be absent in Nematoda[68]. As mentioned, several *C. elegans* globins display significant sequence similarity to human myoglobin, neuroglobin and cytoglobin[32]. *C. elegans* could therefore be a very useful animal model to help further define the functions of these globins. However, the resemblance in protein sequence is only a first indication that these *C. elegans* and human globins are indeed functionally related. Given that human globins are mostly expressed in very specific cell types, it appears useful to compare this to the expression pattern of potential orthologs in *C. elegans*. For example, the *C. elegans* globins GLB-1, GLB-8 and GLB-20 are all expressed in body wall muscle cells[31] and could therefore be functionally related to myoglobin. This relationship is less clear for potential orthologs of neuroglobin and cytoglobin: almost all *C. elegans* globins are present in limited sets of neuronal cells, while at this moment no globins are known to be expressed in cells similar to fibroblast (i.e hypodermal cells in *C. elegans*). It should be noted however that the observed expression pattern for the *C. elegans* globins in our first analysis[31] is most likely only partially complete. It could thus very well be that some *C. elegans* globins are indeed expressed in hypodermal cells or show a more ubiquitous expression in the nervous system. Additional expression analysis to more rigourosly determine the expression pattern of *C. elegans* globins that are considered as potential ortholog for human globins can therefore help to further determine a potential functional relationship.

A second prominent difference between human globins is the coordination state of their heme iron; while hemoglobin and myoglobin are pentacoordinated, neuro-, cyto- and androglobin possess a hexacoordinated heme[10,57,68,109,111,115]. The physiological consequences of penta- vs.

hexacoordination are still largely unknown, but clearly influence the globin's biochemical characteristics. Similarity in coordination state is therefore a second characteristic that can indicate a functional connection between *C. elegans* globins and human globins. At this moment, the coordination state of four *C. elegans* globins has been determined, showing that it possesses both penta- (GLB-1) and hexacoordinated (GLB-5, GLB-6, GLB-26) globins[34-36]. While it is too early to determine if any of these hexacoordianted globins are functionally related to neuroglobin or cytoglobin, the pentacoordination of GLB-1 further supports its resemblance to myoglobin.
Finally, the functional analysis of GLB-13 showed that loss of this neuroglobin-ortholog can be rescued by pan-neuronal expression of neuroglobin[33]. This type of experiment forms a strong indication that the two globins share a similar role and further analysis of GLB-13 could provide valuable information on the function of neuroglobin. Comparable experiments have not been performed for other *C. elegans* globins, but it is evident that this type of experiment is ideally suited to determine if human globins and their potential *C. elegans* orthologs fulfil a related role.
In summary, it appears likely that the large and diverse *C. elegans* globin family also includes several members that are functionally related to the different vertebrate and human globins. Detailed analysis of these *C. elegans* globins could aid in the characterization of the latter and help define their function in an *in vivo* animal model.

3. Redox signaling

3.1. Introduction

Reactive oxygen species (ROS) are continuously produced, modified and again removed in all living organisms. 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[133-136]. ROS are indeed prevalent in diseases ranging from cancer over neurodegenerative pathologies to diabetes. On the other hand, ROS can also operate as signaling molecules, a function that, in recent years, has been widely documented but is still often skeptically received. 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, 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. It is increasingly well understood that this type of signaling participates in a wide variety of essential physiological processes[133-139].

3.2. Messengers in redox signaling

ROS is a collective term for the radical and non-radical molecules that are formed by the incomplete reduction of O_2. A radical is defined as a molecule that contains one or more unpaired electrons. O_2 itself is a radical, containing two unpaired electrons, both with a parallel spin. However, O_2 is not a strong oxidizer, and it only reacts fast with other radicals, such as transition metals. In comparison with O_2, ROS show greater chemical reactivity. O_2 is reduced by four single electron transfers (**Figure 11**), subsequently generating superoxide ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2), hydroxyl radical ($OH^{\cdot}$) and water (H_2O). Further reactions within living systems can cause the formation of additional species, such as singlet oxygen ($^1O_2$), lipid peroxides (ROOH) and hypochlorous acid (HOCl). These different types of ROS, each with unique chemical properties, are not all suited to participate in redox signaling. For example, $OH^{\cdot}$ will react rapidly and nonselectively with virtually all types of biological macromolecules, including carbohydrates, nucleic acids, lipids and amino acids, leading to oxidative damage. *In vivo*, $OH^{\cdot}$ is mainly produced by the reaction of H_2O_2 with reduced metal ions, such as copper and iron. Because of its association with oxidative stress, biological systems have developed mechanisms to prevent the formation of $OH^{\cdot}$ by the tight regulation of copper and iron availability within cells.

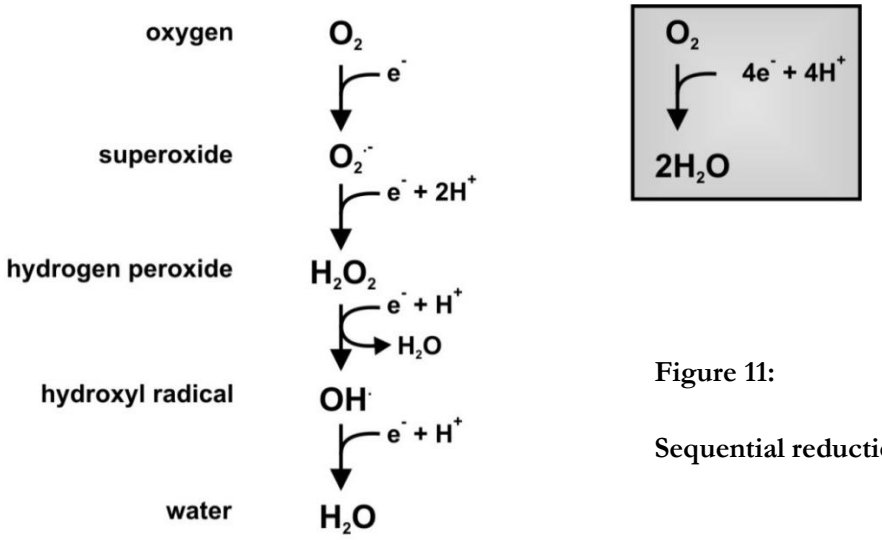


Figure 11:

Sequential reduction of O_2 to H_2O.

At this moment, two types of ROS are recognized to participate in redox signaling, $O_2^{\cdot-}$ and H_2O_2, each having preferred biological targets. $O_2^{\cdot-}$ does not react with most biological molecules, but can quickly react with other radicals and with iron-sulphur (Fe-S) clusters within proteins. The bacterial transcription factor SoxR contains an Fe-S cluster and will exploit this latter reaction to act as an $O_2^{\cdot-}$-sensor[140]. Oxidation of its Fe-S cluster by $O_2^{\cdot-}$ causes a conformational change, resulting in its activation and induction of gene expression involved in $O_2^{\cdot-}$-catabolism. There are several other results that indicate that $O_2^{\cdot-}$ can directly act as a signaling molecule[141-143], however, the main role of $O_2^{\cdot-}$ in redox signaling seems to be as a precursor for H_2O_2. *In vivo*, $O_2^{\cdot-}$ will rapidly and spontaneously dismutate into H_2O_2 ($k=5x10^5 M^{-1}s^{-1}$ at pH 7) and this reaction is further enhanced by superoxide dismutases by 4 orders of magnitude ($k=10^9 M^{-1}s^{-1}$).

H_2O_2 is, because of its uncharged nature, much more diffusible than $O_2^{\cdot-}$. Like $O_2^{\cdot-}$, H_2O_2 is relatively specific in its biochemical sites of action: the most commonly recognized and studied redox posttranslational modification of proteins by H_2O_2 is the oxidation of thiol side chains of cysteine (cys) residues in proteins[106-108]. Cys residues can fulfill a diverse set of roles in proteins, affecting their structure, function and regulation. However, even though H_2O_2 is a potent oxidizer, not all thiol side chains are equal in their ability to become oxidized by H_2O_2 and this reactivity can be substantially tuned and accelerated by the local structure and environment created by the protein[9,133,144-146]. This fact is highlighted by the large differences in reaction rate constants of H_2O_2 with thiols of redox-active peroxiredoxin 2 (PRX2; $2x10^7$ $M^{-1}s^{-1}$)[147] and protein tyrosine phosphatase, nonreceptor type 1B (PTP1B; 20 $M^{-1}s^{-1}$)[148], two major targets of redox signaling in cells. Interestingly, the thiol of glutathione, an essential component in redox biology, will not directly react with H_2O_2 *in vivo*[146]. Also on a proteome-wide scale did the identification of oxidized proteins show that H_2O_2 does not cause random protein thiol oxidation[149,150]. This level of selectivity and specificity by H_2O_2 towards cys oxidation allows the H_2O_2 cys couple to act in redox signaling. In addition, cys residues can be modified in a reversible manner, thus allowing them to cycle between different stable redox forms. Because this latter process is also enzymatically regulated[151], further fine tuning of cys based redox signaling is possible. The H_2O_2 cys based redox interaction thus shows the necessary specificity as well as the capacity for the redox signal to be turned on or off. Finally, the number of discovered cellular targets of H_2O_2 is rapidly growing, making it clear that H_2O_2 cys mediated redox signaling encompasses a wide range of different biological processes. H_2O_2 targets include redox regulated transcription factors, chaperones, metabolic enzymes, proteins involved in signal transduction and antioxidants (reviewed in [9,139]).

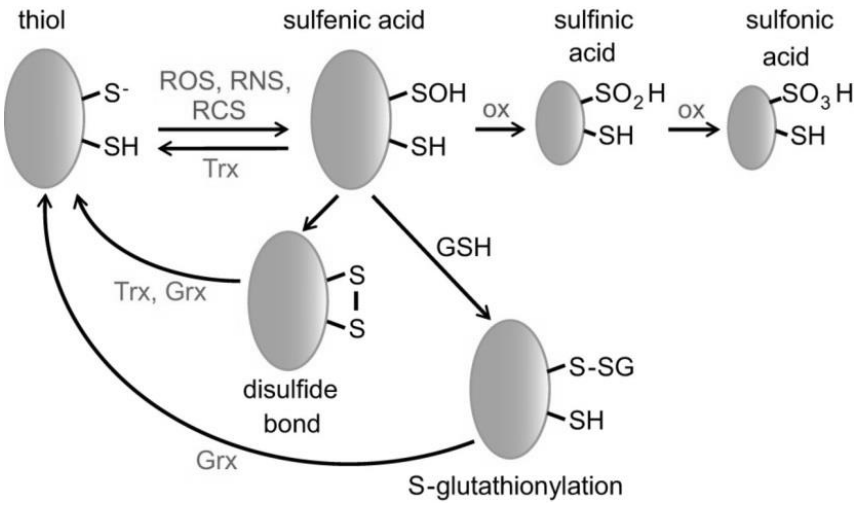


Figure 12: Reversible and irreversible cysteine modifications[9].

When cys thiols become oxidized by reactive oxygen, nitrogen or chlorine species (ROS, RNS, RCS), a highly reactive sulfenic acid intermediate is formed. This intermediate will rapidly interact with another thiol to form inter- or intramolecular disulphide bridges, or with glutathione, leading to S-glutathionylation. Alternatively, sulfenic acids can further oxidize to sulphinic and sulphonic acids. Only the forms of cys that can be reversibly oxidized (sulfenic and occasionally sulfinic acid, disulphide bond, and S-glutathionylation) seem suited to operate in redox signaling. Ox: oxidation; Trx: thioredoxin; Grx: glutaredoxin; GSH: reduced glutathione.

3.3. Enzymatic sources of redox signaling molecules

ROS in biological systems can be produced by both enzymatic and nonenzymatic reactions. The latter include the autoxidation of xenobiotics such as paraquat or of endogenous metabolites such as components of the mitochondrial electron transport chain. These autoxidations are not regulated and it is therefore unlikely that they form a physiological important source of ROS. The enzymatic generation of ROS can be more finely regulated and can therefore lead to a physiological signaling pathway[152].

The majority of $O_2^{\cdot-}$ and H_2O_2 in cells originates from mitochondria. These organelles will transfer electrons along a controlled redox path, named the electron transport chain, which will result in the four-electron reduction of O_2 to H_2O during respiratory ATP synthesis. However, occasionally O_2 will undergo partial reduction and form $O_2^{\cdot-}$ and H_2O_2[153]. It was long assumed that mitochondrial ROS production was an inevitable consequence of an imperfect aerobic respiration system and that cells needed to remove these ROS to avoid oxidative damage. It is now becoming clear that mitochondrial ROS is integrated in the communication between

mitochondria and the host cell by influencing a wide range of signaling pathways between the two[154].

A second important generator of $O_2^{\cdot-}$ and H_2O_2 is a family of membrane-bound enzymes, NADPH-dependent oxidases and their dual oxidase relatives[155-157]. They were first discovered in phagocytic immune cells where they produce $O_2^{\cdot-}$ and H_2O_2 as a cytotoxic agent during the engulfment of pathogens. It is now appreciated that this family is widely expressed and evolutionary conserved and they have been linked to the generation of $O_2^{\cdot-}$ and H_2O_2 as signaling molecules to regulate multiple physiological processes[156,158]. Because of the widespread yet distinct expression of the different isoforms, their use of $O_2^{\cdot-}$ and H_2O_2 in signaling can potentially be placed in the same class as the usage of other ubiquitous small-molecule messengers such as calcium ions (Ca^{2+}) and $NO^{\cdot}$[133].

In addition to mitochondria and NADPH oxidases, $O_2^{\cdot-}$ or H_2O_2 can be produced by a host of other enzymes, such as cytochrome P450 monooxygenases, xanthine oxidases, cyclooxygenases and lipoxygenases, as part of their normal enzymatic function[134]. However, whether ROS produced by these enzymes participate in redox signaling has thus far received very little attention.

3.4. Antioxidant enzymes in redox signaling

The levels of reactive species in living organisms, including ROS levels, are under tight control, either as a way to prevent oxidative damage or to regulate redox biology. A substantial part of this control is mediated by antioxidants, which are very broadly defined as any substance that delays, prevents or removes oxidation of target molecules[137]. Antioxidants include enzymes that catalytically remove reactive species (superoxide dismutase (SOD), catalase, peroxidase), molecules that decrease ROS formation (mitochondrial uncoupling proteins, metal-binding proteins), agents that will, directly or indirectly, act as redox buffer and will be preferentially oxidized (glutathione, thioredoxin, α-tocopherol, ascorbic acid) and systems that prevent and repair oxidative damage (chaperones, proteasome, DNA repair). More strictly defined, antioxidants are agents that will inhibit the oxidation of other molecules by being oxidized themselves. In this definition, antioxidants include enzymes such as SODs and peroxidases that catalytically remove reactive species, as well as reducing agents such as thiols, ascorbic acid and polyphenols[159].

Like its enzymatic generation, the enzymatic removal of ROS can be regulated and therefore seems ideally suited to participate in redox signaling. The majority of enzymatic antioxidants have indeed been implicated in the regulation of redox signaling[139,160,161].

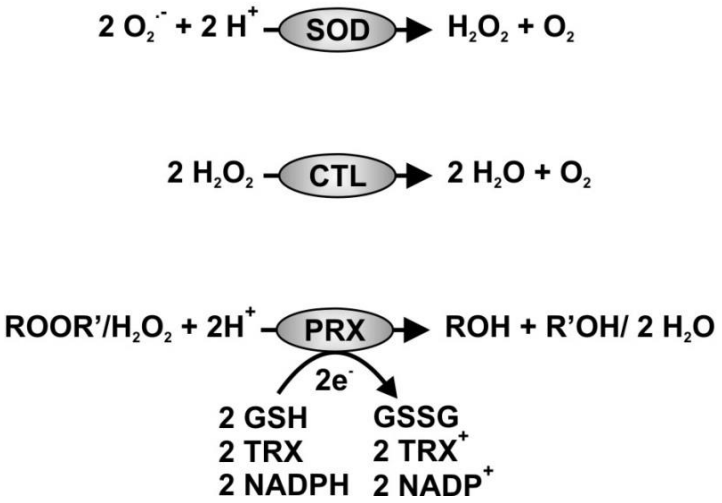


Figure 13: Reaction mechanism of the antioxidant enzymes superoxide dismutase (SOD), catalase (CTL) and peroxidase (PRX).
GSH/GSSG: reduced/oxidized glutathione;
TRX/TRX$^+$: reduced/oxidized thioredoxin;
NADPH/ NADP$^+$: reduced/oxidized nicotinamide adenine dinucleotide phosphate

The spontaneous dismutation of $O_2^{\cdot-}$ into H_2O_2 and O_2 can be strongly enhanced by SODs, a group of oxidoreductases (**Figure 13**). There are several SOD types present, which differ in their metal cofactors and in their subcellular location. Combined, these different SOD types are present in the cytosol, mitochondria and the extracellular space, and probably in other subcellular regions as well, and this in a variety of cell types. H_2O_2 can be further converted to H_2O by two types of enzymes, catalases and peroxidases (**Figure 13**). Because H_2O_2 removal by catalase requires two H_2O_2 molecules at a single active site, this enzyme is very efficient in detoxifying large amounts of H_2O_2 but is considerably slower to remove lower amounts of H_2O_2. Catalase is mainly found in peroxisomes and in general its expression is responsive to oxidative stress. Catalase therefore seems to be implicated in the defense against oxidative stress, rather than in redox signaling. Peroxidases, like peroxiredoxin or glutathione peroxidase, can convert H_2O_2 or hydroperoxides (ROOH) into H_2O or the corresponding alcohol respectively, by oxidizing another substrate (e.g. NADPH, glutathione and thioredoxin). Because their activity is dependent on these substrates, they are directly connected to the cell's metabolism (e.g. NADPH) and its major antioxidant systems (glutathione and thioredoxin). The role of these enzymes in redox signaling is briefly discussed in the following section.

3.5. Spatial and temporal regulation of redox signaling

Besides the level of selectivity present in redox signaling molecules such as H_2O_2, cells will further mediate physiological ROS signaling in a spatial and temporal manner at the subcellular level. Although this is still a very young research area, it is expected that multiple levels of regulation are working together to regulate redox signaling.

3.5.1. Colocalization of redox signaling molecules and targets

Many types of ROS will not be able to migrate far from their source of production because of their inherent instability and reactivity, combined with the redox-buffering and antioxidant capacity of the cell. This limited diffusion capacity allows to regulate the effectiveness of redox signaling by the colocalization of enzymatic sources and downstream targets of ROS[162]. Colocalization also improves selectivity, preventing pathological oxidation of targets such as nucleotides. This type of regulation is best exemplified by NADPH oxidases, which often colocalize with their putative physiological targets, such as phosphatases and kinases, at the plasma membrane within the cell or within cell organelles[163,164]. Also mitochondria appear to regulate their subcellular location to direct redox signaling: a recent report describes the perinuclear redistribution of mitochondria in endothelial cells together with increased nuclear ROS levels that influence the regulation of transcription[168]. Interestingly, multiple reports describe a comparable perinuclear redistribution of mitochondria during oocyte maturation[165-167], but this has not yet been linked to mitochondrial redox signaling. Mitochondria are highly dynamic and their overall cellular structure is determined by the interplay of fusion and division together with movement and positional tethering of mitochondrial units[169-171]. Each of these processes is tightly regulated and integrated with each other as wells as with cellular signaling and stress response pathways. An interesting question in the field of redox signaling is therefore if in general mitochondrial localization is important for the effectiveness of mitochondrial redox signaling, whereby mitochondria will actively change their subcellular location depending on their role in various redox signaling pathways.

3.5.2. 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[133]. 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, 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, for example by its interaction with NOX2, an NADPH oxidase, for the redox activation of NF-κB[172]; 2) for the mitochondrial SOD; for example by its conversion of mitochondrial $O_2^{\cdot-}$ to H_2O_2 to activate a HIF1α-O_2-sensitive K^+ channel pathway[173]; and 3) for the extracellular SOD, for example by its localization at membrane microdomains to promote VEGF receptor type2 signaling[174] (reviewed by Fukai and Ushio-Fukai[160]).

A second example of local redox control is presented by the modular activity of peroxiredoxins, proteins that can convert H_2O_2 to H_2O. Peroxiredoxins typically cycle between a reduced dithiol and an oxidized disulfide form, with oxidation by H_2O_2 and reduction by glutathione[161]. In this manner, low levels of H_2O_2 are quickly and efficiently removed by peroxiredoxins. However, some peroxiredoxin isoforms are susceptible to overoxidation by reaction of the sulfenic acid protein form with a second H_2O_2, resulting in a temporally inactive protein. It is hypothesized that enzymatic production of H_2O_2 at a specific subcellular location can cause local overoxidation and deactivation of these peroxiredoxins, thereby allowing the redox signal to build up in a defined and controlled region. This is termed the floodgate model and would provide a powerful mechanism by which a cell can control localized ROS production for selective cellular redox reactions[161,175]. A variation on this model is shown by a different peroxiredoxin isoform, prx 1, which is less susceptible to overoxidation, but can be deactivated by phosphorylation. The latter has been associated with receptor activation, creating a model in which receptor activation is directly coupled, via kinase signaling, to the modulation of local redox-buffering capacity[175].

3.5.3. 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[141] and aquaporins[133,176,177], respectively. In line with this, it has recently been reported that NADPH oxidases can work

together with aquaporins to regulate transport of extracellularly produced H_2O_2 across the plasma membrane to influence intracellular signaling cascades[162]. 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.

4. Aims and outline of the thesis

Globins constitute a superfamily of heme binding proteins and have well described roles in O_2 transport and O_2 storage. In recent years hundreds of new globin genes have been discovered[20-25,31] and their sequence variability, their presence in organisms living in very different environments and the presence of multiple globin types in single organisms point to a wide array of functions. As a result, several novel roles have been proposed for globins, including other O_2 affiliated roles such as O_2 scavenging or O_2 sensing, roles associated with binding other diatomic gaseous ligands, or participation in electron transfer reactions[12,28-30,56]. However, the exact function for the majority of these newly discovered globins is unknown. The *C. elegans* genome encodes for 33 distinct globins[32]; in addition to their very large number, these globins are diverse in gene and protein structure and expression profiles[16,31,32]. Consequently, this globin family is expected to be diverse in function as well. More detailed analysis of a subset of these globins confirmed their functional variability, with roles that are - or appear to be - related to O_2 storage, O_2 sensing, enzymatic redox reactions and ROS detoxification[27,33-37]. The *C. elegans* globin family thus appears to form a relevant model to study the functional diversity present within the globin superfamily. In addition, *C. elegans* is an established and powerful *in vivo* model organism, allowing detailed functional analysis that is simply not achievable in the majority of other species.

The aim of this thesis was to extend the functional analysis of the *C. elegans* globin family. In more detail, the goal was to describe the molecular mechanisms used by selected *C. elegans* globins to perform their function. To this end, we used two approaches as a starting point.

Firstly, information for several members of the globin family, resulting from previous research, helped us to better define their potential function. These globins were thus interesting candidates for in-depth analysis. We selected three globins to study in more detail.

A family-wide RNAi screening presented a clear and interesting phenotype for a first globin, GLB-12. Depletion of this globin led to sterility, an abnormal vulva and shorter worms, indicating that GLB-12 is involved in reproduction, vulval development and general development. This pleiotropic phenotype appeared to be a very promising lead to decipher the exact function of this globin. In addition, because these processes have already been studied in more detail in *C. elegans*, this enabled us to test several well-defined hypotheses about the role of GLB-12 herein. The ease of use of RNAi would thereby allow us to screen a large number of relevant mutants in a short time. In parallel with this phenotypic characterization, we aimed to

study the physicochemical and structural characteristics of GLB-12 by its recombinant expression. This analysis is described in chapter II.

Previous recombinant expression of a second globin, GLB-26, revealed unusual biochemical characteristics; this globin is hexacoordinated, uncapable of binding O_2 and becomes oxidized when air-exposed[34]. In addition, a previous expression study localized GLB-26 in muscle tissue that is specifically associated with the worm's defecation cycle[31], a relatively well studied rhythmic process. GLB-26 thus appears to participate in a relatively well-defined physiological process, while the availability of a knockout mutant allow us to study this function in more detail. In addition to this phenotypic characterization, we aimed to continue GLB-26' biochemical analysis. Chapter III describes these results.

Previous recombinant expression of a third globin, GLB-1, showed that this is a pentacoordinated globin that reversibly binds O_2[34]. Furthermore, it is one of the most highly expressed globins and appears to localize in head muscle tissue[16,31]. All these characteristics support a role in O_2 supply. The availability of a knockout mutant for this globin allowed us to test this hypothesis by determining if the loss of this globin had an effect on the worm's metabolism. This analysis is part of chapter IV.

Secondly, we analyzed if any *C. elegans* globins are involved in the response to severe O_2 deprivation. In general, the majority of globins that have been studied in more detail are capable of reversible O_2 binding and most likely have a role related to O_2. Furthermore, *C. elegans* is capable of surviving extended periods of hypoxia by activating a conserved transcription factor, HIF-1, that results in the adjustment of worm metabolism and physiology. It can thus be expected that several *C. elegans* globins are involved in the adaptation to O_2 deprivation. We therefore aimed to determine if 1) any *C. elegans* globin gene shows a hypoxia-induced change in expression, and 2) if this change is dependent on HIF-1. Following this first step, we aimed to analyze if RNAi depletion of globins identified in this screen would lead to an obvious phenotype, both during normoxia and hypoxia, in an attempt to further define their role. The presence of a strong phenotype could help us understand in what cellular process these globins are involved and would allow us to construct relevant hypotheses about their function. These results are described in chapter IV.

In conclusion, we aimed to explore the potential functions of the *C. elegans* globin family by 1) examinining those globins for which preliminary data allowed us to better define their potential

role, and 2) determining if any members are involved in the response to O_2 deprivation and analyze these responsive globins in more detail.

PART II: RESULTS

Chapter II:
A redox signaling globin is essential for reproduction in *Caenorhabditis elegans*

Redrafted following submission to Nature Chemical Biology as:

A Redox Signaling Globin is Essential for Reproduction in *Caenorhabditis elegans*

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1. Abstract

Moderate levels of reactive oxygen species (ROS) are now recognized as redox signaling molecules. However, thus far only mitochondria and NADPH oxidases have been identified as cellular sources of ROS in signaling. Here we identify a globin (GLB-12) that produces superoxide, a type of ROS, which serves as an essential signal for reproduction in *C. elegans*. We find that GLB-12 has an important role in the regulation of multiple aspects in germline development, including germ cell apoptosis. We further describe how GLB-12 displays specific molecular, biochemical and structural properties that allow this globin to act as a superoxide generator. In addition, both an intra- and extracellular superoxide dismutase act as key partners of GLB-12 to create a transmembrane redox signal. Our results show that a globin can function as a driving factor in redox signaling, and how this signal is regulated at the subcellular level by multiple control layers.

2. Introduction

ROS-based redox signaling is involved at all levels of cellular organization, from cell differentiation to cell death[134,139]. In this type of signaling, low levels of ROS act as biological messengers by inducing reversible oxidative modifications in downstream proteins and thereby influencing signaling pathways[133,136]. These ROS are enzymatically generated by cells to serve this signaling function. However, in the majority of the reported redox-sensitive signaling pathways, it is often not known which proteins are responsible for generating the redox signal and how cells spatially and temporally link ROS production to specific signaling pathways and so achieve desired cellular outcomes.

Globins are increasingly hypothesized to play a role in redox biology[100]. These proteins are characterized by a common tertiary structure and the presence of a heme group, whereby their function is largely determined by how this heme group is incorporated in the surrounding globin fold. The best-characterized function of globins is oxygen diffusion and transport, as exemplified by vertebrate hemoglobin and myoglobin. The heme iron in these globins is pentacoordinated, leaving the sixth coordination site of the iron free for reversible binding of diatomic ligands, such as O_2. In a second type of globins, named hexacoordinated globins, all six positions of the heme iron are bound[55]. Since their initial discovery two decades ago, these hexacoordinated globins are

now recognized to be broadly present in plants and animals[30]. Because of the hexacoordinated nature of the heme iron, ligand binding/transfer becomes more complex[56] or even absent[35]. On the other hand, this hexacoordinated state of the iron seems to favor electron transfer[27,35,57], indicating that redox reactions involving the heme iron could be a key element in the physiological function of these globins. Therefore, increasing attention has been placed on the potential roles of hexacoordinated globins and globin-like proteins in signaling and redox chemistry, such as protection against ROS and electron transfer to molecular partners[30]. However, even though a fairly detailed understanding on the structure and biochemistry of hexacoordinated globins is now present, it has thus far been extremely difficult to understand what their possible physiological roles are.

C. elegans has thirty-three globin-like proteins, which display different sizes, are differently located and are likely to be functionally diverse[16]. This makes *C. elegans* an attractive model to study the potential roles of globins and globin-like proteins in redox metabolism. A genome-wide RNAi screen in *C. elegans* reported abnormal egg laying and embryonic lethality following RNAi for GLB-12[178], providing a clear phenotype for this so far uncharacterized globin. We therefore analyzed the role of GLB-12 in more detail and discovered that this protein is hexacoordinated, functions as a redox signaling protein and has an essential role in the reproduction of *C. elegans*.

3. Results

GLB-12 is essential for reproduction and acts from the somatic gonad

As a first step to characterize the role of GLB-12, we analyzed the effect of reduced GLB-12 levels on the brood size of *C. elegans*. We observed that *glb-12* RNAi reduced fecundity and increased embryonic lethality in wild type (WT) worms (Fig. 1a). A loss of function mutant for RRF-3, an RNA-directed RNA polymerase that negatively regulates the RNAi response[179], can be used to analyze the effects of enhanced RNAi depletion of GLB-12 in diverse tissues. In this RNAi-hypersensitive strain *rrf-3*, *glb-12* RNAi caused sterility in the majority of worms (Fig. 1a), showing that GLB-12 is essential for reproduction. Expression analysis showed that GLB-12 is present in distinct parts of the somatic reproductive system (Fig. 1b) - the distal gonadal sheath, the proximal part of the spermatheca, and the uterus - and in several head and tail neurons and the vulva. An integrated GLB-12::GFP reporter, which is necessary to allow expression in the germline, showed no visible expression in this tissue, indicating that GLB-12 is not present in the germline (not shown). Further, GLB-12 appears to act directly from the somatic gonad to

regulate reproduction; *glb-12* RNAi only had an effect on reproduction in a soma-specific, but not in a germline- or neuronal-specific RNAi strain (Fig. 1c), while *glb-12* RNAi in the GLB-12 reporter strain reduced reporter levels in the somatic gonad only (Fig. 1d).

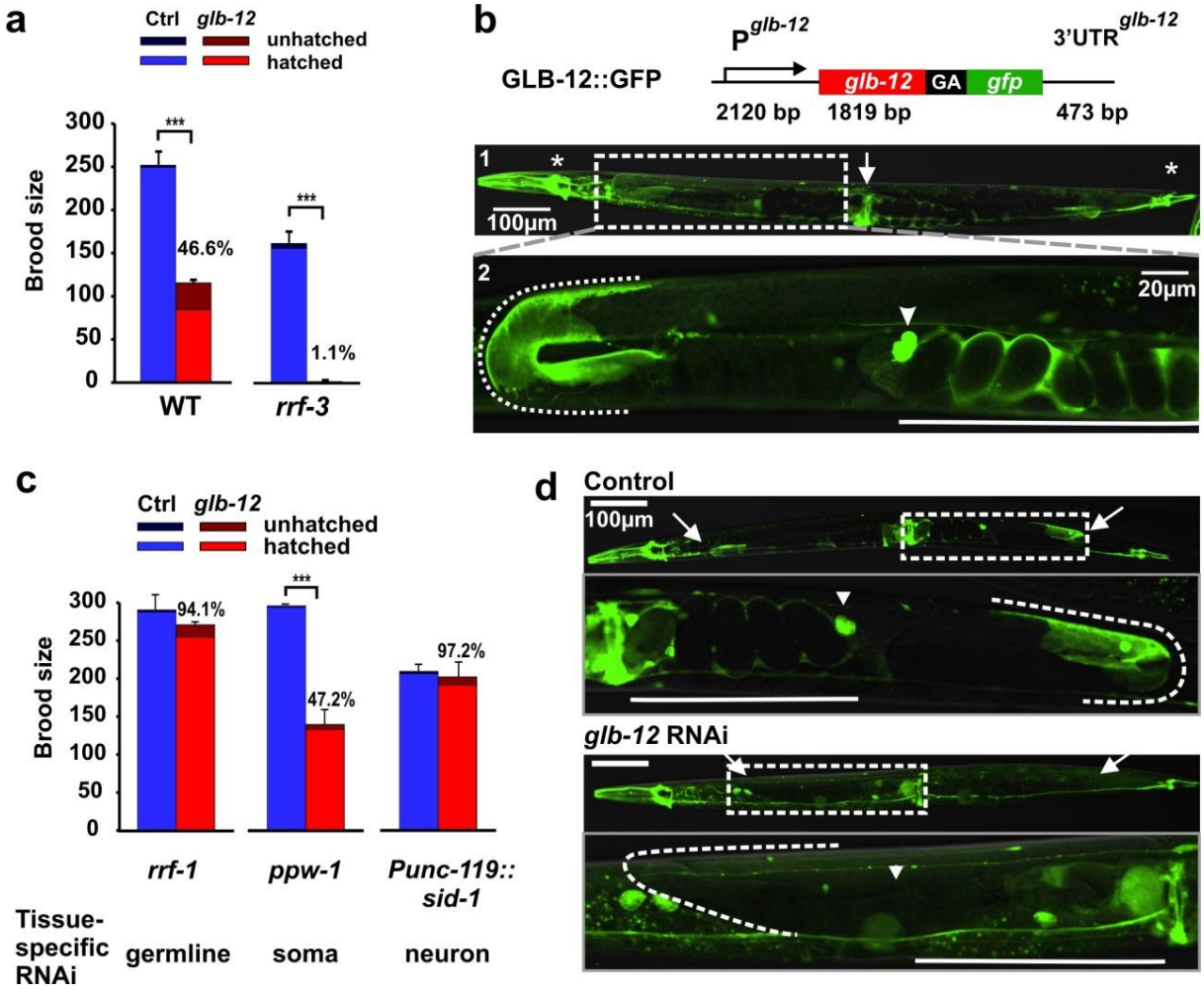


Figure 1. GLB-12 regulates reproduction by acting from the somatic gonad.

(a) *glb-12* RNAi in the WT and RNAi-hypersensitive strain *rrf-3* caused a decreased fecundity. *** P<0.01 Data are represented as mean ± SEM. **(b)** Schematic representation of the reporter construct for *glb-12*. A GA-linker was introduced between GLB-12 and GFP. GLB-12 is expressed in neurons (asterisk), the vulva (arrow) (1), and very specific regions of the somatic gonad (2), i.e. the somatic sheath (dotted line), the proximal part of the spermatheca (arrowhead) and the uterus (full line). **(c)** *glb-12* RNAi reduced fecundity in the soma-RNAi-specific strain *ppw-1*, but not in the germline-RNAi-specific strain *rrf-1* and neuronal-RNAi-specific strain *Punc-119::sid-1*. *** p<0.01. Data are represented as mean ± SEM. **(d)** *glb-12* RNAi reduced expression of the GLB-12 translational reporter only in the somatic gonad. In the control condition, but not following *glb-12* RNAi, expression was seen in the distal gonadal sheath (arrow/dotted lines), the proximal part of the spermatheca (arrowhead) and the uterus (full line).

To understand the impact of GLB-12 on gonad morphology, we used a strain visualizing the germline architecture by fluorescent markers (Fig. 2a and 2b). In the first generation, *glb-12* RNAi caused a range of defects in the adult germline (Fig. 2b): delayed meiotic progression, a considerably smaller rachis (Fig. 2b(1)), increased apoptosis levels (Fig. 2b(2)), and irregular shaped compartments and nuclei (Fig. 2b(3-4)). In the second generation, *glb-12* RNAi led to abnormal germline development and failure to produce oocytes (Fig. 2c). GLB-12 is thus essential for germline development and regulation and appears to be involved in several aspects of reproduction.

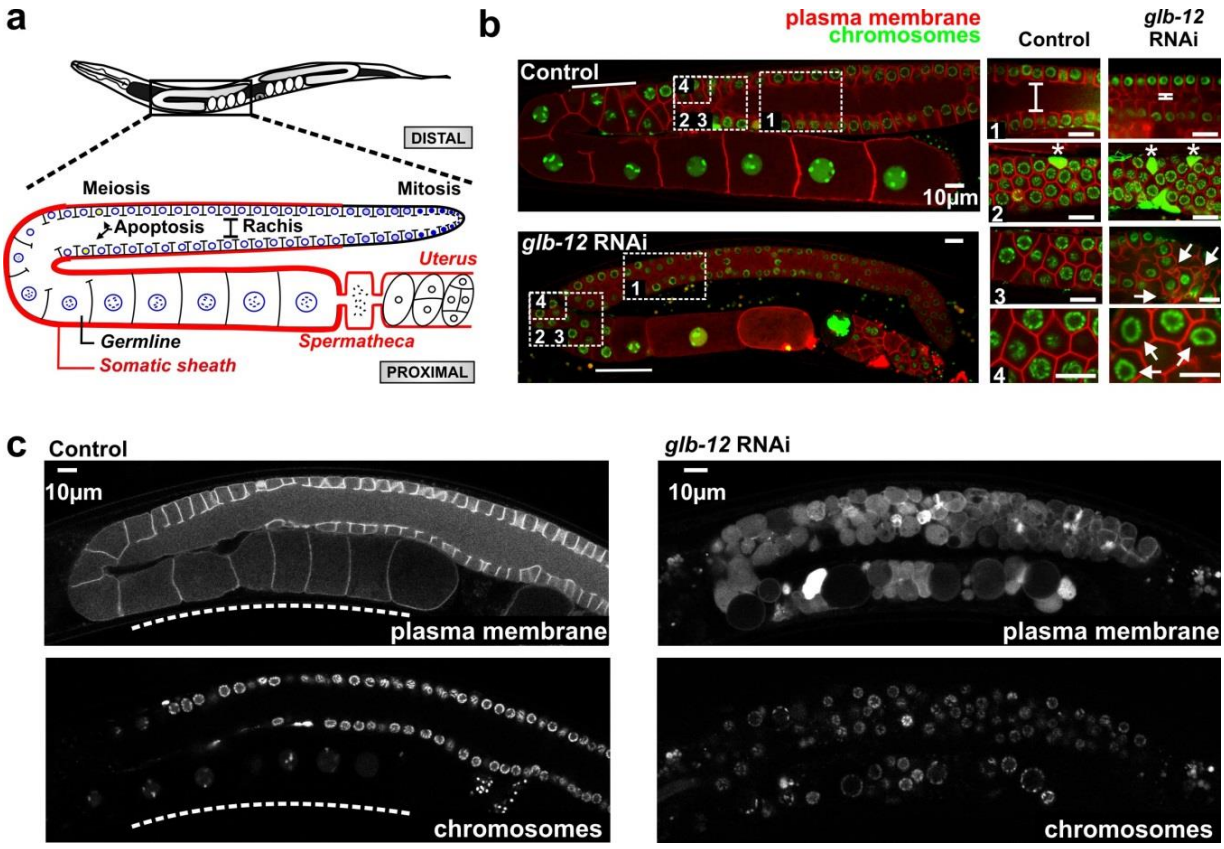
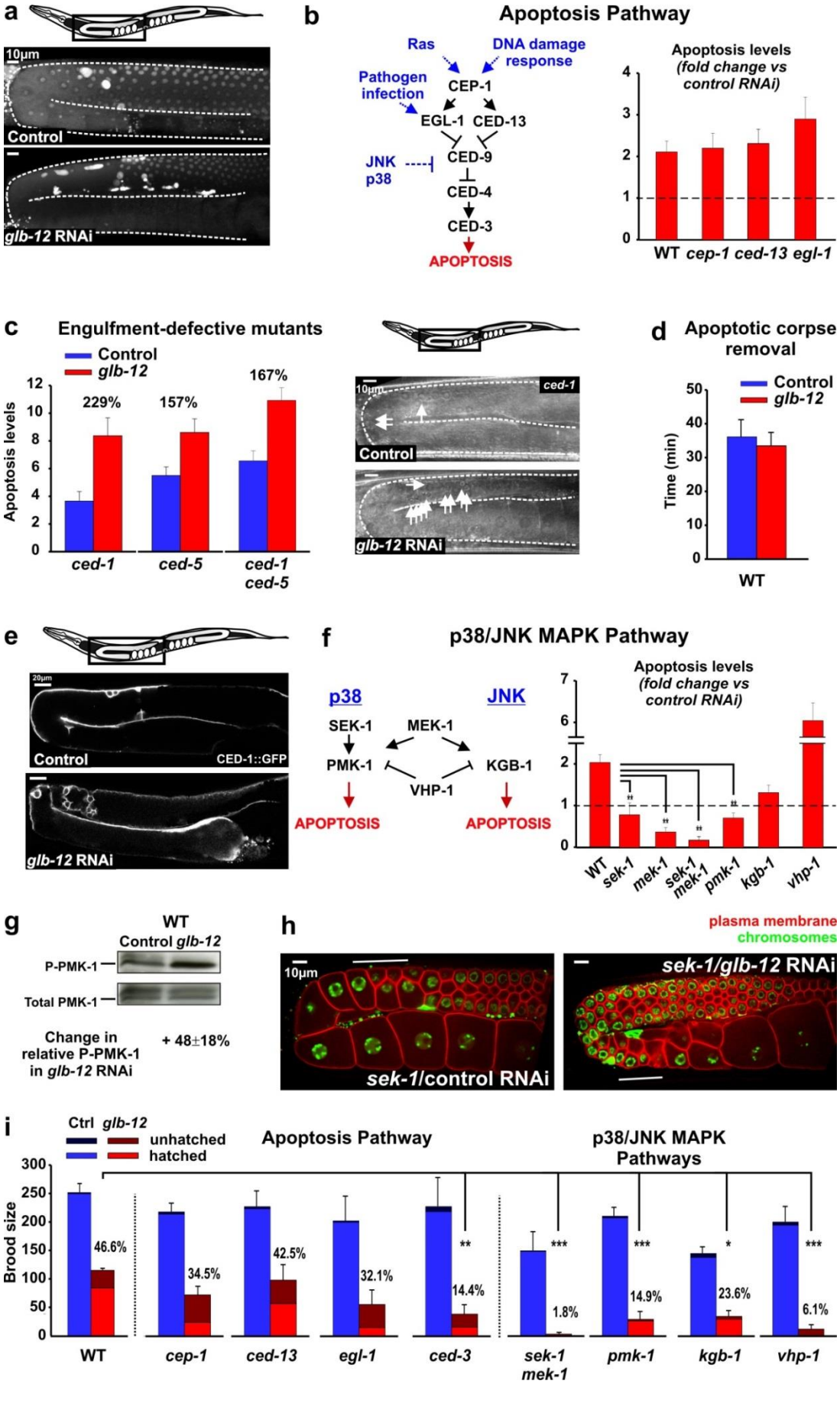


Figure 2. GLB-12 is essential for reproduction.

