Hydrogen Peroxide and Redox Regulation of Development

Christine Rampon[1,2], Michel Volovitch[1,3], Alain Joliot[1] and Sophie Vriz[1,2]

1- Center for Interdisciplinary Research in Biology (CIRB), College de France, CNRS, INSERM, PSL Research University, Paris, France
2- Univ Paris Diderot, Sorbonne Paris Cité, Biology Department, 75205 Paris Cedex 13, France
3 - École Normale Supérieure, Department of Biology, PSL Research University, 75005 Paris, France

Corresponding author:
Prof Sophie Vriz
vriz@univ-paris-diderot.fr

Abstract

Reactive oxygen species (ROS), which were originally classified as exclusively deleterious compounds, have gained increasing interest in the recent years given their action as *bona fide* signalling molecules. The main target of ROS action is the reversible oxidation of cysteines, leading to the formation of disulfide bonds, which modulate protein conformation and activity. ROS endowed with signalling properties are mainly produced by NADPH oxidases at the plasma membrane, but their action also involves a complex machinery of multiple redox-sensitive protein families that differs in their subcellular localization and their activity. Given that the levels and distribution of ROS are highly dynamic in part due to their limited stability, the development of various fluorescent ROS sensors, some of which are quantitative (ratiometric), represents a clear breakthrough in the field and have been adapted to both *ex vivo* and *in vivo* applications. The physiological implication of ROS signalling will be presented mainly in the frame of morphogenetics processes, embryogenesis, regeneration, and stem cell differentiation. Gain and loss of function as well as pharmacological strategies have demonstrated the wide but specific requirement of ROS signalling at multiple stages of these processes and its intricate relationship with other well-known signalling pathways.

1

1. Introduction

For a long time, reactive oxygen species (ROS), including hydrogen peroxide (H_2O_2), were considered deleterious molecules. Emphasis was given to their role in neutrophils where they are produced to contribute to anti-microbial defence [1], and extensive studies have been performed on ROS over-production due to mitochondrial dysfunction in neurological disorders or cancer progression [2-4]. Consistent with these detrimental functions, attention has been almost exclusively focused on their toxicity, and many studies strengthened this aspect of redox biology. However, pioneer works highlighted a new role of ROS in signalling particularly during embryonic development, which led to the emergence of the redox signalling field [for review [5-7]. In 2017, Helmut Sies, a pioneer in redox biology, reviewed the topic and developed the concept of oxidative eustress (physiological redox signalling) and oxidative distress (pathophysiological disrupted redox signalling), bringing the two faces of ROS back together [8]. As recently noted [9], a new reading of the past literature might shed a new light on the tenets of redox signalling. Relevant issues are the nature of the ROS invoked, the accurate localization of its site of production, and its concentration, spreading and dynamics in the context of a defined physiological process. The present review focuses on H_2O_2, a central ROS in redox signalling during development and regeneration in metazoans, and its interplay with the redox machinery.

H_2O_2 is the major ROS produced by cells that acts in signalling pathways as a second messenger [8, 10-12]. H_2O_2 is a by-product of many oxidative reactions, such as oxidative protein folding in the endoplasmic reticulum (ER) and peroxisomal

enzyme activities. For signalling purposes, the main sources of H_2O_2 are the mitochondrial respiratory chain and NADPH oxidases (NOXs) [13]. NOXs are trans-membrane proteins that use cytosolic NADPH as an electron donor. NOXs belong to multi-component complexes that generate either O_2^- (NOX 1, 2, 3 and 5) or H_2O_2 (NOX4, DUOX1 and 2) upon appropriate stimulation (by growth factors, cytokines…) [14, 15]. Even when the primary product of NOX activity is O_2^-, it is largely and immediately transformed into H_2O_2 by a super-oxide dismutase (SOD) enzyme physically associated with NOX, or it dismutates spontaneously at low pH levels. Several NOXs are located at the plasma membrane, which is a hub for cell signalling. In this case, H_2O_2 is delivered in the extracellular space, a somehow puzzling situation considering that most known H_2O_2 targets localize in the cell interior. It was first thought that H_2O_2 could pass from the extracellular to the intracellular milieu by passive diffusion through the plasma membrane, but it was later shown that H_2O_2 has poor lipid membrane diffusion capacities and crosses into cells via aquaporin channels [16-18]. This facilitated transport of H_2O_2 across the plasma membrane is itself subject to redox regulation [19], and further investigations are needed to better understand the role of aquaporins in redox signalling. The unique and specific enzyme for H_2O_2 degradation into H_2O is the ubiquitously expressed protein catalase. It mainly localizes in the peroxisome where it is devoted to the reduction of excess H_2O_2 produced there. However, it can also be secreted by an unknown mechanism and associate with the plasma membrane [20-22] or spread in the extracellular milieu [23].

The main physiological target of H_2O_2 action is the reversible oxidation of cysteine residues in proteins. Modification only occurs on the thiolate anion form (S^-). However, at physiological pH, most cysteines are protonated and thus react weakly

with H_2O_2. However, the pK_a of cysteine greatly depends on its protein environment and can reach several units below ~8.5, the approximate value of cysteine alone [24], making these residues ionized and reactive. H_2O_2 oxidizes the thiolate anion to produce sulfenic acid, which is highly reactive and readily forms a disulfide bond in contact with accessible -SH group. Reciprocally, in reducing conditions, disulfide bonds can be easily cleaved to restore the thiol functions. As oxidative condition increases, sulfenic acid will further oxidize to sulfinic and ultimately sulfonic derivatives. These two reactions are generally irreversible deleterious modifications; however, exceptions were reported for sulfinic derivatives (see below). Redox signalling depends both on the local concentration of H_2O_2 and the state (protonated or deprotonated) of the cysteine. Although some cysteines can be directly oxidized by H_2O_2, most of them require prior activation to be deprotonated, involving additional redox-sensitive relays. The best candidates for this relay function appear to be proteins first identified as antioxidant safe-guarders [25-31] reviews in [32-36], and they will be discussed below. It is now clear that the role of H_2O_2 signalling in oxidative eustress has to integrate the entire redox machine.