(a) Schematic representation of the *C. elegans*' reproductive system anatomy. **(b)** *glb-12* RNAi caused multiple defects in the germline. Solid line indicates the meiotic transition region pachytene - diplotene – diakenesis; bar shows the rachis size(1); asterisks indicate apoptotic cells(2) and arrows indicate irregular compartment junctions(3) and nuclei(4). **(c)** The second generation of worms exposed to of *glb-12* RNAi showed a large number of sterile animals with abnormal germline development and the absence of mature oocytes. In control worms, the region with maturing oocytes is indicated by a dashed line. Because fluorophore expression was weaker in these sterile animals, images for the two fluorophores are presented separately to improve clarity.

GLB-12 modulates germline apoptosis levels through the p38 and JNK MAPK pathways



To identify how GLB-12 influences reproduction, we focused on its role in germline apoptosis, which is regulated by a well-characterized signaling cascade[180]. In WT worms, *glb-12* RNAi led to a doubling in germ cell corpses (Fig. 3a, 3b). In a *ced-3* mutant, in which germline apoptosis is absent, no corpses were present following both control and *glb-12* RNAi (not shown). An increase in germ cell corpses can be caused by an increase in germline apoptosis, or by a decrease in corpse engulfment and removal by the somatic gonad. Because *glb-12* RNAi still caused increased germline apoptosis in engulfment-defective mutants, did not affect the speed of corpse removal by the somatic gonad and had no visible effects on somatic gonad structure (Fig. 3c-3e), we concluded that GLB-12 is directly involved in germline apoptosis. In loss-of-function mutants for the core apoptosis pathway (i.e. *cep-1*, *ced-13* and *egl-1*), *glb-12* RNAi still caused an increase in germline apoptosis (Fig. 3b and Supplementary Fig. 1a), demonstrating that GLB-12

Figure 3. GLB-12 regulates apoptosis via the p38/JNK MAPK pathways.

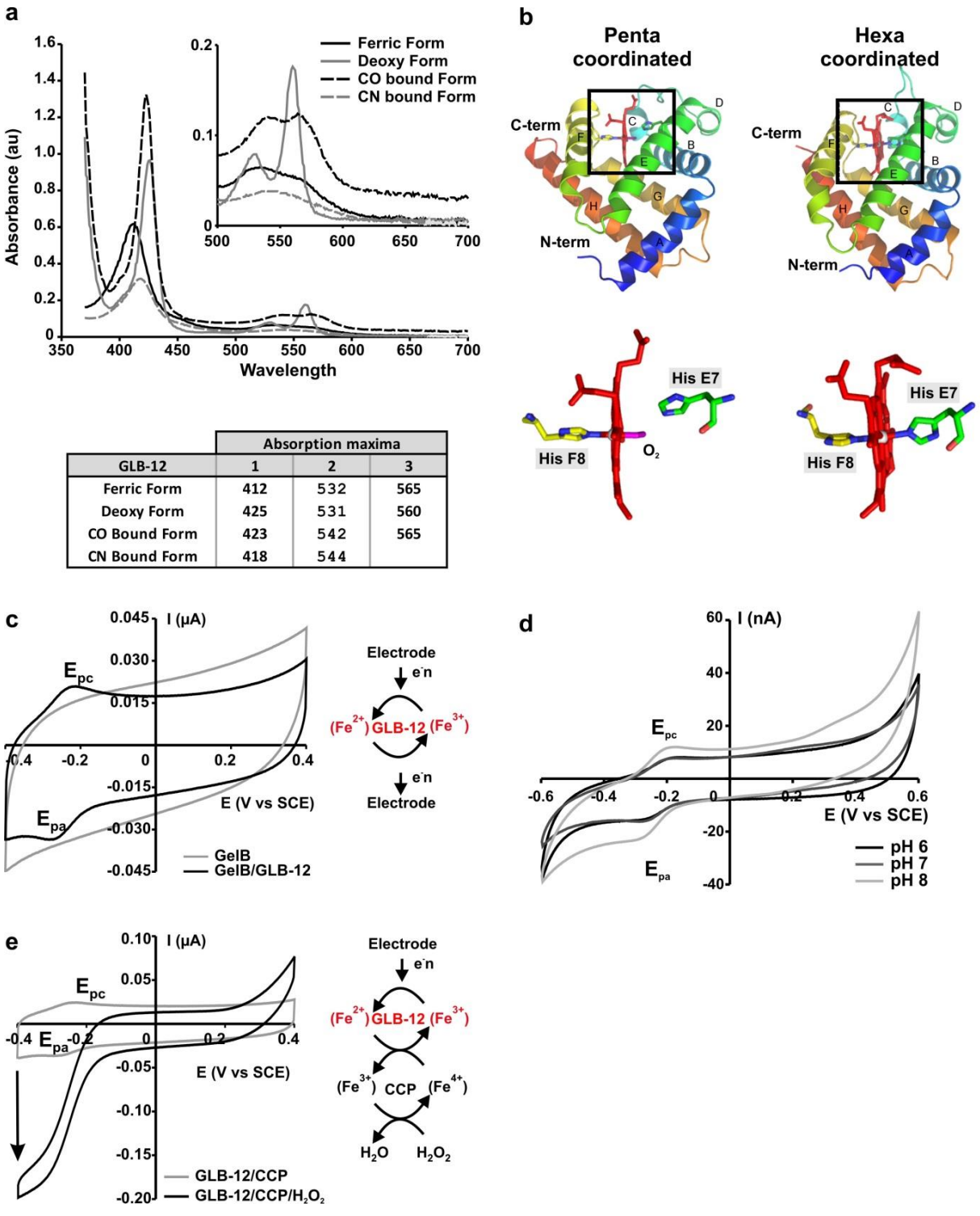
(a) Acridine orange staining was used to visualize the increase in germline apoptosis following *glb-12* RNAi. **(b)** *glb-12* RNAi caused an increase in apoptosis in the WT and in mutants for the core apoptosis pathway. The cartoons show the apoptosis pathway (black) and associated signaling pathways (blue). **(c)** *glb-12* RNAi still caused increased germline apoptosis levels in mutants that are defective for the two parallel pathways that control cell-corpse removal. Because the dye used to score apoptosis in this study, acridin orange, will preferentially label engulfed cell corpses, it could not be used in these mutants. Therefore, apoptotic corpses in these mutants were scored by DIC. A representative image is shown for the *ced-1* mutant, with arrows indicating apoptotic cells. **(d)** No significant effect of *glb-12* RNAi was observed on the speed of cell corpse removal in WT worms. Time-lapse imaging was used to follow the presence of apoptotic corpses. (n=9 corpses for control RNAi and 13 for *glb-12* RNAi, spread over 3 replica's). **(e)** The somatic gonad does not show any obvious defects or abnormalities following *glb-12* RNAi. CED-1::GFP was used as a marker for the somatic gonad. **(f)** *glb-12* RNAi did not cause an increase in apoptosis in mutants for the p38/JNK MAPK pathways. The cartoons show a simplified version of the p38/JNK MAPK pathway. **(g)** Western blot showing that phosphorylated PMK-1 (P-PMK-1) levels are increased following *glb-12* RNAi. **(h)** Effect of *glb-12* and *sek-1* RNAi on the gonadal structure, with membranes in red and histones in green. Full line indicates the transition region pachytene - diplotene – diakenesis. Double RNAi of *glb-12* and *sek-1* still caused several gonadal defects that were also observed following *glb-12* RNAi alone, including delayed meiotic progression and abnormal germline architecture. Similar results were obtained with RNAi against *mek-1*, *pmk-1* and *kgb-1* in combination with *glb-12* RNAi. **(i)** *glb-12* RNAi caused a further decrease in fecundity in a *ced-3* mutant, in which germline apoptosis is absent, and in mutants for the p38/JNK MAPK pathways, but not in mutants for the core apoptosis pathway, i.e. *cep-1*, *ced-13* and *egl-1*. * $p<0.1$; ** $p<0.05$; *** $p<0.01$. Data are represented as mean ± SEM.

does not signal through these proteins. The p38/JNK MAPK pathways act independently of CEP-1, CED-13 and EGL-1[181] (Fig. 3b). *glb-12* RNAi in loss-of-function mutants for these pathways no longer led to an increase in germ cell apoptosis (Fig. 3f and Supplementary Fig. 1b), while in a VHP-1 mutant, an inhibiting phosphatase for these pathways[182], *glb-12* RNAi caused a larger increase in germ cell apoptosis. Further, *glb-12* RNAi in the WT also increased the relative amount of phosphorylated PMK-1 (Fig. 3g). Together, these results strongly indicate that GLB-12 has antiapoptotic effects by inhibiting the p38/JNK MAPK pathways. Surprisingly however, fecundity was even further reduced following *glb-12* RNAi in mutants for CED-3 or for the p38/JNK MAPK pathways, but not so in *cep-1*, *ced-13* and *egl-1* mutants, and several gonadal defects were still observed (Fig. 3h, 3i). GLB-12 mediated apoptosis is thus regulated by the p38/JNK MAPK pathways, while additional GLB-12 effects on reproduction appear to occur independently of these pathways.

GLB-12 is a hexacoordinated globin with redox properties

To determine how GLB-12 could directly influence downstream targets, we analyzed the biochemical and structural properties of the purified protein. Spectroscopic analysis, which allows to discriminate between different globin forms, showed that reduced deoxy (Fe^{2+}) GLB-12 displays a hexacoordinated heme iron (Fig. 4a, 4b). Hexacoordinated globins are present in prokaryotes, plants and animals, but their function is largely unclear[30]. They can potentially bind diatomic ligands such as O_2, CN^- and CO to their heme iron, thereby replacing the endogenous HisE7 ligand. However, even though GLB-12 was able to bind CN^- and CO *in vitro* (Fig. 4a), both reactions proceeded very slowly, requiring several minutes (reaction kinetics not shown). In addition, upon exposure to air, GLB-12 is spontaneously oxidized to the ferric state (Fe^{3+}) (Fig. 4a) and is thus incapable of binding O_2 under physiological conditions. These results argue against a role for GLB-12 in reversible ligand binding and/or transport, the best known function for globins. On the other hand, the spontaneous oxidation of air-exposed GLB-12 is in line with the observation that hexacoordination appears to lower the redox potential and promote electron transfer of the heme iron[27,35,57]. By using cyclic voltammetry to study the electrochemical properties of GLB-12 in more detail, we indeed observed a relatively low reduction potential for the GLB-12 redox couple Fe^{2+}/Fe^{3+} (-0.244V *vs.* SCE), which is largely stable within the physiological pH range (Fig. 4c, 4d). To determine whether GLB-12 could transfer electrons to other molecules, we included cytochrome c peroxidase (CCP) in this experimental setup as an electron acceptor and subsequent catalyst for hydrogen peroxide reduction. It is important to note that CCP by itself shows no electrocatalytic activity under these conditions[183]. When H_2O_2

was added to GLB-12 and CCP, the reduction current increased and the oxidation peak disappeared, indicating very fast GLB-12 oxidation (Fig. 4e). This shows that CCP was capable of reducing H_2O_2 by receiving electrons from GLB-12. GLB-12 can thus transfer electrons to other molecules in a continuous manner.



GLB-12	Absorption maxima 1	2	3
Ferric Form	412	532	565
Deoxy Form	425	531	560
CO Bound Form	423	542	565
CN Bound Form	418	544	

GLB-12 produces $O_2^{\cdot-}$ as a signaling molecule

Because GLB-12 can participate in electron transfer and has a reduction potential lower than the $O_2/O_2^{\cdot-}$ couple[184], it may directly interact with O_2 to generate $O_2^{\cdot-}$. To test this hypothesis, we used an *in vitro* enzymatic reduction system for heme proteins[185] and included lucigenin, which emits light upon reaction with $O_2^{\cdot-}$ (Fig. 5a). We first validated this *in vitro* method using heme proteins with a reduction potential higher and lower than the $O_2/O_2^{\cdot-}$ couple (Fig. 5b). Inclusion of GLB-12 and glucose-6-phosphate, the driving force of this system, led to increased luminescence, showing that GLB-12 can convert O_2 to $O_2^{\cdot-}$ (Fig. 5a). *In vivo*, the $O_2^{\cdot-}$ produced by

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Figure 4. GLB-12 is a hexacoordinated globin with redox properties.

(a) UV-Vis spectra of different GLB-12 forms, with their absorption maxima. The absorption spectrum of purified GLB-12 showed that it is spontaneously oxidized to the ferric state (Fe^{3+}) upon exposure to air. Reducing GLB-12 (Fe^{2+}) resulted in an absorption spectrum that is typical for a hexacoordinated heme iron. Absorption spectra of CN- and CO-bound GLB-12 could be obtained after exposure of GLB-12 to an excess of KCN or CO. **(b)** Penta- and hexacoordination in globins. In globins with a pentacoordinated heme iron, one potential binding site of the iron remains open and reversible binding of diatomic gaseous ligands is a one-step process. The oxygenated structure of sperm whale myoglobin (PDB code 1MBO; [15]) is shown as representative of a pentacoordinated globin with an exogenous ligand bound at the heme distal site. In globins with a hexacoordinated heme iron, all six coordination sites of the iron are occupied. The structure of human neuroglobin (PDB code 1OJ6; [19]) is shown as representative of a hexacoordinated globin. Ligand binding is possible, but requires the dissociation of the sixth coordination and therefore becomes more complex. In both panels the globin structures are shown in ribbon representation, with helices labeled according to the canonical globin fold. The distal HisE7 (green), the proximal HisF8 (yellow), the heme (red, with the Fe-atom in grey) and the oxygen molecule (violet) are shown in stick representation. **(c)** The current potential behavior of a gelatin B (GelB) and a GelB/GLB-12 electrode, with the reduction (E_{pc}) and oxidation peak (E_{pa}), showing that GLB-12 is reversibly oxidized and reduced with a reduction potential of -0.244V (*vs.* SCE). The reversible oxidation/reduction of GLB-12 by cyclic voltammetry is illustrated in the scheme. **(d)** The current potential behavior of a gelatin B electrode containing GLB-12, with the reduction (E_{pc}) and oxidation peak (E_{pa}), at different pH, showing that the redox couple Fe^{2+}/Fe^{3+} couple is largely pH independent within the physiological pH range. **(e)** The current potential behavior of a gelatin B electrode containing GLB-12 and CCP (Cytochrome C Peroxidase), with and without H_2O_2. The reduction (E_{pc}) and oxidation peak (E_{pa}) are indicated for GLB-12/CCP. The arrow points to the increase of the reduction peak when H_2O_2 is added, showing that GLB-12 becomes fully reduced by electron transfer to CCP, making CCP capable of reducing H_2O_2. This reaction is illustrated in the scheme.

GLB-12 could act as a redox signaling molecule. In this hypothesis, RNAi reduces GLB-12 levels and hence the amount of $O_2^{\cdot-}$ produced by GLB-12, causing the reproductive defects. We reasoned that exposing worms to ascorbic acid, a scavenger of $O_2^{\cdot-}$ and other ROS, would further reduce $O_2^{\cdot-}$ levels and thereby increase the severity of the *glb-12* RNAi phenotypes. This was indeed observed (Fig. 5c, 5d and Supplementary Fig. 1c), further indicating that GLB-12 generates $O_2^{\cdot-}$ as a signaling molecule.

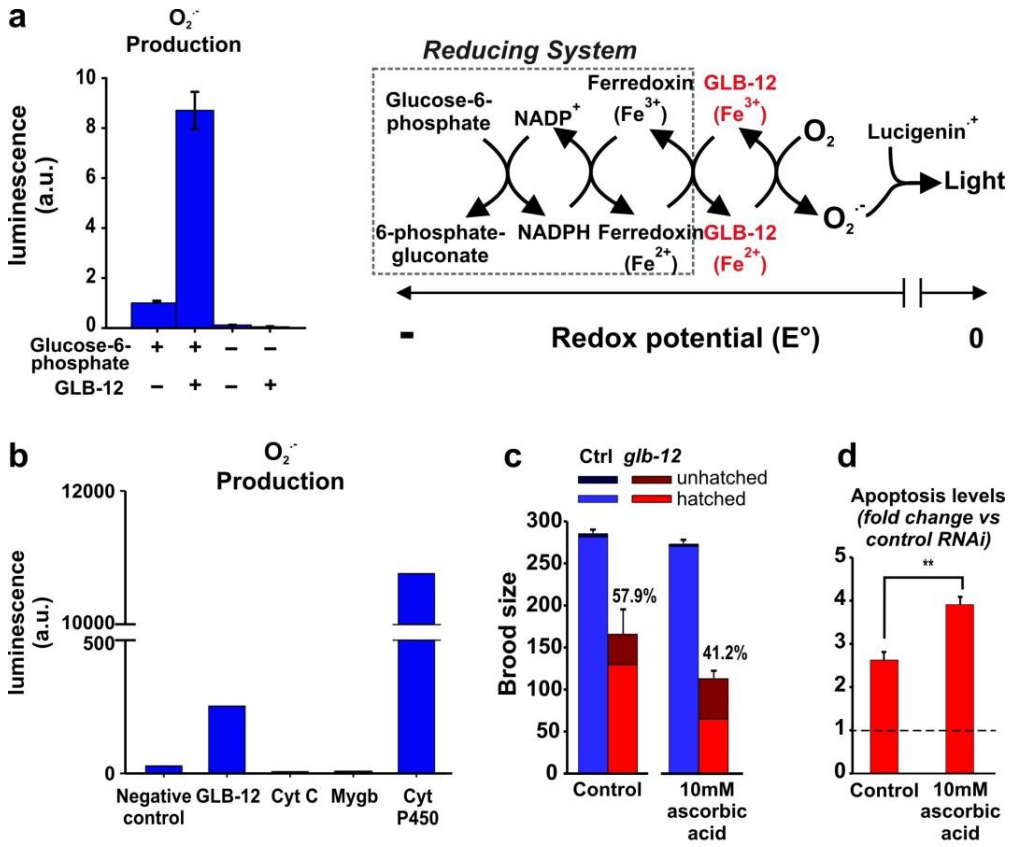


Figure 5. GLB-12 is capable of producing $O_2^{\cdot-}$.

(a) GLB-12 is capable of producing $O_2^{\cdot-}$. (+) indicates the presence, (-) the absence of glucose-6-phosphate and GLB-12. The scheme shows the *in vitro* system used. **(b)** *In vitro* superoxide production by GLB-12, cytochrome C (Cyt C), myoglobin (Mygb) and cytochrome P450 (Cyt P450). Myoglobin and cytochrome c, which have a reduction potential higher than the $O_2/O_2^{\cdot-}$ couple [3,4] and therefore should not be able to produce $O_2^{\cdot-}$, did not lead to an increase in luminescence. Cytochrome P450, which has a reduction potential lower than the $O_2/O_2^{\cdot-}$ couple and can produce high levels of $O_2^{\cdot-}$ [18], lead to a large increase in luminescence. GLB-12 lead to a moderate increase in luminescence, indicating that it produced moderate levels of $O_2^{\cdot-}$. **(c)(d)** The antioxidant ascorbic acid enhances the *glb-12* RNAi phenotypic effects. Data are represented as mean ± SEM.

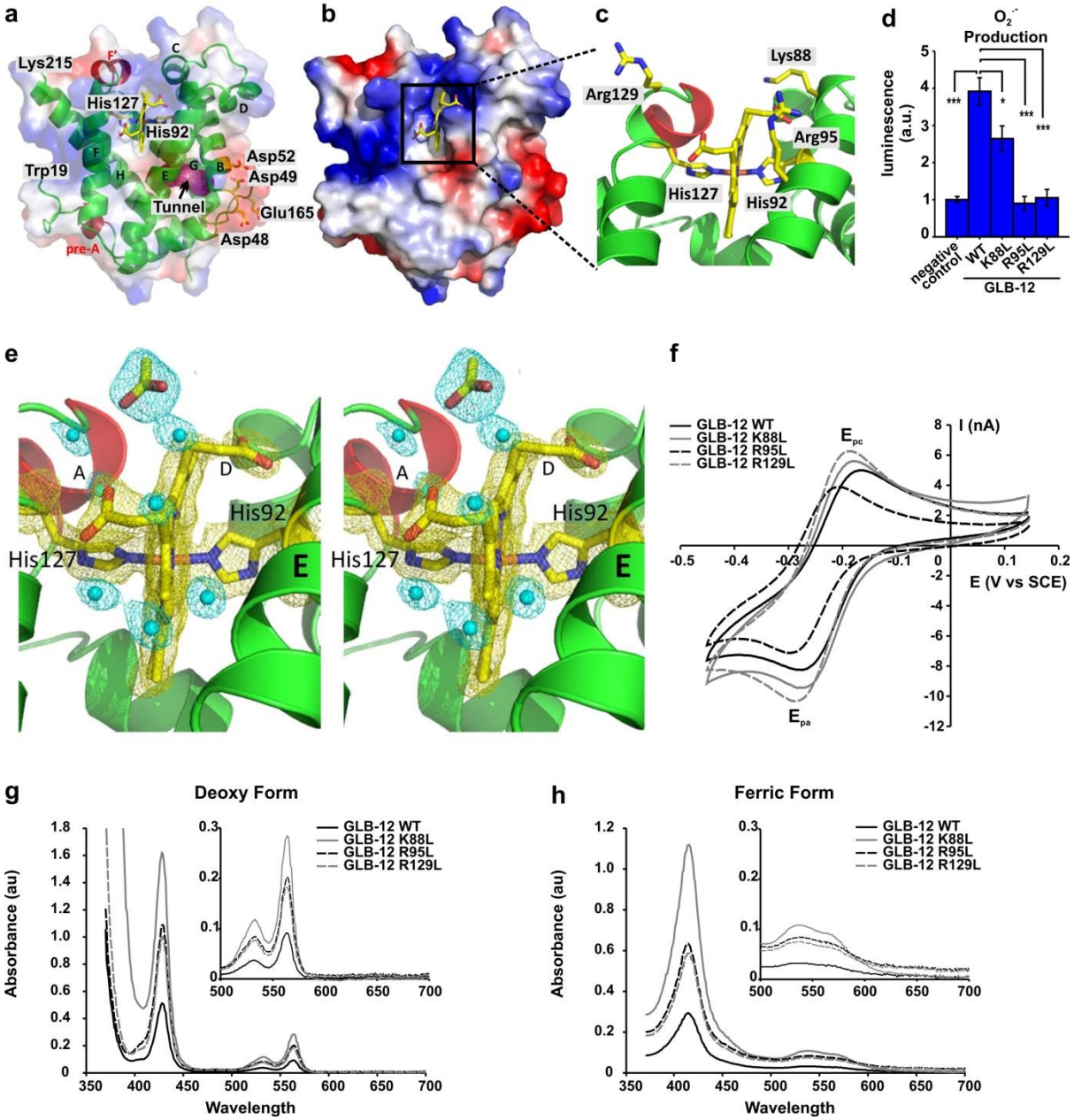
The crystal structure of the GLB-12 globin domain, refined to 1.65 Å resolution (Table 1), further supported our current findings. It confirmed heme hexacoordination, with strong Fe-N bonds with the distal His92 and the proximal His127 residues (Fig. 6a, 6e). Overall, the hexacoordinated GLB-12 globin domain is closely related to the 3D structures of human and murine neuroglobin[19,57,186,187], human cytoglobin[111], nonsymbiotic rice hemoglobin[188], *C. elegans* GLB-6[35] and *Geobacter sulfurreducens* globin-coupled sensor[189], although GLB-12 possesses several unique structural features.

Table 1. GLB-12 Crystal Structure - Data collection and refinement statistics

	GLB-12 native	GLB-12 SAD
Data collection		
Wavelengh (Å)	0.95372	1.72200
Space group	$P6_522$	$P6_522$
Cell dimensions		
a, *b*, *c* (Å)	50.4, 50.4, 245.3	50.4, 50.4, 245.3
α, β, γ (°)	90.0, 90.0, 120.0	90.0, 90.0, 120.0
Resolution (Å)	29.85-1.65 (1.74-1.65)*	49.05-2.70 (2.85-2.70)
No. total reflections	159514	219339
No. unique reflections	23429	5773
R_{merge} (%)	6.4 (56.1)	6.3 (18.6)
$I/\sigma I$	13.9 (3.2)	49.7 (24.8)
Completeness (%)	99.8 (100)	100 (100)
Redundancy	6.8 (6.9)	38.0 (38.0)
Anomalous completeness (%)		100 (100)
Anomalous redundancy		23.0 (21.8)
Refinement		
R_{work}/ R_{free} (%)	17.8 / 23.4	
Protein residues	182 (residues 19-166, 182-215)	
Heme	1	
Water	110	
Sulfate ion	1	
Acetate	4	
B-factors (Å^2):		
Protein	25.4	
…Heme	25.7	
Water	37.4	
….Sulfate ion	47.4	
….Acetate	38.1	
R.m.s deviations:		
Bond lengths (Å)	0.017	
Bond angles (°)	1.6	
Ramachandran plot:		
….most favored regions (%)	92.6	
additional allowed regions (%)	7.4	

*Highest resolution shell is shown in parenthesis.

The proximal His127 is highly exposed to the solvent, which is the result of a hydrogen bond between the A-propionate of the heme to Ser126 OH. As a result, two water molecules are hosted at the entrance of the proximal site (hydrogen bonded to Ser126 OG and to Gln130 NE2, respectively), and four additional water molecules and one acetate ion fall next to the propionate (Fig. 6e). This exposure to a polar aqueous environment would favor oxidation of the ferrous heme, which is consistent with the low GLB-12 redox potential reported.



On the distal site of the heme cavity, the D-propionate stabilizes the location of the E-helix N-terminal region through hydrogen bonding (Fig. 6e). In addition, residues Phe89 and Phe96 (surrounding the distal His92) are part of a wide cluster of aromatic/hydrophobic residues

(Val54, Phe58, Leu59, Val62, Phe73, Phe152) (not highlighted). These could restrict the E-helix movements required to achieve a pentacoordinated heme and would thereby limit ligand binding. This explains the slow CN^- and CO binding we observed. Additionally, this aromatic/hydrophobic residue cluster creates a tunnel of about 50 $Å^3$ in the globin domain, nestled among the B-, E-, and G-helices (Fig. 6a). Interestingly, this otherwise apolar tunnel shows a strong negative charge distribution at the solvent exit (resulting from residues Asp48, Asp49, Asp52, and Glu165) (Fig. 6a, 6b). In the crystal structure the tunnel exit to the solvent appears rather narrow; however, structural fluctuations may vary its diameter, providing a direct connection between the heme distal site and the solvent region. Given this, it is possible that the negative charges located at the tunnel exit would help to remove small and negatively charged species such as $O_2^{\cdot-}$, produced by redox reactions at the distal site, from the protein core.

Figure 6. The globin domain of GLB-12 shows structural characteristics that facilitate $O_2^{\cdot-}$ production.

(a) Ribbon representation of the GLB-12 globin domain, with helices labeled according to the canonical globin fold (green). The GLB-12-specific pre-A helix and F'-helix are highlighted in red. The terminal residues visible in the electron density (19-215) are labeled. The protein electrostatic surface (see also panel b) is displayed in semitransparent colors, allowing the view of underlying secondary structure elements and a distal apolar tunnel (magenta mesh) located between the B-, E-, and G-helices. Acidic residues localized where this apolar tunnel reaches the protein surface are shown in stick representation (yellow) and labeled. **(b)** Electrostatic surface of the GLB-12 globin domain. The blue and red colors highlight positively and negatively charged surfaces, respectively. The heme moiety, with the propionate groups fully exposed to the solvent region, is shown as stick representation (yellow). **(c)** GLB-12 detail of the heme cavity, showing the presence of two histidines and three additional polar amino acids. **(d)** The GLB-12 K88L mutant is less capable of $O_2^{\cdot-}$production, while R95L and R129L mutants are incapable of $O_2^{\cdot-}$production. **(e)** Stereo view of the GLB-12 heme pocket. The distal His92, the proximal His127, and the heme are shown in stick representation, together with one acetate molecule and six water molecules (cyan sphere). His92, His127 and the heme propionates (A and D) are labelled. The electron density $2F_O$-F_C map (contoured at 1 Å) is shown around the heme and the Fe-coordinated His residues (yellow mesh) and around the solvent molecules (cyan mesh). **(f)** The current potential behavior of a gelatin B electrode with WT GLB-12 and the GLB-12 mutants K88L, R95L and R129L. The reduction (E_{pc}) and oxidation peaks (E_{pa}) are indicated. The three mutations cause a small negative shift in reduction potential, from -0.226V (*vs.* SCE) for WT GLB-12, to -0.231V for GLB-12 K88L, -0.255V for GLB-12 R95L and -0.244V for GLB-12 R129L. **(g)(h)** UV-Vis spectra of WT GLB-12, together with GLB-12 K88L, R95L and R129L mutant forms. The absorption spectra show that these three mutants, like WT GLB-12, are spontaneously oxidized to the ferric state (Fe^{3+}) upon exposure to air and have a hexacoordinated heme iron following reduction to a deoxy form (Fe^{2+}).

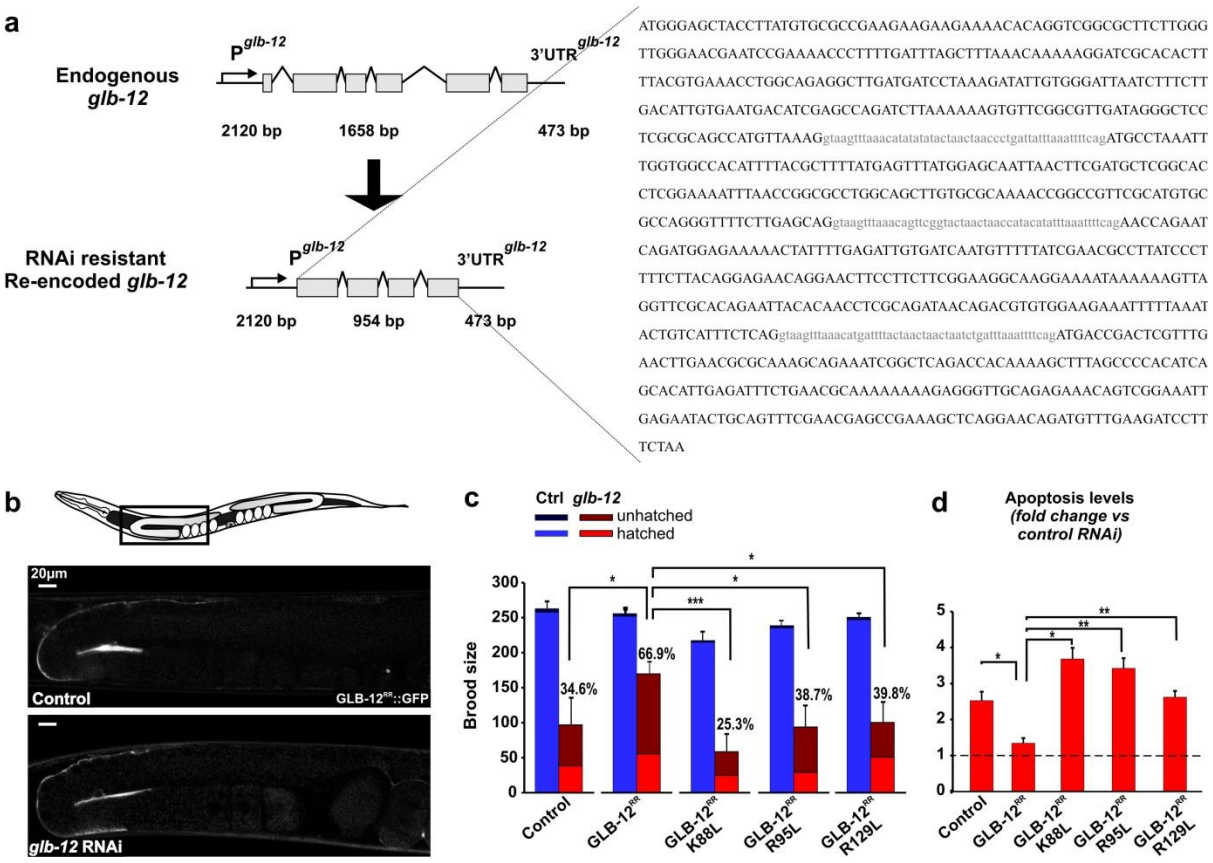


Figure 7. GLB-12 produces $O_2^{\cdot-}$ as a signaling molecule.

(a) The *glb-12* gene was re-encoded to generate the RNAi-resistant *glb-12*^{RR} transgene. The nucleotide sequence of the *glb-12*^{RR} gene is shown. **(b)** *glb-12* RNAi does not deplete the presence of GLB-12^{RR}::GFP in the somatic gonad, while this was the case for the endogenous GLB-12 fused to GFP (see also Fig. 1d). However, the GLB-12^{RR}::GFP construct did not rescue depletion of endogenous GLB-12; therefore, untagged GLB-12^{RR} constructs were used for all functional analyses. **(c)(d)** The GLB-12 mutants K88L, R95L and R129L mutants, when expressed as RNAi Resistant (RR) genes, are not capable of rescuing the *glb-12* RNAi phenotype. ** $p<0.05$; *** $p<0.01$. Data are represented as mean ± SEM.

Further, while the heme pocket in globins usually prevents heme iron oxidation by creating a hydrophobic environment, in GLB-12 three polar amino acids (Lys88, Arg95 and Arg129) are located at the edge of the heme cavity (Fig. 6c), possibly to enhance solvent access to the heme and stimulate heme iron oxidation and $O_2^{\cdot-}$ production. Mutation of these three polar amino acids to the hydrophobic leucine indeed reduced or prevented $O_2^{\cdot-}$ production (Fig. 6d), while other biochemical characteristics remained largely unaffected (Fig. 6f-h). Furthermore, transgene animals bearing these mutations in an RNAi-resistant *glb-12* gene (Fig. 7a, 7b) failed to show rescue of the *glb-12* RNAi phenotypes (Fig. 7c, 7d and Supplementary Fig. 1d). Overall, these

results show that GLB-12 possesses specific structural properties associated with a role in redox biology and further support that GLB-12 produces $O_2^{\cdot-}$ as a signaling molecule.

GLB-12 shows a distinct tissue- and subcellular expression pattern

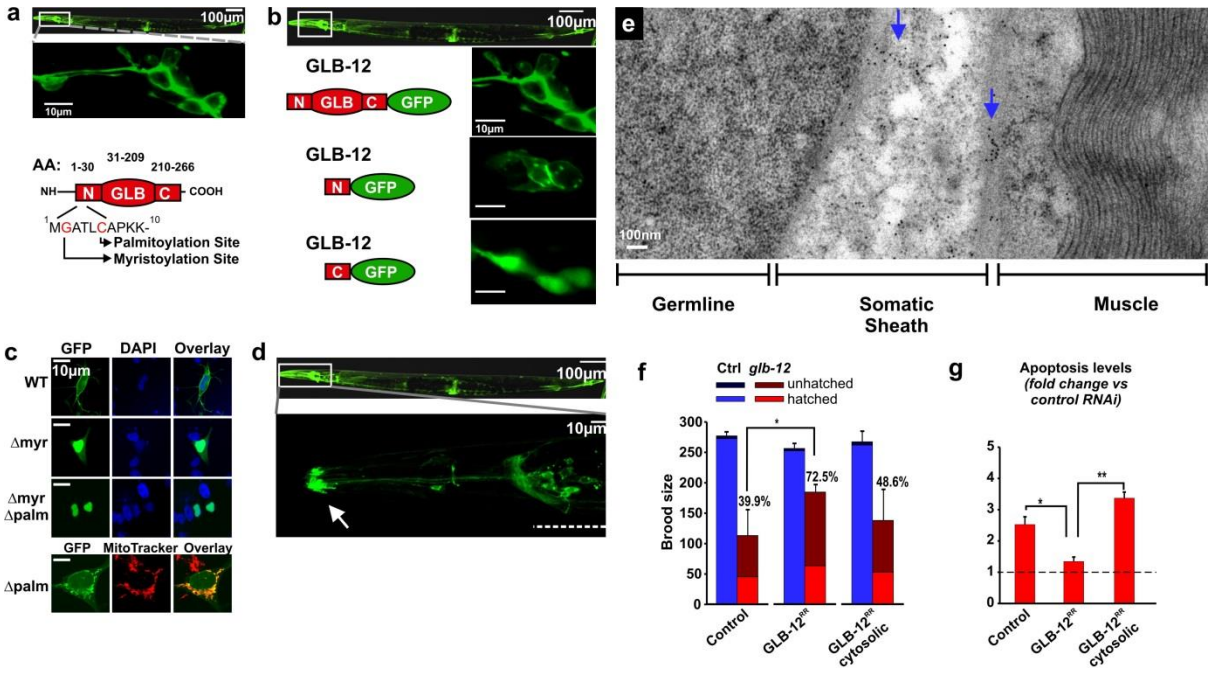


Figure 8. GLB-12 shows a distinct tissue- and subcellular expression pattern.

(a) GLB-12 is membrane-bound, as seen in head neurons. The cartoon shows the presence of a myristoylation and palmitoylation site in the N-terminal region. **(b)** Schematic representation of the reporters for the full-length, N- or C-terminal region of GLB-12, with a representative image of the subcellular expression of these reporters, as seen in the head neurons, showing that GLB-12 is membrane-bound by its N-terminal region. **(c)** Deletion of the myristoylation or palmitoylation site disrupts cell membrane localization, as seen in human neuroblastoma SH-SY5Y cells. **(d)** Subcellular expression pattern of GLB-12::GFP as seen in the head neurons, when the reporter is expressed at lower concentrations. The dotted line indicates the region of the neuronal cell bodies, the arrow points to the most anterior region of these neurons, the neuronal cilia. GLB-12::GFP accumulates at these neuronal cilia. **(e)** Subcellular expression pattern of GLB-12::GFP as seen by immunogold localization. Arrows indicate a clustered distribution. **(f)(g)** A cytosolic RNAi Resistant (RR) GLB-12 is less capable of rescuing the *glb-12* RNAi phenotype. * $p<0.1$; ** $p<0.05$. Data are represented as mean ± SEM.

Because $O_2^{\cdot-}$ is a very short-lived signaling molecule, we reasoned that GLB-12 would show a very specific tissue and subcellular localization to achieve an exact juxtaposition with potential downstream targets. Indeed, besides a very distinct expression pattern in the somatic gonad (Fig.

1b), the GLB-12 reporter was also found to be membrane-bound through a short N-terminal extension (Fig. 8a, 8b). This extension harbors predicted sites for both myristoylation[190] and palmitoylation[191] (Fig. 8a), post-translational modifications that promote stable membrane attachment[192]. Deletion of these sites indeed prevented plasma membrane localization (Fig. 8c). Myristoylation and palmitoylation are specifically associated with protein translocation to membrane rafts, which are dynamic membrane subdomains that compartmentalize cellular processes, including specific redox signaling events[163]. For GLB-12, we indeed observed a clustered distribution, both in the somatic gonad and in the nervous system (Fig. 8d, 8e). Finally, a cytoplasmic version of the RNAi-resistant *glb-12* gene was not able to rescue the increase in germline apoptosis following *glb-12* RNAi, and was less capable of rescuing the decreased fecundity (Fig. 8f, 8g and Supplementary Fig. 1e). Combined, these results support that the localization of GLB-12 serves as a spatial determinant for the $O_2^{\cdot-}$ signal, both on a tissue and intracellular level.