2. The redox machine

The central redox machine contains at least six main protein families: thioredoxin reductases (TrxRs), thioredoxins (Trxs), peroxiredoxins (Prxs), glutathione reductases (GRs), glutaredoxins (Grxs) and glutathione peroxidases (Gpx) (Figure 1) [for general reviews, see [37-40]. The redox state of thiols is regulated by Trxs and Grxs, which are small thiol-disulfide oxidoreductases characterized by a thioredoxin motif (-**C**GP**C**-). Grxs are mainly responsible for S-glutathionylation. They utilize the reducing power of GSH to maintain and regulate the cellular redox state and redox-

dependent signalling pathways [41]. Electron transfer between these family members depends on the NADPH/NADP motor, which is fuelled by the Pentose Phosphate Pathway (PPP). Among the members of the redox machine, it seems that only Prxs and Gpxs can be directly oxidized by H_2O_2. GPx reactivity is high, partly due to the presence of a selenocysteine in its active site (with an approximate pKa ~5.2 for selenocysteine compared to ~8.5 for cysteine) [24]. Prxs (though relying on cysteine) are even more reactive towards H_2O_2 with a rate constant 10^4 to 10^6 higher than proteins bearing low pKa cysteine [42], and they emerge as essential for redox relay [36]. The activity of these enzymes is also subject to regulation [43]. For example, the activity of some Prxs is inhibited by phosphorylation by cdc2 [44] and by sulfinic [45] or sulfonic acid [45] formation in presence of moderate or high H_2O_2 levels, respectively. The reversible inactivation of a subset of Prxs by sulfinyl observed *in vivo* led to the discovery of sulfiredoxine, an enzyme catalysing the reduction of the sulfinyl group back to sulfenyl [46-50]. Moreover, as schematized in Fig. 1, the activities of all enzymes in the redox machine are interconnected (some additional branches between cycles have been omitted), and the final outcome of thiol-oxidation reactions depends on many parameters, making computational modelling useful but hampering genetic approaches.

As mentioned in the introduction, the central redox machine has pleiotropic functions. In addition to detoxification of harmful amounts of ROS, they also act as sensors of oxidant concentrations and can even acquire new functions, such as chaperones activity for some Prxs [51] for a review. This sensor and/or transducer functions are very important given that the vast majority of redox sensitive proteins are poorly sensitive to direct oxidation by H_2O_2 (a possible exception, PTP1B, is discussed in [34]). Prxs have attracted considerable attention as potent mediators redox signals,

as first established in yeast [26, 52-55], and some years after in mammals. Ledgerwood and colleagues demonstrated that Prx1 participates in the propagation of peroxide signals via disulfide exchange with the target kinase ASK1 [56], and the group of Tobias Dick showed that Prx2 forms a redox relay for H_2O_2 signalling together with the transcription factor STAT3 [31]. Very recently, the same group demonstrated that the relay activity of cytosolic Prxs (1 and 2) is not dependent on Trx1 or TrxR1 but is based on transient disulfide conjugates with protein targets and occurs mainly in conditions of fast response to small variations in H_2O_2 [57].

3. Seeing is believing?

A critical step to model redox signalling is to determine the spatiotemporal localization and amount of the different protagonists. Several synthetic dyes were actively used to measure ROS and radical nitrogen species (RNS) [58-60]. However, these dyes are often poorly specific, do not penetrate in tissue, or are unstable. Moreover, their reaction with ROS/RNS is irreversible. In the last decade, a major effort was devoted to develop genetically encoded fluorescent biosensors for the redox machine elements.

3.1. H_2O_2 sensors

For all ROS, *ex vivo* and *in vivo* measurements of H_2O_2 concentration are challenging due to its short half-life, fast spreading and high reactivity. The development of a genetically encoded fluorescent biosensor specific for H_2O_2 revolutionized the field. It provides access to the dynamics of H_2O_2 concentration in living systems and its modulation by genetic or chemical approaches. This goal was first achieved by Vsevolod Belousov who designed the HyPer probe [61]. The HyPer biosensor is based on the fusion of a circularly permutated fluorescent protein

(cpYFP) with the H_2O_2-sensing domain of *E. coli* OxyR. Two cysteines of OxyR moiety form a disulfide bound in presence of H_2O_2 and the resulting conformational change induces a modification of cpYFP spectra, which allows a ratiometric measurement of H_2O_2 levels. Advantages of this probe are its high sensitivity (nanomolar), its reversibility and its fast reaction rate constant. Moreover, ratiometric measurement is independent of the expression level. The main drawback of this sensor is its sensitivity to pH. To circumvent this problem, a cysteine-mutated form of HyPer (SypHer), which is still sensitive to pH but no longer to H_2O_2, can be used as a control or to measure pH *in vivo* [62]. Since the initial version, HyPer probe has evolved, and the HyPer family currently includes members with different spectral and redox properties [63].