GLB-12 interacts with an intracellular and an extracellular SOD to regulate reproduction

In vivo, $O_2^{\cdot-}$ produced by GLB-12 may be converted into the more stable H_2O_2 by superoxide dismutases (SODs), H_2O_2 being an accepted messenger in redox signaling[134,193]. In this hypothesis, reducing GLB-12 and hence $O_2^{\cdot-}$ levels combined with the absence of the downstream SOD would reduce the strength of the H_2O_2 signal and cause even stronger phenotypic defects compared to when the downstream SOD is still present. To test this hypothesis, we reduced GLB-12 levels in mutants for the five *C. elegans sod* genes (*sod-1* to *sod-5*) and found an aggravated effect on fecundity in the main cytoplasmic *sod-1* mutant and, surprisingly, a reduced effect in the extracellular *sod-4* mutant (Fig. 9a, 9b). The effect in a *sod-1;sod-4* and a *sod-4* mutant are comparable, indicating that SOD-4 is epistatic to SOD-1 (Fig. 9a). Comparable but less pronounced results for these three mutant strains were observed for GLB-12 mediated germline apoptosis (Fig. 9c and Supplementary Fig. 1f). Expression analysis of SOD-1 and both isoforms of SOD-4 showed that they are present in the entire somatic gonad (Fig. 9d and Fig. 10a), thus overlapping in expression with GLB-12. When these reporter constructs were expressed in the corresponding *sod* mutant, the *glb-12* RNAi effect on worm fecundity largely reverted to levels observed in the WT (Fig. 10b). Furthermore, expression of the SOD-1 reporter in the *sod-1* mutant significantly suppressed the increase in germline apoptosis compared to WT worms (Fig. 10c and Supplementary Fig. 1g). The intracellular SOD-1 thus most likely catalyzes the conversion of GLB-12-produced $O_2^{\cdot-}$ to H_2O_2 (Fig. 10g). The source of extracellular $O_2^{\cdot-}$ is less clear; GLB-12-produced $O_2^{\cdot-}$ could potentially penetrate the

membrane through anion channels[141] and be converted by the extracellular SOD-4, or $O_2^{\cdot-}$ could be generated extracellularly by an unknown source. To further test the hypothesis that GLB-12/SOD-1 and SOD-4 work in parallel on both sides of the gonadal sheath cell membrane, we targeted PRDX-2, an H_2O_2 scavenger, to both sides of the membrane (Fig. 10d, 10h); following *glb-12* RNAi, the presence of PRDX-2 should then mimic the loss of SOD. This was indeed observed for the *glb-12* RNAi effect on reproduction and on germline apoptosis, with the strongest effect by the intracellular PRDX-2 (Fig. 10e, 10f and Supplementary Fig. 1h). Taken together, these results show that SOD-1 and SOD-4 modulate the downstream effects of GLB-12 in opposite ways.

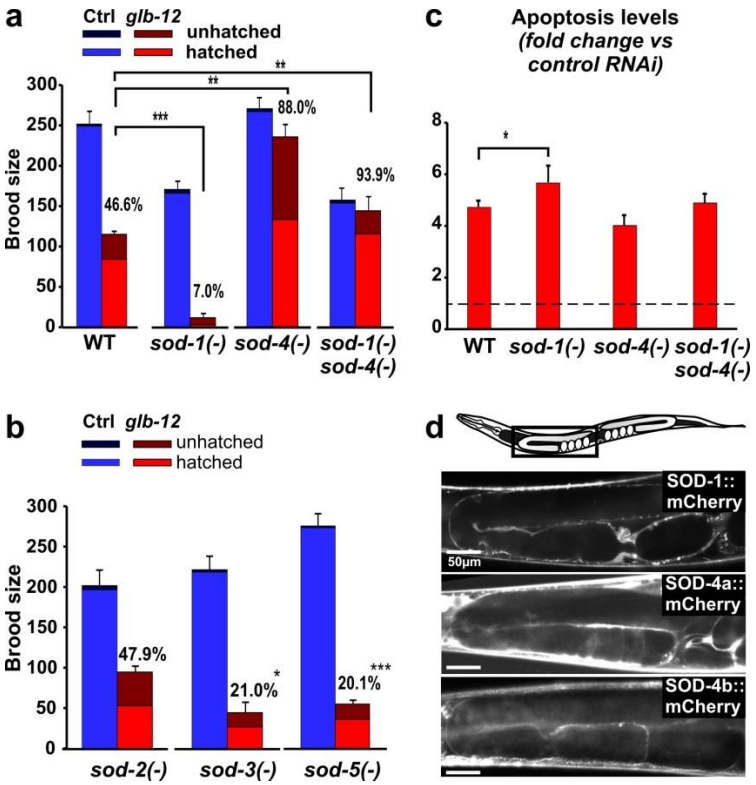


Figure 9. GLB-12 interacts with both an intra- and extracellular superoxide dismutase.
(a)(c) The effects of *glb-12* RNAi were aggravated in a mutant for the main cytoplasmic SOD-1 and reduced in a mutant for the extracellular SOD-4. **(b)** Fecundity following *glb-12* RNAi in loss-of-function mutants for SOD-2, SOD-3 and SOD-5. **(d)** SOD-1 and both isoforms of SOD-4 are present in the somatic gonad.

4. Discussion

In this study, we identified a novel role within the globin superfamily by showing that GLB-12 of *C. elegans* functions as a redox signaling protein in the *C. elegans* reproductive system. Based on our findings, we propose a model whereby GLB-12 acts as a superoxide generator in the somatic gonad after which this $O_2^{\cdot-}$ signal is modulated directly or indirectly by an intracellular and an extracellular SOD, creating a transmembrane H_2O_2 gradient that acts as a redox signal. This

signal then modulates reproduction, including p38/JNK MAPK-dependent germ cell apoptosis (Fig. 11).

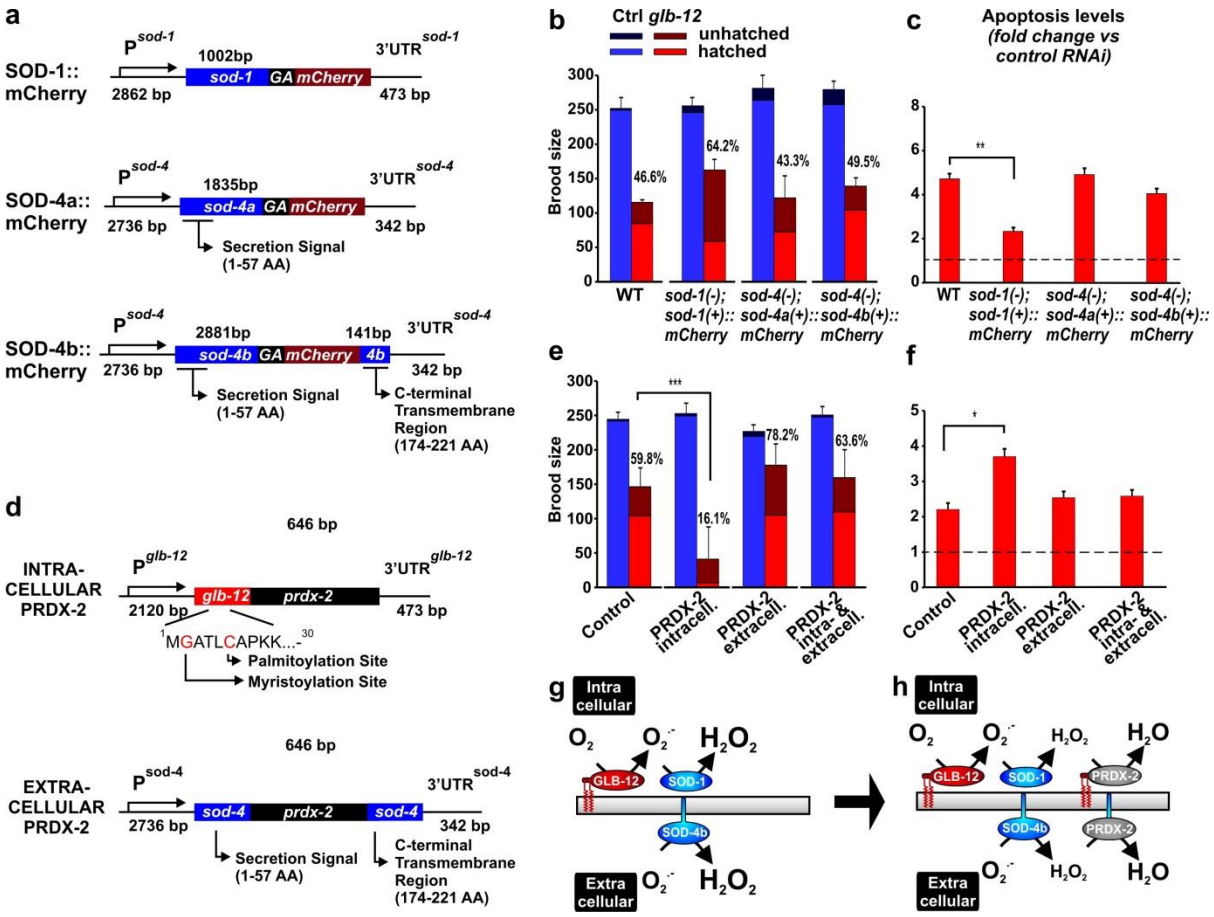


Figure 10. GLB-12, SOD-1 and SOD-4 act together to regulate reproduction.

(a) Schematic representation of the reporter constructs for *sod-1* and the two isoforms of *sod-4*. **(b)(c)** the SOD-1 and SOD-4 reporter constructs, when expressed in the *sod-1* and *sod-4* mutant, respectively, were able to revert the *glb-12* RNAi phenotype to WT levels and, in case of SOD-1, even further decrease germline apoptosis levels. **(d)** Schematic representation of the constructs for *prdx-2* targeted to the intracellular and extracellular side of the plasma membrane. **(e)(f)** Introducing an intracellular and extracellular PRDX-2 mimics the effects seen for *glb-12* RNAi in mutants for SOD-1 and SOD-4, respectively.* $p<0.1$; ** $p<0.05$; *** $p<0.01$. Data are represented as mean ± SEM. **(g)** Cartoon showing the working model for GLB-12 signaling, and **(h)** the anticipated effect of introducing the H_2O_2 scavenger PRDX-2.

The role of GLB-12 in redox chemistry is supported by its biochemical characteristics. We observed that GLB-12 shows a hexacoordinated heme, extremely low ligand affinity, a relatively low redox potential and the ability to transfer electrons to other molecules in a continuous manner. We connected these biochemical characteristics of GLB-12 with a role in redox signaling, by showing that GLB-12 actively converts O_2 to $O_2^{\cdot-}$, after which $O_2^{\cdot-}$ is used as a redox

signaling molecule. Structural and mutational analysis further showed that GLB-12 possesses specific properties that support this role. Heme hexacoordination in globins has previously been associated with increased complexity in ligand binding kinetics and increased redox kinetics for the heme iron[27,35,57], and, as a result, hexacoordinated globins have been hypothesized to function in redox biology[30]. For example, vertebrate neuroglobin has been associated with electron transfer to cytochrome c to protect against apoptosis[194,195], while vertebrate cytoglobin has been functionally linked to lipid oxidation[196]. In *C. elegans*, the hexacoordinated GLB-6 and GLB-26 have been proposed to work as a redox sensor to control behavior[35], and to regulate defecation in response to oxidative stress[132], respectively. In plants, hexacoordinated globins have been linked to maintaining cellular redox status by acting as NO scavengers[197,198]. However, it has thus far been very difficult to link these observations to the biochemical mechanisms used by these globins *in vivo*. Our results on GLB-12 show how a hexacoordinated globin can actively support redox signaling, providing a reference model for the function of globins and globin-like proteins in other organisms.

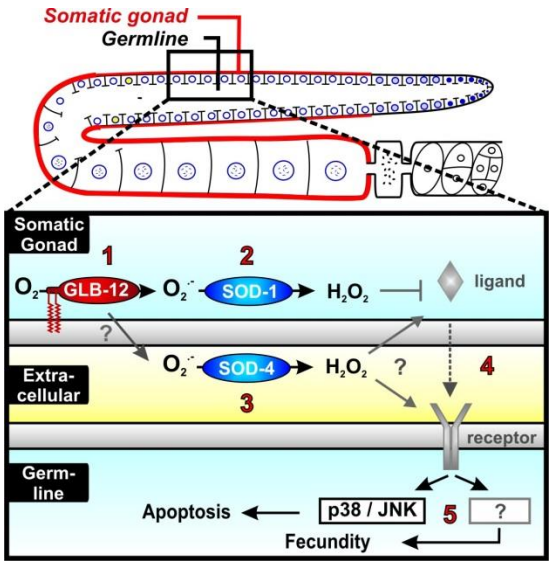


Figure 11. GLB-12 working model.

GLB-12 is expressed in the somatic gonad and is capable of converting O_2 to O_2^{-} (1) after which this signal is modulated by an intracellular SOD (2), and influenced by an extracellular SOD (3). Combined, GLB-12, SOD-1 and SOD-4 influence germline functioning, including p38 and JNK MAPK-mediated apoptosis (4-5).

While it is appreciated that redox signals can originate from endogenous sources, such as membrane-bound NADPH oxidases or mitochondria, the control mechanisms on ROS as endogenous signaling molecules are poorly understood. One way whereby specificity could be achieved is by compartmentalization of the ROS signal: by the tightly controlled, tissue- and subcellular-specific expression of the proteins producing these messengers, exact colocalization of ROS with the downstream target can be realized[133]. This specificity in expression is indeed observed for GLB-12: (1) on a tissue level, GLB-12 is only present in a specific part of the somatic gonad, whereby it acts from this tissue to regulate germline reproduction; (2) on a

subcellular level, GLB-12 is membrane-bound by myristoylation and palmitoylation, whereby membrane localization increases the effectiveness of the GLB-12 signal. In addition, these types of protein acylation will localize proteins to dynamic membrane subdomains that have been associated with specific redox signaling events[163]. Given that GLB-12 produces $O_2^{\cdot-}$ and that $O_2^{\cdot-}$ is diffusible and short lived, we therefore reason that the location of GLB-12 serves as a spatial determinant for downstream signals, both on a tissue and intracellular level.

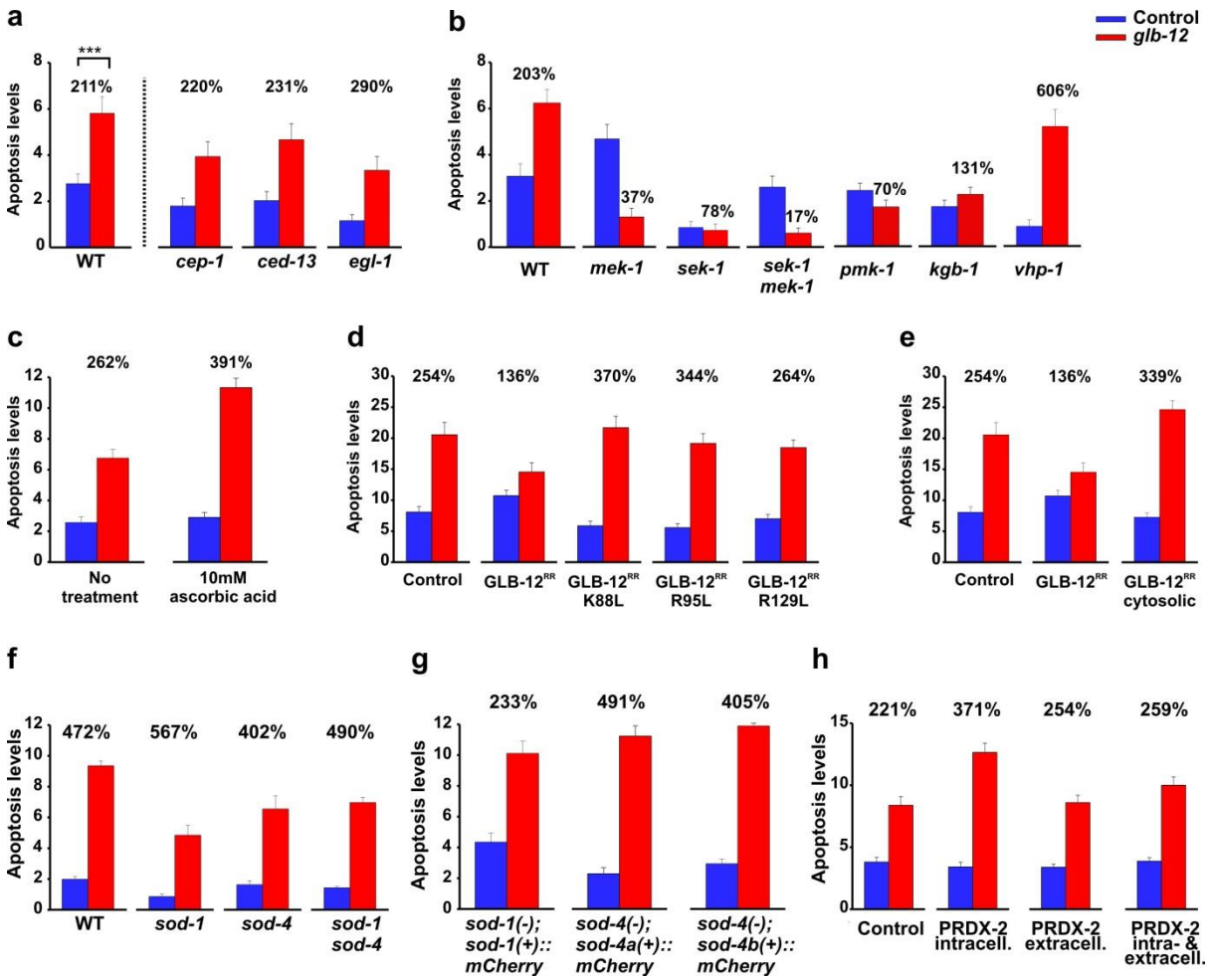
On top of localizing the enzymatic source of a redox signal with its potential downstream target, an additional layer of regulation can be achieved by alterations in the local redox buffer capacity[133]. For example, the presence of SODs enhances the spontaneous dismutation of $O_2^{\cdot-}$ into O_2 and H_2O_2, which could thus influence the efficiency of a H_2O_2 redox signal. We observed that GLB-12 acts together with the main cytoplasmic SOD-1, whereby this interaction indeed increases the effectiveness of the redox signal. Fascinatingly, also the extracellular SOD-4 influences the GLB-12 signal and this in an opposite manner compared to SOD-1. These results clearly show that SODs have essential roles in the regulation of redox signaling and are not merely antioxidants. We hypothesize that H_2O_2 produced by GLB-12/SOD-1 influences ligand release from the somatic gonad, while H_2O_2 produced by SOD-4 could influence ligand release or affect receptor activity on the germline plasma membrane (Fig. 11). These observations are in line with the increasing amount of evidence that the intra- and extracellular redox states work in concert to influence intracellular signaling[152,199]. Furthermore, the interaction between GLB-12 and these two SODs adds a surprisingly third level of regulation in this redox signal, whereby the amount of $O_2^{\cdot-}$ and H_2O_2 on the intra- and extracellular side of the plasma membrane are important determinants of the downstream signal. It is interesting to note that, while plasma membranes form a physical barrier for ROS, ROS transport across membranes could potentially be regulated by the presence of selective membrane channels. 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[141] and aquaporins[133,176,177]. This active ROS transport across membranes has therefore been hypothesized to participate in the regulation of redox signaling and could potentially also be involved in GLB-12 mediated signaling. Overall, our results show that redox signaling by GLB-12 is regulated by multiple control layers. Interesting to note is that NADPH oxidase, another currently known enzymatic generator of ROS signals, also displays a very specific subcellular location that is related to its function, and also appears to interact with SOD enzymes[160,163]. These shared characteristics between GLB-12 and NADPH oxidases indicate that redox signaling proteins function according to similar principles to achieve physiological redox signaling. Furthermore, the presence of a

transmembrane redox gradient in GLB-12 mediated signaling presents a fascinating additional principle in how redox signaling can be further modulated.

We were able to link this redox signaling function of GLB-12 with an essential role in *C. elegans* reproduction. When GLB-12 levels were reduced, a decreased fecundity, smaller gonads, increased germline apoptosis levels and several defects during oocyte development were observed. We further showed that GLB-12 has antiapoptotic effects by inhibiting the p38 and JNK MAPK pathways. The highly conserved p38 and JNK MAPK pathways downstream of GLB-12 have been shown to be sensitive to environmental stress, including oxidative stress, in several organisms[200,201]. Also in *C. elegans*, the combined role of these pathways in modulating germ cell apoptosis in response to stressors such as oxidative and heavy metal toxicity has been demonstrated[181]. The results presented here provide a link between a redox signaling protein and p38 and JNK MAPK-mediated germline apoptosis. Because our results show that GLB-12 acts specifically from the somatic gonad and not from the germline, GLB-12 likely influences the release of ligands and/or the activation of receptors of the p38 and JNK MAPK pathways, thereby regulating their activity level. In other organisms, it has been observed that ROS can influence receptor activation upstream of the p38 and JNK MAPK pathways[200]. When these ligands and receptors of the p38 and JNK MAPK pathways in the *C. elegans'* germline will have been identified, our results presented here indicate that they could form an interesting model to study how GLB-12 mediated redox signaling can directly influence the activity of downstream signaling cascades.

In conclusion, we have identified a globin that acts as a source of redox signaling. The specific biochemical characteristics of GLB-12 show how a globin can fulfill this function, while the precise subcellular localization and the interaction with the SOD antioxidant enzymes increase the effectiveness of the redox signal. The opposite effect of the intra- and extracellular SOD on GLB-12 signaling is particularly intriguing; it indicates the importance of transmembrane redox gradients and further deepens the concepts of redox signaling compartmentalization. Finally, given the widespread occurrence of globins, the majority with unknown function, our results add an important member to the group of proteins that drive redox signaling.

5. Supplementary Figures



Supplementary Figure 1. Absolute germline apoptosis levels

(a) Absolute germline apoptosis levels following control and *glb-12* RNAi in the WT and in mutants for the apoptosis pathway. These results are associated with Figure 3b. **(b)** Absolute germline apoptosis levels following control and *glb-12* RNAi in the WT and in mutants for the p38/JNK MAPK pathways. These results are associated with Figure 3f. **(c)** Absolute germline apoptosis levels following control and *glb-12* RNAi in WT worms that were exposed to ascorbic acid. These results are associated with Figure 5d. **(d)** Absolute germline apoptosis levels following control and *glb-12* RNAi in transgenic worms carrying the GLB-12^{RR} constructs. These results are associated with Figure 7d. **(e)** Absolute germline apoptosis levels following control and *glb-12* RNAi in transgenic worms carrying the GLB-12^{RR} constructs. These results are associated with Figure 8g. **(f)** Absolute germline apoptosis levels following control and *glb-12* RNAi in mutants for SOD-1 and SOD-4. These results are associated with Figure 9c. **(g)** Absolute germline apoptosis levels following control and *glb-12* RNAi in mutants for SOD-1 and SOD-4 that carry the SOD-1 and SOD-4 reporter constructs, respectively. These results are associated with Figure 10c. **(h)** Absolute germline apoptosis levels following control and *glb-12* RNAi in transgenic worms carrying the PRDX-2 constructs. These results are associated with Figure 10f. All data are represented as mean ± SEM.

6. Materials and Methods

Nematode strains

Worm strains used in this study were Bristol N2 wild type, NL2099 [*rrf-3 (pk1426)* II], TU3311 [uIs60], NL3511 [*ppw-1(pk1425*) I], RB798 [*rrf-1(ok589)* I], OD95 [*unc-119(ed3)* III*; ltIs37* IV; *ltIs38*], MT1522 [*ced-3(n717)* IV], FX536 [*ced-13(tm536)* X], TJ1 [*cep-1(gk138)* I], *MT8735* [*egl-1(n1084n3082)* V], FK171 [*mek-1(ks54)* X], KU4 [*sek-1(km4)* X], *mek-1(ks54) sek-1(km4)*, JT366 [*vhp-1(sa366)* II], KB3 [*kgb-1(um3)* IV], KU25 [*pmk-1(km25)* IV], GA187 [*sod-1(tm776)* II], GA184 [*sod-2(tgk257)* II], GA186 [*sod-3(tm760)* X], GA416 [*sod-4(gk101)* III], GA502 [*sod-5(tm1146)* II] and HT1593 [*unc-119(ed3)* III], which were provided by the *Caenorhabditis* Genetics Center funded by the National Institutes of Health National Center for Research Resources. Maintenance of *C. elegans* was carried out according to standard procedures. *C. elegans* strains were cultured at 20°C on cholesterol-supplemented nutrient agar (OXOID) plates containing a lawn of freshly grown *Escherichia coli* K12 cells. To obtain synchronized cultures, gravid adults were lysed by hypochlorite treatment and eggs were allowed to hatch overnight in S buffer.

Molecular Biology

Translational reporters and genetic constructs were made using fusion PCR, as described by Hobert [202]. The translational reporters contain the target gene, and approximately 3 kb upstream and 0.5 kb downstream of the target gene, to include endogenous promoter and 3'UTR elements. The *gfp* or *mCherry* gene was fused at the 3' side of the target gene. A PCR product coding for the *unc-119* gene was used as a co-injection marker. Final PCR products were injected into the gonads of young adult hermaphrodites, at a concentration of 50 ng/µl for the translational reporters and genetic constructs and 20 ng/µl for the *unc-119* gene. Transformed reporter lines were analyzed using a Nikon Eclipse TE2000-5 confocal microscope.

An RNAi-resistent *glb-12* gene (*glb-12*^{RR}) was generated by following the guidelines described in Green *et al.*[203]. In short, the nucleotide sequence was recoded by shuffling alternative codons encoding the same amino acid, without changing the original amino acid sequence or the overall codon bias, until there were no long (>9bp) stretches of homology between the original and recoded *glb-12* gene. The five *glb-12* introns were replaced by 3 synthetic introns. The *glb-12*^{RR} gene was fused to the same up- and downstream regions used for the *glb-12* translational reporter. Because we observed that the *glb-12* introns were essential for somatic gonad expression, these introns were co-injected as short PCR fragments. The *glb-12*^{RR} gene was injected at 25 ng/µl, 20 ng/µl for the *unc-119* gene, and introns at 2-6 ng/µl.

For localization in human neuroblastoma SH-SY5Y cells, cDNAs of wt *glb-12*, *glb-12*Δmyr, *glb-12*Δpalm, and *glb-12*ΔmyrΔpalm were cloned into the pEGFP-N1 vector (Clontech) using BglII and HindIII restriction enzymes (Biolabs, Westburg). Primers used are described in Table S2. Ligation of cDNAs in the vectors was performed using T4 DNA ligase (Novagen). The myristoylation site, glycine at position 2, was modified to alanine and is annotated as GLB-12Δmyr. The palmitoylation site, cysteine at position 6, was mutated to alinine and is annotated as GLB-12Δpalm. The mutant where both fatty acylation sites are mutated to alanine is annotated as GLB-12ΔmyrΔpalm. All mutations were done with the Quickchange™ site directed mutagenesis kit (Stratagene).

Human neuroblastoma SH-SY5Y cells (ATCC® CRL-2266™) were cultured as recommended by the manufacturer's protocol. Cells were transfected with 3 µl of lipofectamine™ 2000 and 0.5 µg of pEGFP-N1 plasmide. After 4 hours, transfection medium was replaced by growth medium and SH-SY5Y-cells were allowed to express the GFP-tagged proteins for 24 hours. For GLB-12Δpalm, colocalization was performed with the MitoTracker® Deep Red probe (Invitrogen) according to the manufacturer's protocol. Localization was examined with an UltraVIEW Vox ERS microscope (PerkinElmer), and images were created with the Volocity 6.0.1 software.

Feeding RNAi

RNAi was applied by feeding bacteria expressing dsRNA to the worms. RNAi induction was performed as described by Timmons *et al*[204]. Synchronized L1 worms were placed on NGM plates seeded with freshly induced RNAi bacteria.

Fecundity assay

Two days after synchronized L1 worms were placed on plates containing RNAi bacteria, individual L4 hermaphrodite were transferred to NGM plates with a 25µl spot of 4 times concentrated, induced RNAi bacteria and maintained at 20°C, except for the temperature-sensitive strain *rrf-3 (pk1426)*, which was kept at 17°C. Animals were transferred to fresh plates each day until they stopped laying eggs. 24 hours after the adult was transferred, hatched larvae and unhatched eggs on each plate were counted. The fecundity of each worm was calculated as the total number of hatched and unhatched eggs produced. At least 18 worms spread over at least 3 independent replicas were analyzed for each strain.

Germline apoptosis assay

Apoptotic germ cells were visualized by acridin orange (AO) staining. 200µl AO (1mg/ml) was added to a 9cm diameter NGM plate with 0 day old adult animals for overnight incubation. The following day, worms were rinsed from the plate, washed, mounted under coverslips in 20 µl of a 12.5 mM sodium azide solution, and immediately analyzed. Apoptotic cells observed in the late pachytene region of the germline were scored. At least 10 worms per biological replicate were scored, one gonadal arm per animal was used, and at least 3 biological replicates were performed. Apoptotic germ cells in engulfment-defective mutants were quantified by contrast microscopy. Analysis of removal speed of apoptotic corpse was carried out by time-lapse contrast microscopy. 1 day old adult animals were anaesthetized with 10mM levamisole for 10 min, after which they were mounted on agar pads for analysisis. Through-focus z-stack images were taken every 4 min, for a total of 75 min.

Ascorbic acid treatment

Ascorbic acid was added into NGM media from a freshly made 100mM stock solution the day before the plates were used. Worms were placed on plates with ascorbic acid starting from the fourth larval stage.

Western blotting

Western blotting was performed according to standard procedures. Synchronyzed populations were grown on NGM with induced RNAi, collected as 1 day old adults, and flash frozen in 100µl volumes. After worm homogenization, protein content was determined using the bicinchoninic acid method. Equal amounts of total protein content were loaded on a 12.5% SDS-PAGE gel. The primary rabbit polyclonal antibodies anti-PMK-1, and anti-phospho-p38 MAPK monoclonal antibody 28B10 (Cell Signaling) were used. The specificity of anti-PMK-1 and anti-phospho-p38 MAPK was determined by using the PMK-1 mutant *KU25 [pmk-1(km25) IV]*.

Superoxide measurement

To test the capacity of heme proteins to produce superoxide *in vitro*, we applied the method of Hayashi *et al.*[185] to create a reducing environment for heme proteins, and included lucigenin that produces light upon reaction with superoxide, to quantify superoxide production. In brief, the components of the reduction system are an NADPH-generating system, consisting of glucose-6-phosphate, glucose-6-phosphate dehydrogenase and $NADP^+$, and an electron-mediating system, consisting of ferredoxin and ferredoxin-$NADP^+$ reductase. We omitted catalase from the original

system, as this might lead to quick removal of the produced superoxide. Concentrations of the different components, including the different heme proteins tested here, were as described in the original paper. Heme proteins tested were myoglobin from horse heart (Sigma M1882), cytochrome P450 2B4 from rabbit liver (Sigma C7552) and cytochrome C from horse heart (Sigma C2506). Lucigenin was added to a final concentration of 100μM. Measurements were performed in a 200μl working volume in a 96 well microtiter plate. Glucose-6-phosphate was added last to start the reduction. Immediately following this, light emission was recorded in a Victor2 1420 Multilabel Counter (Perkin–Elmer, Wellesley, MA).

Recombinant expression of GLB-12

C. elegans worms were grown, total RNA was isolated, and cDNA was prepared as described previously [34]. Primers used are described in Table S2. cDNA was cloned into the pET23a-vector (Novagen) using NdeI and XhoI restriction enzymes (Biolabs).

UV/Vis

UV-Vis spectra were measured in a 250-700 nm range on a Cary-5 UV-Vis-NIR spectrophotometer (Varian). Ferrous CO-bound and reduced deoxy protein samples were prepared by flushing 1 ml of 100 mM potassium phosphate buffer pH 7.0 for 15 minutes with CO- and N_2-gas respectively in a sealed cuvette. After addition of 10 μl of a satured solution of sodium dithionite, highly concentrated, recombinant purified protein was added to obtain a final concentration of 50 μM. Ferric CN-ligated GLB-12 was prepared by adding an excess of 40 moles of KCN.

Cyclic Voltammetry

Cyclic Voltammetry was performed as described earlier [183]. The gelatin B solution was prepared by mixing the gelatin B powder and the HEPES buffer solution at approximately 40 °C. These electrodes are referred to in the text as GelB. If a GLB and/or CCP solution is incorporated in the gelatin matrix, the final electrodes are denoted as GLB|CCP|GelB (1.5:1.5:7 ratio), GLB|GelB (3:7 ratio) or CCP|GelB (3:7 ratio). The concentration of GLB, CCP and gelatin is 0.05 mmol.L^{-1}, 0.05 mmol.L^{-1}, 5 m% respectively unless stated otherwise.

Crystallization and structure determination.

Crystals of GLB-12 complex were grown at 4 °C by the hanging-drop vapour diffusion method, by mixing 1μl protein solution with 1μl crystallization well solution containing 2 M $(NH_4)_2SO4$,

0.2 M Na-acetate, 0.1 M Na-acetate pH 4.6. Crystals usually grew in about two months, and were cryo-protected with the same crystallization well solution supplemented with 30% glycerol, prior to cryo-cooling in liquid nitrogen. The crystals belong to the space group $P6_522$, with unit cell parameters a = 50.4 Å, b = 50.4 Å, c = 245.3 Å, γ = 120°, and one GLB12 molecule per asymmetric unit. Diffraction data were collected to 1.65 Å resolution using synchrotron radiation (BM14 beamline, ESRF, Grenoble, France). Raw data were processed with Mosflm and Scala (Supplemental Table 1).

The structure was solved by single-wavelength anomalous dispersion (SAD) method, based on the heme Fe atom anomalous signal. The heme Fe atom position and the initial phases were calculated by using the AutoSol pipeline implemented in PHENIX. The GLB-12 model was built with the program COOT and restrained refined to the maximum resolution using REFMAC. At the end of the refinement stages (including anisotropic B-factor refinement), 110 water molecules, 1 sulfate ion, and 4 acetate molecules were located through inspection of difference Fourier maps. The final Rfactor value was 17.8 %, and Rfree 23.4 %. No electron density was detected for residues 1-18, 167-181, and 216-266. The programs Procheck and Surfnet were used to assess the stereochemical quality of the protein structures and to explore the protein matrix cavities.

TEM - High-pressure freezing and freeze-substitution

To analyze the subcellular localization of GLB-12, the GLB-12 translational reporter in combination with anti-GFP antibodies was used. N2 WT worms were included as negative control.

Copper membrane carriers (1,5 mm x 0,2 mm) (Leica, Microsystems, Vienna, Austria) treated with 1% lecithin were used and filled with 20% (w/v) Bovine Serum Albumin (BSA). Three to four one-day-old adult nematodes were transferred to the membrane carrier, immersed in the BSA and then immediately frozen in a high-pressure freezer (EM PACT, Leica, Microsystems).

Freeze substitution was carried out using a Leica EM AFS (Leica, Microsystems). Carriers were transferred from EM pact to AFS under liquid nitrogen and placed in an eppendorf filled with dry acetone. Over a period of five days, animals were freeze-substituted as follows: -90°C for 27 hours, 2°C per hour increase for 15 hours, -60°C for 12 hours, 2° C per hour increase for 15 hours, -30°C for 32 hours, 2°C per hour increase for 17 hours. At 4°C carriers were rinsed 3 times with dry acetone for 20 min each time.

TEM - Embedding and sectioning

Samples were infiltrated stepwise in LR-White, hard grade (London, Resin, Basingstoke, UK) and embedded in closed capsules. Polymerisation was performed by UV illumination of the AFS for 24 hours at 0°C, 2°C per hour increase for 10 hours followed by 24 hours at 20°C, 2°C per hour increase for 8 hours followed by 72 hours at 37°C. Ultrathin (70 nm) sections were cut using a Leica Ultracut S ultramicrotome (Leica, Vienna, Austria) with a diamond knife (Diatome, Ltd., Biel, Switzerland) and collected on formvar-coated copper single slot grids (Agar Scientific, Stansed, UK).

TEM - Immunogold labeling

All steps of the immunolabeling were performed in a humid chamber at room temperature. Grids were floated upside down on 25µl of aliquots of blocking solution (5% BSA, 1% Fish skin gelatin in Phosphate buffered saline (PBS)) for 30 min followed by a wash step for 5 times 5 min in incubation buffer (IB: 1% BSA in PBS). Incubation of primary antibodies for 120 min, Goat anti-GFP-biotin antibody, 1:300 (Rockland 600-106-215) followed by washing 5 times 5 min in IB. The grids were then incubated with unconjugated bridging antibodies, Rabbit anti biotin, 1:10000 (Rockland 100-4198) for 30 min. After washing 5 times 5 min in IB, the grids were incubated in Protein A gold (PAG) (10 nm, Cell Biology, Utrecht University) and washed twice, 5 min each, with IB; 3 times 5 min with PBS; and 5 times 2 min with double distilled water. Control experiments consisted of treating sections with bridging antibodies and/or PAG 10nm alone.

After post-staining in a Leica EM AC20 (Leica, Microsystems) for 30 min in uranyl acetate at 20°C and for 7 min in lead citrate at 20°C sections were examined with a JEOL JEM 1010 (Jeol, Ltd, Tokyo, Japan) transmission electron microscope operating at 60kV. Pictures were digitized using a Ditabis system (Pforzheim, Germany).

Phylogenetic analysis

Sequences of *C. elegans* globins and their orthologs were obtained from Wormbase, vertebrate globin sequences were obtained from GenBank. All encoded globin sequences were aligned manually as described previously [31]. Bayesian inference trees were obtained employing MrBayes version 3.1.2 [205]; to generate the phylogenetic tree for all GLB-12 orthologs, four chains were run simultaneously for 5×10^6 generations, trees were sampled every 1000 generations, the first 2000 trees were discarded, generating a total of 3000 trees. To generate the phylogenetic tree for six *C. elegans* globins and their orthologs, four chains were run simultaneously for 2×10^6 generations,

trees were sampled every 1000 generations, the first 1000 trees were discarded, generating a total of 1000 trees. The final average standard deviations of split frequencies for both trees were stationary and in the range of about 0.003 and 0.01, respectively. Posterior probabilities were estimated on these final trees. The appropriate model of amino acid sequence evolution for both trees was selected by ProtTest 2.4 [206] using the Akaike Information Criterion (AIC), and were the JTT[207] and Vt[208] model, respectively.

Statistical Analysis

Statistical comparisons of fecundity and germline apoptosis levels were performed using a two-sided *t*-test, assuming unequal variance.

7. Acknowledgments

Atomic coordinates and structure factors determined from the GLB-12 crystal have been deposited in the Protein Data Bank, with entry code 4bja. We thank Dr. K. Matsumoto and Dr. T. Mizuno for kindly providing the *mek-1(ks54) sek-1(km4)* double mutant, the antibody anti-PMK-1 and technical advice on antibody use; Dr. D. Kim for kindly providing the pDK177 RNAi strain; Dr. M. Ubbink and Dr. Q. Bashir for providing CCP; Dr. K. Oegema for sharing technical expertise; and M. Couvreur for assistance in generating transgenic lines. Some strains were provided by the CGC, which is funded by the NIH Office of Research Infrastructure Programs (P40 OD010440). SDH is a Ph.D. fellow of the Fund for Scientific Research (FWO). Financial support to SD and LM was provided by the University of Antwerp (BOF UA TOP 2006) and to SD, LM, BPB, by FWO project G.0247.09.

Chapter III:

An N-Myristoylated Globin with a Redox-Sensing Function That Regulates the Defecation Cycle in *Caenorhabditis elegans*

Personal Contribution: Generation of transgenic line
Microscopy
Oxidative stress assays
Writing the manuscript (10%)

Adapted following publication as:

An N-Myristoylated Globin with a Redox-Sensing Function That Regulates the Defecation Cycle in *Caenorhabditis elegans*

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1. Abstract

Globins occur in all kingdoms of life where they fulfill a wide variety of functions. In the past they used to be primarily characterized as O_2 transport/storage proteins, but since the discovery of new members of the globin family like neuroglobin and cytoglobin, more diverse and complex functions have been assigned to this heterogeneous family. Here we propose a function for a membrane-bound globin of *C. elegans*, GLB-26. This globin was predicted to be myristoylated at its N-terminus, a post-translational modification only recently described in the globin family. *In vivo*, this globin is found in the membrane of the head mesodermal cell and in the tail stomato-intestinal and anal depressor muscle cells. Since GLB-26 is almost directly oxidized when exposed to O_2, we postulate a possible function as electron transfer protein. Phenotypical studies show that GLB-26 takes part in regulating the length of the defecation cycle in *C. elegans* under oxidative stress conditions.

2. Introduction

Myristoylation is a modification by which a 14-carbon fatty acid myristate is covalently attached to the N-terminal glycine residue of proteins[209-211]. The modification is catalyzed by the enzyme N-myristoyltransferase, and occurs most commonly on glycine residues exposed during co-translational N-terminal methionine removal[212]. Myristoylation also occurs post-translationally, for example when internal glycine residues become exposed by caspase cleavage during apoptosis[213-218]. N-myristoylation promotes weak and reversible protein-membrane and protein-protein interactions[219,220], and is important for the *in vivo* localization and the role that proteins can play in e.g. signal transduction, oncogenesis and viral replication. A wide range of proteins is known to be myristoylated at the N-terminus, like the catalytic subunit of PKA cAMP-dependent protein kinase[221], calcineurin B[222], and the non-receptor tyrosine kinase c-Src[223]. Recently, acylation sites were also discovered in other classes of proteins, e.g. the globin family.

Globins occur in all kingdoms of life where they participate in a wide variety of processes, like O_2 sensing[224], NO detoxification[225,226], and behavior[35,36]. While globin association with the cell membrane (like *Vitreoscilla* hemoglobin) has been identified in some bacteria[227], only a few globins were known to be anchored in it through covalent attachment of fatty acids. This view changed in 2010 when the hemoglobin of the green shore crab *Carcinus maenas* was reported to possess a myristoylation site at its N-terminus, localizing it in the membrane of the gill's chief cells, where it could play a role in the protection of membrane lipids from ROS[228]. Globin X,

found in fish and amphibians, and some of the globins of the acorn worm *Saccoglossus kowalevskii*, possess a myristoylation and a palmitoylation site that are both required for correct targeting and membrane localization[21,229].