When expressed in *Xenopus laevis* oocytes, HyPer revealed an oscillating production of H_2O_2 induced by fertilization. This production of H_2O_2 is of mitochondrial origin, dependent on calcium waves initiated by fertilization and involved in cell cycle progression at the beginning of development [64]. HyPer was also expressed by transgenesis in two animal models (nematode and fish), where it revealed a highly dynamic fluctuation in H_2O_2 levels during embryonic and post-embryonic development. In *Caenorhabditis elegans* (where HyPer expression was under the control of the ubiquitous RPL-21 promoter), H_2O_2 levels were high during larval development (in the head, notably in the pharynx and neurons), strongly decreased at the transition to adult stage, and remained low during most of the reproductive period [65]. A similar pattern was observed in zebrafish transgenic animals with high levels of H_2O_2 during development and a massive reduction at 3 days post fertilization (dpf) when most of developmental programmes have ended.

Notably, in fish and nematode, HyPer revealed a highly dynamic pattern of H_2O_2 levels in the developing nervous system [66] (Figure 2).

Another type of H_2O_2 sensor was developed from a fusion between roGFP2 (a redox sensitive GFP) and Orp1, the yeast H_2O_2 sensor and modulator of redox-sensitive transcription factor Yap1 [29]. Orp1 is sensitive to H_2O_2; once oxidized, Orp1 promotes the nearby oxidation of roGFP2 (as it does for Yap1), resulting in a shift of roGFP2 spectral properties. Compared with HyPer, this biosensor is insensitive to pH but less sensitive to H_2O_2. This lower affinity for H_2O_2 was overcome by fusion of roGFP2 to the yeast Prx Tsa2 (Tsa2ΔC_R) [67]. roGFP2-Orp1 has been successfully used to measure H_2O_2 in developing and adult *Drosophila* [68]. One of the advantages of genetically encoded sensors is their ability to be addressed to a cell specific compartment upon fusion with appropriate targeting sequences. Differential targeting into either the cytosol or the mitochondria allowed Albrecht et al. to demonstrate the heterogeneity of H_2O_2 levels depending on the tissue and that H_2O_2 level is not coupled with the redox state of glutathione during development [68]. Cytosol/mitochondria expression of roGFP2-Orp1 in the germline of *Caenorhabditis elegans* revealed an increase in H_2O_2 levels in the proximal side of the germline and a peak within the oocytes and in the zygote [69]. An elegant approach preserving the redox status of roGFP2-Orp1 during tissue cryo-section allowed H_2O_2 measurements in mammalian development and adult tissues [70]. This strategy is very promising to acquire redox maps of non-optically accessible tissue. roGFP2-Orp1 was also targeted to zebrafish cardiomyocytes in different compartments (nucleus, mitochondria and cytosol) to follow H_2O_2 level variations during cardiac function and upon pharmaceutical treatments, demonstrating the interest of this H_2O_2 probe to score oxidant or antioxidant molecules [71].

3.2. glutathione redox potential sensors

Glutathione plays a key role in cellular thiol-disulfide exchange reactions, and the GSH/GSSG ratio is considered a good indicator of redox balance (E_{GSH})(Figure 1). A fluorescent sensor for E_{GSH} was generated by fusion of the roGFP2 with the human glutaredoxin-1 (Grx1). roGFP2 alone exhibits a slow response to redox changes. Grx1 fusion to roGFP2 resulted in a rapid equilibrium between the GSH/GSSG couple and the reporting redox couple (roGFP2red and roGFP2ox), thus reflecting the level of E_{GSH} [72]. This sensor was introduced in several species. In *Drosophila*, it was addressed to mitochondria and cytosol to compare E_{GSH} with H_2O_2 levels in developing structures and adults [68]. Live imaging of the third-instar larvae revealed high variations in mitochondrial E_{GSH} amongst different tissues, whereas the cytosolic E_{GSH} was almost constant [68] . Transgenic *C. elegans* expressing a cytosolic form of Grx1-roGFP2 under an ubiquitous promoter was used to analyse E_{GSH} during development [73]. E_{GSH} decreases globally during development and then remains constant in adult except in the spermathecae where fertilization occurs [73].

3.3 NADPH sensor: iNap

NADPH is a key element in the redox machine as a final electron donor for thiol oxidation by H_2O_2. A genetically encoded fluorescent indicator for NADPH (iNap sensor) was developed by mutagenesis of the ligand binding site of the NADH/NAD+ sensor SoNar to switch the selectivity from NADH to NADPH [74]. iNap can be used *in vivo,* and a proof of concept experiment was performed in a wound healing assay in zebrafish larvae. In combination with a red version of HyPer, the iNap biosensor revealed the concomitant decrease of NADPH levels after tissue wounding with an

increase in H_2O_2 levels, which is consistent with NADPH consumption by NOXs during wound healing [74].

All these sensors provide invaluable information on cellular redox status *in vivo*. However, it is worth reminding that all H_2O_2 sensors consume H_2O_2 to measure its concentration. Thus, there is room to improve these systems in particular through increased sensitivity. Only one molecule of H_2O_2 is consumed to modify each molecule of sensor for both HyPer and roGFP2-Orp1. However, for the later, it also depends on the local redox potential. This is a clear illustration of a common difficulty: seeing is modifying.

4. Redox signalling in animal development and regeneration

H_2O_2 is generated in response to many stimuli, including cytokine or growth factors, which are involved in embryonic development and adult homeostasis. Given redundancy in the redox machinery components, most single KOs in mice are viable, and embryonic development generally occurs normally [75]. Metazoan development can be divided into 3 phases: (1) fertilization and cleavage period, (2) gastrulation, and (3) morphogenesis. At the adult stage, most tissues are continuously renewed and, in some species, rebuilt after amputation. This section will describe the state of the art for the role of redox signalling during these processes. It will not address molecular targets of redox signalling, which is well reviewed by Covarrubias [6, 7]. The role of reactive nitrogen species and oxidative stress in pathologies of the nervous system, which are not discussed here, are extensively reviewed in [76-78]. Finally, it is worth mentioning that some developmental effects of redox signalling in the brain only become apparent much later (as for instance in the case of critical periods [79] or mental illnesses, such as schizophrenia [80]. These effects have only

been analysed in the context of their dysregulation, but their normal progress certainly warrants better examination.