In our search for myristoylation sites in the group of 33 globins of the nematode *Caenorhabditis elegans* (*C. elegans*)[16,31,32,230], we found that GLB-26, already partially characterized by our group[27,34], was predicted to be myristoylated at its N-terminus. Recombinant GLB-26 is a globin that exhibits hexacoordination of the heme iron and was purified in the ferric low-spin state. A stable oxygenated species could not be produced since the heme iron atom, when exposed to O_2, was almost directly oxidized, thereby reducing the bound diatomic ligand[34]. Therefore, a function as O_2 transporter was excluded and other possible roles were suggested. Given the fast oxidation of the heme iron atom, a role in redox reactions seemed most plausible. A model of the 3D structure of GLB-26 was constructed, supporting this idea[230]. Several possible reaction mechanisms were suggested. In the case that O_2 is reduced, the superoxide ion O_2^- can participate in redox reactions or can dismutate into O_2 and H_2O_2. The latter can then act as a signaling molecule *in vivo*[231-233]. In this case, GLB-26 could serve a role as oxidase. Localization studies where *glb-26* promotor::*gfp* fusion constructs were generated and injected into the gonads of young adult hermaphrodites[31], localized GLB-26 in the head mesodermal cell and the tail stomato-intestinal muscle cell. Since these are the sites where food intake and defecation take place, a role of GLB-26 in the defecation cycle of *C. elegans* can be emphasised. Defecation in *C. elegans* is a three-part motor program (DMP) that recurs every 45.3 (±4.3) seconds in well-fed wild type worms[234-236]. Three sets of muscles are activated sequentially, triggered by an endogenous ultradian clock[235,237]. First the posterior body-wall muscles near the tail contract simultaneously, pressurizing and pushing gut contents through it (pBoc). These muscles then relax and about 2 seconds later anterior body-wall muscles simultaneously contract, forcing the pharynx back against the intestinal lumen (aBoc). Immediately following the anterior contraction, muscles that open the anus contract to release the pressurized gut contents, which is called the expulsion (exp)[236]. The study of motor program mutants and motor neuron mutants indicated that the execution of the defecation motor program, i.e. activation of the defecation muscles, activation of the motor neurons, completion of the motor program, and release of gut pressure, are not necessary for generation of the normal DMP rhythm[235]. Instead, the defecation cycle is controlled by an endogenous clock which continues to run in the absence of DMP execution. This is further illustrated by the finding that animals that stop the defecation cycle in the absence of food resume defecating in phase with their previous rhythm upon returning to food. Later research showed that calcium oscillations in the intestine, which occur about every 50 s, act as

the endogenous clock and trigger rhythmic contractions of the defecation muscles through downstream GABAergic neurons that innervate these muscles[238-240]. In addition to this endogenous clock, specific proteins collaborate to regulate the defecation cycle in a positive or negative manner. These include proteins encoded by class 1 *flr*-genes, like FLR-1, an ion channel of the epithelial sodium channel/degenerin superfamily, and FLR-4, a protein kinase with a hydrophobic domain at the carboxyl terminus[237].

We proved that myristoylation plays a crucial part in targeting GLB-26 to the cell membrane. We also show that GLB-26 is present in the head mesodermal cell, the tail stomato-intestinal muscle cell and the anal depressor muscle cell. We further suggest that this globin is involved in the defecation cycle of *C. elegans*, and that it serves a role in regulating the length of this rhythmic behavior under oxidative stress conditions.

3. Results

In silico search for N-myristoylation and subcellular localization of GLB-26

Using the Expasy Myristoylator tool[190], GLB-26 was predicted to be myristoylated on the glycine residue at position 2 with a probability of 99% (Figure 1).

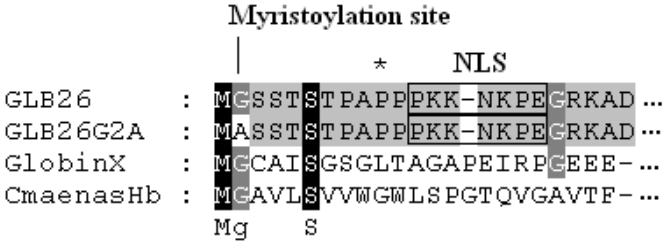

Figure 1. Alignment of the myristoylated globins GLB-26, GLB-26G2A, Globin X, and *Cmaenas*Hb. The myristoylation sites of all globins and the predicted NLS of GLB-26 are indicated.

Myristoylation is assumed to target GLB-26 to the membrane of a subcellular compartment. To predict which compartment, we launched its primary sequence in different software programs. With the WoLF PSORT software[241] the classical nuclear localization signal PKKNKPE was found starting at position 12, and a probability of 59% that GLB-26 is transported to the nucleus *in vivo* was predicted. This was confirmed by other prediction programs. The Subnuclear Compartments Prediction System was run to predict in which part of the nucleus GLB-26 is localized, yielding the presence of GLB-26 in the nuclear lamina[242,243].

In vitro subcellular localization of GLB-26*

Expression of the *pglb-26* - egfp* - N1 plasmid in neuroblastoma cells (SH-SY5Y) showed that GLB-26* is predominantly localized in the lamina of the nucleus, as predicted by the software

programs used (Figure 2A). Occasionally GLB-26* was also observed in the cellular membrane (data not shown), although expression in the nuclear lamina was more prevalent. Deletion of the myristoylation site in the *glb-26** gene by site directed mutagenesis resulted in the expression of GLB-26*G2A in the nucleoplasm of the cells (Figure 2B), proving that GLB-26* is transported into the nucleus and that myristoylation of the N-terminal glycine residue is responsible for the insertion of the wild-type protein into the nuclear membrane.

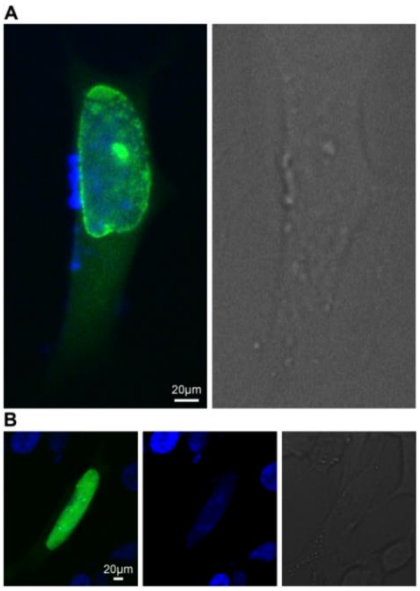

Figure 2. *In vitro* localization of GLB-26. Neuroblastoma SH-SY5Y cells were transfected with A - 0.5 µg of the p*glb-26**- *egfp*-N1 plasmid, and B – 0.5 µg of the p*glb-26**G2A- *egfp*-N1 plasmid. GLB-26* was expressed in the nuclear lamina, GLB-26*G2A was localized inside the nucleus (green fluorescence). The nucleus was visualized with DAPI staining (blue fluorescence), and presented in a separate image for clarity.

In vivo localization of GLB-26

A translational reporter for GLB-26 was created to determine the gene product expression pattern in *C. elegans*. This reporter includes the endogenous promoter, introns and 3'UTR, and has GFP fused to the C-terminus of GLB-26.

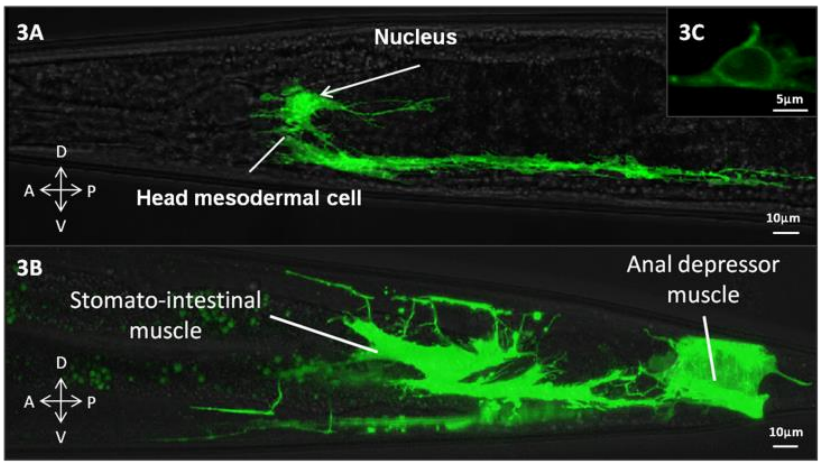

Figure 3. *In vivo* expression pattern of the GLB-26 translational reporter. A) In the head region expression is seen in the head mesodormal cell, with apparent enrichment in the nucleus, B) In the tail region expression is present in the stomato-intestinal and anal depressor muscle cells, C) Detailed analysis shows that GLB-26 is membrane bound. Arrows indicate the orientation of the cells with A : Anterior side, D : Dorsal side, P : Posterior side, V : Ventral side.

Confocal images showed that GLB-26 is expressed in the head mesodermal cell (Figure 3A), and in the tail in the stomato-intestinal and anal depressor muscle cell (Figure 3B). GLB-26 was also found to be expressed in the male anal depressor muscle (not shown). The expression pattern observed here is in accordance with the results of a localization study conducted by Hoogewijs and coworkers, who used a transcriptional reporter[31]. Finally, this reporter also shows that GLB-26 is membrane bound (Figure 3C).

EPR measurements

The CW-EPR spectrum of as-purified (ferric) GLB-26 (Figure 4) is dominated by a signal stemming from a low-spin (S = 1/2) ferric heme.

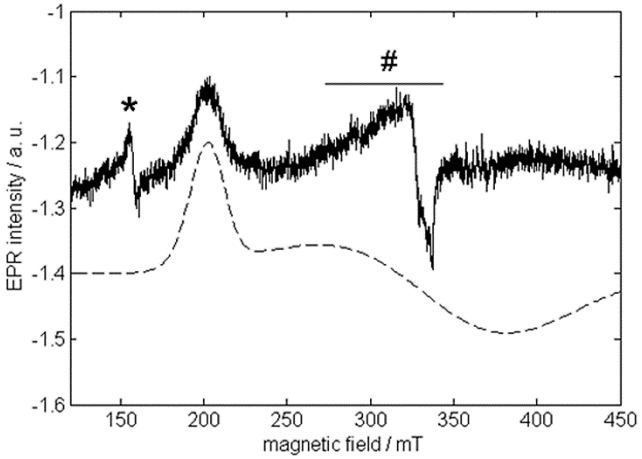

Figure 4. CW-EPR spectrum of ferric GLB-26. Top: Experimental X-band CW-EPR spectrum of ferric GLB-26 in a phosphate buffer (pH7). The peaks indicated by * are due to extra heme iron. # indicates a Cu(II) background signal from the cavity. The spectrum was recorded at a temperature of 10 K. Bottom: Simulation of the dominant low-spin ferric heme contribution.

In the low-field part of the spectrum (not shown) a minor contribution stemming from a high-spin (S = 5/2) ferric heme is found. The latter can be assigned to a small (partially) denatured portion of GLB-26, as observed for example in cytochrome c-554 from *Nitrosomonas europaea*[244]. Only the low-field feature (corresponding to g = 3.25 (±0.01)) of the low-spin contribution can be clearly discerned. This observation, combined with the large value of g (considerably higher than 3) is typical for so-called 'large g_{max}' or type-I EPR signals, also referred to as the highly anisotropic low-spin (HALS)-type signals[245]. Such EPR signals have been reported previously for bis-ligated ferric porphyrins and ferric cytochromes[246-251]. To our knowledge, GLB-26 is a rare case of a globin with a type-I (HALS) EPR spectrum. Although the g_{max} value of ferric human neuroglobin and human cytoglobin is also significantly larger than 3[252], the EPR features are still more typical of a normal rhombic or type-II EPR signal. One of the three ferric heme forms found for hemoglobin 1 of *Drosophila melanogaster* (*Dm*Hb1) is characterized by a HALS-type EPR spectrum with even larger g_{max} (3.50), but little is known about the specific ligation of the heme in this form or about the function of this form[253]. In the case of GLB-26, a bis-histidine

coordination of the heme group (binding of E7His and F8His) is assumed[34]. For bis-imidazole-coordinated ferric porphyrins, large g_{max} signals are indicative of dihedral angles between the imidazole planes larger than ~60°[254]. Detailed information on the orientation of the imidazole planes relative to each other and to the heme can be obtained from a pulsed EPR analysis[253,255]. However, since only the low-field EPR signal is clearly resolved in the case of GLB-26 (Figure 4), relevant pulsed EPR spectra could only be obtained at this field position. Figure 5 shows the HYSCORE spectrum and its simulation for this observer position. The HYSCORE spectrum reflects the nuclear frequencies stemming from the interaction of the unpaired electron with the ^{14}N nuclei of the heme and His ligands. Table 1 gives the nitrogen hyperfine and nuclear quadrupole components derived from the simulations for GLB-26 in comparison to other globins. While the parameters of the heme nitrogens of GLB-26 are similar to those of other globins, there is quite a strong variation in the hyperfine values stemming from the Fe-coordinating His nitrogens for the different globins indicative of the marked difference in the imidazole orientation and N_{His}-Fe binding strength (Table 1). The A_{zz} value of the His ^{14}N of GLB-26 not only differs strongly from that of the globin domain of the globin-coupled sensor of *Geobacter sulfurreducens*, a globin in which the two axial heme ligating His groups have almost coplanar imidazole planes[256], it is also smaller than for the neuroglobin and cytoglobin cases[255,257].

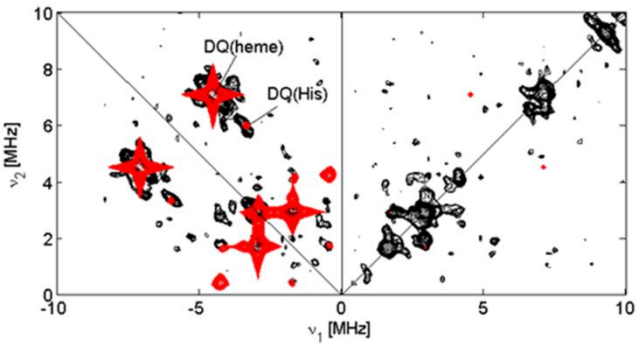

Figure 5. Experimental (black) and simulated (red) HYSCORE spectra taken at the magnetic field position corresponding with $g = g_{max}$. The double-quantum (DQ) cross peaks of the heme ^{14}N and His ^{14}N nuclear frequencies are indicated. Both simulated and experimental spectra are sums of the spectra with τ = 96, 120 and 192 ns.

		Heme ^{14}N nuclei		His ^{14}N nuclei		
	g_{max}	$\|A_{zz}\|$/MHz	$\|Q_{zz}\|$/MHz	$\|A_{zz}\|$/MHz	$\|Q_{zz}\|$/MHz	Ref.
GLB-26	3.25 (±0.01)	5.65 (±0.20)	0.42 (±0.05)	4.65 (±0.10)	0.90 (±0.10)	This work
CYGB	3.20	5.45	0.42	5.00	0.90	[68]
NGB ΔCys	3.10	5.70	0.43	4.90	0.85	[66]
*Gs*GCS[162]	2.925	5.40	0.46	5.90	0.75	[67]

Table 1. Nitrogen hyperfine and nuclear quadrupole components derived from the simulations for GLB-26 in comparison to other globins.

Redox chemistry

To unravel the redox potential of GLB-26 and its function, we encapsulated the protein in a biocompatible gelatin matrix on top of a MOH modified gold electrode. Figure 6 represents the current-potential behavior of a GLB-26|GelB|MOH|Au electrode with different GLB-26 concentrations (curve 1–3) in a 10 mmol L^{-1} HEPES buffer solution in a potential window from −0.6 to 0.4 V with a scan rate of 50 mVs^{-1}. Gelatin is not electrochemically active in this potential range[183]. The redox process at ca. −0.47 V vs SCE (−0.27 V vs SHE), observed in curve 1–3, can be explained as the reaction of one of the heme groups (Fe^{3+}/Fe^{2+}) present in the globin protein, since no other redox active elements are present in the protein. When the GLB-26 concentration increases, the redox current increases as well, following the Randles-Sevcik equation[258,259]. It is shown that after adding hydrogen peroxide, no electrocatalytic wave is observed at this redox potential, indicating that this low-potential heme site doesn't show electrocatalytic properties towards hydrogen peroxide. Since the applied potential is highly negative, stressing the native conformation of GLB-26, this redox couple might be due to a (partially) denatured fraction of the protein sample. This is consistent with the minor high-spin fraction of GLB-26 observed by CW-EPR measurements. However, at a higher potential, a sigmoidal wave with a limiting current appears, after addition of hydrogen peroxide, corresponding to an enzymatic velocity, and this wave is centered at a potential E_{cat}. As given in the inset of Figure 6, the peaks are centered at an E_{cat} of ca. −0.17 V vs SCE (+0.03 V vs the standard hydrogen electrode, SHE). Therefore, this high-potential heme group functions as the electron relay site.

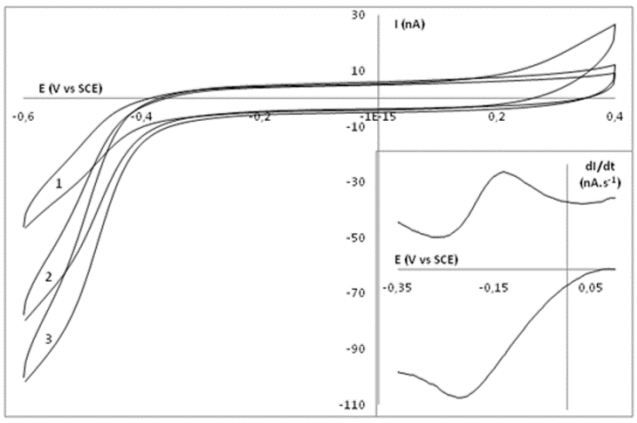

Figure 6. The current-potential behavior of a GLB-26|GelB|MOH|Au electrode with different GLB-26 concentrations (mmolL^{-1}): 0.1 (1), 0.2 (2) and 0.4 (3) in a 10 mmol L^{-1} HEPES pH 7 buffer solution with a scan rate of 50 mVs^{-1}. Inset: Derivative of the current-potential behavior of a GLB-26|GelB|MOH|Au electrode in the presence of hydrogen peroxide.

To investigate the possible formation of a ferryl-oxo[a] group by the reaction of ferric GLB-26 with hydrogen peroxide, a process often observed in peroxidases[261] as well as in globins[260], H_2O_2 was added in equimolar proportion to ferric GLB-26, and the reaction was followed spectroscopically. No change in absorbance was measured indicating the absence of the formation of a ferryl intermediate (not shown).

Phenotypical characterization of the *glb-26* knock-out strain

The *glb-26 (tm4837)* strain is homozygous viable and has no obvious phenotypic defects. The *glb-26 (tm4837)* mutation removes 221 of the 552 coding nucleotides of GLB-26 and is likely a null mutation. Because GLB-26 is expressed in muscles involved in the defecation cycle of *C. elegans*, we compared the defecation rates of the *glb-26(tm4837)* and the wild type strain. No significant difference in defecation rate was seen under normal conditions between the wild type and *glb-26 (tm4837)* strain, indicating that GLB-26 is not necessary for the control of the defecation cycle under normal conditions (Figure 7A).

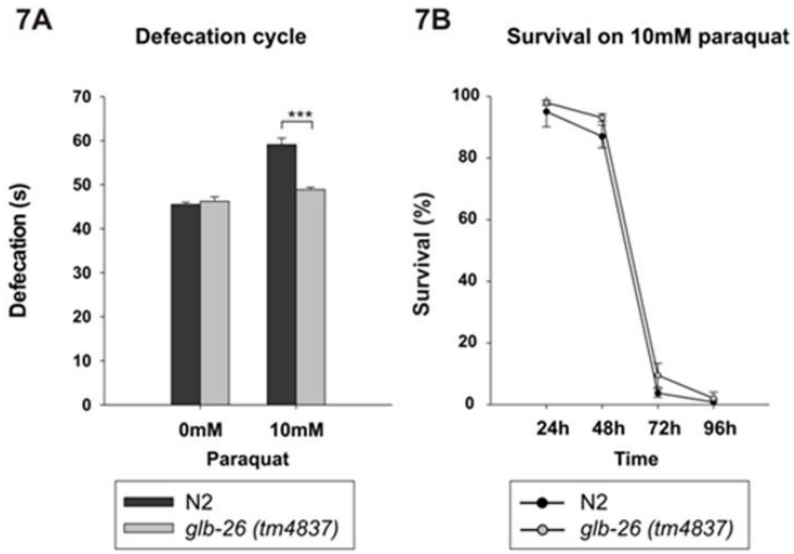

Figure 7. Influence of PQ on wild type N2 and *glb-26(tm4837)*. A) Defecation cycle under normal conditions does not significantly differ between the wild type N2 and *glb-26(tm4837)*. When exposed to 10 mM PQ, the wild type shows a strong slowing down of the defecation cycle, whereas this slowing down is weaker in *glb-26(tm4837)*. Error bars indicate standard error (n = 3), * $p<0.05$; *** $p<0.001$. **B**) Survival of N2 and *glb-26 (tm4837)* on 10 mM PQ does not significantly differ. Error bars indicate standard error (n = 3).

[a] For the reaction of ferric globin with hydrogen peroxide, two reducing equivalents are required to break the O-O bond. The first of these is provided by the ferric iron, which is converted into a ferryl (Fe^{4+}=O) species. The second electron comes from the heme ring itself, forming a *pi*-cation radical, after which the oxidizing equivalent is transferred to the globin moiety of the globin[260].

Geuens and coworkers showed that GLB-26 cannot function as an O_2 carrier, and instead suggested that it might function in redox reactions/signaling[34]. Therefore, the defecation cycle was analyzed in worms exposed to PQ, an environmental toxin that induces oxidative stress. *In vivo*, PQ will become part of redox cycling, leading to the generation of superoxide on one hand, and to the oxidation of reducing equivalents (e.g., NADPH, reduced glutathione) on the other hand[262]. This makes it a good compound to study any form of redox signaling or detoxification of reactive compounds. Interestingly, exposure of the worms to 10 mM PQ significantly ($p = 3E-9$) slowed down the defecation cycle in the wild type strain, while in the *glb-26 (tm4837)* strain only limited slowing down was seen ($p = 0.03$) (Figure 7A). GLB-26 thus seems to be involved in the defecation cycle when worms are exposed to conditions that alter the redox status. To test if GLB-26 could specifically be involved in detoxification of reactive compounds, the survival rate of worms continuously exposed to 10 mM PQ was analyzed (Figure 7B). After 24 h, the first negative effects on survival could be seen, and after 72 h, the majority of worms had died. In this case, however, no significant difference was seen between the wild type and *glb-26 (tm4837)*, although the latter appeared to survive slightly better than the wild type.

4. Discussion

Given the fast autoxidation and strong hexacoordination of GLB-26, this globin has previously been suggested to have a redox function *in vivo*[16,27,31,32,34,230]. This was confirmed by EPR and electrochemical experiments performed in this work. Indeed, the ^{14}N-His hyperfine parameters of GLB-26 vary from those of other bis-His-ligated globins (Figure 5, Table 1) and the CW-EPR spectrum of ferric GLB-26 (Figure 4) resembles those reported for bis-histidine-coordinated cytochromes[247,251], indicating dihedral angles between the imidazole planes of GLB-26 of 55-60°. Moreover, it was shown that homodimeric GLB-26 possesses two low-spin, high potential heme sites (Figure 6) comparable to the high potential heme site of cytochrome bc_1 of the purple bacteria *Blastochloris*[263], and cytochrome c peroxidase of *Paracoccus denitrificans*[264]. The two high potential heme sites of GLB-26 show electrocatalytic activity towards H_2O_2, a type of ROS (Figure 8). Moreover, earlier work showed that GLB-26 can transfer an electron to cytochrome c at the same rate as mitochondrial redox carriers *in vitro*[27]. Therefore a function as a member of an electron transport chain, involving H_2O_2 is feasible.

To identify which process(es) GLB-26 might be involved in, the *in vivo* expression pattern was determined. GLB-26 is mainly expressed in the cellular and nuclear membrane of the head mesodermal cell and in the tail stomato-intestinal and anal depressor muscle cells (Figure 3). The

function of the head mesodermal cell is unknown, but some ideas have been postulated based on its connections with neighboring cells[265]. The head mesodermal cell has several processes on the dorsal and ventral side of the body wall. The ventral posterior arm runs in conjunction with ventral body wall muscle arms and the hypodermal ridge and makes gap junctions with ventral body wall muscle arms. The dorsal posterior process runs some distance adjacent to the dorsal hypodermal ridge and makes gap junctions with arms from dorsal muscles. The head mesodermal cell thus stays in close contact with the body wall muscles and during defecation, these body wall muscles contribute to the control of internal pressure and concentration of the gut contents before the expulsion of the waste material.

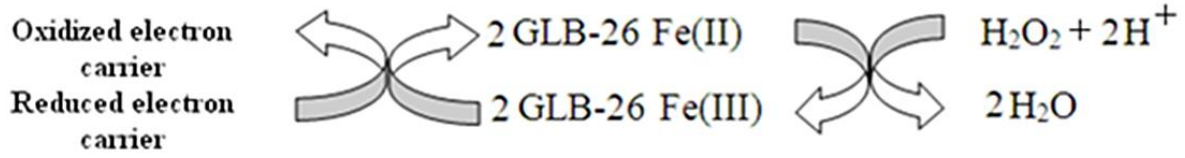

Figure 8. Reduction cycle of GLB-26. GLB-26 can transfer an electron to H_2O_2, and can in turn be reduced by a yet unknown electron carrier. This electron shuttle results in a prolonged defecation cycle in *C. elegans*.

Stomato-intestinal muscles are two sheet-like cells that connect the surfaces of the intestinal cells to the ventral body wall. Contraction of these muscles promotes defecation by pressurizing the intestinal contents near the posterior end of the intestine. The anal depressor muscle is a sexual dimorphic muscle. In hermaphrodites it is involved in the defecation cycle, while in males it is specialized to function as an auxiliary spicule muscle[6].

Given the specific localization pattern of GLB-26 in sister cells of the gut, associated with defecation, and the aforementioned reactivity towards H_2O_2, a role for GLB-26 in the defecation cycle under oxidative stress conditions was suggested. Indeed, after addition of 10 mM PQ, a source of ROS, the defecation cycle in the wild type *C. elegans* strain was prolonged, while in the *glb-26* knock out strain this prolongation was far less pronounced, suggesting a role for GLB-26 in the regulation of the periodicity of this cycle (Figure 7A). The addition of 10 mM PQ had no significantly different effect on the lifespan of both the *glb-26* knock-out strain and the wild type strain, indicating that GLB-26 is not involved in general protection against oxidative stress (Figure 7B).

Other genes affecting the periodicity of the defecation cycle of *C. elegans* have been identified in the past and generally act in the gut by altering some of its properties[266-270]. To date, only one gene, *dsc-1*, has been identified that acts in the enteric muscle cells, i.e. the stomato-intestinal muscles and anal depressor muscle cell, through a feedback mechanism to the gut. *dsc-1* encodes

a Paired-like homeodomain protein, a class of transcription factors previously associated with the terminal differentiation of neurons in *C. elegans*[270]. One type of feedback mechanism works through gut expansion and may involve a humoral signal that acts to coordinate the Ca^{2+} signal generated in the gut with the timed contractions of the various muscles types, thereby regulating the length of the defecation cycle[270].

At present, it is unclear if a similar feedback mechanism is utilized by GLB-26 and that it as such works as a signaling molecule, or its role is purely enzymatic, or both. An enzymatic function is supported by the fact that relative quantification of the *in vivo* expression levels of all globin genes of *C. elegans* showed that *glb-26* was expressed about 40 fold higher than the other globin genes under normal conditions[16,30].

At this point we cannot assign the exact role of GLB-26 in this process however, but some suggestions can be postulated based on its reactivity towards H_2O_2 and the physiological significance of this ROS generation.

Several sources of ROS have been identified and one of them involves both the nematode's immune defense and the defecation cycle. *C. elegans* feeds on bacteria, but when pathogens are ingested, the innate immune defense is initiated through germ-line encoded pattern-recognition receptors to restrict the damage and to ensure the organism's survival[271]. This response is pathogen-dependent[272] and includes the excretion of ROS in the intestinal lumen of the nematode[273,274]. The dual oxidase Ce-Duox-1/BLI-3 was shown to play a role in the defense mechanism against pathogens as it possesses an NADPH oxidase domain to generate H_2O_2, an EF hand domain for enzyme activation by the binding of Ca^{2+}[275], and an extracellular peroxidase domain, that might be important for the binding of H_2O_2 [276,277]. However, the role of the latter domain is still unknown[278].

In addition to the protection against pathogens, ROS increase also stimulates inositol trisphosphate (IP_3)-mediated Ca^{2+} mobilization by increasing cytosolic Ca^{2+} accumulation through the endoplasmic reticulum, and by stimulating Ca^{2+} influx through Ca^{2+} channels. Two types of Ca^{2+} binding proteins include the IP_3 receptor (ITR-1), and calreticulin (CTR-1), a molecular chaperone, that are expressed predominantly in the intestine of *C. elegans*. *Ctr-1* genetically interacts with *itr-1*, and shows a synergic effect on the length of the defecation cycle[239,279,280]. An elongation of the defecation cycle of *C. elegans* was also observed upon pathogenic ingestion[281], possibly through the same Ca^{2+} signaling pathways, and this could play an important role in microbial infections.

GLB-26 is expressed in the cellular and nuclear membrane as a result of N-myristoylation, and is therefore in close proximity of the membrane bound Ce-Duox-1/BLI-3 (Figure 2A), that was

suggested to act downstream of IP_3[282]. As such, GLB-26 could act as an electron donor for H_2O_2 and signal the presence of this reactive compound by oxidizing another electron carrier. Its dual localization in the cellular and nuclear membrane might be crucial for the fast propagation of the detection in the change of oxidative stress conditions (Figure 7A), and to elucidate an appropriate response in the nucleus, possibly involving the modulation of transcription factors. Future experiments to identify interaction partners are necessary and will hopefully gain insight in the complex regulation of this rhythmic behavior.

5. Conclusions

GLB-26 is a hexacoordinated globin that is attached to the cellular and nuclear membrane by myristoylation of its N-terminus. GLB-26 may act as a heme peroxidase, signaling the presence of H_2O_2 in the nucleus and in the cytosol of the head mesodermal cell, stomato-intestinal muscle, and anal depressor muscle cells. When oxidative stress conditions are induced, the redox status of the cytoplasm and hence of the nucleus is altered. This is sensed by GLB-26, and is translated in a prolonged defecation cycle of *C. elegans*. The dual expression of GLB-26 in the cytosol and in the nucleus may contribute to the fast translation of the changed redox status in the cell.

6. Materials and Methods

Prediction of N-terminal myristoylation and subcellular localization of GLB-26

The prediction of the post-translational modification was done by the Myristoylator Prediction Program (www.expasy.org/tools). The subcellular localization of GLB-26 was predicted using the General Eukaryotic Localization Prediction Program WoLF PSORT[241], the Euk-mPLoc server version 2.0[283-288], the Balanced Subcellular Localization Predictor[289], and the Subnuclear Compartments Prediction System[242,243].

Cloning of *glb-26*

C. elegans worms were grown, total RNA was isolated, and cDNA was prepared as described previously[32,34]. The cDNA of wild type *glb-26* was amplified using the gene specific forward and reverse primers 5'– GGAAGATCTCATGGGCTCCTCTACTTCGACTCCTGC–3' and 5'–CCCAAGCTTCTCCTCATCGTCTTCTTTTGTTTC–3' respectively. Wild type GLB-26 contains two cysteine residues that can cause aggregation due to the formation of disulfide bridges. To avoid this, both cysteine residues were mutated to serine (GLB-26*) using the Quickchange™ site directed mutagenesis kit (Stratagene) as described earlier[57], using mutation primers 5'–GGACCGTCAAACTCTGGAAGTACAATAACG–3', and 5'–CGTTATTGTACTTCCAGAGTTTGACGGTCC–3' for the first cysteine, and 5'–CGAAACTTTCTCAAGAAATCGGC–3' and 5'–GCCGATTTCTTGAGAAAGTTTCG–3' for the second cysteine. To prove myristoylation of GLB-26*, the myristoylation site, glycine at position 2, was modified to alanine, with the mutation primers 5'–GATCTCATGGCCTCCTCTACTTCG–3' and 5'–CGAAGTAGAGGAGGCCATGAGATCTGA–3'. This mutant is annotated as GLB-26*G2A. Both cDNAs were cloned into the pEGFP-N1 vector (Clontech) using BglII and HindIII restriction enzymes (Biolabs, Westburg), resulting in two expression constructs : p*glb-26*-egfp*-N1 and p*glb-26**G2A-*egfp*-N1. Ligation of cDNA in the vector was performed using T4 DNA ligase (Novagen).

***In vitro* sublocalization of GLB-26**

Human neuroblastoma SH-SY5Y cells (ATCC® CRL-2266™) were cultured as per manufacturer's protocol. Cells were seeded in 10 mm cell culture dishes with glass bottom (Greiner bio-one) one day before transfection at a density of 5×10^4 cells per cm^2. They were transfected with 3 µl of lipofectamine™ 2000 and 0.5 µg of pEGFP-N1 plasmid containing the

cDNA for *glb-26* or *glb-26G2A* according to the manufacturer protocol. After four hours, the transfection medium was replaced by growth medium and SH-SY5Y-cells were allowed to express the GFP-tagged GLB-26 proteins for 24 hours. Finally, the sublocalization was examined with an Ultra*VIEW* VoX microscope (PerkinElmer), and images were obtained with the Volocity 6.0.1 software. GFP was excitated at 488 nm with a solid state laser and a bandpass filter was used to allow emission light between 500 nm and 550 nm.

In vivo **localization of GLB-26**

The translational reporter for *glb-26* was constructed using fusion PCR, as described by Hobert and coworkers[202]. The reporter contains 2.12 kb upstream and 0.90 kb downstream of the *glb-26* gene, to include endogenous promoter and 3'UTR elements. The *gfp* gene was amplified from the vector pPD95.75 (Fire Lab), and fused at the 3' side of the *glb*-26 coding gene, thereby preceding the *glb-26* 3'UTR region. For *glb-26*, the primers used were the forward primer 5'–TGAAGATGGTGGTACAAAGT–3' to amplify the promoter sequence, the forward nested primer 5'–GTAAAACTTTGGGTTGGTCT–3' for the promoter sequence, the reverse primer 5'–AGTTCTTCTCCTTTACTCAACTCCTCATCGTCTTCTTTTG–3' for the *glb-26* gene, the *glb-26* 3'UTR forward primer 5'–GCATGGATGAACTATACAAATGAATGTGTGATTTTTTGAT–3', the *glb-26* 3'UTR reverse primer 5'–GAAATGTGCTCTCTATGAGG–3', and the *glb-26* 3'UTR reverse nested primer 5'–GCACTTGTGACGTTTTCTAT–3'. For the *gfp* gene, the forward primer 5'–TTGAGTAAAGGAGAAGAAC–3' and the reverse primer 5'–TTTGTATAGTTCATCCATGCC–3' were used.

The *unc-119* gene, including a 2.189 kb upstream region and a 1.228 kb downstream region of the *unc-119a* isoform, was amplified with the forward primer 5'– TCAGTAAAAGAAGTAGAAT–3' and reverse primer 5'–GAATTTTAACAATACTTC–3'. The PCR product was used as a co-injection marker, and rescued the locomotion defect of the *unc-119(ed3)* strain, i. e. the strain used for microinjection.

The final PCR products were injected into the gonads of young adult hermaphrodites using an AxioVert 135 (Zeiss) microscope and FemtoJet microinjection system (Eppendorf), at a concentration of 50 ng/µl for the *glb-26* reporter and 20 ng/µl for the *unc-119* gene. Transformed lines were analyzed using a Nikon Eclipse TE2000-5 confocal microscope.

Electron Paramagnetic Resonance measurements

The X-band continuous-wave (CW) Electron Paramagnetic Resonance (EPR) experiments were performed on a Bruker ESP300E spectrometer (microwave (mw) frequency 9.45 GHz) equipped with a gas-flow cryogenic system, allowing operation from room temperature down to 2.5 K. The magnetic field was measured with a Bruker ER035M NMR gaussmeter. The spectra were recorded at a temperature of 10 K, a microwave power of 2 mW, a modulation amplitude of 1 mT and a modulation frequency of 100 kHz.

X-band pulsed EPR experiments were performed on a Bruker Elexsys instrument equipped with Helium cryostat (Oxford Inc.). The measurements were done at 7 K. The HYSCORE (hyperfine sublevel correlation) spectrum[290] was recorded using the pulse sequence: $\pi/2$-τ-$\pi/2$-t_1-π-t_2-$\pi/2$-τ-echo, with $t_{\pi/2}$ = 16 ns and t_{π} = 32 ns; t_1 and t_2 were varied in step of 16 ns (matrix dimension [350×350]). Spectra were recorded for τ = 96, 120 and 192 ns. A four-step phase cycle was performed in all cases to remove the unwanted echoes. The HYSCORE traces were baseline corrected using a third-order polynomial, apodized with a Hamming window and zero-filled. After Fourier transformation the absolute-value spectra were computed and the spectra recorded at different τ-values were added together.

All EPR simulations were done with the EasySpin program, a Matlab toolbox developed for EPR simulations[291]. The HYSCORE spectra were simulated assuming two-spin $S = 1/2$, $I = 1$ systems using the parameters in Table 1. The simulated spectral contributions for the heme and His nitrogens were then added together. Simulations were performed of the different experimental τ-values.

Reduction potential - Chemicals and solutions.

Mercaptohexanol (MOH), 2-[4-(2-hydroxyethyl)-piperazinyl]ethanesulfonicacid (HEPES) and sodium hydroxide were purchased from Sigma-Aldrich. The HEPES buffer solution of 10 mmol L^{-1} was set to pH 7.0 using a 0.15 mol L^{-1} NaOH solution. Type B gelatin (Gel, IEP = 5, Bloom strength = 257), isolated from bovine skin by the alkaline process, was kindly supplied by Tessenderlo Chemie (Belgium).

Reduction potential - Electrode preparation.

The three-electrode system consists of a saturated calomel reference electrode (SCE, Radiometer Analytical, France), a graphite counter electrode and a gold inlaid disc electrode. The gold working electrodes of 1.6 mm diameter were pretreated by mechanical polishing and modification. Before its first use, the electrode surface was briefly polished on an aluminum

oxide film disc of small particles to obtain a smooth and clean surface. To remove any adherent Al_2O_3 particles the electrode surface was rinsed thoroughly with deionised water in an ultrasonic bath and dried with a tissue. Secondly, the gold electrode was modified by immobilization of MOH by hanging the bare electrode for 12 hours in a 14 mmol L^{-1} MOH solution. This electrode is denoted as MOH|Au.

To immobilize a drop dried layer of gelatin B onto a MOH|Au electrode, 10 μL of a gelatin B solution (5 w%)/HEPES mixture was placed on the surface by means of a pipette and was left to dry to air at 4°C. The gelatin B solution was prepared by mixing the gelatin B powder and the HEPES buffer solution at approximately 40°C, as was described earlier[183,292]. These electrodes are referred to in the text as GelB|MOH|Au. If a GLB solution is incorporated in the gelatin matrix, the final electrodes are denoted as GLB-26|GelB|MOH|Au (3:7 ratio). The concentration of gelatin is 5 m%.

Reduction potential - Apparatus.

A μ-Autolab potentiostat controlled by GPES 4.9 007 software package (Metrohm, The Netherlands) was used for recording the cyclic voltametric curves. The solutions were thoroughly deoxygenated by bubbling with nitrogen for at least 30 minutes, and again for approximately 5 minutes in the cell itself before usage.

Functional characterization of GLB-26 - *C. elegans* strains.

The *C. elegans* strains Bristol N2 (wild type strain) and *unc-119(ed3)* were obtained from the *Caenorhabditis* Genetics Center (CGC, University of Minnesota). The *glb-26 (tm4837)* knockout strain was provided by the Japanese National Bioresource Project. *glb-26 (tm4837)* was outcrossed eight times with N2 to remove adventitious mutations. Primers used to genotype *glb-26 (tm4837)* were 5' - AGCGGGCTCGATACGAATAA – 3' and 5' – CACATACTGCTCGAACTGCA – 3'. Strains were cultured and experiments were carried out at 20°C on nutrient agar plates seeded with *Escherichia coli* OP50.

Functional characterization of GLB-26 -Paraquat sensitivity.

For paraquat (1,1'-dimethyl-4,4'-bipyridinium dichloride, PQ) sensitivity assays, worms were synchronized by hypochlorite treatment, and isolated eggs were allowed to hatch at 20°C overnight. Hatched larvae were grown to 1- day-old worms, after which they were transferred to nutrient agar plates seeded with a spot of *E. coli* OP50, and with a final concentration of 10 mM paraquat. This concentration was used since at lower concentrations no effect on the defecation

cycle was observed. At higher concentrations worms were severely affected, showing almost no movement, no pharyngeal pumping and no defecation cycle. The strongest response was found at 10 mM paraquat, and was therefore chosen in this study. Survival was monitored every 24 hours. Worms that failed to respond to repeated touching with a platinum wire were considered to be dead. PQ sensitivity assays were performed in triplicate, with approximately 200 worms per strain per trial. The values for the individual time points of the three replicates were averaged, and were used to generate survival curves. Student's t-test was used for statistical analysis.

Functional characterization of GLB-26 -Defecation cycle length.

One-day-old adult hermaphrodites were obtained as described in the PQ sensitivity assay, and were used to measure the defecation cycle length as the average time between consecutive pBoc contractions. Adults were picked to a fresh nutrient agar plate seeded with a spot of *E. coli* OP50, after which they were allowed to settle down for at least 10 minutes. In the assays where PQ was included, a final concentration of 10 mM PQ was added to the plate 3 h before worms were transferred. Individual worms were followed for approximately 5 minutes, after which the average defecation cycle length was calculated. Results represent 3 independent trials with at least 5 worms per trial. Student's t-test was used for statistical analysis.