4.1.　Embryonic development

Given the importance of NOXs in anti-microbial defence, inflammation, disorders including cancers, and more generally in the maintenance of redox balance, most studies on these enzymes in metazoans focus on adult expression. Some NOXs exhibit broad distribution among tissues (NOX2 was first believed to be exclusively expressed in neutrophils and macrophages and is currently known to present the largest distribution). Others are more restricted (NOX3 is predominantly expressed in the inner ear), but none of them is ubiquitously expressed. Constitutive expression coexists with induction phenomena (for review see [14, 81]. NOX expression also greatly varies during development (Table 1). The most detailed study was performed by qPCR and *in situ* hybridization in developing zebrafish [82]. Unlike the uniform and homogeneous expression of Nox2, Nox1 and Nox5 expression is high during gastrulation and then decreases to a basal level upon morphogenesis. During this period of development, Nox1 expression is increased in the brain. The expression of dual oxidase (Duox), which is a member of the NOX family, is increased during late morphogenesis [82]. Though useful, these expression data did not provide a clear picture on the physiological function of these enzymes.

The use of the HyPer probe in a live animal revealed a surprising high level of oxidation during zebrafish development, and H_2O_2 levels proved to be heterogeneous and dynamic in space and time [66] (Figure 2). H_2O_2 levels occasionally exhibit a graded distribution with clear functional outcome. This distribution occurs in the embryonic tectum where the organization of the retinotectal projections is impaired by Pan NOX inhibition [66]. Nox2 invalidation using the CRISPR/Cas9 strategy

induced the same phenotype [83], confirming NOX2 involvement in axon pathfinding during zebrafish development. A role for Nox enzymes was also evidenced *ex vivo* for the establishment of rat hippocampal neuron polarity in culture [84, 85], the *in vitro* maturation of rat cerebellar granule neurons [86], and growth cone dynamics in *Aplysia* neuron [87-89].

Catalase, SOD1 and GPx expression and activities have been analysed during mice development from 8 days of gestation to adulthood [90]. mRNAs of these 3 proteins increase during somitogenesis. Then, catalase activity increases after birth, whereas SOD1 and GPx activities reach a plateau. In zebrafish, SOD activity is globally constant throughout morphogenesis until 7 days post fertilization, whereas an increase in catalase activity is observed from 48 hpf onward when morphogenesis is almost completed [66], (Figure 2). In *Drosophila*, catalase protein is minimally detectable during embryogenesis and enhanced during the third instar larval stage and after the first day of pupal development [91]. It thus appears that low levels of catalase expression during development and high levels in mature tissues represent a general property that has been verified in mice, *Drosophila* and zebrafish.

GSH/GSSG balance is a crucial redox parameter during development [for review: [92]. Embryos from mutant mice invalidated for the enzymes responsible for GSH synthesis fail to gastrulate, do not form mesoderm, develop distal apoptosis, and die before day 8.5 [93, 94]. In zebrafish, the total amount of glutathione doubles during embryonic development. The redox potential (E_{GSH}) is high in eggs and late larval stage but very low during morphogenesis [95]. This study defined 4 GSH contexts during embryonic development: (1) high E_{GSH} and low GSH in mature oocytes, (2) low E_{GSH} and low GSH from mid-blastula to 24 hpf, (3) high E_{GSH} and high GSH during organogenesis (30-48 hpf) and (4) high E_{GSH} and high GSH in mature larvae.

GRX patterns of expression during development have not been systematically investigated, but expression of Grx2 and its isoforms in vertebrate tissues at various stages (mostly adults) is described in [96-99]. Their role during development has been demonstrated in different models. One of the first hint (though outside metazoan) was that Grx1 affects cell fate decision in the culmination process in *Dictyostelium discoideum* [100]. It was subsequently shown that knocking down cytosolic Grx2 in zebrafish embryo impaired the development of the central nervous system. Grx2 is indeed essential for neuronal differentiation, survival and axon growth [101]. In mammals, Grx2 controls axon growth via a dithiol-disulfide switch affecting the conformation of CRMP2, a mediator of semaphorin-plexin signalling pathway [102]. The same Grx2 isoform is also required for correct wiring of embryonic vasculature in zebrafish by de-glutathionylation of the active site of the NAD-dependent deacetylase Sirtuin-1 [103]. Grx2 is also implicated in embryonic heart formation. Grx2 knock down (KD) in zebrafish embryo prevented neural crest cell migration into the primary heart field, impairing heart looping [104]. The role of other members of the Grx family was also described in erythropoiesis. Grx5 is essential to zebrafish haeme synthesis through assembly of the Fe-S cluster, and this role is apparently conserved in humans [105]. Grx3 is also crucial to red blood cell formation in zebrafish as demonstrated by the reduced number of erythrocytes in embryos treated with Grx3 morpholinos and is required in human cells for the biogenesis of Fe-S proteins, as demonstrated by silencing Grx3 expression in HeLa cells [106]. Grx3 KO in mice induces a delay in development and eventually death approximately 12.5 days of gestation. *Ex vivo* analysis of Grx3$^{-/-}$ cells reveals impaired growth and cell cycle progression at the G2/M transition [107]. The loss of Grx3 also disturbs the development of mammary alveoli during pregnancy and

lactation [108]. In addition to these roles during development proper, Grx proteins are also involved in various physio-pathological processes (for review [41]).

Two distinct thioredoxin/thioredoxin reductase systems, Trx1 and Trx2, are present in mammalian cells in the cytosol and mitochondria. Trx1 and Trx2 are ubiquitously expressed, but Trx2 is expressed at higher levels in metabolically active tissues, such as heart, brain, and liver [109]. Interestingly, in mice, Trx1 (the cytosolic and nuclear Trx) KO is embryonic lethal early after implantation [110] and presents a dramatically reduced proliferation of inner mass cells. Trx2 (the mitochondrial form) KO is also embryonic lethal. Embryos die later during development (between 10.5 and 12.5 dpf), and lethality is associated with increased in apoptosis and exencephaly development [111]. Trx2 KD in zebrafish increases apoptosis and induces developmental defects in the liver [112]. In chick, Trx2 KD impairs post mitotic neurons and induces massive cell death [113]. Thioredoxin reductases (TrxRs) KO reveals that TrxR1 (non-mitochondrial form) is embryonic lethal (E10.5) with multiple abnormalities in all organs except heart [114], whereas TrxR2 KO leads specifically to haematopoietic and heart defects [115]. These works demonstrate a clear dichotomy in the tissue specificity of TrxR1 and 2 actions during development: broad or restricted to heart and haematopoietic lineage development. In addition, Trxs play critical roles in immune response, cancer (for review [116]), and various pathologies in the nervous system (for reviews [76, 78]).

The peroxiredoxins (Prxs) family includes 6 members in vertebrates. These enzymes have been mostly studied in physio-pathological contexts or *ex vivo* where it was demonstrated that Prxs are major regulators of cell adhesion and migration [117]. *In vivo*, Prx regulates cadherin expression during early *Drosophila* development [118]. Prxs are typically broadly expressed in embryos with mild specificity amongst

isoforms. However, an exhaustive analysis in *Xenopus laevis* reveals maternal expression of prx1, 2, 3 and 6, which persists through all developmental stages. In contrast, prx4 mRNA becomes detectable at gastrulation and increases afterwards, and prx5 mRNA is always detected but at low levels. Additional specificities are revealed by *in situ* hybridization. Specifically, prx1 is expressed in anterior structures, and prx4, 5 and 6 expression is preferentially detected in somites [119]. This pattern of expression is not completely conserved between *Xenopus* and other vertebrates. In zebrafish, Prx1 is expressed in developing vessels, and prx1 KD induces vascular defects [120]. In mice, proteomic analysis suggested that prx1 is involved in digit formation where it regulates interdigit apoptosis [121]. One of the best examples of Prxs' roles during normal differentiation is the involvement of Prx1 and Prx4 in the formation of motor neurons in the spinal cord of chicken and mouse [122, 123].

Sexual reproduction and more precisely gamete formation is an unusual process regarding redox signalling. NOXs are involved in spermatogonia and germline stem cell renewal [124]. Moreover, a significant amount of H_2O_2 is needed to favour disulfide bridge formation during spermatozoa maturation and capacitation (for review [125]). Conversely, ROS insult should be neutralized to protect DNA and maintain genomic integrity from generation to generation. Several GPx members are involved in these opposite aspects of redox signalling [126].

4.2 Adult stem cells and tissue homeostasis

During life, adult stem cells replenish damaged and lost tissues. In recent years, H_2O_2 appeared to be a major component of stem cell niche, stem cell renewal and recruitment for differentiation (for review [127, 128]). In some studies, an increase in H_2O_2 is responsible for stem cell differentiation. In *Drosophila*, increased H_2O_2 induces the differentiation haematopoietic progenitor cells, whereas a reduction

delays the expression of differentiation markers [129]. In mammals, an increase in H_2O_2 induces vascular muscle cell [130] or blood stem cell [131] differentiation. In an apparent contradiction, high H_2O_2 levels are associated with stem cell renewal and proliferation. The renewal of intestinal stem cells in *Drosophila* is dependent on H_2O_2 [132], and self-renewal of neural stem cells is under the control of NOX activity in the mouse [133, 134]. These opposite effects of H_2O_2 illustrate our limited understanding of cell fate regulation by redox signalling but both strengthen the relevance of ROS levels in the control of stem cell behaviour and the need for their tight regulation. Some clues come from the iPSC field where H_2O_2 increase is essential during the early phase of iPSC generation. Reduction of H_2O_2 levels by NOX KD or antioxidant treatment suppresses nuclear reprogramming [135]. NOX2 is also involved in induced pluripotent stem cell (iPSc) differentiation into endothelial cells [136].

4.3. Regeneration

Some species have the ability to regenerate damaged or removed body parts at adulthood [137-139]. Regeneration is probably the best paradigm of adult morphogenesis. The first step of regeneration is wound repair and the formation of a wound epidermis. Shortly after wound epidermis formation, progenitor cells are generated via stem cells that are recruited and the dedifferentiation of differentiated cells [138, 140, 141]. The newly formed progenitors (and stem cells in some systems) migrate to the wound epithelium to form a mass of undifferentiated cells called the blastema. The entire missing structure will be formed by differentiation and morphogenesis of blastema cells. The process of regeneration can be divided into 3 modules: (1) injury immediate response (wound healing), (2) regeneration induction

(blastema formation), (3) formation of the missing structure (blastema growth, differentiation and patterning) [142] (Figure 3A).