Chapter IV:

Analysis of hypoxia-responsive globin genes in *C. elegans*

Not submitted:

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1. Abstract

Oxygen is essential for most multicellular organisms. Consequently, O_2 deprivation leads to an adjustment of the organism's metabolism and physiology, including changes in cell turnover, modulation of stress signaling pathways and alteration of the organism's behavior. Globins have a well-established role in the efficient uptake and transport of O_2 and seem capable of additional functions related to O_2 metabolism, such as O_2 sensing and protection against oxidative stress. We studied the role of the diverse *C. elegans* globin family in hypoxia and observed that the expression of eleven globin genes is sensitive to O_2 deprivation. In animals, the hypoxia-inducible factor (HIF) transcription factors are responsible for most of the hypoxia-induced changes in gene expression. In this study, we indeed found that a subset of the hypoxia-sensitive globin genes in *C. elegans* is regulated by HIF-1. The majority of these hypoxia-responsive globins are expressed in neurons, which suggests a function in O_2 sensing, while one globin appears to facilitate O_2 uptake under hypoxia. Overall, these expression results indicate that several globin genes in *C. elegans* are involved in the adaptation to hypoxia.

2. Introduction

Almost all multicellular organisms require O_2 for survival. However, during normal development and homeostasis and in many pathological conditions, cells and tissues can be challenged by O_2 levels that are too low to support normal physiological functions. This condition is termed hypoxia. To survive and sustain a normal metabolism during hypoxic periods, metazoa have developed a number of mechanisms. The conserved transcription factor Hypoxia-Inducible Factor 1 (HIF-1) is one of the key regulators of O_2 homeostasis and is essential to cope with the effects of moderate hypoxia[45,46]. At normal O_2 concentrations, HIF-1 is hydroxylated by a prolyl-hydroxylase (EGL-9 in *C. elegans* and EGLN/PHD in mammals). Once hydroxylated, HIF-1 binds an E3 ubiquitin ligase, termed the von Hippel Lindau tumour suppressor (VHL-1 in *C. elegans* and VHL in mammals), and is targeted for degradation[293,294]. In hypoxic conditions, the proline hydroxylation and degradation of HIF-1 are decreased, allowing HIF-1 to relocate to the nucleus and activate target genes. HIF-1 activation plays a central role in metabolic adaptation and modulation of signal transduction and tissue repair[295,296], as well as in the development of various types of cancer[297].

The central role of globins in O_2 metabolism is well understood by the detailed study of vertebrate hemoglobin and myoglobin, showing that these proteins function in O_2 transport and

O_2 storage (reviewed by Rahaman and Straub[83]). It is also appreciated that globin genes in a wide range of organisms show inducible changes in expression following O_2 deprivation[38-44], indicating that the function of many globins is intrinsically related to O_2 metabolism. At the same time, variation in functional properties of different globins and globin isoforms, such as ligand affinity, expression pattern and subcellular locations, has made clear that these proteins can be involved in a multitude of functions. Additional roles related to O_2 metabolism have therefore been proposed and include participating in stress signaling, for example by acting as O_2 or redox sensors, and protecting against oxidative stress during and following hypoxia[12]. The *C. elegans* genome encodes for thirty-three globin-like proteins, which display different sizes, are differently located, and are likely to be functionally diverse[16]. This makes *C. elegans* an attractive model to study the potential roles of globins.

To identify *C. elegans* globin genes that are responsive to hypoxia and that are regulated by HIF-1, we analyzed the expression of the globin gene family following O_2 deprivation in wild-type and *hif-1* deficient worms, as well as in *egl-9* and *vhl-1* deficient worms at normal O_2 concentrations. About one-third of this globin family is indeed responsive to hypoxic stress, with both HIF-1-dependent and independent changes in globin gene expression.

3. Results

To identify hypoxia-induced changes in globin gene expression, we exposed one-day old adult worms[b] to three hypoxic conditions that are associated with HIF-1 activation, namely 0.1%, 0.5% and 1% O_2, and compared these with normoxic worms. F22B5.4, a gene responsive to hypoxia and dependent on HIF-1[296], was used as positive control (Fig. 1). We observed that eleven globin genes showed a significant change in expression for at least one of the three hypoxic conditions. Remarkably, only two globin genes displayed a significant change for all three hypoxic conditions, *glb-1* and *glb-15*, together with the positive control. Two additional globin genes, *glb-22* and *glb-24*, showed a consistent upregulation following all three hypoxic conditions, but this was only significant in two of the three conditions tested. Three additional globin genes, *glb-11*, *glb-13* and *glb-21* showed a significant upregulation at the most severe hypoxic condition (0.1% O_2), while the remaining four globin genes, *glb-8*, *glb-14*, *glb-31* and *glb-32*, showed a significant downregulation at this condition.

[b] When cultivating *C. elegans* at 16-24°C, the age of adult worms is defined as the time following the transition from the fourth and last larval stage to adulthood. For example, adult worms are day-zero in the 24 hours following the transition from the fourth larval stage to adulthood, and day-one between 24 and 48 hours following this event.

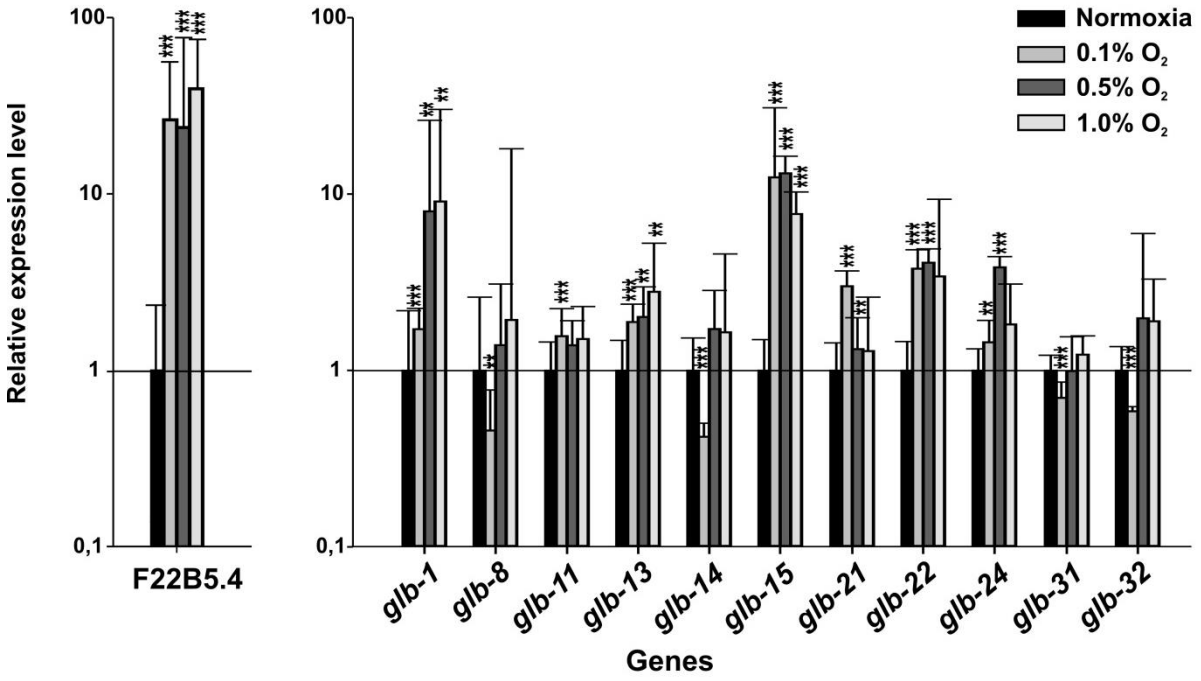

Figure 1: Expression analysis of globin genes in WT worms in normoxic and hypoxic conditions. Eleven globin genes show a significant change in expression in young adult WT animals following 12 h of hypoxia. Error bars indicate 95% confidence intervals (n=4), ** $p<0.05$; *** $p<0.01$.

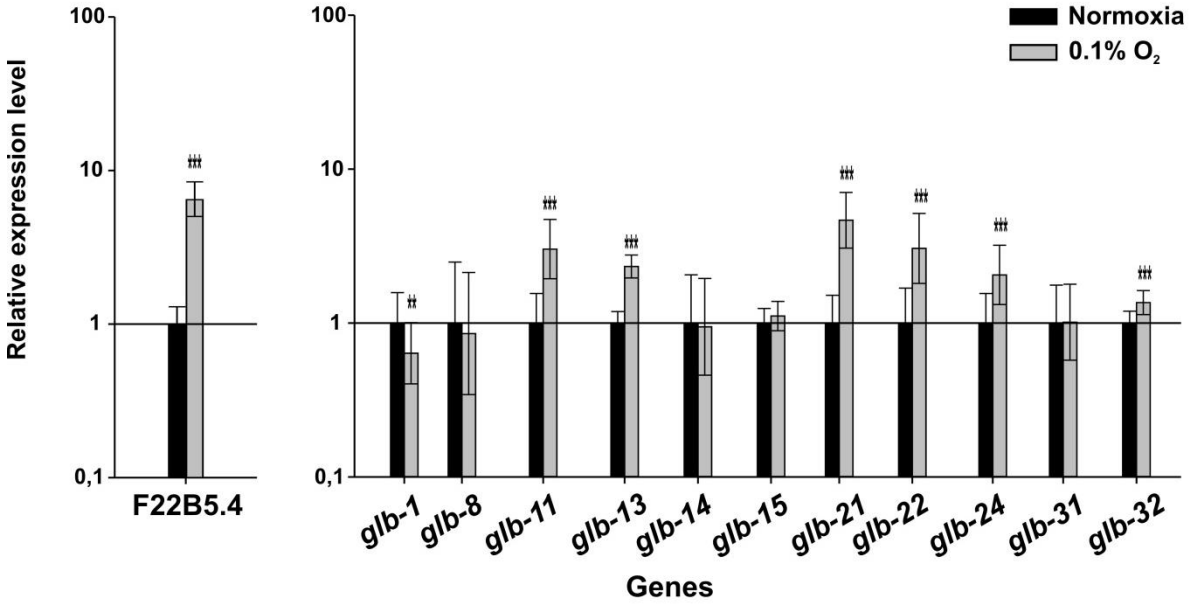

Figure 2: Expression analysis of hypoxia-sensitive globin genes in a *hif-1* mutant in normoxic and hypoxic conditions (12h 0.1% O_2). Error bars indicate 95% confidence intervals (n=3), ** $p<0.05$; *** $p<0.01$.

To determine if these gene expression changes were HIF-1-dependent, we compared normoxic and hypoxic expression levels in a *hif-1* knockout strain. We chose to expose worms to severe hypoxia (0.1% O_2), because all eleven globin genes showed significant changes at this condition in wild type worms. In the *hif-1* knockout strain, five globin genes showed no (*glb-8*, *glb-14* and *glb-15*) or a different (*glb-1* and *glb-32*) change following hypoxia, suggesting that they are HIF-1 dependent (Fig. 2). Note that the positive control F22B5.4 is still induced in the *hif-1* mutant following hypoxia, but that this induction is substantially lower than in WT worms exposed to hypoxia (Fig. 1&2). This result therefore does not argue against the HIF-1 regulated expression of F22B5.4. However, it does indicate that this gene is influenced by additional transcription factors besides HIF-1 during hypoxia. The detection of this limited induced expression of F22B5.4 in the *hif-1* mutant following hypoxia, which was not described in the microarray study of Shen and colleagues[296], can be explained by the greater sensitivity of real-time RT-PCR compared to microarray hybridization.

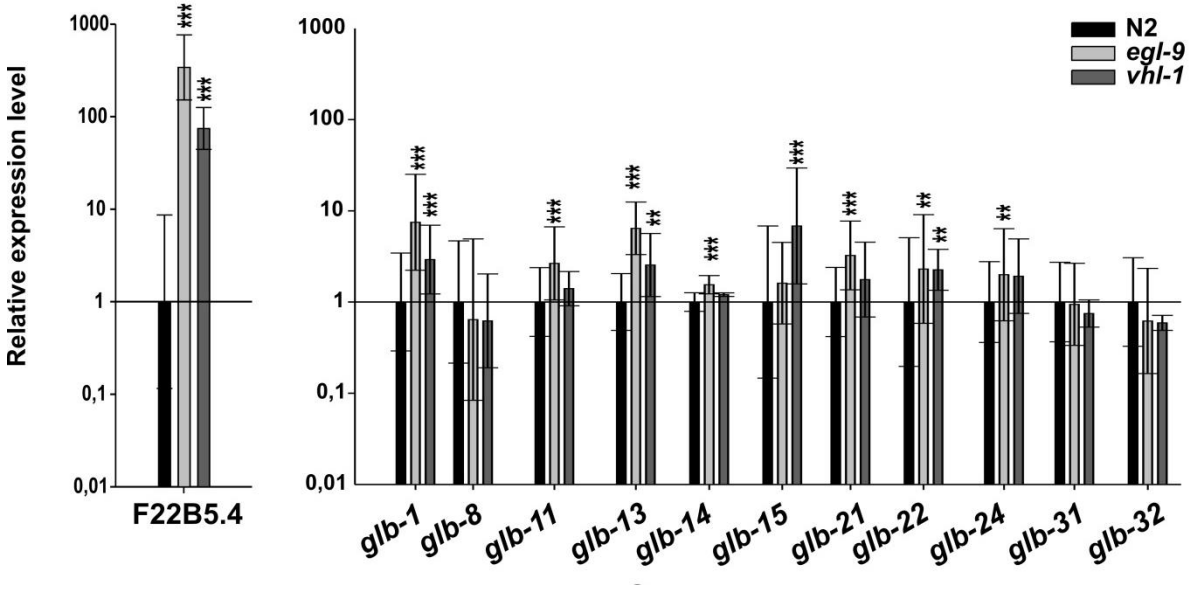

Figure 3: Expression analysis of hypoxia-sensitive globin genes in wild-type, *egl-9* and *vhl-1* worms in normoxic conditions. Error bars indicate 95% confidence intervals (n=3), ** $p<0.05$; *** $p<0.01$.

By using *vhl-1* and *egl-9* mutants, which have continuously active HIF-1, we were able to further test this potential HIF-1 dependency. Note that the positive control F22B5.4 indeed shows highly elevated expression levels in both mutants. We found that three of the potential HIF-1 dependent globin genes (*glb-1*, *glb-14* and *glb-15*) showed a significant change in expression in at

least one of the two mutants, further supporting their HIF-1 dependency (Fig. 3). The two other potential HIF-1 dependent globin genes, *glb-8* and *glb-32*, did not significantly change in expression in these mutants compared to WT worms, making it unclear if these genes are truly HIF-1 regulated. Five additional hypoxia-sensitive globin genes (*glb-11*, *glb-13, glb-21, glb-22* and *glb-24*) also displayed a significant change in expression in at least one of the two mutants. Because this last group of globin genes were still induced in *hif-1* mutants under hypoxia, these results might be an indication that the expression of these globin genes is partially HIF-1 dependent during O_2 deprivation. Overall, these results provide a first indication on the potential HIF-1 dependent regulation of these hypoxia-sensitive globin genes. However, given the discrepancy in results obtained with the *hif-1* knockout mutant and the mutants with constitutively active HIF-1, these findings need to be further validated.

Based on these results, *glb-1* and *glb-15* seemed the two most promising targets for further analysis: they are both significantly upregulated at all three hypoxic conditions in WT worms and showed HIF-1-dependency in the *hif-1* knockout and *hif-1* overexpressor mutants. A previous study using transcriptional reporters[16] showed that *glb-1* is expressed in several neurons and in hypodermal or muscle tissue in the head region, whereas the *glb-15* reporter failed to show expression. These transcriptional reporters were generated using the globin gene's promoter, fused to GFP and a commonly used 3'UTR (3'UTR$^{unc\text{-}54}$). Regulatory domains essential for proper transcription can also be present in a gene's introns or own 3'UTR, potentially explaining why the *glb-15* reporter showed absence of expression. We therefore generated translational reporters for both *glb-15* and *glb-1*, including their introns and endogenous 3'UTR.

The novel *glb-15* translational reporter indeed showed expression and indicates that GLB-15 is exclusively expressed in a limited number of head, tail and nerve cord neurons (Fig. 4).

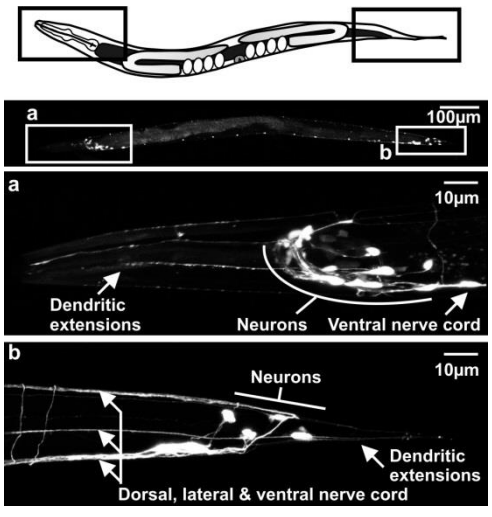

Figure 4: Expression pattern of the *glb-15* translational reporter. Expression is observed in head (a) and tail (b) neurons.

A substantial number of these neurons are sensoric, with long dendritic regions extending anteriorly in the head and posteriorly in the tail. The majority of *C. elegans* globin genes is expressed in neuron subsets and are therefore hypothesized to fulfill a sensory role[16]. GLB-15 could potentially act as an O_2 sensor, allowing rapid behavioral responses to changes in O_2 availability, or influencing normal physiology and development during hypoxic conditions. However, *glb-15* RNAi in an RNAi-hypersensitive strain did not result in any obvious defects in development, egg production and embryonic survival, both in normoxia or hypoxia (0.5% O_2) (data not shown). It is possible that *glb-15* RNAi does not reduce GLB-15 levels sufficiently to provide a clear phenotype or that redundant globins compensate for the reduction in GLB-15 levels. In this context, it is worth noting that RNAi against the majority of *C. elegans* globins does not result in an obvious phenotype. Because of the absence of a clear phenotype for *glb-15* RNAi, we focused our attention on GLB-1.

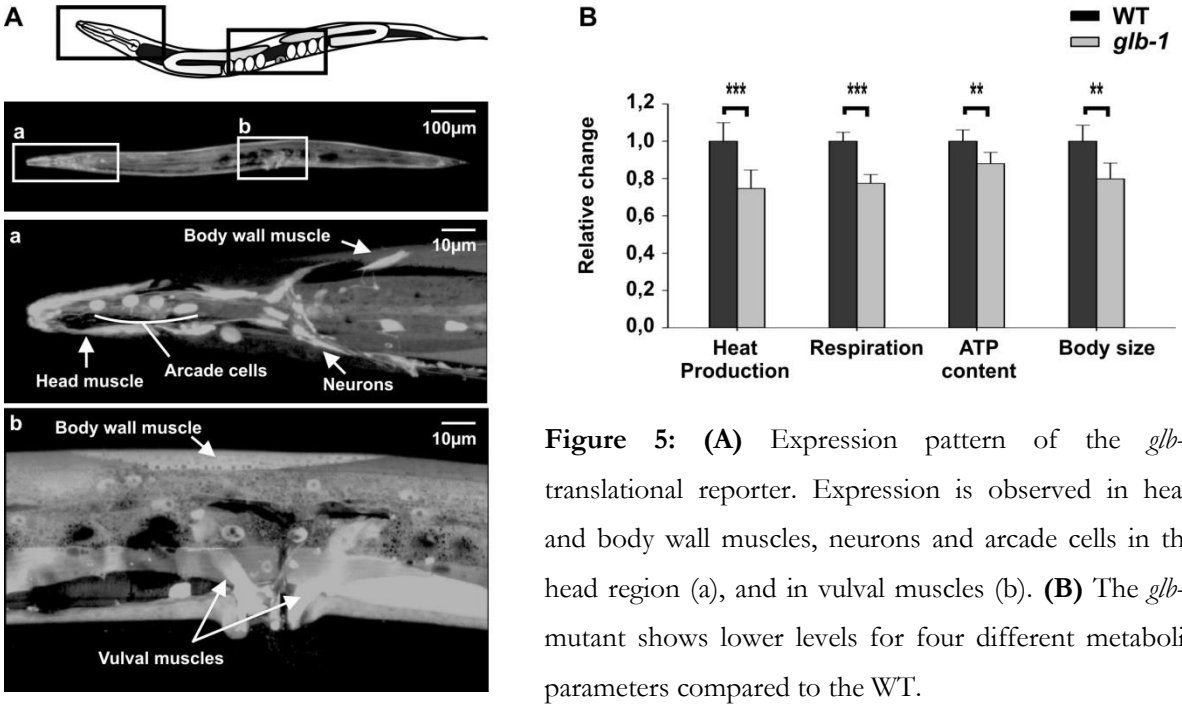

Figure 5: (A) Expression pattern of the *glb-1* translational reporter. Expression is observed in head and body wall muscles, neurons and arcade cells in the head region (a), and in vulval muscles (b). **(B)** The *glb-1* mutant shows lower levels for four different metabolic parameters compared to the WT.

GLB-1 is one of the most highly expressed globins in *C. elegans*[16]. Previous biochemical and structural analysis showed that GLB-1 is pentacoordinated and can reversibly bind O_2 with high affinity[34]. A role in O_2 metabolism has been proposed for this globin, potentially serving to maintain a constant cellular O_2 concentration. *glb-1*'s induced expression in hypoxia is in further support of this role. A *glb-1* translational reporter showed expression in body wall, head and vulval muscles, as well as in several head neurons and in arcade cells, the latter being specialized head epithelial cells (Fig. 5A). The predominant presence of GLB-1 in muscles suggests that it

may serve a role comparable to vertebrate myoglobin, i.e. functioning as an O_2 storage to sustain aerobic metabolism. In addition, the broader expression pattern of the translational reporters for *glb-1* and *glb-15* compared to their transcriptional reporters[16] indicates that additional regulatory regions are indeed present in the introns or 3'UTR of these genes.

A *glb-1* knockout mutant, in which the entire gene is deleted, was used to study the effects of GLB-1 loss on several metabolic parameters. We observed that the *glb-1* mutant showed reduced respiration rate, heat production, ATP content and body length (Fig. 5B), which further supports a role in O_2 metabolism for this globin.

Interestingly, in addition to being regulated by HIF-1, *glb-1* is also a major target of the DAF-2 insulin-like signaling pathway. This pathway regulates many aspects of the worm's physiology, including development, metabolism, longevity, stress responses and immunity[298]. The insulin-like receptor DAF-2 exerts these effects through negative regulation of DAF-16, a FOXO transcription factor. Partial loss of function of *daf-2* results in an increased expression of *glb-1*, and this increase is dependent on *daf-16*[16]. This result was later also confirmed on a protein level[299]. In addition to its regulation by DAF-2/DAF-16, increased levels of GLB-1 were reported following infection of *C. elegans* with the pathogenic bacterium *Aeromonas hydrophila*[300]. Noteworthy, GLB-1 was part of a small group of proteins (65 in total) that were identified in this screen, strongly indicating that this globin is important for the worm's defense system against pathogens. Finally, a recent report showed increased *glb-1* expression following exposure of the worm to (non-pathogenic) bacterially derived NO[301]. Several globins, including vertebrate myoglobin, are known to function in NO metabolism[12]. Remarkably, DAF-16 seemed to have a repressive instead of a positive effect on *glb-1* expression in this experiment, as *glb-1* levels further increased in an *daf-16* mutant following bacterial exposure. Taken together, these results indicate that *glb-1* expression is sensitive to a number of environmental conditions and that GLB-1 could be involved in protection against environmental stress.

We therefore decided to compare the stress sensitivity of the *glb-1* knockout mutant with WT worms. However, while an initial screen indicated that the *glb-1* knockout mutant was less sensitive to oxidative stress (not shown), these results were highly variable over several biological replicates. PCR analysis to test potential contamination of the mutant strain *glb-1(ok2747)* showed that it was still homozygous for the deletion (Fig. 6A and 6B). Note that the *(ok2747)* deletion removes the *glb-1* gene completely. However, further screening indicated that the full *glb-1* gene was still present in this mutant (Fig. 6B) and surprisingly even expressed at almost normal levels (Fig. 6C). Several additional *C. elegans* strains in which the mutation *(ok2747)* was crossed in also still carried the full *glb-1* gene (not shown). We therefore reasoned that the mutation process

to create the *glb-1(ok2747)* mutant removed the entire *glb-1* gene, together with predicted regulatory regions immediately up- and downstream of the gene, only for it to become inserted at another location in the genome.

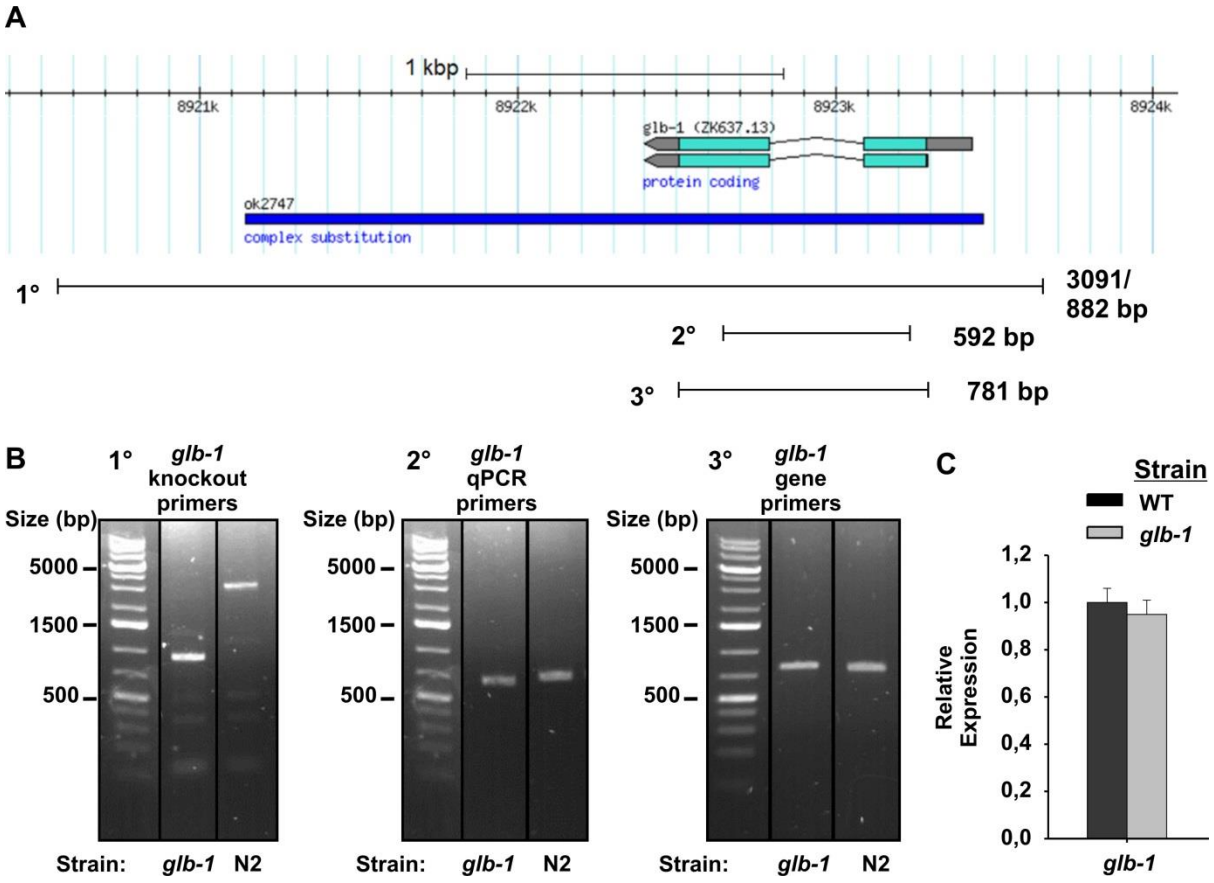

Figure 6: (A) Schematic overview of *glb-1* gene structure and the mutation present in the *glb-1(ok2747)* mutant used in this study. 1-3 show the PCR amplicon sites and amplicon length of the PCR products that were used to analyze the *glb-1(ok2747)* mutant in **(B).** Note that the panels 1, 2 and 3 in (B) are composed of cropped images; only the relevant lanes are shown, other lanes have been removed. **(C)** Expression analysis of *glb-1* in WT worms and the *glb-1(ok2747)* mutant, showing that *glb-1* is still expressed in the *glb-1(ok2747)* mutant.

We hypothesize that the presence of small and adjacent regulatory regions are capable of inducing expression of the relocated *glb-1* gene under normal conditions, but are not always sufficient to regulate expression following stress conditions, explaining the observed variation in our stress response screen. It is remarkable however that the *glb-1* mutant showed a reduced metabolism, which seemed in line with a role for GLB-1 in O_2 metabolism. However, this effect could equally be caused by *glb-1*'s relocation and disruption of a gene involved in metabolism. In effect, these results made clear that the *glb-1(ok2747)* mutant cannot be used for further analysis.

RNAi against *glb-1*, while effective in reducing *glb-1* levels, was unable to support a role for GLB-1 in stress response (not shown). However, similar to GLB-15, residual levels of GLB-1 could still be sufficient for its function. An additional limitation of RNAi is that it is not suited to grow large numbers of worms, which are needed for the metabolic assays used here. For these reasons, we decided to halt further analysis on *glb-1*, and for future research try and generate a new and "true" *glb-1* mutant strain.

4. Discussion

To survive periods of decreased O_2 availability, aerobic metazoa induce a range of cellular adaptions, thereby achieving a more efficient O_2 uptake and compensating for the lack of available O_2. In animals, adaptations to hypoxia are mainly regulated by the highly conserved transcription factor HIF-1[45,46]. Also hypoxia-induced changes in globin expression have been associated with HIF-1 activity, both in vertebrates and invertebrates[39,40,47,48]. While several hypoxia-sensitive globins have well-defined functions in O_2 uptake and storage[83,120], other hypoxia-sensitive globins seem to carry out novel functions. In this study we analyzed if members of the *C. elegans* globin family are involved in the response to hypoxia. Identified hypoxia-sensitive globin genes were tested for HIF-1 dependency, as well as for potential phenotypes associated with O_2 deprivation.

We identified eleven out of the thirty three globin genes that are responsive to hypoxia, showing either up- or downregulation following O_2 deprivation. Only two globin genes, *glb-1* and *glb-15*, were responsive to all three hypoxic conditions tested. A subset of the hypoxia-sensitive genes seems to be completely dependent on HIF-1 for their expression, including *glb-1* and *glb-15*, while other globin genes appear to be either partially dependent or completely independent of HIF-1 activity. A previous whole genome microarray-based approach in *C. elegans* to study hypoxia-induced expression changes identified 110 hypoxia-regulated genes, less than one percent of all genes[296]. One-third of the *C. elegans* globin gene family is hypoxia-sensitive, which is considerably larger and could point to an important role for this globin family in the worm's adaptation to O_2 deprivation. However, it should be noted that the method used here to detect expression level changes, i.e. real-time RT-PCR, is much more sensitive compared to microarray analysis. It therefore appears very likely that a much larger fraction of all genes is responsive to hypoxia than was identified in this previous genome-wide study. In line with this, none of the hypoxia-sensitive globin genes identified here were reported in the earlier microarray screen. Also, while these results give a first indication of HIF-1-dependency of these globin genes,

further analysis is necessary to support these findings. It is thereby important to note that the two mutants with constitutively active HIF-1 that were used in this study, *egl-9* and *vhl-1*, display phenotypic abnormalities that are not necessarily associated with HIF-1 activation[302-304]. These defects could potentially influence globin gene expression and complicate the interpretation of results obtained with these strains.

The majority of the hypoxia-sensitive globins, including GLB-15, is expressed in small subsets of neurons[31]. Nerve globins have been proposed to function as temporary O_2 storage, supporting aerobic metabolism in the metabolic highly active neuronal cells during hypoxic periods[117,305]. In addition, nerve globins could be directly involved in protection against oxidative stress during hypoxia and subsequent reoxygenation[12,306]. Both functions seem unlikely for the hypoxia-sensitive globins in *C. elegans*, given their expression in only a very limited number of neurons. Instead, their distinct expression pattern seems to indicate that these globins could function in O_2 sensing. One *C. elegans* globin, GLB-5, has already been identified to participate in O_2 dependent behavior at atmospheric O_2 concentrations[36,37]. Consistent with a sensory function, GLB-5 is expressed in a small number of O_2 sensing neurons. Likewise, several of the hypoxia-sensitive globins are clearly expressed in sensory neurons. These globins could be directly or indirectly sensitive to changes in O_2 concentration, inducing behavioral changes or metabolic and developmental adaptations when the environment becomes hypoxic.

One hypoxia-sensitive globin, GLB-1, could fulfill a similar role as myoglobin in vertebrates. Like myoglobin, GLB-1 is predominantly expressed in muscle tissue and can reversibly bind O_2 within physiological O_2 concentration limits[34]. GLB-1 could thus act as a temporary O_2 storage and stimulate intracellular O_2 diffusion when O_2 is limiting. In addition to its role in O_2 metabolism, myoglobin can function as NO dioxygenase, preventing excessive NO to inhibit mitochondrial respiration[83]. Also this function seems possible for GLB-1; while *C. elegans* is not capable of producing endogenous NO, bacterially derived NO influences worm physiology, including the induction of *glb-1* expression[301]. In addition, GLB-1 possesses a glutamine residue at the distal side of the heme group (GlnE7)[34], a property that has been observed in multiple invertebrate globins. Its presence increases O_2 affinity, but has also been associated with an increased binding of NO to the heme iron. Finally, similar to myoglobin, GLB-1 could potentially function as ROS scavenger during periods of oxidative stress. The expression of *glb-1* is regulated by both HIF-1 and DAF-16[16,299], two transcription factors that are known to induce the expression of genes associated with oxidative stress defense. Overall, it appears plausible that GLB-1 and myoglobin are functionally related and carry out multiple functions.

In conclusion, we observed that one-third of the *C. elegans* globin genes is hypoxic-responsive. A subset of these genes appears to be regulated by the transcription factor HIF-1. Because RNAi for these hypoxia-sensitive globins did not cause an obvious phenotype, we were unable to test their potential functions. However, the number of available *C. elegans* mutants is continuously increasing and loss-of-function mutants for these hypoxia-sensitive globins are expected to become available. This will allow more detailed analysis of these proteins, including the examination of the here proposed functions.

5. Material and methods

Worm culturing

Culturing of *C. elegans* was carried out according to standard procedures. *C. elegans* strains were cultured at 20°C on cholesterol-supplemented nutrient agar (OXOID) plates containing a lawn of freshly grown *Escherichia coli* K12 cells[307]. To obtain synchronized cultures, gravid adults were lysed by hypochlorite treatment and eggs were allowed to hatch overnight in S buffer[307]. Strains used were Bristol N2 wild-type worms, ZG31 (*hif-1*(*ia04*)), JT307 (*egl-9*(*sa307*)), and CB5602 (*vhl-1*(*ok161*)).

Expression analysis under hypoxia

For hypoxia treatments, plates containing synchronized young adult worms were placed either in room air (normoxia control) or in a hypoxic chamber (MIC-101, Billups-Rothenberg Inc.) with constant gas flow. Worms were incubated for 12 h in 21% O_2 (normoxic) or the indicated O_2 concentrations, at 20°C. After 12h, animals were quickly harvested in S-buffer (43.55 mM KH_2PO_4, 6.45 mM K_2HPO_4 and 100 mM NaCl in distilled water, pH 6) and samples were stored at -80°C. RNA was extracted using the RNeasy Midi kit (Qiagen) according to the manufacturer's instructions. All samples were treated with DNase (Zymo Research). A NanoDrop ND 1000 spectrophotometer (Isogen) was used to analyze RNA concentration and purity. First strand cDNA was synthesized from 2 µg RNA using an oligo(dT) primer and Moloney murine leukemia virus reverse transcriptase (Fermentas) at 42°C for 1 hr. Quantitative RT-PCR was carried out using a Rotor-Gene 2000 centrifugal real-time cycler (Corbett Research) using the Platinum SYBR Green qPCR Super-Mix-UDG (Invitrogen) as described previously[16]. A single melt peak for each reaction confirmed the identity of each PCR product. The threshold cycle (Ct) values of the Rotor-Gene software version 6.0 (Corbett Research) were exported to qBase version 1.3.5[308] for further analysis. All measurements were produced in duplicate, and for each primer set, reaction efficiency estimates were derived from standard curves that were generated using serial dilutions of a cDNA pool of normoxic and hypoxic nematode samples. These were then used by qBase to transform the Ct values to relative quantities that were normalized using the geometric mean of three reference genes (*tba-1*, *csq-1* and *cdc-2*) identified by the geNorm 3.4 software from a set of 8 candidate control genes[309]. The exposure of N2 worms to three different hypoxic conditions was performed in four biological replicates; the experiments involving the strains *hif-1*(*ia04*), *egl-9*(*sa307*) and *vhl-1*(*ok161*) were varied out in triplicate. Statistical analysis was performed by using a two-way student's t-test.

Molecular biology

Translational reporters were made using fusion PCR, as described by Hobert [202]. These reporters contain the target gene, and approximately 3 kb upstream and 0.5 kb downstream of the target gene, to include endogenous promoter and 3'UTR elements. 5' upstream and 3' downstream sequences for *glb-1* and *glb-15* were extracted from the UCSC Genome Browser database (http://genome.ucsc.edu). The *gfp* gene was fused at the 3' side of the target gene. A PCR product coding for the *unc-119* gene was used as a co-injection marker. Final PCR products were injected into the gonads of young adult hermaphrodites, at a concentration of 50 ng/µl for the reporter and 20 ng/µl for the *unc-119* gene. Microinjection was carried out by injecting DNA into the gonads of young adult hermaphrodites using an Axio-Vert 135 (Zeiss) microscope and FemtoJet microinjection system (Eppendorf). For each promoter-GFP fusion construct 20 to 30 wild-type N2 worms were injected. For imaging, worms were mounted on agarose pads and immobilized with 10mM sodium azide. Images were taken using a D-Eclipse C1 Confocal Microscope (Nikon).

Metabolic rate measurements, and ATP and body volume quantification

Respiration and heat dissipation rates by living animals were performed and corrected to whole worm protein content as previously described[310]. ATP quantification of homogenised worm samples was carried out as previously described[311]. Analysis of length and width of young gravid worms was performed with a particle analyzer (RapidVue; Beckman Coulter Inc., Miami, FL, USA), and 2000 worms per strain were measured. Body volume was estimated by approximating the worm as a cylinder.

Feeding RNAi

RNAi was applied by feeding bacteria expressing dsRNA to the worms. RNAi induction was performed as described by Timmons *et al*[204].

PART III: DISCUSSION

Chapter V:
General discussion and conclusion

1. Introduction

The globin superfamily, characterized by a common tertiary structure and the presence of a heme group, is an ancient and ubiquitous group of proteins[23,25,67]. The increasing availability of genome and transcriptome sequence data revealed that most organisms contain genes with homology to globins. This also showed that considerable variation is present in both gene and protein sequences within this family. Detailed physicochemical and structural analyzes of different globin types has made clear that structural variations and conformational changes of the globin fold can greatly influence ligand affinity of the heme group and therefore most likely has a major impact on the function of globins[27,29,34,56,73,230]. At the same time, it has been difficult to link this knowledge to a better understanding on the *in vivo* physiological role of these globins. However, it is clear that these proteins are capable of fulfilling a wide range of functions, from O_2 transport over O_2 sensing to enzymatic redox reactions[12].

A detailed *in silico* analysis of the complete genome sequence of *C. elegans* provided a set of 33 potential globin genes, an unusually high number[32]. Several of these genes show clear homology with vertebrate globins, making them particularly interesting candidates for in-depth analysis. Further research confirmed the expression of all 33 *C. elegans* globins, showed that they are very diverse in gene and protein structure and that they are expressed in a variety of cell types[16,31]. It was therefore hypothesized that this group would be diverse in function as well. More detailed analysis of individual members of this family has thus far supported this hypothesis; globins have been shown or predicted to function in O_2 storage (GLB-1)[34], O_2 sensing (GLB-5, GLB-6)[35-37], redox sensing and signaling (GLB-6, GLB-12 and GLB-26) (this thesis and [34,35,132]), and protection against oxidative stress (GLB-13)[33]. In addition, expression analysis has indicated that specific subsets of globins are responsive to O_2 shortage or show modified expression in specific life stages, such as the dauer stage (this thesis and [16]). In the following, the characteristics and known and potential functions of the *C. elegans* globins will be discussed in more detail.

2. Globins in redox biology

Three *C. elegans* globins, GLB-6, GLB-12 and GLB-26, are or appear to be implicated in redox biology (this thesis and [35,132]). Interestingly, these 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 two histidine side chains. Initial analysis showed that the distal histidine side chain in these types of globins is capable of reversible dissociation to allow the stable binding of gaseous ligands, like CO, NO and O_2[30]. However, GLB-6 is almost incapable of binding ligands, while GLB-12 and GLB-26 bind CO only at reduced rates. This indicates that the heme iron is very tightly hexacoordinated and that these globins 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 points to a function in redox reactions for all three globins. The absence of ligand binding would thereby help to keep the reduction potential of the heme iron unaffected.

The crystal structure for the GLB-6[35] and GLB-12 (this thesis) globin domain has been solved, while the GLB-26 three-dimensional structure has been modelled using GLB-6 as template[230]. These results show that the three globins also show remarkably 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 on the distal side of the heme, which is expected to hamper ligand binding and thus corresponds to the observed ligand binding characteristics.

The results presented in this thesis have confirmed a role in redox signaling for GLB-12 and support such a function for GLB-26[132]. GLB-26 is exclusively expressed in two muscle cells and one cell in the head region of the worm, 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. Interestingly, GLB-26 localizes to both the cell and the nuclear membrane. Further research should help clarify these observations.

GLB-12 is present in several neurons, the developing vulva and parts of the somatic gonad. From the somatic gonad, GLB-12 has an essential role in germline development and regulation by producing $O_2^{\cdot-}$ as a signaling molecule. Fascinating, GLB-12 interacts with two SODs and these three proteins appear to form a redox signaling module, with signaling occurring on both sides of the cell membrane. We hypothesize that the separation by the cell membrane provides an additional level of regulation in the GLB-12 mediated redox signaling pathway, a property

that might also be a more general principle in the field of redox signaling. In addition to its effect on reproduction, *glb-12* RNAi infrequently led to worms with a protruding vulva. It appears likely that the expression of GLB-12 in the vulva is associated with the developmental regulation of this tissue, perhaps even with a similar molecular mechanisms as to what is observed in the somatic gonad. To analyze the role of neuronal GLB-12, *glb-12* RNAi has also been performed in strains that show both enhanced and exclusive neuronal RNAi, but this did not lead to obvious behavioral phenotypes. It is therefore unclear what the role is of GLB-12 in neurons, but a sensory function seems plausible.

GLB-6 has not yet received functional analysis. 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[31], 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 two 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 cell biological 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 three globins supports a spatially confined function, such as cell signaling. We indeed 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. This spatial restriction is assumed to be a general principle for redox signaling proteins[133]. Interestingly, a membrane localization dependent on the presence of fatty acids has also been identified for several other globins outside of *C. elegans*[21,228,229]. 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[122]. The authors 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 three hexacoordinated globins which share several biochemical and structural characteristics that associates them with a role in redox biology. Additional functional analysis of GLB-12 showed 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 three *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 more 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.

3. Hypoxia-sensitive globins

The natural habitat of *C. elegans* remains largely unknown, but the worm is found in antropogenic habitats such as compost and garden soil. In general, *C. elegans* is thought to live in decaying organic material and other nutrient-rich environments, where it feeds on bacteria and other microorganisms. In these settings, *C. elegans* is expected to move between micro-environments that show variation in environmental characteristics, such as temperature, humidity and O_2 concentration. The worm is capable of adapting to these varying conditions by a number of stress signaling cascades that alter its metabolism and physiology[296,312,313]. These stress response pathways also allow *C. elegans* to surviving harsh hypoxic and even anoxic periods[314]. Expression analysis showed that several globin genes change expression following both anoxic and hypoxic conditions (this thesis and [16]). Remarkably, there is little overlap in globin genes that are anoxia-sensitive and hypoxia-sensitive, indicating that these globins are part of distinct stress responses. It is indeed recognized that at least three separate signaling cascades are activated in response to different levels of O_2 shortage[314]. One of these cascades leads to activation of the transcription factor HIF-1[45,296] and several hypoxia-sensitive globins indeed appear to be regulated by HIF-1. Whereas the traditional view would be that these hypoxia-sensitive globins have a function

directly related to O_2 metabolism, such as O_2 storage and facilitation of O_2 diffusion, this is most likely not the case for the majority of *C. elegans* globins. Because of its small size, every cell in the worm is in close proximity to the outside environment, and O_2 exchange under normal conditions is probably achieved by simple diffusion[303]. Consistent with this hypothesis, *C. elegans* lacks both a specialized respiratory and circulatory system, which would be necessary to transport O_2 throughout the body. Consequently, the majority of globins in this small organism do not seem to act as O_2 transporters. It appears more likely that several of these globins are involved in sensing varying O_2 concentrations, given that the majority of them are expressed in distinct subsets of neurons. As O_2 sensors, they can transmit this information to downstream signaling cascades, resulting in behavioral or physiological adaptation to the prevalent O_2 concentrations. Two globins, GLB-5 and GLB-6, have indeed been implicated in O_2 sensing that is related to behavioral changes[35-37]. However, there appears to be at least one globin that fulfills the classic roles in O_2 metabolism, namely GLB-1. This globin shares several functional characteristics with myoglobin; it can reversibly bind O_2[34], is mainly expressed in muscle tissues and is responsive to O_2 deprivation. In addition to its well-studied role in O_2 metabolism, myoglobin is increasingly being appreciated to function in NO metabolism as well[83]. Interestingly, GLB-1 expression is also sensitive to changes in environmental NO levels[301]. GLB-1 could thus be of great value to better understand the multiple roles carried out by vertebrate myoglobin. In general, the anoxia- and hypoxia-sensitive globins could become very informative to appreciate the range of functions that can be carried out by globins and will most likely reveal how these proteins can function as O_2 sensors. Equally informative will be how these globin functions are integrated with stress signaling pathways. Because these stress responses are often well conserved, insight in their mode of action can also potentially be translated into a better understanding of these processes in higher organisms.

4. Globins in dauers

A previous report from our group showed that nine globin genes, including GLB-12, are upregulated in dauers relative to young adults, while one globin, GLB-26, displayed a non-significant downregulation[16]. Dauers are an alternative and arrested third larval stage that will develop in response to harsh environmental conditions[125]. The decision to develop to the dauer stage hinges on the integration of three environmental parameters: population density, reflected by pheromone concentration, food supply, and ambient temperature[315]. In line with this, the presence of chemosensory neurons with functional cilia is important for the ability to develop to

the dauer stage. It is assumed that dauer arrest depends on both stimulatory and inhibitory signals from different sensory neurons[316]. The nine globin genes that show an increased expression in dauers are all expressed in subset of neurons[16]. Consequently, it appears plausible that some of these globins could be directly involved in the decision to dauer formation by acting in the detection of environmental cues. One way to asses this is by determining if knockout or knockdown of these globins directly influences dauer formation. Additionally, it could be valuable to determine if any of these globins are involved in the correct development or functioning of the neurons they are expressed in, e.g. by assessing if knockdown or knockout of these globins influences the morphology of neurons or neuronal cilia, or by performing specific functional and behavioral assays to identify mutant worms with defective cilia. In addition to the switch to dauer development, dauer larvae show several characteristics that are distinct from larvae that develop in non-stressful conditions: they possess a dauer-specific cuticle that protects them from harsh environmental conditions, do not feed, have a blocked buccal cavity and a pharynx that is constricted and non-pumping, utilize the glyoxylate cycle to generate carbohydrates from lipid stores instead of using aerobic respiration, arrest germline development, are more stress resistant and show elevated levels of antioxidant enzymes[316]. The downregulation in dauer larvae of GLB-26, which is involved in the regulation of the defecation cycle, can be explained by the fact that dauers are non-feeding. It is at this moment hard to determine how the globin genes upregulated in dauers are involved in the large number of unique morphological and physiological traits that are associated to this larval stage. However, four distinct pathways have been identified that regulate dauer arrest, i.e. a guanylyl cyclase pathway, a TGFβ-like pathway, an insulin-like pathway and a steroid hormone pathway[316]. It could therefore be informative to assess if any of these globin genes are regulated by these pathways, which might help in determining in which biological process these globins are involved. Previous analysis from our group, whereby globin gene expression was assessed in a *daf-2* mutant in which this insulin-like pathway is activated, did not show any changes in expression for these nine globin genes relative to WT worms, indicating that they are not regulated by this specific signaling cascade. A similar approach could be followed to determine if any of the other pathways are involved in globin expression and so aid in the further analysis of these globins associated with the dauer stage. Of final note, there is little overlap in the globin genes that are differentially regulated following dauer formation, following anoxia and following hypoxia relative to control worms; GLB-23 is upregulated in both dauers and following anoxia, and GLB-24 is upregulated in dauers and following hypoxia. No globin genes are differentially

regulated in all three conditions. The functional relevance of these results is however unclear at this moment.

5. ROS scavenging globins

A recent report indicated that the *C. elegans* globin GLB-13 is involved in protection against oxidative stress[33]. This globin is expressed in a limited number of head and tail neurons[31] and shows homology to vertebrate neuroglobin, based on similarity in protein sequence[32]. Because the latter has been implicated in protection of neurons against oxidative stress[30,306,317,318], it was further analyzed by Ren and colleagues if such a role is also possible for GLB-13. They indeed found that a ROS-producing reagent increases *glb-13* expression and hampers development of a *glb-13* mutant in comparison with WT worms[33]. Fascinating, they were able to rescue the ROS-sensitive phenotype of the *glb-13* mutant by pan-neuronal expression of vertebrate neuroglobin. While these results suggest that GLB-13 and neuroglobin are functionally related, it does not necessarily mean that both globins are directly responsible for ROS detoxification. Also the limited expression of GLB-13 in only a handful of neurons argues against such a role; a ROS-scavenging globin would be expected to be present in most, if not all neurons. Alternatively, it is possible that GLB-13 indirectly plays a role in oxidative stress levels, by influencing cell biological processes that are associated with ROS production, e.g. mitochondrial functioning, or by influencing stress signaling associated with oxidative stress protection. Mitochondria are the major cellular source of ROS and are actively involved in controlling oxidative stress levels[154]. Furthermore, neuroglobin appears to localize near mitochondria[108], while increased neuroglobin levels are associated with preserved mitochondrial function (reviewed by Yu *et al.*[319]). It is therefore hypothesized that neuroglobin can have neuroprotective roles through mitochondria-mediated pathways. Mitochondria and mitochondria-associated stress response pathways are heavily researched in *C. elegans* and it could be fruitful to apply the methods and knowledge developed in this research to assess the potential relationship between GLB-13, mitochondria and oxidative stress responses. Ren and colleagues further determined if antioxidant activity (SOD activity, SOD/catalase ratio and glutathione levels on whole worm homogenate) was increased in the *glb-13* mutant, but could not detect any difference with WT worms. These methods have their limitations though, such as loss of spatial resolution, and also here additional and complementary methods are available that could be very informative. Overall, the relationship between GLB-13, neuroglobin and the response to oxidative stress is very intriguing and deserves further attention.

6. Unusual globin extensions

The additional interhelical and N- and C-terminal extensions of variable length found in various *C. elegans* globins create a wide diversity among this globin family. Internal GH interhelical extensions of unusual length are seen in 6 globins (up to 40 amino acids in GLB-17), N-terminal extensions are found in 12 globins (up to 372 amino acids in GLB-33) and C-terminal extensions are found in 10 globins (up to 120 amino acids in GLB-4)[32]. Their functional relevance is however largely unclear. Such extensions have also been observed in vertebrate cytoglobin, androglobin and globin X, as well as in several invertebrate globins and in fungal flavohemoglobin-like proteins[22,68,110,228,320,321]. The structure of vertebrate androglobin is particularly interesting, showing an N-terminal calpain-like domain and an IQ calmodulin-binding motif flanking the internal globin domain, while the latter is split into two parts and permutated, with helices C to H followed by helices A and B[68]. Androglobin however appears to be absent in nematodes. Of the *C. elegans* globins with N- and C-terminal extensions, only GLB-33 carries a recognizable second protein domain, namely a G-coupled receptor-like domain. It is hypothesized that both the G-coupled receptor-like domain and the globin domain can act as sensors, respectively to a peptide ligand and gaseous ligand. This information is then most likely passed on by the G-coupled receptor-like domain to a downstream signaling cascade. Several other *C. elegans* globins carry extensions of sufficient length to harbor an additional protein domain[32], but such domains have thus far not been identified. Alternatively, some of these extensions might function as signal sequences to direct subcellular location, which appears to be the case for GLB-26, or facilitate membrane anchoring, as observed for GLB-12 and GLB-26 (this thesis and [132]). Bioinformatic tools indeed predict with varying confidence such roles for several of these N- and C-terminal regions (personal observation). These extensions, together with a very specific expression pattern, could thus be important for globins that function in signaling, like GLB-12, or have other organelle-specific functions. Furthermore, some extensions might carry regulatory post-translational modification sites or could be important to mediate protein-protein interactions. It has been observed that signal sequences participating in protein-protein interactions commonly lack a stable secondary and tertiary structure, which allows for the highly specific binding of a great variety of proteins[322]. This lack of a stable secondary and tertiary structure appears to be in line with the absence of a recognizable protein domain in these N- and C-terminal regions (personal observation and [31]). Finally, given that the interhelical loops are directly located within the globin domain, they could have an important structural role in the overal globin fold and so directly influence the functional properties of the latter. Likewise, some

N- or C-terminal extensions could similarly participate in the regulation of the functional properties of the globin domain by influencing its structure.

7. Globin diversification in nematodes

Finally, why do nematodes like *C. elegans* have such an unusual high number of globins? While initially it appeared that only *C. elegans* had 33 globin genes, more recent sequence data from other nematode species, including distantly related nematode species, shows that a large number of these globin genes is conserved within this phylum (personal observation and [31]). One possibility for this diversification is that nematode evolution has exploited the potential roles of globins to their full extent. Because small changes in globin structure can lead to profound effects on functional properties of these proteins, evolutional variation could have quickly generated multiple globin types, each with different functional properties. Together with cell-specific expression patterns, this diversification could have allowed for these globins to fulfill an array of functions and become integrated in the organism's physiology. For example, it can be hypothesized that in nematodes multiple globins function as O_2 sensors, each with their own sensitivity range. Given the ecology and small size of many nematodes, these animals can encounter dramatic changes in O_2 concentration moving from one micro-environment to another. Several O_2 sensing globins combined would allow these small organisms to detect subtle differences in O_2 over a wide O_2 range, and thus be able to respond with suitable behavioral or physiological responses. In the same line, a group of nematode globins, each with small differences in functional properties, could be integrated in the redox biology of these organisms, catalysing different types of redox reactions or functioning as a set of redox sensors. This last hypothesis already appears to be supported by the analysis of GLB-6 and GLB-12, both present in sensory neurons and showing only minor biochemical differences.

Direct homologs for these thirty-three globins cannot be straightforwardly detected in animal groups other than nematodes[31]. It thus appears that this large evolutionary radiation of globins is specific to nematodes and occurs in a more limited form in most other organisms. Alternatively, it could be that the full width of globin diversity in other organisms has not yet been detected, due to lack of sequence data. One exeption is formed by the globin family of diverse chironomid species, with more than 40 globin genes in the genome of *Chironomus tentans* [323]. This is the largest globin gene family reported so far in any organism. Unlike in *C. elegans*, there is almost no functional data on the expression or biochemical characteristics of these globins, leaving the presence of this large family thus far unexplained at the functional level. It is however known

that globin synthesis in Chironomidae appears to be largely restricted to the aquatic larval and pupal stages. Fascinatedely, several globins have also been detected in adult ovaries and eggs[324,325]. These globins have been suggested to serve a nutritional function during embryogenesis, as their concentration was found to decrease throughout embryonic development. However, while most organisms do not have large numbers of globins, they do possess multiple globin types, each most likely with unique properties. In addition, single globin types, like vertebrate myoglobin, can fulfill multiple functions. It can thus be expected that globins in many organism will be involved in a range of physiological processes. Research on the *C. elegans* globin family has already uncovered novel functions for these proteins and has, to this date, confirmed that these globins are functionally diverse. It therefore appears likely that *C. elegans* can also become a model organism for the functional diversity that exists within the globin superfamily.

8. Perspectives

The discovery and family-wide characterization of the large and diverse globin family in the model organism *C. elegans* presented a compelling opportunity to study the variety of roles that can be performed by globins. The subsequent analysis of individual members of this globin family however proved to be quite difficult: RNAi depletion for the majority of these globins did not result in a detectable phenotype and knockout mutants were not available at first. This made it challenging to decipher the potential function for any individual member of this family. Since the start of this thesis, additional genome-wide RNAi screenings presented highly-specific phenotypes for several globins and multiple knockout mutants became available. For example, an RNAi screen for muscle defects showed that GLB-10 depletion resulted in abnormal myosin organization in body wall muscles[326] and three different mutant forms are currently available for this globin. A second example is presented by GLB-3, for which a knockout mutant has become available that shows that this globin is involved in reproduction. Similar to the analysis of GLB-12 and GLB-26 in this thesis, the availability of a phenotype and/or knockout mutants for a particular globin will allow analyzing its function with much more focus in future research. Given the inherent diversity of this globin family, this will most likely reveal additional unexpected functions for these proteins and will therefore be of utmost value to advance our understanding on globin functioning. Such a novel function can for example already be expected from the analysis of GLB-33, in which the globin domain is coupled to a G protein-coupled

receptor. This unusual combination indicates a role in gaseous ligand sensing (the globin domain) linked to a signaling function (the G protein-coupled receptor domain).

As mentioned in the introduction, it remains at this moment rather unclear which *C. elegans* globins can serve as direct functional homologes of the globins present in higher vertebrates, including humans. However, this relationship will become clearer as additional functional data becomes available for the *C. elegans* globins. At present, two globins in the worm appear to be functionally related to vertebrate globins. *C. elegans* GLB-1 shares several functional characteristics with vertebrate myoglobin and is most likely a good model for the latter. Also, because of the relationship between GLB-1 and several conserved stress response pathways, it can be expected that in-depth analysis of GLB-1 will help us understand additional functions for muscle globins, more specifically how they can be integrated in stress response pathways and what their role is herein. The relationship between *C. elegans* GLB-13 and neuroglobin might be even more revealing, whereby detailed study of GLB-13 provides an opportunity to study the role of neuroglobin in a relatively simple animal model. In addition to these two globins, several other *C elegans* globins show sequence similarity with vertebrate globins[32] and should be considered as valuable targets for future research.

Finally, the characterization of GLB-12 as a redox signaling protein has added a novel and unexpected role in redox biology for globins. While it can be anticipated that future analysis of *C. elegans* globins will reveal additional functions in redox biology for these proteins, e.g how GLB-26 functions on a molecular level, this research will most likely also support existing principles and present novel hypotheses for the field of redox biology. For example, the similarity in biochemical characteristics between GLB-6 and GLB-12 combined with their expression in sensory neurons indicates that these proteins function together as a set of sensors, catalyzing redox reactions to fulfil their role. Also the characteristics of GLB-26 are intruiging, e.g. its combined presence at both the cytoplasmic and nuclear membrane together with its reactivity towards H_2O_2 could point to a signaling-associated role. Future research on the *C. elegans* globin family could therefore also provide important contributions to the field of redox signaling.

9. Conclusion

In conclusion, the research carried out in this thesis has increased our understanding on the functions that can be fulfilled by globins. In recent years, novel globins and globin-like proteins

have been identified and selected globins have received more detailed analysis. The majority of research in this field has primarily focused on the *in vitro* biochemical properties of these proteins, but it has been very challenging to understand their true physiological roles. Our research on GLB-12 and to a lesser extent GLB-26 connects a detailed biochemical, electrochemical and structural analysis with a phenotypic and molecular characterization. In doing so, we uncover the strong relationship between the properties of these globins and their *in vivo* role in redox biology. Given that globins form a large and evolutionary conserved superfamily, our findings provide a reference model for the function of these proteins in other organisms.

In addition, we describe that one-third of the *C. elegans* globin family is sensitive to hypoxia. Previous expression analysis already showed that several globin genes change expression following anoxic conditions[16]. In future research, these anoxia- and hypoxia-sensitive globins could become very informative to appreciate the range of functions that can be carried out by globins and that are associated with O_2 deprivation. It appears very likely that this research will also reveal how these proteins can function as O_2 sensors.

Overall, the results presented in this thesis expand the currently known functions for globins by showing that they can actively participate in redox signaling. Furthermore, they confirm the functional diversity of the *C. elegans* globin family and provide promising hypotheses on novel globin functions. These hypotheses will form a valuable starting point for further experimental investigations.

ADDENDUM

Chapter VI:
Monitoring H_2O_2 levels in a Complex and Dynamic Tissue

1. Introduction

The functional analysis of GLB-12 and GLB-26 carried out in this thesis showed that globins can act as redox signaling proteins. In addition, this research, together with several results from the literature, strongly indicated that redox signaling forms an integral part of *C. elegans* reproduction. These are discussed in more detail below. Furthermore, the worm's reproductive system is a particularly interesting model to analyze dynamic cell biological processes such as redox signaling. In parallel with this research, the application of genetically encoded redox sensors in *C. elegans* had successfully been introduced in our lab over the last years. Combined, this presented a unique and promising opportunity to study redox signaling in the *C. elegans* reproductive system by using these genetically-encoded redox sensors. The techniques and results in this study are described below. This research initially also assessed the potential involvement of selected globins in *C. elegans* reproduction, by analyzing the effects of their RNAi depletion on reproductive capacity and gonad morphology. However, no additional globins with a strong effect on reproduction besides GLB-12 were identified. While this does not necesseraly rule out the involvement of other *C. elegans* globins in reproduction, the lack of a clear phenotype prevented further research on their potential role herein. Therefore, because this research does not directly involve the study of globins, it has been placed in addendum to this thesis.

2. Monitoring H_2O_2 levels in the *C. elegans* reproductive system

The importance of redox signaling in many aspects of cell biology has been well established over the last 20 years[134,135,138,139,327]. In addition, it is now clear that the redox state of essential molecules in redox biology, e.g. glutathione, or the presence of redox signaling messengers, e.g. H_2O_2, can vary within and between different subcellular compartments and the extracellular space, as well as over time. It is also observed that the redox state of individual redox couples can change independently of each other [1,328]. These spatiotemporal variations of redox couples are assumed to be actively regulated and to play a role in modulating cell signaling[133,134,152]. Many colorimetric, fluorometric and luminescent probes with high accuracy for several distinct redox couples exist and have been widely used for measurements based on whole cell lysates. However, these results do not allow drawing conclusions about the redox parameters present in individual

cellular compartments. At the same time, intracellular dyes for redox parameters have performed poorly and have suffered from many artifacts. It has therefore been extremely challenging to visualize the spatiotemporal character of redox biology and redox signaling *in vivo*[329,330].

In the last years, a large number of genetically-encoded sensors for ions, small molecules and enzymatic activities have been developed, including redox sensors for H_2O_2 and the glutathione redox state. Because these sensors are genetically-based, they can be targeted to specific subcellular compartments. Furthermore, several of these sensors show very high specificity and allow dynamic observations in living cells. Consequently, these sensors have widely opened the possibilities in the field of redox signaling, as they make it possible to study *in vivo* redox signaling in real time, and this at a subcellular level[329,330].

The reproductive system of *C. elegans* is a valuable tissue for the analysis of dynamic biological processes; it shows a high turnover-rate and a complex cellular architecture, and it has become relatively easy to visualize cell biological processes in this organ. In addition, several observations indicate that redox signaling is intimately associated with the regulation of *C. elegans* reproduction; maturing oocytes display a ROS increase that is associated with mitochondrial activity, SOD-1 activity and fertilization[331]; the spermatheca clearly shows a more reduced glutathione redox status compared to other tissues[328]; an altered redox chemistry of a mitochondrial mutant influences several signaling cascades that are associated with germline development[266]; the research presented in this thesis showes that a redox signaling globin is essential for normal germline functioning; and finally our observation that the main intracellular SOD is expressed in the entire somatic gonad, while the extracellular, membrane-bound SOD is present in large parts of the somatic gonad. The *C. elegans* reproductive system could thus also become a powerful model for studying the dynamic aspects of redox signaling in a complex tissue. However, to this date, a holistic analysis on the role of redox signaling in this organ, or in a highly dynamic tissue in general, has not yet been performed.

During my doctoral research, I had the opportunity to participate in the lab of Prof. Dr. Karen Oegema at the Ludwig Institute at the University of California, San Diego, as a visiting scholar. I was able to expand my knowledge on how cell biological processes can be studied *in vivo* and to develop a first set of tools for the analysis of redox signaling within the *C. elegans* reproductive system. The latter was achieved by expressing the probe roGFP2-ORP1 within the germline. roGFP2-ORP1, which visualizes relative changes in H_2O_2 levels, consists of a redox-sensitive fluorescent protein, roGFP2, and a yeast peroxidase, ORP1[332](Fig. 1).

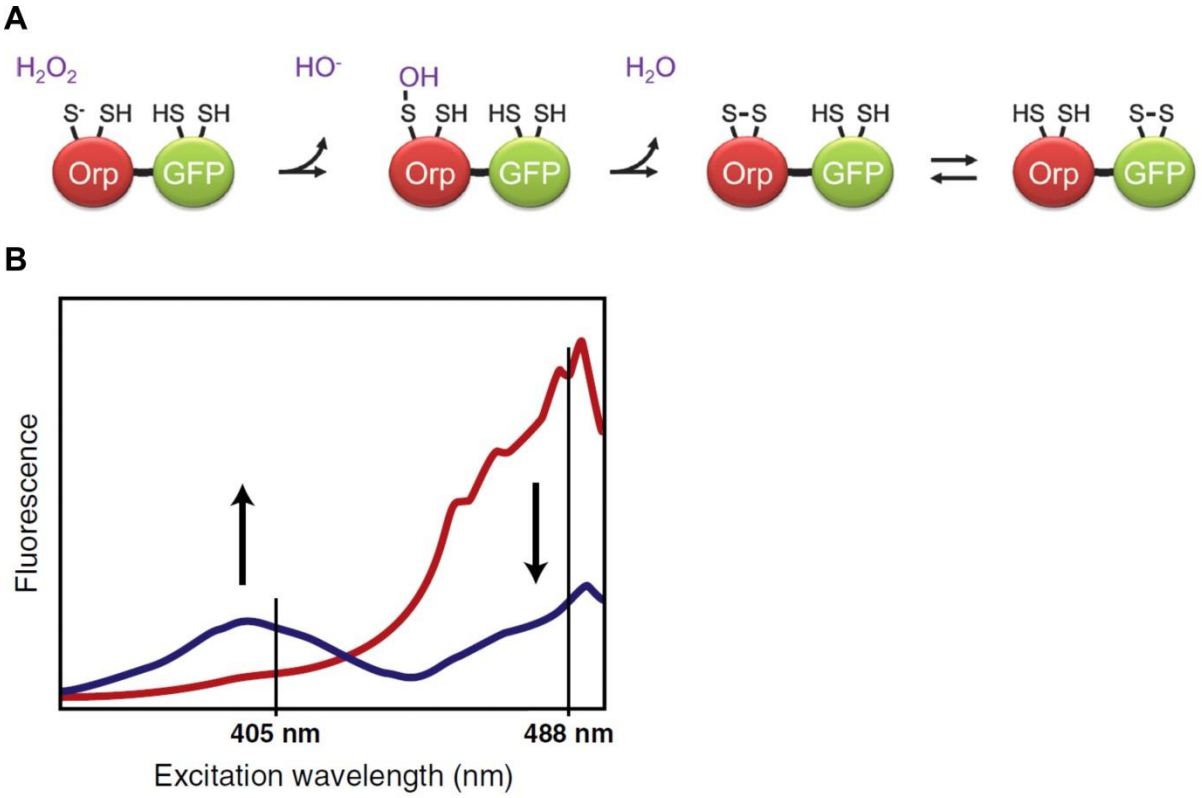

Figure 1: (A) Molecular mechanism of H_2O_2 sensitivity of the roGFP2-ORP-1 probe[1,2]: H_2O_2 oxidizes one cysteine in ORP1, creating a cysteinyl sulfenic acid, after which this reacts with the second ORP-1 cysteine, forming an intramolecular disulfide bridge. This bond is then passed on to roGFP2, influencing the spectral properties of the latter. **(B)** roGFP2 has two excitation peaks, corresponding to the protonated and anionic form of the chromophore[11]. Oxidation of roGFP2, leading to disulfide-bridge formation, results in an increase in the protonated form excitation band (400 nm) and a decrease in the anionic form excitation band (490 nm). The presence of two excitation peaks allows ratiometric fluorescence readout.

The redox-sensitivity of roGFP2 is caused by two cysteine residues present on its surface; these allow the reversible formation of a disulfide bridge, which changes the fluorescent properties of the protein and thus permits discrimination between the oxidized and reduced form (Fig. 1B). While roGFP2 by itself is primarily sensitive to the glutathione redox status and shows a slow equilibration with the surrounding glutathione pool, the addition of ORP1 makes it a highly specific and relatively fast sensor for submicromolar changes in H_2O_2 levels. H_2O_2 oxidation of ORP1 results in the formation of a cysteinyl sulfenic acid and a subsequent intramolecular disulfide bond in this protein. This bond is then passed on to roGFP2 via a thiol-disulfide exchange reaction, thus creating a H_2O_2 sensor with the spectral properties of roGFP2 (Fig.1A). It was demonstrated that ORP1 catalyzes near stoichiometric conversion of H_2O_2 to disulfides in roGFP2 when the two are fused together[332].

The expression of transgenes in the *C. elegans* reproductive system is particularly difficult due to germline silencing of genes lacking the necessary regulatory sequences. Therefore, the gene coding for roGFP2-ORP1 was optimized to allow its expression in this tissue. This approach was based on the thoroughly described optimization of eGFP and mCherry for *C. elegans* (germline) expression[203]. In short, this consisted of 1) introducing artificial intronic sequences, identical to those used in eGFP and mCherry, and 2) adapting codon usage to achieve higher expression. The latter approach has more recently also been described as a way to control protein levels, whereby consistent results were obtained that showed that the relative amount of optimal codons determines the expression level of the protein[333]. Finally, based on the results of Merritt *et al.*[334], a promoter and 3'UTR combination that leads to expression in the entire germline was chosen to drive expression of the gene coding for roGFP2-ORP1 (Fig.2A and 2B).
To study H_2O_2 levels in the *C. elegans* reproductive system, roGFP2-ORP1 was targeted to both the cytosol and the mitochondrial matrix within the germline (Fig.2A and 2B). While roGFP2-ORP1 has an approximately 8-fold dynamic range under ideal conditions[332], the experimental dynamic range can be influenced by a number of parameters, such as microscope sensitivity, interfering background fluorescence and probe expression levels. The experimental dynamic range was determined as described by Morgan *et al.*[11], and included measuring the emission intensity and optimizing microscope settings following complete reduction and oxidation using an excess of DTT and H_2O_2, respectively (Fig.2C).
Following determination of the dynamic range, both the cytosolic and mitochondrial probe were used to visualize H_2O_2 levels within the germline under normal, non-stressful conditions (Fig. 2D and Fig. 4B and C). Interestingly, both probes showed that spatial differences exist in germline H_2O_2 levels. More specifically, both cytosolic and mitochondrial H_2O_2 levels increase towards the proximal side of the germline and peak within oocytes before fertilization and in the zygote directly following fertilization. From the two-cell embryo on, H_2O_2 levels decrease again. The H_2O_2 increase in the proximal germline corresponds with the previously described ROS increase detected in maturing oocytes[331]. Noteworthy, this ROS increase was also dependent on SOD-1 activity, suggesting that a considerable amount of this ROS consists of $O_2^{\cdot-}$ or H_2O_2. In addition, the cytosolic-targeted probe is also present in the nuclei and clearly shows that nuclear H_2O_2 levels are lower compared to cytosolic levels (Fig. 2D, 3A and 3C). This absence of H_2O_2 diffusion between cellular compartments has been observed before[330], and it is assumed that the H_2O_2 permeability of plasma membranes is actively regulated and used for compartmentalization of redox processes[133,152,199]. Overall, these results show that spatial differences in H_2O_2 levels are

present within the *C. elegans* germline under normal conditions, and appear to form an additional indication that H_2O_2 based redox signaling is present in this tissue.

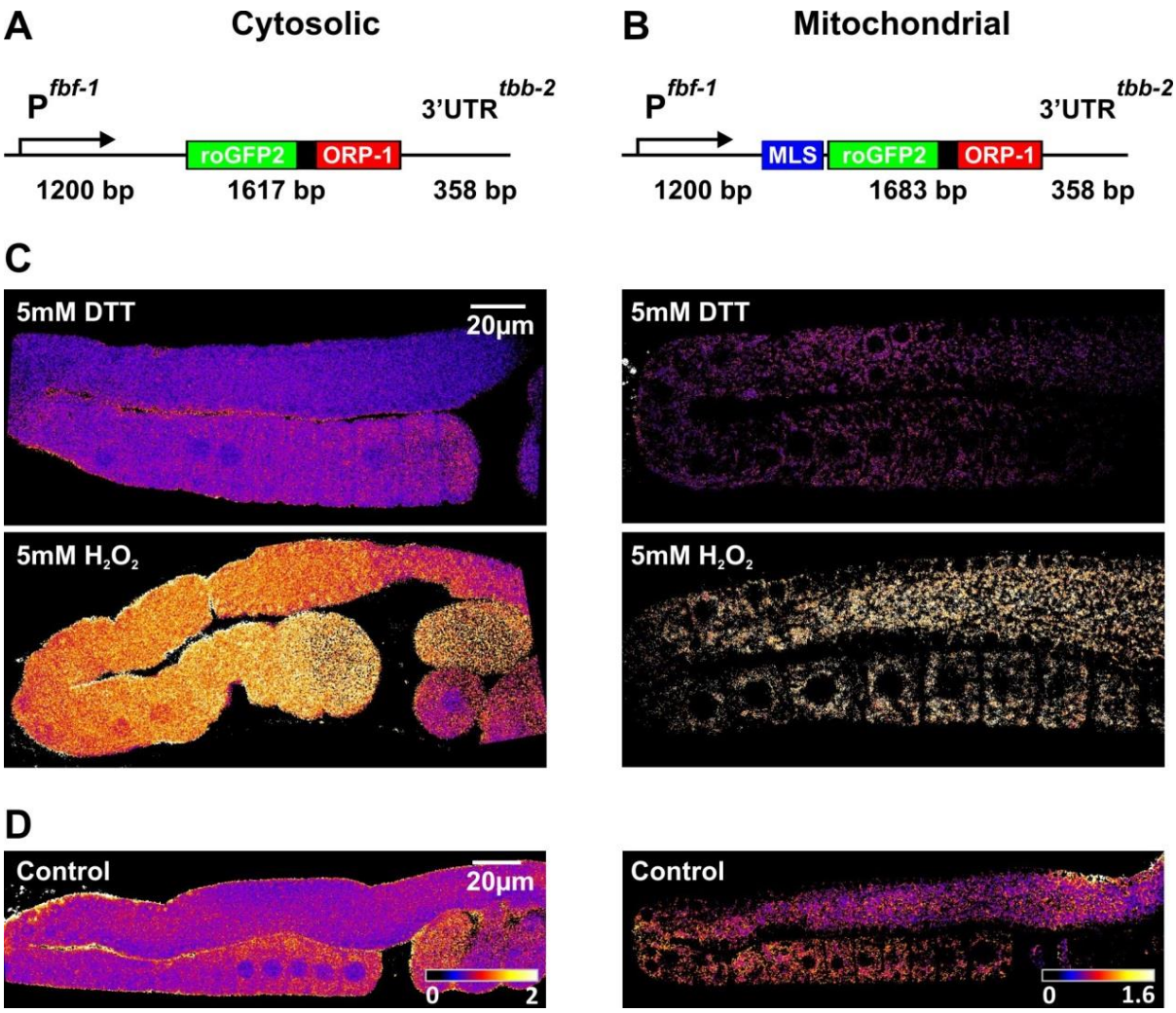

Figure 2: Schematic overview of **(A)** the cytosolic and **(B)** the mitochondrial roGFP2-ORP-1 gene construct (MLS: mitochondrial localization sequence), **(C)** their full reduction and oxidation when expressed in the germline by an excess of DTT and H_2O_2, respectively, and **(D)** their ratio image under non-stressful control conditions.

Next, we analyzed if 1) paraquat and 2) RNAi against a mitochondrial protein would lead to detectable changes in H_2O_2 levels. Paraquat can act as a redox cycling compound and causes increased $O_2^{\cdot-}$ production within mitochondria[335,336]. This leads to oxidative stress and premature death when paraquat is present at high levels, but results in lifespan extension when used at low levels, potentially by modifying redox-sensitive signaling pathways[337,338]. Paraquat could therefore be a useful compound to analyze both the beneficial and detrimental effects of ROS. The second approach, depletion of mitochondrial proteins, generally causes mitochondrial stress and is often

associated with increased mitochondrial ROS production. Like paraquat, partial depletion of mitochondrial proteins can have beneficial effects, while stronger depletion is almost always harmful[339]. However, the level of depletion to induce beneficial effects is *a priori* not known, and varies between different mitochondrial proteins.

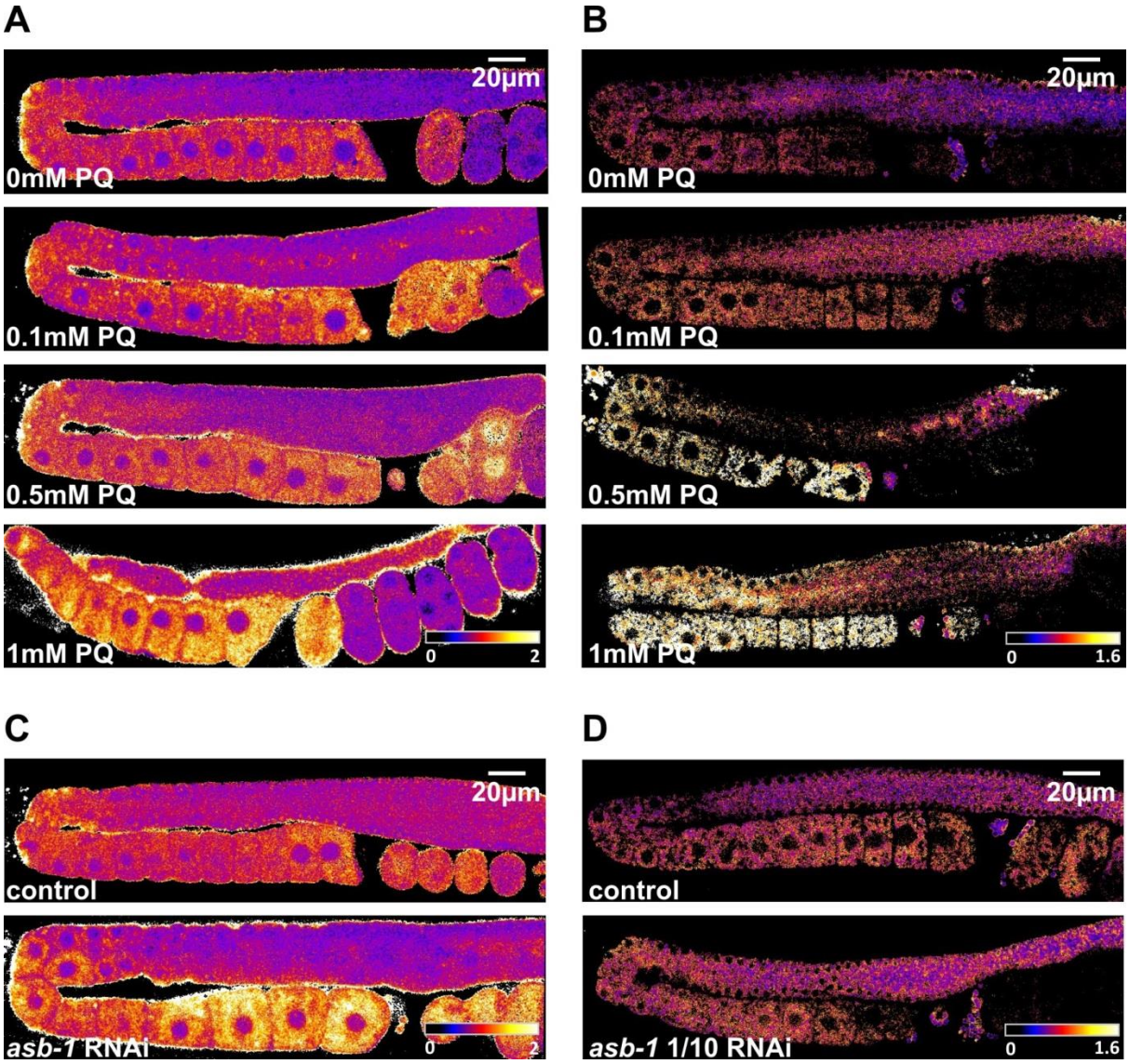

Figure 3: Ratio images of **(A and C)** the cytosolic and **(B and D)** the mitochondrial roGFP2-ORP-1 probe, under control conditions and following exposure to paraquat and following RNAi against the mitochondrial protein ASB-1.

For this analysis, we chose to use both beneficial (~0.1-0.25mM) and harmful (>0.25mM) concentrations of paraquat[337,338] and to deplete the mitochondrial protein ASB-1, a germline-specific ATP-synthase subunit. Because ASB-1 is only present in the germline, ASB-1 depletion does not affect somatic mitochondria, worms develop at a normal rate and do not show any

defects other than those of the germline (personal observation and [340]). However, it is not known if partial depletion of this protein can have beneficial effects in the organism.

Both treatments resulted in increased cytosolic H_2O_2 levels, which were most pronounced in the proximal germline and the one-cell embryo (Fig. 3A, 3C and 4B). Paraquat treatment also caused increased H_2O_2 levels in the mitochondrial matrix, with a spatial distribution that is comparable to this of cytosolic H_2O_2 levels (Fig. 3B and 4C). No changes in H_2O_2 levels in the mitochondrial matrix were observed following ten times diluted *asb-1* RNAi (Fig. 4C). Less diluted *asb-1* RNAi decreases or stops mitochondrial protein import, including roGFP2-ORP1 import (not shown). While the disturbed mitochondrial import clearly shows that *asb-1* RNAi interferes with normal mitochondrial functioning, this also makes it impossible to determine if H_2O_2 levels change within the mitochondrial matrix at these high RNAi concentrations.