It has been known for a long time that wounds generate ROS, specifically H_2O_2, in phylogenetically diverse organisms, such as *Drosophila melanogaster*, *C. elegans*, zebrafish, [143-146], and plants [147-149]. In zebrafish, H_2O_2 production triggered by wounding is restricted to the first two hours following injury in larva [144] and adult [150]. After a lesion is generated, a gradient of H_2O_2 formed by NOX activity is required for leukocyte recruitment to the wound [144, 151]. The role of H_2O_2 is not restricted to wounding and healing but extends to other steps of regeneration. When a body part is removed, induction of the regenerative programme correlates with a sustained production of H_2O_2 for several hours (Figure 3B) [150]. It has been demonstrated in different model organisms that after amputation, H_2O_2 signalling not only modulates the regeneration process but is indispensable for launching it (Table 2). Planarian and Hydra regenerate not only body parts but also their body axis [139, 152]. In these animals, ROS are detected at the level of the amputation plane shortly after amputation, and reduction of H_2O_2 levels impairs regeneration [153, 154] (Figure 3B). In *Drosophila*, regeneration can be observed in imaginal discs during larval development and adult gut regeneration [155-157]. In both systems, regeneration is redox-dependent [132, 155, 156, 158-161]. In the *Xenopus* tadpole tail regeneration model, amputation-induced H_2O_2 production is necessary to activate Wnt/β-catenin, Fgf signalling and acetylation of lysine 9 of histone 3 (H3K9ac) [162, 163] (Figure 3B). In the newt, ROS production is also necessary for neural stem cell proliferation, neurogenesis, and regeneration of dopamine neurons [164]. Moreover, recent data demonstrate that H_2O_2 activates voltage-gated Na^+ channels, linking redox signalling to bioelectric signalling during regeneration [165]. During gecko tail

regeneration, H_2O_2 is produced by skeletal muscles and is also required for successful tail regeneration. In this case, H_2O_2 levels control autophagy in the skeletal muscles and consequently the length of the regenerated tail [166]. In adult zebrafish heart, caudal fin and superficial epithelial cells regeneration models, perturbation of ROS levels through the inhibition of NOX or overexpression of catalase impairs regeneration (Figure 3B) [150, 167-169]. Different targets were identified at the cellular (neural cells), functional (apoptosis), and molecular levels (MAP kinases and Sonic Hedgehog). In mammals, regeneration at adulthood is very limited [170]. The comparison of regeneration of large circular defects through the ear pinna between regenerative mammals (*Acomys cahirinus*) and non-regenerative mammals (*Mus musculus*) revealed a strong correlation between H_2O_2 levels after injury and regenerative capacities (Figure 3B) [171]. In rat, it was further demonstrated that H_2O_2 participates in liver regeneration after partial hepatectomy. In this case, sustained and elevated H_2O_2 levels activate MAP kinase signalling that triggers the shift from quiescence to proliferation [172]. Recently, it has been demonstrated that H_2O_2 produced shortly after inguinal fat pad damage is responsible for its regeneration in MRL mice (Figure 3B). In the non-regenerative C57BL/6 strain, artificial enhancement of H_2O_2 leads to regeneration [173].

5. Conclusions: towards the redox code

Redox signalling interacts directly or indirectly with most of the signalling pathways that control embryonic development. However, we are only starting to perceive the tip of the iceberg [174]. A comprehensive understanding of developmental redox biology will benefit from a better characterization of thiol targets. For this purpose, the optimization of redox proteomics and the *in silico* identification of reactive thiols susceptible to redox regulation based on 3D rather than 2D models are two

promising strategies [175]. One can note that unlike other posttranslational modifications (i.e., phosphorylation or ubiquitination), which behave as binary switches, thiol modifications are diverse, including the formation of sulfonic acids (-S-OH), S-nitro groups (S-NO) and disulfides bridges [176]. Each of these modifications could confer a specific status to the targeted protein, thus extending the spectrum of regulation provided by redox signalling [177]. Finally, because the different members of the redox machinery are interconnected, the modification of a specific thiol likely depends on the equilibrium of the entire redox machine. Modelling the entire process by integrating dynamic and quantitative information of the different redox machinery members would greatly help to decipher the physiological role of redox signalling.

Acknowledgements

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Tables

Table 1: Expression of the redox machinery genes during zebrafish development
No results have been reported for gpx2, gpx3, gpx9, nox4, sod3a, txnrd2-1, and txnrd2-2.
CNS: central nervous system; DS: digestive system; SS: sensory system.

Enzyme	Gene	Cellular localization	Gene expression	Reference
Catalase	cat	mitochondria, peroxisome	brain, DS, gill, muscle, SS	Tisse et al. ZFIN 2004
Glutaredoxin	glrx2	cytoplasm	whole organism	[101]
	glrx3	cytoplasm	brain, heart, SS	Thisse et al. ZFIN. 2004
	glrx5	cytoplasm	blood island, DS, heart, SS	Kudoh et al. ZFIN. 2001
Glutathione peroxidase	gpx1a	nd	DS, muscle, SS	Thisse et al. ZFIN. 2001
	gpx1b	cytoplasm	DS, SS	Thisse et al. ZFIN. 2004
	gpx4a	nd	DS, peridermis	Thisse et al. ZFIN. 2001, Mendieta-Serrano et al. Gene exp patterns. 2015
	gpx4b	nd	blastoderm, DS, epidermis, epiphysis, muscle, pharyngeal arch, pronephric duct, SS	Thisse et al. ZFIN. 2001, [178]
	gpx7	nd	notochord, splanchnocranium	Thisse et al. ZFIN. 2004
	gpx8	membrane	notochord, pharyngeal arch, SS	Thisse et al. ZFIN. 2004
Glutathione reductase	gsr	cytoplasm	DS, macrophage	Thisse et al. ZFIN. 2004
NADPH oxidase	nox1	membrane	brain, spinal cord, SS	[82]
	nox2/Cybb	membrane	blood, brain, spinal cord, SS	[82] Thisse et al. 2004
	nox5	membrane	brain, spinal cord, SS	[82]
	duox	membrane	brain, DS, epidermis, spinal cord, SS, swim bladder, thyroid,	[82]
	duox2	ER membrane	brain, DS, thyroid	[179]
Peroxiredoxin	prdx1	cytoplasm	brain, neural crest derivatives, vessels	[120]
	prdx2	nd	blood, CNS, DS, pharyngeal arch, SS	Rauch et al. ZFIN 2003; Tisse et al. ZFIN 2001
	prdx3	nd	blood, DS, myotome, pharyngeal arch, SS	Tisse et al. ZFIN 2001
	prdx4	nd	DS, hatching gland, pharyngeal arch, SS	Tisse et al. ZFIN 2004
	prdx5	nd	macrophage, pronephric duct, SS	Tisse et al. ZFIN 2004
	prdx6	nd	DS, rhombomere, SS	Tisse et al. ZFIN 2004
Superoxide dismutase	sod1	cytoplasm	whole organism	Tisse et al. ZFIN 2004
	sod2	mitochondria	blood, brain, DS, gill, kidney, muscle, SS	Tisse et al. ZFIN 2001
	sod3b	cytoplasm	whole organism	
Thioredoxin	txn	nd	DS, Hypophysis, spinal cord, SS, tegmentum	Tisse et al. ZFIN 2004
	txn2	mitochondria	whole organism	Tisse et al. ZFIN 2004
Thioredoxin reductase	txn	cytoplasm	brain, DS, spinal cord, SS, tegmentum	Tisse et al. ZFIN 2004;
	txnrd3	mitochondria	blood, CNS, DS, muscle, pharyngeal arch, spinal cord, SS	Tisse et al. ZFIN 2001

Table 2: Redox regulation of regeneration among Phyla
APO: apocynin; DHE: dihydroethidium; dpa: days post amputation; hpa: hours post amputation. ZFIN: https://zfin.org/

	Animals/ Species	Stage	ROS detection	Profil	ROS modulation	Organ/Appendage	ROS targets	reference
Cnidaria	Hydra		DMPO	wound edge				[153]
Platyhelminthes	Planarian (*Schmidtea mediterranea*)		H2DCFDA	burst at the wound site	DPI, APO	central nervous system	neuroregeneration	[154]
Arthropoda	Drosophila (*Drosophila melanogaster*)	Larvae	CellRox green	burst after apoptosis induction	NAC, vitamin C, Trolox, SOD, CAT	wing imaginal disc	p38 pathway / JNK pathway	[155]
		Larvae	DHE, H2DCFDA	up to 24 h after apoptosis induction	misexpression of extracellular CAT	eye and wing imaginal disc	macrophages	[156]
		Adult	H2DCFDA	burst after oral administration of HgCl2	Vitamin E	midgut		[157]
Amphibians	Xenopus (*Xenopus laevis or tropicalis*)	Tadpole	HyPer	production 6 h - 4 dpa	DPI, APO, MCI-186	Tail	Wnt/b-catenin pathway / FGF pathway	[162]
			DHE	nd	DPI, MCI, VAS, H_2O_2		bioelectric activity	[165]
			H2DCFDA	nd	APO	Notochord in tail	acetylation of H3K9	[163]
Squamata	Gecko (*Gekko japonicus*)	Adult	H2DCFDA	production (0 - 7 dpa), skeletal muscles	DPI, APO	tail	autophagy in skeletal muscles (via ULK and MAPK activities)	[166]
Teleost fish	Zebrafish (*Danio rerio*)	Larvae	HyPer	nd	DPI	caudal fin	Src family kinase	[180]
		Adult	H2DCFDA	production 0 - 16 hpa	VAS2870, DPI		Apoptosis / JNK pathway	[150]
			H2DCFDA, HyPer		VAS2870, H_2O_2		Hh pathway / nerve	[169]
			Myl7:HyPer, Redox sensor cc-1	production 3 - 14 dpa epicardium and adjacent myocardium, max 7 dpa	DPI, APO, Catalase	heart	ERK pathway	[167]
			CellRox green	production 2h - 12 hpa	VAS2870	superficial epithelial cells (SECs) in caudal fin	nd	[168]
Mammals	Rat (*Rattus norvegicus*)	Adult	H2DCFDA, red H_2O_2 assay kit, amplex red H_2O_2 assay kit	production 1h - 3 dpa	GOX, CAT	liver	ERK pathway / p38 pathway	[172]
	Mice (*Mus musculus*)	Adult	luminol	MRL mice (production 0 - 72 h pa, max 12h), C57Bl6 (no significant production)		inguinal fat pad		[173]
			lucigenin	no significant production 3h - 10 dpa		ear		[171]
			luminol	production 3h - 4 dpa				
	Spiny mice (*Acomys cahirinus*)	Adult	lucigenin	production 3h - 5 dpa				
			luminol	no significant production 3h - 10 dpa				

Figure legends:

Figure 1: The redox machinery

Interconnection of redox couples from H_2O_2 to thiol targets are represented. H_2O_2 is a by-product of oxidative reactions. Major