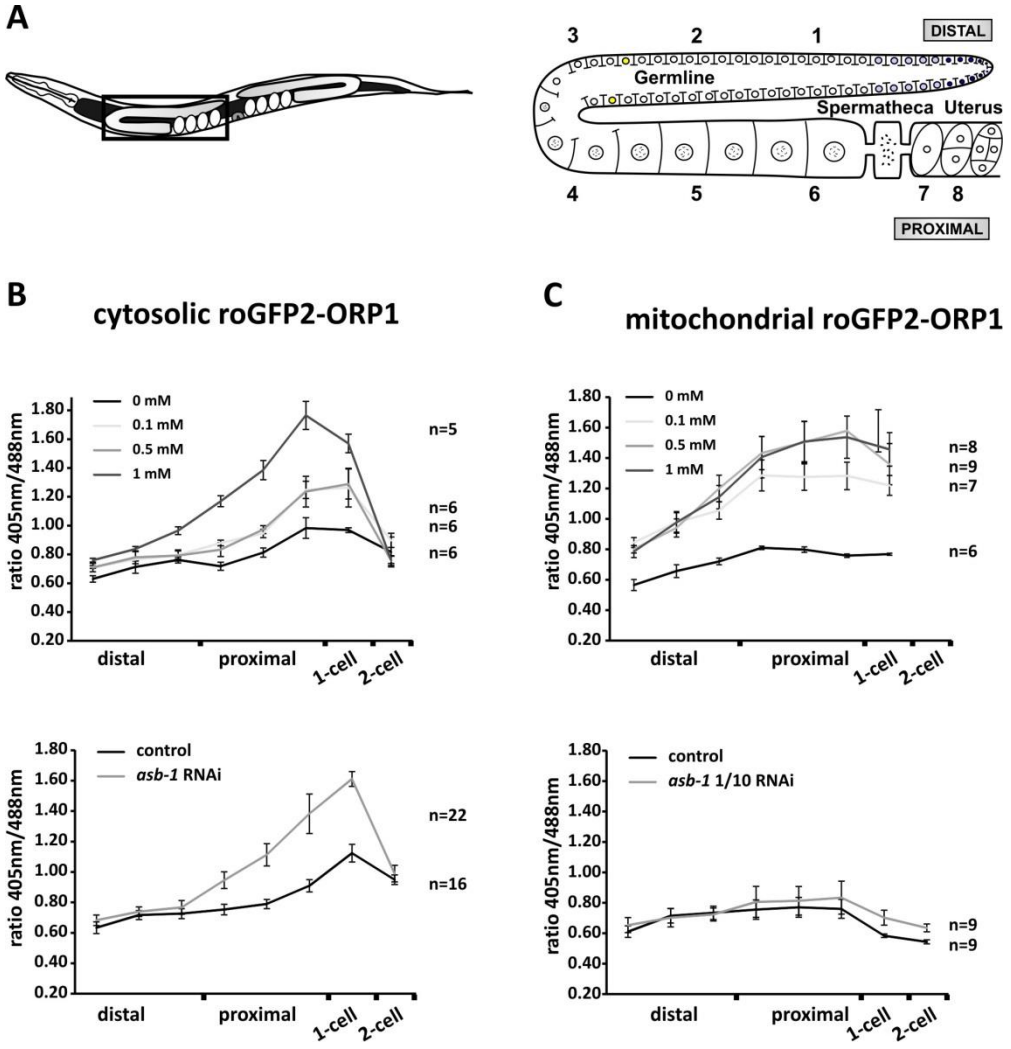

Figure 4: (A) Schematic overview of the *C. elegans* germline, showing the regions where relative H_2O_2 levels were quantified. Region 1-3 corresponds to measurements in the distal germline, 4-6 to the proximal germline, 7 to one-cell embryos and 8 to two-cell embryos. Following this quantification, relative H_2O_2 levels of **(B)** the cytosolic and **(C)** the mitochondrial roGFP2-ORP-1 probe were plotted.

Overall, H_2O_2 measurements following both treatments were quantifiable and reproducible (Fig. 4), confirming the advantages of this reporter. The measured increase in H_2O_2 levels following the lowest paraquat concentration also indicate that the roGFP2- ORP1 probe in the *C. elegans* germline can be used to study changes in H_2O_2 levels that are associated with redox signaling. In other systems, the roGFP2-ORP1 probe has already been show to detect H_2O_2 changes associated with redox signaling[1,332]. In addition, because the additional H_2O_2 increase at higher, harmful paraquat concentrations is also within the dynamic range of the roGFP2- ORP1 probe, this system could potentially be used to analyze both the effects of H_2O_2 in redox signaling and in oxidative stress.

In conclusion, these first results support the potential of the *C. elegans* reproductive system as a model to study redox signaling in a complex tissue. Spatial differences in H_2O_2 levels are visible under normal conditions and small H_2O_2 increases can be reliably detected, both of which might be associated with redox signaling. However, a current limitation of this model is that it is not possible to make dynamic measurements; the laser intensity for excitation at 405nm that is needed for a sufficiently strong emission in this organ also results in rapid probe bleaching. There are potential solutions for this problem, such as an increased sensitivity of the detection system and/or increased expression of the probe.

A second potential improvement to this model is to increase the sensitivity of the roGFP2-ORP1 probe by bringing it closer to the sites of H_2O_2 generation; while enzymatically generated H_2O_2 will be present in spatially defined regions, the cytosolic probe and, to a lesser extent, the probe in the mitochondrial matrix are free to diffuse. As a result, probes that are oxidized at a specific H_2O_2 rich region will diffuse out, which will result in a weaker and spatially less defined signal. This problem can be overcome by targeting the probe to specific membrane regions, such as membrane subdomains associated with signaling or, particularly relevant in this case, the cytosolic side of the outer mitochondrial membrane and the matrix side of the inner mitochondrial membrane. The cytosolic probe used here is already able to detect spatial differences in H_2O_2 levels and targeting the probe to specific subcellular regions is expected to further improve its sensitivity. Targeting the mitochondrial probe to the inner mitochondrial membrane could potentially also improve its sensitivity.

An additional important consideration is that, even though roGFP2-ORP1 is highly specific to H_2O_2 oxidation, subsequent probe reduction depends on interaction with another redox pair, which is most likely the glutathione couple[332]. This means that roGFP2-ORP1 is sensitive to both H_2O_2 and the glutathione redox status, complicating the interpretation of results acquired with this probe. However, there is currently no better alternative for dynamic H_2O_2

measurements. On the other hand, this probe behavior closely mimics how many proteins are redox-regulated inside the cell (H_2O_2 dependent oxidation and subsequent reduction through the glutathione or thioredoxin system) and the probe can therefore be considered as appropriate to study conditions of H_2O_2 based redox signaling[329]. With this in mind, a roGFP2-based probe that is specific for the glutathione-couple also exists and would be very informative to use in parallel. A probe specific for the thioredoxin-couple does not exist yet.

Overall, the use of the roGFP2-ORP1 probe in the *C. elegans* reproductive system has produced several promising first results. In addition, the use of this probe within this tissue allows room for improvement and can potentially be complemented by other genetically-encoded sensors. The potential research questions that can be addressed with this system are vast: from the role of redox signaling in development, cell differentiation, cell and organelle movement, fertilization and stress signaling, to the effects of oxidative stress and the potential therapeutic use and efficacy of antioxidant compounds.

3. Material and methods

C. elegans culture

Maintenance of *C. elegans* was carried out according to standard procedures[307]. *C. elegans* strains were cultured at 20°C on cholesterol-supplemented nutrient agar (OXOID) plates containing a lawn of freshly grown *Escherichia coli* OP50 cells.

Molecular Biology

The roGFP2-ORP1 gene was optimized for expression by changing the overall codon usage to 60% of most optimal codon usage in *C. elegans*, and by introducing five introns in the gene. This was carried out by using the software "*C. elegans* Codon Adapter" (http://worm-srv3.mpi-cbg.de/codons/cgi-bin/optimize.py). Based on the results of Merritt *et al.*[334], the *fbf-1* promoter and *tbb-2* 3'UTR region were selected to achieve expression in the entire germline. Engineered transgenes were cloned into pCFJ150 and injected into strain EG6699 to create integrated single-copy roGFP2-ORP1 transgenes using the MosSCI method[341].

Paraquat treatment

A paraquat stock solution was freshly made before each experiment and was added into NGM media the day before the plates were used. Worms were placed on plates with paraquat starting from the fourth larval stage.

Feeding RNAi

RNAi was applied by feeding bacteria expressing dsRNA to the worms. RNAi induction was performed as described by Timmons *et al.*[204]. Synchronized fourth larval stage worms were placed on NGM plates seeded with freshly induced RNAi bacteria.

Live Imaging

One day old adult hermaphrodites were removed from the plate, anesthetized in a fresh mixture of 1 mg/ml Tricane (ethyl 3-aminobenzoate methanesulfonate salt) and 0.1 mg/ml of tetramisole hydrochloride (TMHC) dissolved in M9 for 15-30 min before transferring them to an agarose pad under a coverslip for imaging. Gonads were imaged by collecting an 10 x 2 mm z-series following excitation at 405 nm and 488 nm. Microscopy was performed with a Nikon Eclipse TE2000-5 confocal microscope.

Image Processing

Image processing was carried out as described by Morgan *et al.*[11]. In brief, images were saved as 16-bit tiff files and processed by ImageJ. Images were first converted to 32-bit tiff files, background was subtracted using an upper and lower threshold and background values were set to "not a number"(NaN). Subsequently, 405 nm images were divided by 488 nm images pixel by pixel to create the ratio images, and displayed in false colors using the lookup table "Fire". Relative H_2O_2 levels in these processed images were quantified by selecting 8 equally sized rectangle areas in a single focal plane, followed by the calculation of the mean ratio in each of the selected areas.

References

1. Albrecht, S.C., Barata, A.G., Grosshans, J., Teleman, A.A. & Dick, T.P. In vivo mapping of hydrogen peroxide and oxidized glutathione reveals chemical and regional specificity of redox homeostasis. *Cell Metab* **14**, 819-29 (2011).
2. WormClassroom. (Laboratory for Optical and Computational Instrumentation, University of Wisconsin-Madison).
3. Battistuzzi, G. et al. Redox reactivity of the heme Fe3+/Fe 2+ couple in native myoglobins and mutants with peroxidase-like activity. *J Biol Inorg Chem* **12**, 951-8 (2007).
4. Raphael, A.L. & Gray, H.B. Axial ligand replacement in horse heart cytochrome c by semisynthesis. *Proteins* **6**, 338-40 (1989).
5. Altun, Z.F. & Hall, D.H. Introduction. Vol. 2009 (Wormatlas).
6. Lints, R. & Hall, D.H. Male introduction. (WormAtlas, 2009).
7. The Bodner Group, D.o.C.E., Purdue University. The Chemistry of Hemoglobin and Myoglobin.
8. Fielenbach, N. & Antebi, A. C. elegans dauer formation and the molecular basis of plasticity. *Genes Dev* **22**, 2149-65 (2008).
9. Cremers, C.M. & Jakob, U. Oxidant sensing by reversible disulfide bond formation. *J Biol Chem* **288**, 26489-96 (2013).
10. Pesce, A. et al. Neuroglobin and cytoglobin. Fresh blood for the vertebrate globin family. *EMBO Rep* **3**, 1146-51 (2002).
11. Morgan, B., Sobotta, M.C. & Dick, T.P. Measuring E(GSH) and H2O2 with roGFP2-based redox probes. *Free Radic Biol Med* **51**, 1943-51 (2011).
12. Vinogradov, S.N. & Moens, L. Diversity of globin function: enzymatic, transport, storage, and sensing. *J Biol Chem* **283**, 8773-7 (2008).
13. Perutz, M.F. Regulation of oxygen affinity of hemoglobin: influence of structure of the globin on the heme iron. *Annu Rev Biochem* **48**, 327-86 (1979).
14. Kapp, O.H. et al. Alignment of 700 globin sequences: extent of amino acid substitution and its correlation with variation in volume. *Protein Sci* **4**, 2179-90 (1995).
15. Phillips, S.E. Structure and refinement of oxymyoglobin at 1.6 A resolution. *J Mol Biol* **142**, 531-54 (1980).
16. Hoogewijs, D. et al. Wide diversity in structure and expression profiles among members of the Caenorhabditis elegans globin protein family. *BMC Genomics* **8**, 356 (2007).
17. Altun, Z.F. & Hall, D.H. Hermaphrodite anatomy. General introduction. (Wormatlas 1.0).
18. Shumyantseva, V.V. et al. A new format of electrodes for the electrochemical reduction of cytochromes P450. *J Inorg Biochem* **100**, 1353-7 (2006).
19. Pesce, A. et al. Human brain neuroglobin structure reveals a distinct mode of controlling oxygen affinity. *Structure* **11**, 1087-95 (2003).
20. Vinogradov, S.N., Tinajero-Trejo, M., Poole, R.K. & Hoogewijs, D. Bacterial and archaeal globins - a revised perspective. *Biochim Biophys Acta* **1834**, 1789-800 (2013).
21. Hoffmann, F.G. et al. Evolution of the globin gene family in deuterostomes: lineage-specific patterns of diversification and attrition. *Mol Biol Evol* **29**, 1735-45 (2012).
22. Hoogewijs, D., Dewilde, S., Vierstraete, A., Moens, L. & Vinogradov, S.N. A phylogenetic analysis of the globins in fungi. *PLoS One* **7**, e31856 (2012).
23. Vázquez-Limón, C., Hoogewijs, D., Vinogradov, S.N. & Arredondo-Peter, R. The evolution of land plant hemoglobins. *Plant Sci* **191-192**, 71-81 (2012).
24. Vinogradov, S.N. et al. A model of globin evolution. *Gene* **398**, 132-42 (2007).
25. Vinogradov, S.N. et al. A phylogenomic profile of globins. *Bmc Evolutionary Biology* **6**, 17 (2006).
26. Mörner, K.A.H. Beobachtungen über den Muskelfarbstoff. Vol. 30 1-8 (Nord. Med. Ark., 1897).
27. Kiger, L. et al. Electron transfer function versus oxygen delivery: a comparative study for several hexacoordinated globins across the animal kingdom. *PLoS One* **6**, e20478 (2011).
28. Hankeln, T. et al. Neuroglobin and cytoglobin in search of their role in the vertebrate globin family. *J Inorg Biochem* **99**, 110-9 (2005).
29. Weber, R.E. & Vinogradov, S.N. Nonvertebrate hemoglobins: functions and molecular adaptations. *Physiol Rev* **81**, 569-628 (2001).
30. Kakar, S., Hoffman, F.G., Storz, J.F., Fabian, M. & Hargrove, M.S. Structure and reactivity of hexacoordinate hemoglobins. *Biophys Chem* **152**, 1-14 (2010).
31. Hoogewijs, D. et al. The Caenorhabditis globin gene family reveals extensive nematode-specific radiation and diversification. *BMC Evol Biol* **8**, 279 (2008).
32. Hoogewijs, D. et al. Genome-wide analysis of the globin gene family of C. elegans. *IUBMB Life* **56**, 697-702 (2004).

33. Ren, C. et al. GLB-13 is associated with oxidative stress resistance in Caenorhabditis elegans. *IUBMB Life* **65**, 423-34 (2013).
34. Geuens, E. et al. Globin-like proteins in Caenorhabditis elegans: in vivo localization, ligand binding and structural properties. *BMC Biochem* **11**, 17 (2010).
35. Yoon, J. et al. Structure and properties of a bis-histidyl ligated globin from Caenorhabditis elegans. *Biochemistry* **49**, 5662-70 (2010).
36. Persson, A. et al. Natural variation in a neural globin tunes oxygen sensing in wild Caenorhabditis elegans. *Nature* **458**, 1030-3 (2009).
37. McGrath, P.T. et al. Quantitative mapping of a digenic behavioral trait implicates globin variation in C. elegans sensory behaviors. *Neuron* **61**, 692-9 (2009).
38. Fraser, J. et al. Hypoxia-inducible myoglobin expression in nonmuscle tissues. *Proc Natl Acad Sci U S A* **103**, 2977-81 (2006).
39. Fordel, E., Geuens, E., Dewilde, S., De Coen, W. & Moens, L. Hypoxia/ischemia and the regulation of neuroglobin and cytoglobin expression. *IUBMB Life* **56**, 681-7 (2004).
40. Gorr, T.A., Cahn, J.D., Yamagata, H. & Bunn, H.F. Hypoxia-induced synthesis of hemoglobin in the crustacean Daphnia magna is hypoxia-inducible factor-dependent. *J Biol Chem* **279**, 36038-47 (2004).
41. Roesner, A., Mitz, S.A., Hankeln, T. & Burmester, T. Globins and hypoxia adaptation in the goldfish, Carassius auratus. *FEBS J* **275**, 3633-43 (2008).
42. Roesner, A., Hankeln, T. & Burmester, T. Hypoxia induces a complex response of globin expression in zebrafish (Danio rerio). *J Exp Biol* **209**, 2129-37 (2006).
43. Schmidt, M. et al. Cytoglobin is a respiratory protein in connective tissue and neurons, which is up-regulated by hypoxia. *J Biol Chem* **279**, 8063-9 (2004).
44. Awenius, C., Hankeln, T. & Burmester, T. Neuroglobins from the zebrafish Danio rerio and the pufferfish Tetraodon nigroviridis. *Biochem Biophys Res Commun* **287**, 418-21 (2001).
45. Jiang, H., Guo, R. & Powell-Coffman, J.A. The Caenorhabditis elegans hif-1 gene encodes a bHLH-PAS protein that is required for adaptation to hypoxia. *Proc Natl Acad Sci U S A* **98**, 7916-21 (2001).
46. Yu, R., Gao, L., Jiang, S., Guan, P. & Mao, B. Association of HIF-1alpha expression and cell apoptosis after traumatic brain injury in the rat. *Chin J Traumatol* **4**, 218-21 (2001).
47. Kristiansen, G. et al. Endogenous myoglobin in breast cancer is hypoxia-inducible by alternative transcription and functions to impair mitochondrial activity: a role in tumor suppression? *J Biol Chem* **286**, 43417-28 (2011).
48. Haines, B. et al. Hypoxia-inducible factor-1 and neuroglobin expression. *Neurosci Lett* **514**, 137-40 (2012).
49. Vinogradov, S.N. et al. A model of globin evolution. *Gene* **398**, 132-142 (2007).
50. KENDREW, J.C. Myoglobin and the structure of proteins. *Science* **139**, 1259-66 (1963).
51. Perutz, M.F. Myoglobin and haemoglobin: role of distal residues in reactions with haem ligands. *Trends Biochem Sci* **14**, 42-4 (1989).
52. Bolognesi, M. et al. Reactivity of ferric Aplysia and sperm whale myoglobins towards imidazole. X-ray and binding study. *J Mol Biol* **158**, 305-15 (1982).
53. Bolognesi, M., Bordo, D., Rizzi, M., Tarricone, C. & Ascenzi, P. Nonvertebrate hemoglobins: structural bases for reactivity. *Prog Biophys Mol Biol* **68**, 29-68 (1997).
54. Royer, W.E., Knapp, J.E., Strand, K. & Heaslet, H.A. Cooperative hemoglobins: conserved fold, diverse quaternary assemblies and allosteric mechanisms. *Trends Biochem Sci* **26**, 297-304 (2001).
55. Duff, S.M., Wittenberg, J.B. & Hill, R.D. Expression, purification, and properties of recombinant barley (Hordeum sp.) hemoglobin. Optical spectra and reactions with gaseous ligands. *J Biol Chem* **272**, 16746-52 (1997).
56. de Sanctis, D. et al. Structure-function relationships in the growing hexa-coordinate hemoglobin sub-family. *IUBMB Life* **56**, 643-51 (2004).
57. Dewilde, S. et al. Biochemical characterization and ligand binding properties of neuroglobin, a novel member of the globin family. *J Biol Chem* **276**, 38949-55 (2001).
58. Forrester, M.T. & Foster, M.W. Protection from nitrosative stress: a central role for microbial flavohemoglobin. *Free Radic Biol Med* **52**, 1620-33 (2012).
59. Martínková, M., Kitanishi, K. & Shimizu, T. Heme-based globin-coupled oxygen sensors: linking oxygen binding to functional regulation of diguanylate cyclase, histidine kinase, and methyl-accepting chemotaxis. *J Biol Chem* **288**, 27702-11 (2013).
60. Gilles-Gonzalez, M.A. & Gonzalez, G. Heme-based sensors: defining characteristics, recent developments, and regulatory hypotheses. *J Inorg Biochem* **99**, 1-22 (2005).
61. Freitas, T.A., Saito, J.A., Hou, S. & Alam, M. Globin-coupled sensors, protoglobins, and the last universal common ancestor. *J Inorg Biochem* **99**, 23-33 (2005).
62. Nardini, M., Pesce, A., Milani, M. & Bolognesi, M. Protein fold and structure in the truncated (2/2) globin family. *Gene* **398**, 2-11 (2007).
63. Bonamore, A. et al. A novel chimera: the "truncated hemoglobin-antibiotic monooxygenase" from Streptomyces avermitilis. *Gene* **398**, 52-61 (2007).
64. Vuletich, D.A. & Lecomte, J.T. A phylogenetic and structural analysis of truncated hemoglobins. *J Mol Evol* **62**, 196-210 (2006).

65. Ouellet, H. et al. Reaction of Mycobacterium tuberculosis truncated hemoglobin O with hydrogen peroxide: evidence for peroxidatic activity and formation of protein-based radicals. *J Biol Chem* **282**, 7491-503 (2007).
66. Pawaria, S. et al. Intracellular growth and survival of Salmonella enterica serovar Typhimurium carrying truncated hemoglobins of Mycobacterium tuberculosis. *Microb Pathog* **42**, 119-28 (2007).
67. Vinogradov, S.N. et al. Three globin lineages belonging to two structural classes in genomes from the three kingdoms of life. *Proceedings of the National Academy of Sciences of the United States of America* **102**, 11385-11389 (2005).
68. Hoogewijs, D. et al. Androglobin: a chimeric globin in metazoans that is preferentially expressed in Mammalian testes. *Mol Biol Evol* **29**, 1105-14 (2012).
69. Ronda, L., Bruno, S. & Bettati, S. Tertiary and quaternary effects in the allosteric regulation of animal hemoglobins. *Biochim Biophys Acta* **1834**, 1860-72 (2013).
70. Molé, P.A. et al. Myoglobin desaturation with exercise intensity in human gastrocnemius muscle. *Am J Physiol* **277**, R173-80 (1999).
71. Gödecke, A. Myoglobin: safeguard of myocardial oxygen supply during systolic compression? *Cardiovasc Res* **87**, 4-5 (2010).
72. Endeward, V., Gros, G. & Jürgens, K.D. Significance of myoglobin as an oxygen store and oxygen transporter in the intermittently perfused human heart: a model study. *Cardiovasc Res* **87**, 22-9 (2010).
73. Helbo, S., Weber, R.E. & Fago, A. Expression patterns and adaptive functional diversity of vertebrate myoglobins. *Biochim Biophys Acta* **1834**, 1832-9 (2013).
74. Kooyman, G.L. & Ponganis, P.J. The physiological basis of diving to depth: birds and mammals. *Annu Rev Physiol* **60**, 19-32 (1998).
75. Helbo, S. & Fago, A. Functional properties of myoglobins from five whale species with different diving capacities. *J Exp Biol* **215**, 3403-10 (2012).
76. Ponganis, P.J. Diving mammals. *Compr Physiol* **1**, 447-65 (2011).
77. Wittenberg, B.A. & Wittenberg, J.B. Transport of oxygen in muscle. *Annu Rev Physiol* **51**, 857-78 (1989).
78. Sidell, B.D. Intracellular oxygen diffusion: the roles of myoglobin and lipid at cold body temperature. *J Exp Biol* **201**, 1119-28 (1998).
79. Wittenberg, J.B. & Wittenberg, B.A. Myoglobin function reassessed. *J Exp Biol* **206**, 2011-20 (2003).
80. Cole, R.P. Myoglobin function in exercising skeletal muscle. *Science* **216**, 523-5 (1982).
81. Levy, M.J., Livingston, D.J., Criddle, R.S. & Brown, W.D. Isolation and characterization of metmyoglobin reductase from yellowfin tuna (Thunnus albacares). *Comp Biochem Physiol B* **81**, 809-14 (1985).
82. Livingston, D.J., McLachlan, S.J., La Mar, G.N. & Brown, W.D. Myoglobin: cytochrome b5 interactions and the kinetic mechanism of metmyoglobin reductase. *J Biol Chem* **260**, 15699-707 (1985).
83. Rahaman, M.M. & Straub, A.C. The emerging roles of somatic globins in cardiovascular redox biology and beyond. *Redox Biol* **1**, 405-410 (2013).
84. Cassoly, R. & Gibson, Q. Conformation, co-operativity and ligand binding in human hemoglobin. *J Mol Biol* **91**, 301-13 (1975).
85. Doyle, M.P. & Hoekstra, J.W. Oxidation of nitrogen oxides by bound dioxygen in hemoproteins. *J Inorg Biochem* **14**, 351-8 (1981).
86. Eich, R.F. et al. Mechanism of NO-induced oxidation of myoglobin and hemoglobin. *Biochemistry* **35**, 6976-83 (1996).
87. Moncada, S. The 1991 Ulf von Euler Lecture. The L-arginine: nitric oxide pathway. *Acta Physiol Scand* **145**, 201-27 (1992).
88. Vallance, P., Collier, J. & Moncada, S. Effects of endothelium-derived nitric oxide on peripheral arteriolar tone in man. *Lancet* **2**, 997-1000 (1989).
89. Brown, G.C. & Cooper, C.E. Nanomolar concentrations of nitric oxide reversibly inhibit synaptosomal respiration by competing with oxygen at cytochrome oxidase. *FEBS Lett* **356**, 295-8 (1994).
90. Shiva, S. et al. Deoxymyoglobin is a nitrite reductase that generates nitric oxide and regulates mitochondrial respiration. *Circ Res* **100**, 654-61 (2007).
91. Gladwin, M.T. & Kim-Shapiro, D.B. The functional nitrite reductase activity of the heme-globins. *Blood* **112**, 2636-47 (2008).
92. Hendgen-Cotta, U.B. et al. Nitrite reductase activity of myoglobin regulates respiration and cellular viability in myocardial ischemia-reperfusion injury. *Proc Natl Acad Sci U S A* **105**, 10256-61 (2008).
93. Totzeck, M. et al. Nitrite regulates hypoxic vasodilation via myoglobin-dependent nitric oxide generation. *Circulation* **126**, 325-34 (2012).
94. Stuehr, D.J. et al. Radical reactions of nitric oxide synthases. *Biochem Soc Symp*, 39-49 (2004).
95. Hendgen-Cotta, U.B., Kelm, M. & Rassaf, T. A highlight of myoglobin diversity: the nitrite reductase activity during myocardial ischemia-reperfusion. *Nitric Oxide* **22**, 75-82 (2010).
96. Castiglione, N., Rinaldo, S., Giardina, G., Stelitano, V. & Cutruzzolà, F. Nitrite and nitrite reductases: from molecular mechanisms to significance in human health and disease. *Antioxid Redox Signal* **17**, 684-716 (2012).
97. Lundberg, J.O. et al. Nitrate and nitrite in biology, nutrition and therapeutics. *Nat Chem Biol* **5**, 865-9 (2009).
98. Rifkind, J.M., Ramasamy, S., Manoharan, P.T., Nagababu, E. & Mohanty, J.G. Redox reactions of hemoglobin. *Antioxid Redox Signal* **6**, 657-66 (2004).

99. Reeder, B.J. & Wilson, M.T. Hemoglobin and myoglobin associated oxidative stress: from molecular mechanisms to disease States. *Curr Med Chem* **12**, 2741-51 (2005).
100. Reeder, B.J. The redox activity of hemoglobins: from physiologic functions to pathologic mechanisms. *Antioxid Redox Signal* **13**, 1087-123 (2010).
101. Flögel, U., Gödecke, A., Klotz, L.O. & Schrader, J. Role of myoglobin in the antioxidant defense of the heart. *FASEB J* **18**, 1156-8 (2004).
102. Whitburn, K.D. The interaction of oxymyoglobin with hydrogen peroxide: the formation of ferrylmyoglobin at moderate excesses of hydrogen peroxide. *Arch Biochem Biophys* **253**, 419-30 (1987).
103. Widmer, C.C. et al. Hemoglobin can attenuate hydrogen peroxide-induced oxidative stress by acting as an antioxidative peroxidase. *Antioxid Redox Signal* **12**, 185-98 (2010).
104. Bonaventura, C. et al. Extreme differences between hemoglobins I and II of the clam Lucina pectinalis in their reactions with nitrite. *Biochim Biophys Acta* **1804**, 1988-95 (2010).
105. Kraus, D.W. & Wittenberg, J.B. Hemoglobins of the Lucina pectinata/bacteria symbiosis. I. Molecular properties, kinetics and equilibria of reactions with ligands. *J Biol Chem* **265**, 16043-53 (1990).
106. Numoto, N. et al. Structure of an extracellular giant hemoglobin of the gutless beard worm Oligobrachia mashikoi. *Proc Natl Acad Sci U S A* **102**, 14521-6 (2005).
107. Bailly, X. & Vinogradov, S. The sulfide binding function of annelid hemoglobins: relic of an old biosystem? *J Inorg Biochem* **99**, 142-50 (2005).
108. Burmester, T., Weich, B., Reinhardt, S. & Hankeln, T. A vertebrate globin expressed in the brain. *Nature* **407**, 520-3 (2000).
109. Trent, J.T., Watts, R.A. & Hargrove, M.S. Human neuroglobin, a hexacoordinate hemoglobin that reversibly binds oxygen. *J Biol Chem* **276**, 30106-10 (2001).
110. Burmester, T., Ebner, B., Weich, B. & Hankeln, T. Cytoglobin: a novel globin type ubiquitously expressed in vertebrate tissues. *Mol Biol Evol* **19**, 416-21 (2002).
111. de Sanctis, D. et al. Crystal structure of cytoglobin: the fourth globin type discovered in man displays heme hexa-coordination. *J Mol Biol* **336**, 917-27 (2004).
112. Kugelstadt, D., Haberkamp, M., Hankeln, T. & Burmester, T. Neuroglobin, cytoglobin, and a novel, eye-specific globin from chicken. *Biochem Biophys Res Commun* **325**, 719-25 (2004).
113. Roesner, A., Fuchs, C., Hankeln, T. & Burmester, T. A globin gene of ancient evolutionary origin in lower vertebrates: evidence for two distinct globin families in animals. *Mol Biol Evol* **22**, 12-20 (2005).
114. Fuchs, C., Burmester, T. & Hankeln, T. The amphibian globin gene repertoire as revealed by the Xenopus genome. *Cytogenet Genome Res* **112**, 296-306 (2006).
115. Burmester, T. & Hankeln, T. Function and evolution of vertebrate globins. *Acta Physiol (Oxf)* (2014).
116. Hamdane, D. et al. The redox state of the cell regulates the ligand binding affinity of human neuroglobin and cytoglobin. *J Biol Chem* **278**, 51713-21 (2003).
117. Fago, A. et al. Allosteric regulation and temperature dependence of oxygen binding in human neuroglobin and cytoglobin. Molecular mechanisms and physiological significance. *J Biol Chem* **279**, 44417-26 (2004).
118. Green, B.N. et al. Observation of large, non-covalent globin subassemblies in the approximately 3600 kDa hexagonal bilayer hemoglobins by electrospray ionization time-of-flight mass spectrometry. *J Mol Biol* **309**, 553-60 (2001).
119. Suzuki, T. & Vinogradov, S.N. Globin and linker sequences of the giant extracellular hemoglobin from the leech Macrobdella decora. *J Protein Chem* **22**, 231-42 (2003).
120. Gerke, P., Börding, C., Zeis, B. & Paul, R.J. Adaptive haemoglobin gene control in Daphnia pulex at different oxygen and temperature conditions. *Comp Biochem Physiol A Mol Integr Physiol* **159**, 56-65 (2011).
121. Kloek, A.P., Yang, J., Mathews, F.S. & Goldberg, D.E. Expression, characterization, and crystallization of oxygen-avid Ascaris hemoglobin domains. *J Biol Chem* **268**, 17669-71 (1993).
122. Blank, M. & Burmester, T. Widespread occurrence of N-terminal acylation in animal globins and possible origin of respiratory globins from a membrane-bound ancestor. *Mol Biol Evol* **29**, 3553-61 (2012).
123. Félix, M.A. & Braendle, C. The natural history of Caenorhabditis elegans. *Curr Biol* **20**, R965-9 (2010).
124. Brenner, S. The genetics of Caenorhabditis elegans. *Genetics* **77**, 71-94 (1974).
125. Cassada, R.C. & Russell, R.L. The dauerlarva, a post-embryonic developmental variant of the nematode Caenorhabditis elegans. *Dev Biol* **46**, 326-42 (1975).
126. Hall, D.H. et al. Ultrastructural features of the adult hermaphrodite gonad of Caenorhabditis elegans: relations between the germ line and soma. *Dev Biol* **212**, 101-23 (1999).
127. McCarter, J., Bartlett, B., Dang, T. & Schedl, T. Soma-germ cell interactions in Caenorhabditis elegans: multiple events of hermaphrodite germline development require the somatic sheath and spermathecal lineages. *Dev Biol* **181**, 121-43 (1997).
128. Sulston, J.E., Schierenberg, E., White, J.G. & Thomson, J.N. The embryonic cell lineage of the nematode Caenorhabditis elegans. *Dev Biol* **100**, 64-119 (1983).
129. White, J.G., Southgate, E., Thomson, J.N. & Brenner, S. The structure of the nervous system of the nematode Caenorhabditis elegans. *Philos Trans R Soc Lond B Biol Sci* **314**, 1-340 (1986).

130. Consortium, C.e.S. Genome sequence of the nematode C. elegans: a platform for investigating biology. *Science* **282**, 2012-8 (1998).
131. Jorgensen, E.M. & Mango, S.E. The art and design of genetic screens: caenorhabditis elegans. *Nat Rev Genet* **3**, 356-69 (2002).
132. Tilleman, L. et al. An N-Myristoylated Globin with a Redox-Sensing Function That Regulates the Defecation Cycle in Caenorhabditis elegans. *PLoS One* **7**, e48768 (2012).
133. Dickinson, B.C. & Chang, C.J. Chemistry and biology of reactive oxygen species in signaling or stress responses. *Nat Chem Biol* **7**, 504-11 (2011).
134. Finkel, T. Signal transduction by reactive oxygen species. *J Cell Biol* **194**, 7-15 (2011).
135. Finkel, T. Oxidant signals and oxidative stress. *Curr Opin Cell Biol* **15**, 247-54 (2003).
136. D'Autréaux, B. & Toledano, M.B. ROS as signaling molecules: mechanisms that generate specificity in ROS homeostasis. *Nat Rev Mol Cell Biol* **8**, 813-24 (2007).
137. Halliwell, B. & Gutteridge, J. *Free Radicals in Biology and Medicine*, (Oxford University Press, 2007).
138. Veal, E. & Day, A. Hydrogen peroxide as a signaling molecule. *Antioxid Redox Signal* **15**, 147-51 (2011).
139. Ray, P.D., Huang, B.W. & Tsuji, Y. Reactive oxygen species (ROS) homeostasis and redox regulation in cellular signaling. *Cell Signal* **24**, 981-90 (2012).
140. Hidalgo, E. & Demple, B. An iron-sulfur center essential for transcriptional activation by the redox-sensing SoxR protein. *EMBO J* **13**, 138-46 (1994).
141. Madesh, M. et al. Selective role for superoxide in InsP3 receptor-mediated mitochondrial dysfunction and endothelial apoptosis. *J Cell Biol* **170**, 1079-90 (2005).
142. Madesh, M. & Hajnóczky, G. VDAC-dependent permeabilization of the outer mitochondrial membrane by superoxide induces rapid and massive cytochrome c release. *J Cell Biol* **155**, 1003-15 (2001).
143. Devadas, S., Zaritskaya, L., Rhee, S.G., Oberley, L. & Williams, M.S. Discrete generation of superoxide and hydrogen peroxide by T cell receptor stimulation: selective regulation of mitogen-activated protein kinase activation and fas ligand expression. *J Exp Med* **195**, 59-70 (2002).
144. Poole, L.B., Karplus, P.A. & Claiborne, A. Protein sulfenic acids in redox signaling. *Annu Rev Pharmacol Toxicol* **44**, 325-47 (2004).
145. Gilbert, H.F. Molecular and cellular aspects of thiol-disulfide exchange. *Adv Enzymol Relat Areas Mol Biol* **63**, 69-172 (1990).
146. Winterbourn, C.C. & Metodiewa, D. Reactivity of biologically important thiol compounds with superoxide and hydrogen peroxide. *Free Radic Biol Med* **27**, 322-8 (1999).
147. Peskin, A.V. et al. The high reactivity of peroxiredoxin 2 with H(2)O(2) is not reflected in its reaction with other oxidants and thiol reagents. *J Biol Chem* **282**, 11885-92 (2007).
148. Denu, J.M. & Tanner, K.G. Specific and reversible inactivation of protein tyrosine phosphatases by hydrogen peroxide: evidence for a sulfenic acid intermediate and implications for redox regulation. *Biochemistry* **37**, 5633-42 (1998).
149. Leichert, L.I. et al. Quantifying changes in the thiol redox proteome upon oxidative stress in vivo. *Proc Natl Acad Sci U S A* **105**, 8197-202 (2008).
150. Le Moan, N., Clement, G., Le Maout, S., Tacnet, F. & Toledano, M.B. The Saccharomyces cerevisiae proteome of oxidized protein thiols: contrasted functions for the thioredoxin and glutathione pathways. *J Biol Chem* **281**, 10420-30 (2006).
151. Holmgren, A. et al. Thiol redox control via thioredoxin and glutaredoxin systems. *Biochem Soc Trans* **33**, 1375-7 (2005).
152. Fisher, A.B. Redox signaling across cell membranes. *Antioxid Redox Signal* **11**, 1349-56 (2009).
153. Murphy, M.P. How mitochondria produce reactive oxygen species. *Biochem J* **417**, 1-13 (2009).
154. Sena, L.A. & Chandel, N.S. Physiological roles of mitochondrial reactive oxygen species. *Mol Cell* **48**, 158-67 (2012).
155. Suh, Y.A. et al. Cell transformation by the superoxide-generating oxidase Mox1. *Nature* **401**, 79-82 (1999).
156. Lambeth, J.D. & Neish, A.S. Nox enzymes and new thinking on reactive oxygen: a double-edged sword revisited. *Annu Rev Pathol* **9**, 119-45 (2014).
157. Geiszt, M., Kopp, J.B., Várnai, P. & Leto, T.L. Identification of renox, an NAD(P)H oxidase in kidney. *Proc Natl Acad Sci U S A* **97**, 8010-4 (2000).
158. Bedard, K. & Krause, K.H. The NOX family of ROS-generating NADPH oxidases: physiology and pathophysiology. *Physiol Rev* **87**, 245-313 (2007).
159. Sies, H. Oxidative stress: oxidants and antioxidants. *Exp Physiol* **82**, 291-5 (1997).
160. Fukai, T. & Ushio-Fukai, M. Superoxide dismutases: role in redox signaling, vascular function, and diseases. *Antioxid Redox Signal* **15**, 1583-606 (2011).
161. Wood, Z.A., Poole, L.B. & Karplus, P.A. Peroxiredoxin evolution and the regulation of hydrogen peroxide signaling. *Science* **300**, 650-3 (2003).
162. Miller, E.W., Dickinson, B.C. & Chang, C.J. Aquaporin-3 mediates hydrogen peroxide uptake to regulate downstream intracellular signaling. *Proc Natl Acad Sci U S A* **107**, 15681-6 (2010).