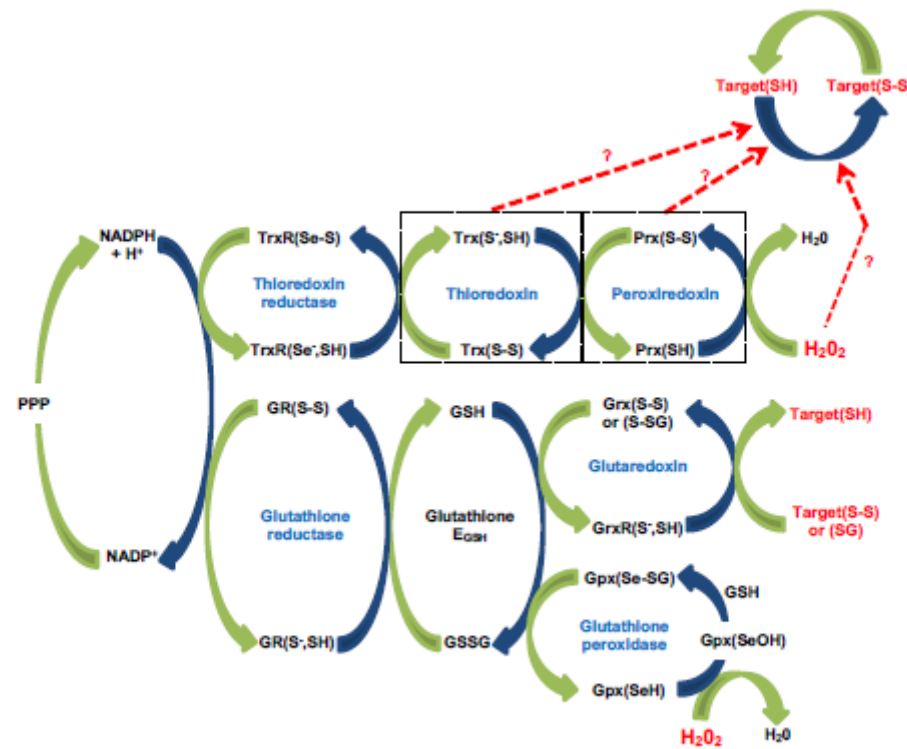sources include mitochondrial respiratory chain and NOXs [for review [13]. PPP: Pentose Phosphate Pathway

Figure 2: H$_2$O$_2$ detection during development

Upper panel: H$_2$O$_2$ detection during *C. elegans* development. Adapted from Bach 2012 Free rad biol med and Knoefler 2012 Mol

Cell.

Middle panel: H$_2$O$_2$ levels and catalase activity during zebrafish development. Adapted from [Gauron 2016 Dev Biol].

Lower panel: Zebrafish HyPer fish reveal spatio-temporal dynamic and gradients of H$_2$O$_2$ during neural development. hpf: hours
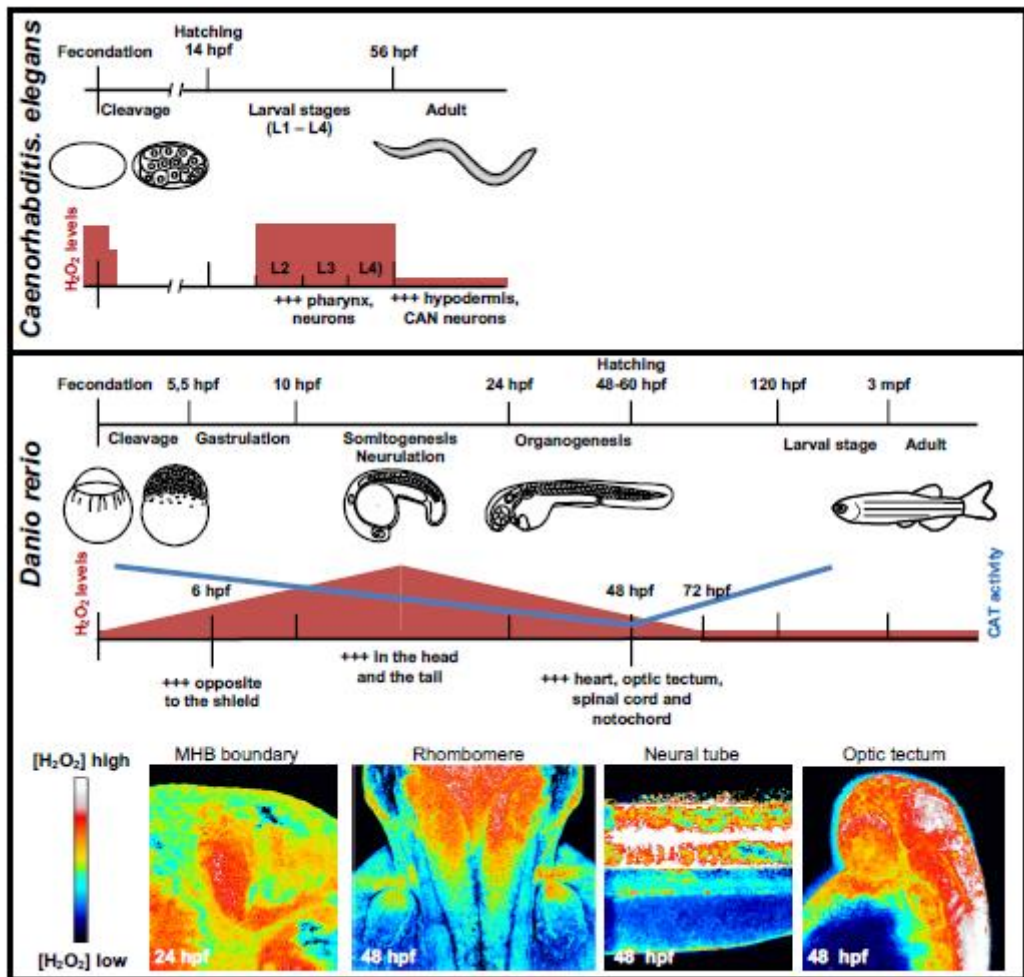
post fertilization.

Figure 3: H$_2$O$_2$ detection during metazoan regeneration

A: regeneration is divided in three modules. **B:** H$_2$O$_2$ levels during regeneration in different models and organs. dpa: days post

amputation. Adapted from [154] [162] [150] [167] [171] [173] [181].