163. Ushio-Fukai, M. Compartmentalization of redox signaling through NADPH oxidase-derived ROS. *Antioxid Redox Signal* **11**, 1289-99 (2009).
164. Ushio-Fukai, M. Localizing NADPH oxidase-derived ROS. *Sci STKE* **2006**, re8 (2006).
165. Yu, Y., Dumollard, R., Rossbach, A., Lai, F.A. & Swann, K. Redistribution of mitochondria leads to bursts of ATP production during spontaneous mouse oocyte maturation. *J Cell Physiol* **224**, 672-80 (2010).
166. Bavister, B.D. The mitochondrial contribution to stem cell biology. *Reprod Fertil Dev* **18**, 829-38 (2006).
167. Katayama, M. et al. Mitochondrial distribution and microtubule organization in fertilized and cloned porcine embryos: implications for developmental potential. *Dev Biol* **299**, 206-20 (2006).
168. Al-Mehdi, A.B. et al. Perinuclear mitochondrial clustering creates an oxidant-rich nuclear domain required for hypoxia-induced transcription. *Sci Signal* **5**, ra47 (2012).
169. Hoppins, S. The regulation of mitochondrial dynamics. *Curr Opin Cell Biol* **29C**, 46-52 (2014).
170. Lackner, L.L. Determining the shape and cellular distribution of mitochondria: the integration of multiple activities. *Curr Opin Cell Biol* **25**, 471-6 (2013).
171. Rafelski, S.M. Mitochondrial network morphology: building an integrative, geometrical view. *BMC Biol* **11**, 71 (2013).
172. Mumbengegwi, D.R., Li, Q., Li, C., Bear, C.E. & Engelhardt, J.F. Evidence for a superoxide permeability pathway in endosomal membranes. *Mol Cell Biol* **28**, 3700-12 (2008).
173. Archer, S.L. et al. Mitochondrial metabolism, redox signaling, and fusion: a mitochondria-ROS-HIF-1alpha-Kv1.5 O2-sensing pathway at the intersection of pulmonary hypertension and cancer. *Am J Physiol Heart Circ Physiol* **294**, H570-8 (2008).
174. Oshikawa, J. et al. Extracellular SOD-derived H2O2 promotes VEGF signaling in caveolae/lipid rafts and post-ischemic angiogenesis in mice. *PLoS One* **5**, e10189 (2010).
175. Woo, H.A. et al. Reversing the inactivation of peroxiredoxins caused by cysteine sulfinic acid formation. *Science* **300**, 653-6 (2003).
176. Bienert, G.P. et al. Specific aquaporins facilitate the diffusion of hydrogen peroxide across membranes. *J Biol Chem* **282**, 1183-92 (2007).
177. Dynowski, M., Schaaf, G., Loque, D., Moran, O. & Ludewig, U. Plant plasma membrane water channels conduct the signaling molecule H2O2. *Biochem J* **414**, 53-61 (2008).
178. Kamath, R.S. et al. Systematic functional analysis of the Caenorhabditis elegans genome using RNAi. *Nature* **421**, 231-7 (2003).
179. Simmer, F. et al. Loss of the putative RNA-directed RNA polymerase RRF-3 makes C. elegans hypersensitive to RNAi. *Curr Biol* **12**, 1317-9 (2002).
180. Gartner, A., Boag, P.R. & Blackwell, T.K. Germline survival and apoptosis. *WormBook*, 1-20 (2008).
181. Salinas, L.S., Maldonado, E. & Navarro, R.E. Stress-induced germ cell apoptosis by a p53 independent pathway in Caenorhabditis elegans. *Cell Death Differ* **13**, 2129-39 (2006).
182. Mizuno, T. et al. The Caenorhabditis elegans MAPK phosphatase VHP-1 mediates a novel JNK-like signaling pathway in stress response. *EMBO J* **23**, 2226-34 (2004).
183. De Wael, K. et al. Electrochemical study of gelatin as a matrix for the immobilization of horse heart cytochrome c. *Talanta* **82**, 1980-5 (2010).
184. Petlicki, J. & van de Ven, T.G.M. The equilibrium between the oxidation of hydrogen peroxide by oxygen and the dismutation of peroxyl or superoxide radicals in aqueous solutions in contact with oxygen. Vol. 94 2763-2767 (*Journal* of the Chemical Society, *Faraday*, 1999).
185. Hayashi, A., Suzuki, T. & Shin, M. An enzymic reduction system for metmyoglobin and methemoglobin, and its application to functional studies of oxygen carriers. *Biochim Biophys Acta* **310**, 309-16 (1973).
186. Couture, M., Burmester, T., Hankeln, T. & Rousseau, D.L. The heme environment of mouse neuroglobin. Evidence for the presence of two conformations of the heme pocket. *J Biol Chem* **276**, 36377-82 (2001).
187. Vallone, B., Nienhaus, K., Brunori, M. & Nienhaus, G.U. The structure of murine neuroglobin: Novel pathways for ligand migration and binding. *Proteins* **56**, 85-92 (2004).
188. Hargrove, M.S. et al. Crystal structure of a nonsymbiotic plant hemoglobin. *Structure* **8**, 1005-14 (2000).
189. Pesce, A. et al. HisE11 and HisF8 provide bis-histidyl heme hexa-coordination in the globin domain of Geobacter sulfurreducens globin-coupled sensor. *J Mol Biol* **386**, 246-60 (2009).
190. Bologna, G., Yvon, C., Duvaud, S. & Veuthey, A.L. N-Terminal myristoylation predictions by ensembles of neural networks. *Proteomics* **4**, 1626-32 (2004).
191. Ren, J. et al. CSS-Palm 2.0: an updated software for palmitoylation sites prediction. *Protein Eng Des Sel* **21**, 639-44 (2008).
192. Shahinian, S. & Silvius, J.R. Doubly-lipid-modified protein sequence motifs exhibit long-lived anchorage to lipid bilayer membranes. *Biochemistry* **34**, 3813-22 (1995).
193. Veal, E.A., Day, A.M. & Morgan, B.A. Hydrogen peroxide sensing and signaling. *Mol Cell* **26**, 1-14 (2007).
194. Brittain, T., Skommer, J., Raychaudhuri, S. & Birch, N. An antiapoptotic neuroprotective role for neuroglobin. *Int J Mol Sci* **11**, 2306-21 (2010).
195. Fago, A., Mathews, A.J., Moens, L., Dewilde, S. & Brittain, T. The reaction of neuroglobin with potential redox protein partners cytochrome b5 and cytochrome c. *FEBS Lett* **580**, 4884-8 (2006).

196. Reeder, B.J., Svistunenko, D.A. & Wilson, M.T. Lipid binding to cytoglobin leads to a change in haem co-ordination: a role for cytoglobin in lipid signaling of oxidative stress. *Biochem J* **434**, 483-92 (2011).
197. Dordas, C. et al. Expression of a stress-induced hemoglobin affects NO levels produced by alfalfa root cultures under hypoxic stress. *Plant J* **35**, 763-70 (2003).
198. Ohwaki, Y., Kawagishi-Kobayashi, M., Wakasa, K., Fujihara, S. & Yoneyama, T. Induction of class-1 non-symbiotic hemoglobin genes by nitrate, nitrite and nitric oxide in cultured rice cells. *Plant Cell Physiol* **46**, 324-31 (2005).
199. Chaiswing, L. & Oberley, T.D. Extracellular/microenvironmental redox state. *Antioxid Redox Signal* **13**, 449-65 (2010).
200. McCubrey, J.A., Lahair, M.M. & Franklin, R.A. Reactive oxygen species-induced activation of the MAP kinase signaling pathways. *Antioxid Redox Signal* **8**, 1775-89 (2006).
201. Wada, T. & Penninger, J.M. Mitogen-activated protein kinases in apoptosis regulation. *Oncogene* **23**, 2838-49 (2004).
202. Hobert, O. PCR fusion-based approach to create reporter gene constructs for expression analysis in transgenic C. elegans. *Biotechniques* **32**, 728-30 (2002).
203. Green, R.A. et al. Expression and imaging of fluorescent proteins in the C. elegans gonad and early embryo. *Methods Cell Biol* **85**, 179-218 (2008).
204. Timmons, L., Court, D.L. & Fire, A. Ingestion of bacterially expressed dsRNAs can produce specific and potent genetic interference in Caenorhabditis elegans. *Gene* **263**, 103-12 (2001).
205. Huelsenbeck, J.P. & Ronquist, F. MRBAYES: Bayesian inference of phylogenetic trees. *Bioinformatics* **17**, 754-5 (2001).
206. Abascal, F., Zardoya, R. & Posada, D. ProtTest: selection of best-fit models of protein evolution. *Bioinformatics* **21**, 2104-5 (2005).
207. Jones, D.T., Taylor, W.R. & Thornton, J.M. The rapid generation of mutation data matrices from protein sequences. *Comput Appl Biosci* **8**, 275-82 (1992).
208. Müller, T. & Vingron, M. Modeling amino acid replacement. *J Comput Biol* **7**, 761-76 (2000).
209. Boutin, J.A. Myristoylation. *Cell Signal* **9**, 15-35 (1997).
210. Farazi, T.A., Waksman, G. & Gordon, J.I. The biology and enzymology of protein N-myristoylation. *J Biol Chem* **276**, 39501-39504 (2001).
211. Hayashi, N. & Titani, K. N-myristoylated proteins, key components in intracellular signal transduction systems enabling rapid and flexible cell responses. *Proc Jpn Acad Ser B Phys Biol Sci* **86**, 494-508 (2010).
212. Towler, D.A., Eubanks, S.R., Towery, D.S., Adams, S.P. & Glaser, L. Amino-terminal processing of proteins by N-myristoylation. Substrate specificity of N-myristoyl transferase. *J Biol Chem* **262**, 1030-1036 (1987).
213. Zha, J., Weiler, S., Oh, K.J., Wei, M.C. & Korsmeyer, S.J. Posttranslational N-myristoylation of BID as a molecular switch for targeting mitochondria and apoptosis. *Science* **290**, 1761-1765 (2000).
214. Utsumi, T., Sakurai, N., Nakano, K. & Ishisaka, R. C-terminal 15 kDa fragment of cytoskeletal actin is posttranslationally N-myristoylated upon caspase-mediated cleavage and targeted to mitochondria. *FEBS Lett* **539**, 37-44 (2003).
215. Sakurai, N. & Utsumi, T. Posttranslational N-myristoylation is required for the anti-apoptotic activity of human tGelsolin, the C-terminal caspase cleavage product of human gelsolin. *J Biol Chem* **281**, 14288-14295 (2006).
216. Vilas, G.L. et al. Posttranslational myristoylation of caspase-activated p21-activated protein kinase 2 (PAK2) potentiates late apoptotic events. *Proc Natl Acad Sci U S A* **103**, 6542-6547 (2006).
217. Martin, D.D.O. et al. Rapid detection, discovery, and identification of post-translationally myristoylated proteins during apoptosis using a bio-orthogonal azidomyristate analog. *FASEB J* **22**, 797-806 (2008).
218. Yap, M.C. et al. Rapid and selective detection of fatty acylated proteins using omega-alkynyl-fatty acids and click chemistry. *J Lipid Res* **51**, 1566-1580 (2010).
219. Peitzsch, R.M. & McLaughlin, S. Binding of acylated peptides and fatty acids to phospholipid vesicles: pertinence to myristoylated proteins. *Biochemistry* **32**, 10436-10443 (1993).
220. Murray, D., Ben-Tal, N., Honig, B. & McLaughlin, S. Electrostatic interaction of myristoylated proteins with membranes: simple physics, complicated biology. *Structure* **5**, 985-989 (1997).
221. Carr, S.A., Biemann, K., Shoji, S., Parmelee, D.C. & Titani, K. n-Tetradecanoyl is the NH2-terminal blocking group of the catalytic subunit of cyclic AMP-dependent protein kinase from bovine cardiac muscle. *Proc Natl Acad Sci U S A* **79**, 6128-6131 (1982).
222. Aitken, A. et al. Identification of the NH2-terminal blocking group of calcineurin B as myristic acid. *FEBS Lett* **150**, 314-318 (1982).
223. Patwardhan, P. & Resh, M.D. Myristoylation and membrane binding regulate c-Src stability and kinase activity. *Mol Cell Biol* **30**, 4094-4107 (2010).
224. Hou, S. et al. Globin-coupled sensors: a class of heme-containing sensors in Archaea and Bacteria. *Proc Natl Acad Sci U S A* **98**, 9353-9358 (2001).
225. Gardner, P.R., Gardner, A.M., Martin, L.A. & Salzman, A.L. Nitric oxide dioxygenase: an enzymic function for flavohemoglobin. *Proc Natl Acad Sci U S A* **95**, 10378-10383 (1998).

226. Flogel, U., Merx, M.W., Godecke, A., Decking, U.K. & Schrader, J. Myoglobin: A scavenger of bioactive NO. *Proc Natl Acad Sci U S A* **98**, 735-740 (2001).
227. Ramandeep et al. Vitreoscilla hemoglobin. Intracellular localization and binding to membranes. *J Biol Chem* **276**, 24781-24789 (2001).
228. Ertas, B., Kiger, L., Blank, M., Marden, M.C. & Burmester, T. A membrane-bound hemoglobin from gills of the green shore crab Carcinus maenas. *J Biol Chem* **286**, 3185-93 (2011).
229. Blank, M. et al. A membrane-bound vertebrate globin. *PLoS One* **6**, e25292 (2011).
230. Tilleman, L. et al. Globins in Caenorhabditis elegans. *IUBMB Life* **63**, 166-74 (2011).
231. Iruthayanathan, M., O'Leary, B., Paul, G. & Dillon, J.S. Hydrogen peroxide signaling mediates DHEA-induced vascular endothelial cell proliferation. *Steroids* **76**, 1483-1490 (2011).
232. Park, J., Lee, J. & Choi, C. Mitochondrial network determines intracellular ROS dynamics and sensitivity to oxidative stress through switching inter-mitochondrial messengers. *PLoS One* **6**, e23211 (2011).
233. Rice, M.E. H2O2: a dynamic neuromodulator. *Neuroscientist* **17**, 389-406 (2011).
234. Croll, N. & Smith, J. Integrated Behavior In The Feeding Phase Of *Caenorhabditis Elegans* (Nematoda). Vol. 184 507–517 (J Zool (Lond), 1978).
235. Liu, D.W. & Thomas, J.H. Regulation of a periodic motor program in C. elegans. *J Neurosci* **14**, 1953-1962 (1994).
236. Thomas, J.H. Genetic analysis of defecation in Caenorhabditis elegans. *Genetics* **124**, 855-872 (1990).
237. Kobayashi, Y., Kimura, K.D. & Katsura, I. Ultradian rhythm in the intestine of Caenorhabditis elegans is controlled by the C-terminal region of the FLR-1 ion channel and the hydrophobic domain of the FLR-4 protein kinase. *Genes Cells* **16**, 565-575 (2011).
238. Teramoto, T. & Iwasaki, K. Intestinal calcium waves coordinate a behavioral motor program in C. elegans. *Cell Calcium* **40**, 319-27 (2006).
239. Dal, S., Logan, M., Chisholm, A. & Jorgensen, E. The Inositol Trisphosphate Receptor Regulates A 50-Second Behavioral Rhythm In *C. Elegans*. Vol. 98 757–767 (Cell, 1999).
240. Beg, A.A. & Jorgensen, E.M. EXP-1 is an excitatory GABA-gated cation channel. *Nat Neurosci* **6**, 1145-52 (2003).
241. Horton, P. et al. WoLF PSORT: protein localization predictor. *Nucleic Acids Res* **35**, 585-587 (2007).
242. Lei, Z. & Dai, Y. Assessing protein similarity with Gene Ontology and its use in subnuclear localization prediction. *BMC Bioinformatics* **7**, 491-491 (2006).
243. Lei, Z. & Dai, Y. An SVM-based system for predicting protein subnuclear localizations. *BMC Bioinformatics* **6**, 291-291 (2005).
244. Andersson, K.K., Lipscomb, J.D., Valentine, M., Munck, E. & Hooper, A.B. Tetraheme cytochrome c-554 from Nitrosomonas europaea. Heme-heme interactions and ligand binding. *J Biol Chem* **261**, 1126-1138 (1986).
245. Walker, F. Magnetic Spectroscopic (EPR, ESEEM, Mossbauer, MCD And NMR) Studies Of Low-Spin Ferriheme Centers And Their Corresponding Heme Proteins. Vol. 185–186 471–543 (Coordination Chemistry Reviews, 1999).
246. Leigh, J.S. & Erecinska, M. Thermodynamic and EPR characterization of mitochondrial succinate-cytochrome c reductase-phospholipid complexes. *Biochim Biophys Acta* **387**, 95-9106 (1975).
247. Salerno, J.C. Cytochrome electron spin resonance line shapes, ligand fields, and components stoichiometry in ubiquinol-cytochrome c oxidoreductase. *J Biol Chem* **259**, 2331-2336 (1984).
248. Malkin, R. & Vanngard, T. An EPR study of cytochromes from spinach chloroplasts. *FEBS Lett* **111**, 228-231 (1980).
249. Orme-Johnson, N.R., Hansen, R.E. & Beinert, H. Electron paramagnetic resonance-detectable electron acceptors in beef heart mitochondria. Ubihydroquinone-cytochrome c reductase segment of the electron transfer system and complex mitochondrial fragments. *J Biol Chem* **249**, 1928-1939 (1974).
250. Walker, F., Huyn, B., Scheidt, W. & Osvath, S. Models Of The Cytochromes B. Effect Of Axial Ligand Lane Orientation In EPR And Mo¨ssbauer Spectra Of Low-Spin Ferrihemes. Vol. 108 5288–5297 (Journal Of The American Chemical Society, 1986).
251. Zoppellaro, G. et al. Review: studies of ferric heme proteins with highly anisotropic/highly axial low spin (S = 1/2) electron paramagnetic resonance signals with bis-histidine and histidine-methionine axial iron coordination. *Biopolymers* **91**, 1064-1082 (2009).
252. Vinck, E., Van Doorslaer, S., Dewilde, S. & Moens, L. Structural change of the heme pocket due to disulfide bridge formation is significantly larger for neuroglobin than for cytoglobin. *J Am Chem Soc* **126**, 4516-4517 (2004).
253. Ioanitescu, A.I., Van Doorslaer, S., Dewilde, S. & Moens, L. Unusual flexibility of distal and proximal histidine residues in the haem pocket of Drosophila melanogaster haemoglobin. *Metallomics* **1**, 256-264 (2009).
254. Yatsunyk, L.A., Dawson, A., Carducci, M.D., Nichol, G.S. & Walker, F.A. Models of the cytochromes: crystal structures and EPR spectral characterization of low-spin bis-imidazole complexes of (OETPP)Fe(III) having intermediate ligand plane dihedral angles. *Inorg Chem* **45**, 5417-5428 (2006).
255. Vinck, E. et al. Analyzing heme proteins using EPR techniques: the heme-pocket structure of ferric mouse neuroglobin. *J Biol Inorg Chem* **11**, 467-475 (2006).

256. Desmet, F. et al. The heme pocket of the globin domain of the globin-coupled sensor of Geobacter sulfurreducens--an EPR study. *J Inorg Biochem* **104**, 1022-1028 (2010).
257. Ioanitescu, A., Van Doorslaer, S., Dewilde, S., Endeward, B. & Moens, L. Probing The Heme –Pocket Structure Of The Paramagnetic Forms Of Cytoglobin And A Distal Histidine Mutant Using Electron Paramagnetic Resonance. Vol. 105 2073–2086 (Molecular Physics, 2007).
258. Sevcik, A. Nanostructured Materials In Electrochemistry. 349–354 (Collection Of Czechoslovak Chemical Communications, 1948).
259. Randles, J. A Cathode Ray Polarograph. Part II.—The Current-Voltage Curves. Vol. 44 327–338 (Transactions Of The Faraday Society, 1948).
260. Egawa, T., Shimada, H. & Ishimura, Y. Formation of compound I in the reaction of native myoglobins with hydrogen peroxide. *J Biol Chem* **275**, 34858-66 (2000).
261. Berglund, G.I. et al. The catalytic pathway of horseradish peroxidase at high resolution. *Nature* **417**, 463-468 (2002).
262. Moran, J.M., Ortiz-Ortiz, M.A., Ruiz-Mesa, L.M. & Fuentes, J.M. Nitric oxide in paraquat-mediated toxicity: A review. *J Biochem Mol Toxicol* **24**, 402-409 (2010).
263. Yoshida, M., Shimada, K. & Matsuura, K. The Photo-Oxidation Of Low-Potential Hemes In The Tetraheme Cytochrome Subunit Of The Reaction Center In Whole Cells Of Blastochloris Viridis. Vol. 40 192–197 (Plant Cell Physiology, 1999).
264. Gilmour, R. et al. Spectroscopic characterization of cytochrome c peroxidase from Paracoccus denitrificans. *Biochem J* **294 (Pt 3)**, 745-752 (1993).
265. Kniazeva, M. et al. Suppression of the ELO-2 FA elongation activity results in alterations of the fatty acid composition and multiple physiological defects, including abnormal ultradian rhythms, in Caenorhabditis elegans. *Genetics* **163**, 159-169 (2003).
266. Shibata, Y., Branicky, R., Landaverde, I.O. & Hekimi, S. Redox regulation of germline and vulval development in Caenorhabditis elegans. *Science* **302**, 1779-82 (2003).
267. Take-uchi, M., Kobayashi, Y., Kimura, K.D., Ishihara, T. & Katsura, I. FLR-4, a novel serine/threonine protein kinase, regulates defecation rhythm in Caenorhabditis elegans. *Mol Biol Cell* **16**, 1355-1365 (2005).
268. Take-Uchi, M. et al. An ion channel of the degenerin/epithelial sodium channel superfamily controls the defecation rhythm in Caenorhabditis elegans. *Proc Natl Acad Sci U S A* **95**, 11775-11780 (1998).
269. White, J.G., Southgate, E., Thomson, J.N. & Brenner, S. The structure of the ventral nerve cord of Caenorhabditis elegans. *Philos Trans R Soc Lond B Biol Sci* **275**, 327-348 (1976).
270. Branicky, R. & Hekimi, S. Specification of muscle neurotransmitter sensitivity by a Paired-like homeodomain protein in Caenorhabditis elegans. *Development* **132**, 4999-5009 (2005).
271. Lee, M.S. & Kim, Y.-J. Pattern-recognition receptor signaling initiated from extracellular, membrane, and cytoplasmic space. *Mol Cells* **23**, 1-10 (2007).
272. Rae, R., Witte, H., Rodelsperger, C. & Sommer, R.J. The importance of being regular: Caenorhabditis elegans and Pristionchus pacificus defecation mutants are hypersusceptible to bacterial pathogens. *Int J Parasitol* **42**, 747-753 (2012).
273. Chavez, V., Mohri-Shiomi, A., Maadani, A., Vega, L.A. & Garsin, D.A. Oxidative stress enzymes are required for DAF-16-mediated immunity due to generation of reactive oxygen species by Caenorhabditis elegans. *Genetics* **176**, 1567-1577 (2007).
274. Garsin, D.A. et al. Long-lived C. elegans daf-2 mutants are resistant to bacterial pathogens. *Science* **300**, 1921-1921 (2003).
275. Geiszt, M. & Leto, T.L. The Nox family of NAD(P)H oxidases: host defense and beyond. *J Biol Chem* **279**, 51715-8 (2004).
276. Chavez, V., Mohri-Shiomi, A. & Garsin, D.A. Ce-Duox1/BLI-3 generates reactive oxygen species as a protective innate immune mechanism in Caenorhabditis elegans. *Infect Immun* **77**, 4983-4989 (2009).
277. Edens, W.A. et al. Tyrosine cross-linking of extracellular matrix is catalyzed by Duox, a multidomain oxidase/peroxidase with homology to the phagocyte oxidase subunit gp91phox. *J Cell Biol* **154**, 879-891 (2001).
278. Lambeth, J.D. NOX enzymes and the biology of reactive oxygen. *Nat Rev Immunol* **4**, 181-189 (2004).
279. Park, B.J. et al. Calreticulin, a calcium-binding molecular chaperone, is required for stress response and fertility in Caenorhabditis elegans. *Mol Biol Cell* **12**, 2835-2845 (2001).
280. Walker, D.S., Ly, S., Gower, N.J.D. & Baylis, H.A. IRI-1, a LIN-15B homologue, interacts with inositol-1,4,5-triphosphate receptors and regulates gonadogenesis, defecation, and pharyngeal pumping in Caenorhabditis elegans. *Mol Biol Cell* **15**, 3073-3082 (2004).
281. Dhakal, B.K. et al. Caenorhabditis elegans as a simple model host for Vibrio vulnificus infection. *Biochem Biophys Res Commun* **346**, 751-757 (2006).
282. Pellegriti, K. Increased Susceptibility Of *C. elegans* To *Candida Albicans* Infection After Tissue-Specific RNAi Knockdown Of An ROS-Generating NADPH Oxidase. (2011).
283. Chou, K.C. Using amphiphilic pseudo amino acid composition to predict enzyme subfamily classes. *Bioinformatics* **21**, 10-9 (2005).

284. Chou, K.-C. & Shen, H.-B. Cell-PLoc: a package of Web servers for predicting subcellular localization of proteins in various organisms. *Nat Protoc* **3**, 153-162 (2008).
285. Chou, K.-C. & Shen, H.-B. Euk-mPLoc: a fusion classifier for large-scale eukaryotic protein subcellular location prediction by incorporating multiple sites. *J Proteome Res* **6**, 1728-1734 (2007).
286. Shen, H.-B. & Chou, K.-C. Ensemble classifier for protein fold pattern recognition. *Bioinformatics* **22**, 1717-1722 (2006).
287. Chou, K.C. & Shen, H.B. A new method for predicting the subcellular localization of eukaryotic proteins with both single and multiple sites: Euk-mPLoc 2.0. *PLoS One* **5**, e9931 (2010).
288. Chou, K.C. & Shen, H.B. Predicting eukaryotic protein subcellular location by fusing optimized evidence-theoretic K-Nearest Neighbor classifiers. *J Proteome Res* **5**, 1888-97 (2006).
289. Pierleoni, A., Martelli, P.L., Fariselli, P. & Casadio, R. BaCelLo: a balanced subcellular localization predictor. *Bioinformatics* **22**, 408-416 (2006).
290. Ho¨fer, P., Grupp, A., Nebenfu¨hr, H. & Mehring, M. Hyperfine Sublevel Correlation (Hyscore) Spectroscopy: A 2D ESR Investigation Of The Squaric Acid Radical. Vol. 132 279–282 (Chemical Physics Letters, 1986).
291. Stoll, S. & Schweiger, A. EasySpin, a comprehensive software package for spectral simulation and analysis in EPR. *J Magn Reson* **178**, 42-55 (2006).
292. De Wael, K. et al. Electrochemical determination of hydrogen peroxide with cytochrome c peroxidase and horse heart cytochrome c entrapped in a gelatin hydrogel. *Bioelectrochemistry* **83**, 15-18 (2012).
293. Epstein, A.C. et al. C. elegans EGL-9 and mammalian homologs define a family of dioxygenases that regulate HIF by prolyl hydroxylation. *Cell* **107**, 43-54 (2001).
294. Kaelin, W.G. & Ratcliffe, P.J. Oxygen sensing by metazoans: the central role of the HIF hydroxylase pathway. *Mol Cell* **30**, 393-402 (2008).
295. Benizri, E., Ginouvès, A. & Berra, E. The magic of the hypoxia-signaling cascade. *Cell Mol Life Sci* **65**, 1133-49 (2008).
296. Shen, C., Nettleton, D., Jiang, M., Kim, S.K. & Powell-Coffman, J.A. Roles of the HIF-1 hypoxia-inducible factor during hypoxia response in Caenorhabditis elegans. *J Biol Chem* **280**, 20580-8 (2005).
297. Semenza, G.L. Targeting HIF-1 for cancer therapy. *Nat Rev Cancer* **3**, 721-32 (2003).
298. Depuydt, G., Vanfleteren, J.R. & Braeckman, B.P. Protein metabolism and lifespan in Caenorhabditis elegans. *Adv Exp Med Biol* **694**, 81-107 (2010).
299. Depuydt, G. et al. Reduced insulin/insulin-like growth factor-1 signaling and dietary restriction inhibit translation but preserve muscle mass in Caenorhabditis elegans. *Mol Cell Proteomics* **12**, 3624-39 (2013).
300. Bogaerts, A. et al. A differential proteomics study of Caenorhabditis elegans infected with Aeromonas hydrophila. *Dev Comp Immunol* **34**, 690-8 (2010).
301. Gusarov, I. et al. Bacterial nitric oxide extends the lifespan of C. elegans. *Cell* **152**, 818-30 (2013).
302. Shen, C., Shao, Z. & Powell-Coffman, J.A. The Caenorhabditis elegans rhy-1 gene inhibits HIF-1 hypoxia-inducible factor activity in a negative feedback loop that does not include vhl-1. *Genetics* **174**, 1205-14 (2006).
303. Bishop, T. et al. Genetic analysis of pathways regulated by the von Hippel-Lindau tumor suppressor in Caenorhabditis elegans. *PLoS Biol* **2**, e289 (2004).
304. Chang, A.J. & Bargmann, C.I. Hypoxia and the HIF-1 transcriptional pathway reorganize a neuronal circuit for oxygen-dependent behavior in Caenorhabditis elegans. *Proc Natl Acad Sci U S A* **105**, 7321-6 (2008).
305. Geuens, E. et al. Nerve globins in invertebrates. *IUBMB Life* **56**, 653-6 (2004).
306. Fordel, E., Thijs, L., Moens, L. & Dewilde, S. Neuroglobin and cytoglobin expression in mice. Evidence for a correlation with reactive oxygen species scavenging. *FEBS J* **274**, 1312-7 (2007).
307. Sulston, J.E. & Hodgkin, J. Methods, in: The Nematode *Caenorhabditis elegans*. (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1988).
308. Hellemans, J., Mortier, G., De Paepe, A., Speleman, F. & Vandesompele, J. qBase relative quantification framework and software for management and automated analysis of real-time quantitative PCR data. *Genome Biol* **8**, R19 (2007).
309. Vandesompele, J. et al. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biol* **3**, RESEARCH0034 (2002).
310. Braeckman, B.P., Houthoofd, K., De Vreese, A. & Vanfleteren, J.R. Assaying metabolic activity in ageing Caenorhabditis elegans. *Mech Ageing Dev* **123**, 105-19 (2002).
311. Brys, K., Castelein, N., Matthijssens, F., Vanfleteren, J.R. & Braeckman, B.P. Disruption of insulin signaling preserves bioenergetic competence of mitochondria in ageing Caenorhabditis elegans. *BMC Biol* **8**, 91 (2010).
312. Van Voorhies, W.A. & Ward, S. Broad oxygen tolerance in the nematode Caenorhabditis elegans. *J Exp Biol* **203**, 2467-78 (2000).
313. Rodriguez, M., Snoek, L.B., De Bono, M. & Kammenga, J.E. Worms under stress: C. elegans stress response and its relevance to complex human disease and aging. *Trends Genet* **29**, 367-74 (2013).
314. Powell-Coffman, J.A. Hypoxia signaling and resistance in C. elegans. *Trends Endocrinol Metab* **21**, 435-40 (2010).

315. Golden, J.W. & Riddle, D.L. The Caenorhabditis elegans dauer larva: developmental effects of pheromone, food, and temperature. *Dev Biol* **102**, 368-78 (1984).
316. Hu, P.J. Dauer. *WormBook*, 1-19 (2007).
317. Sun, Y. et al. Neuroglobin protects the brain from experimental stroke in vivo. *Proc Natl Acad Sci U S A* **100**, 3497-500 (2003).
318. Sun, Y., Jin, K., Mao, X.O., Zhu, Y. & Greenberg, D.A. Neuroglobin is up-regulated by and protects neurons from hypoxic-ischemic injury. *Proc Natl Acad Sci U S A* **98**, 15306-11 (2001).
319. Yu, Z., Poppe, J.L. & Wang, X. Mitochondrial mechanisms of neuroglobin's neuroprotection. *Oxid Med Cell Longev* **2013**, 756989 (2013).
320. Burmester, T., Storf, J., Hasenjäger, A., Klawitter, S. & Hankeln, T. The hemoglobin genes of Drosophila. *FEBS J* **273**, 468-80 (2006).
321. Hankeln, T., Klawitter, S., Krämer, M. & Burmester, T. Molecular characterization of hemoglobin from the honeybee Apis mellifera. *J Insect Physiol* **52**, 701-10 (2006).
322. Putker, M. et al. Redox-dependent control of FOXO/DAF-16 by transportin-1. *Mol Cell* **49**, 730-42 (2013).
323. Burmester, T. & Hankeln, T. The respiratory proteins of insects. *J Insect Physiol* **53**, 285-94 (2007).
324. Trewitt, P.M., Saffarini, D.A. & Bergtrom, G. Multiple clustered genes of the haemoglobin VIIB subfamily of Chironomus thummi thummi (Diptera). *Gene* **69**, 91-100 (1988).
325. Trewitt, P.M., Boyer, D.R. & Bergtrom, G. Characterization of maternal haemoglobins in the eggs and embryos of *Chironomus thummi*. Vol. 32 963–969 (Journal of Insect Physiology, 1986).
326. Meissner, B. et al. An integrated strategy to study muscle development and myofilament structure in Caenorhabditis elegans. *PLoS Genet* **5**, e1000537 (2009).
327. Herrmann, J.M. & Dick, T.P. Redox Biology on the rise. *Biol Chem* **393**, 999-1004 (2012).
328. Back, P. et al. Exploring real-time in vivo redox biology of developing and aging Caenorhabditis elegans. *Free Radic Biol Med* **52**, 850-9 (2012).
329. Ezeriņa, D., Morgan, B. & Dick, T.P. Imaging dynamic redox processes with genetically encoded probes. *J Mol Cell Cardiol* (2014).
330. Lukyanov, K.A. & Belousov, V.V. Genetically encoded fluorescent redox sensors. *Biochim Biophys Acta* **1840**, 745-56 (2014).
331. Yang, Y., Han, S.M. & Miller, M.A. MSP hormonal control of the oocyte MAP kinase cascade and reactive oxygen species signaling. *Dev Biol* **342**, 96-107 (2010).
332. Gutscher, M. et al. Proximity-based protein thiol oxidation by H2O2-scavenging peroxidases. *J Biol Chem* **284**, 31532-40 (2009).
333. Redemann, S. et al. Codon adaptation-based control of protein expression in C. elegans. *Nat Methods* **8**, 250-2 (2011).
334. Merritt, C., Rasoloson, D., Ko, D. & Seydoux, G. 3' UTRs are the primary regulators of gene expression in the C. elegans germline. *Curr Biol* **18**, 1476-82 (2008).
335. Castello, P.R., Drechsel, D.A. & Patel, M. Mitochondria are a major source of paraquat-induced reactive oxygen species production in the brain. *J Biol Chem* **282**, 14186-93 (2007).
336. Blanco-Ayala, T., Andérica-Romero, A.C. & Pedraza-Chaverri, J. New insights into antioxidant strategies against paraquat toxicity. *Free Radic Res* (2014).
337. Van Raamsdonk, J.M. & Hekimi, S. Superoxide dismutase is dispensable for normal animal lifespan. *Proc Natl Acad Sci U S A* **109**, 5785-90 (2012).
338. Lee, S.J., Hwang, A.B. & Kenyon, C. Inhibition of respiration extends C. elegans life span via reactive oxygen species that increase HIF-1 activity. *Curr Biol* **20**, 2131-6 (2010).
339. Rea, S.L., Ventura, N. & Johnson, T.E. Relationship between mitochondrial electron transport chain dysfunction, development, and life extension in Caenorhabditis elegans. *PLoS Biol* **5**, e259 (2007).
340. Kawasaki, I. et al. ASB-1, a germline-specific isoform of mitochondrial ATP synthase b subunit, is required to maintain the rate of germline development in Caenorhabditis elegans. *Mech Dev* **124**, 237-51 (2007).
341. Frøkjaer-Jensen, C. et al. Single-copy insertion of transgenes in Caenorhabditis elegans. *Nat Genet* **40**, 1375-83 (2008).

List of abbreviations

BCA: bicinchoninic acid

Cys: cysteine

DAF: dauer formation abnormal

Fe^{2+}: ferrous

Fe^{3+}: ferric

Fe^{4+}: ferryl

GLB: globin

GSH: glutathione

GSSG: glutathione disulfide

H_2O_2: hydrogen peroxide

HIF-1: Hypoxia-inducible factor-1

JNK: c-Jun N-terminal kinase

MAPK: Mitogen-activated protein kinases

NAD: Nicotinamide adenine dinucleotide

NADP: Nicotinamide adenine dinucleotide phosphate

NO: nitric oxide

O_2: oxygen

O_2^{-}: superoxide

PDB: protein data bank

qRT-PCR: quantitative reverse transcription polymerase chain reaction

Redox: reduction/oxidation

RNAi: RNA interference

roGFP: redox-sensitive green fluorescent protein

ROS: reactive oxygen species

SOD: superoxide dismutase

TEM: transmission electron microscopy

Curriculum Vitae

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II. Education & Research Experience

2007 - 2014 **Ph.D. program, Biological Sciences** - Ghent University, Belgium

2012 - 2013 **Visiting Scholar** - University of California, San Diego, USA

2006 - 2007 **M.S. Medical Biotechnology** - Ghent University, Belgium

Greatest Distinction - Ranked First of Class

2004 - 2006 **M.S. Biology** - Ghent University, Belgium

Great Distinction - Award for Best M.S. Dissertation

2002 - 2004 **B.S. Biology** - Ghent University, Belgium

Distinction

III. Publications

- **De Henau S**, Tilleman L, Luyckx E, Trashin S, Pauwels M, Germani F, Vlaeminck C, Vanfleteren JR, Bert W, Pesce A, Nardini M, Bolognesi M, De Wael K, Moens L, Dewilde S, Braeckman BP. A Redox Signaling Globin is Essential for Reproduction in *Caenorhabditis elegans* - Submitted
- Ren C, Li Y, Han R, Gao D, Li W, Shi J, Hoogewijs D, Braeckman BP, **De Henau S**, Lu Y, Qu W, Gao Y, Wu Y, Li Z, Liu H, Wang Z, Zhang C. (2013) GLB-13 is associated with oxidative stress resistance in Caenorhabditis elegans. *IUBMB Life* 65(5):423-34
- Tilleman L, **De Henau S**, Pauwels M, Nagy N, Pintelon I, Braeckman BP, De Wael K, Van Doorslaer S, Adriaensen D, Timmermans JP, Moens L, Dewilde S. (2012) An N-myristoylated globin with a redox-sensing function that regulates the defecation cycle in Caenorhabditis elegans. *PLOS ONE* **7**(12)

- Tilleman L, Germani F, **De Henau S**, Geuens E, Hoogewijs D, Braeckman BP, Vanfleteren JF, Moens L and Dewilde S. (2011) Globins in *Caenorhabditis elegans*. *IUBMB Life* **63**:166-74
- Geuens E, Hoogewijs D, Nardini M, Vinck E, Pesce A, Kiger L, Fago A, Tilleman L, **De Henau S**, Marden MC, Weber RE, Van Doorslaer S, Vanfleteren J, Moens L, Bolognesi M, Dewilde S. (2010) Globin-like proteins in *Caenorhabditis elegans*: *in vivo* localization, ligand binding and structural properties. *BMC Biochem.* **2:**11-17
- Hoogewijs D, **De Henau S**, Dewilde S, Moens L, Couvreur M, Borgonie G, Vinogradov SN, Roy SW, Vanfleteren JR. (2008) The *Caenorhabditis* globin gene family reveals extensive nematode-specific radiation and diversification. *BMC Evol Biol.* **8**:279

IV. Abstracts

- **De Henau S**, Tilleman L, Pauwels M, Pesce A, Nardini M, Bolognesi M, De Wael K, Moens L, Dewilde S, Braeckman BP. A Redox Signaling Globin Regulates Germ Cell Apoptosis in *Caenorhabditis elegans*. 19th International *C. elegans* Meeting, Los Angeles, USA, June 26 - June 30, 2013 – Oral presentation
- **De Henau S**, Tilleman L, Germani F, Pauwels M, Vlaeminck C, Vanfleteren JR, De Wael K, Moens L, Dewilde S, Braeckman BP. Globin 12 of *Caenorhabditis elegans* regulates p38 and JNK MAPK-mediated germ cell apoptosis through redox signaling. XVII International meeting "Oxygen binding and sensing proteins", Parma, Italy, August 29 – September 1, 2012 – Poster presentation
- **De Henau S,** Tilleman L, Germani F, Vlaeminck C, Vanfleteren JR, Moens L, Dewilde S, and Braeckman BP. Globin 12 of *Caenorhabditis elegans* Regulates the p38 and JNK MAPK Pathways through Redox Signaling to Control Germline Apoptosis. *C. elegans* Development, Cell Biology, & Gene Expression Meeting 2012, Madison, USA, June 7- June 10– Oral presentation
- **De Henau S,** Tilleman L, Germani F, Moens L, Dewilde S, Vanfleteren JR and Braeckman BP. A globin of *Caenorhabditis elegans* regulates reproduction by acting in a redox pathway. UGent Doctoraatssymposium 2012, 22 maart 2012 – Oral presentation
- **De Henau S**, Tilleman L, Hoogewijs D, Moens L, Dewilde S, Vanfleteren JR and Braeckman BP. *Caenorhabditis elegans'* GLB-12 regulates germline apoptosis levels and vulval development. 18th International *C. elegans* Meeting, Los Angeles, USA, June 22 - June 26, 2011 – Poster presentation

- **De Henau S**, Braeckman BP and Vanfleteren JR. The role of the *Caenorhabditis elegans* globin family in the response to hypoxia. European *C. elegans* Neurobiology, Crete, Greece, 9-11 October, 2010 – Poster presentation
- **De Henau S**, Braeckman BP and Vanfleteren JR. Globins of the nematode *Caenorhabditis elegans*: How diverse are their functions? XVIth International Conference on Oxygen Binding and Sensing Proteins, Antwerp, Belgium, 22 -26 August, 2010 – Oral presentation
- **De Henau S**, Braeckman BP and Vanfleteren JR. Wide diversity in structure and expression profiles among members of the *Caenorhabditis elegans* globin protein family. European Conference on Nematode Neurobiology, Cambridge, United Kingdom, 21-23 September, 2009 – Poster presentation
- **De Henau S**, Braeckman BP and Vanfleteren JR. Wide diversity in structure and expression profiles among members of the *Caenorhabditis elegans* globin protein family. 17th International *C. elegans* Meeting, Los Angeles, USA, June 24 - June 28, 2009 – Poster presentation – *Award for 3rd place in poster competition, section Evolution*
- **De Henau S**, Braeckman BP and Vanfleteren JR. Quantitative expression analysis of the globin family of Caenorhabditis elegans during hypoxia and NO-stress. XVth International Conference on Oxygen Binding and Sensing Proteins, Aarhus, Denmark, 17-21 August, 2008 – Poster presentation

V. Teaching related activities

- **Practical courses:**
    - Introduction to Animal Physiology (3rd Bachelor in Biology), Prof. Dr. Bart P. Braeckman
    - Nematodes as Model Organisms (Master of Nematology), Prof. Dr. Bart P. Braeckman
    - Limnetic and Terrestrial Field Biology (3rd Bachelor in Biology), Prof. Dr. Maurice Hoffman
    - Biological Model Organisms (1st Bachelor in Biomedical Sciences), Prof. Dr. Ann Huysseune

- **Bachelor projects (3rd year Bachelor Biology)**

2008 Gijs De Cort

Kwantitatieve expressieanalyse van de globine familie van *Caenorhabditis elegans* bij hypoxische condities

2009 Ellie Himschoot

Analyse van de rol van de globinefamilie van *Caenorhabditis elegans* bij hypoxische condities

2010 Barbara Burger

The role of GLB-1 in the oxygen metabolism of *Caenorhabditis elegans*

2010 Vital De Coninck

Is GLB-26 van *Caenorhabditis elegans* betrokken bij de bescherming tegen vrije zuurstofradicalen?

- **Master projects (Master of Biology)**

2010 Glenn Vermote

Aanmaak reporterconstructen, lokalisatie en expressieanalyse van de globinefamilie bij *Caenorhabditis elegans*

- **Master projects (Master of Nematology)**

2010 Nilesh Shanmugam

Functional analysis of the globin gene family of *Caenorhabditis elegans* under low oxygen conditions

2011 Remegie Ntowenimana

Functional Analysis of globin-1 of the nematode Caenorhabditis elegans under oxidative stress

2012 Katarzyna Składanowska

Functional characterization of the two-domain SCP-like VAP-1 protein in the nematode *Caenorhabditis elegans*

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