Review

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Genetically encoded thiol redox-sensors in the zebrafish model: lessons for embryonic development and regeneration

<https://doi.org/10.1515/hsz-2020-0269>

Received July 31, 2020; accepted September 28, 2020;

published online October 15, 2020

Abstract: Important roles for reactive oxygen species (ROS) and redox signaling in embryonic development and regenerative processes are increasingly recognized. However, it is difficult to obtain information on spatiotemporal dynamics of ROS production and signaling *in vivo*. The zebrafish is an excellent model for *in vivo* bioimaging and possesses a remarkable regenerative capacity upon tissue injury. Here, we review data obtained in this model system with genetically encoded redox-sensors targeting H₂O₂ and glutathione redox potential. We describe how such observations have prompted insight into regulation and downstream effects of redox alterations during tissue differentiation, morphogenesis and regeneration. We also discuss the properties of the different sensors and their consequences for the interpretation of *in vivo* imaging results. Finally, we highlight open questions and additional research fields that may benefit from further application of such sensor systems in zebrafish models of development, regeneration and disease.

Keywords: embryo; glutathione; hydrogen peroxide; imaging; regeneration; zebrafish.

Introduction: why zebrafish?

The zebrafish (*Danio rerio*), a small sweet water fish originating from India, has received steadily growing attention

as a model for vertebrate biology over the past 40 years (Grunwald and Eisen 2002). Initially mainly used to tackle the genetics of vertebrate development, an ever increasing number of research topics has benefited from zebrafish studies, including neurobiology (Bollmann 2019), cardiovascular research (Gut et al. 2017), endocrinology (Lohr and Hammerschmidt 2011), toxicology (Bambino and Chu 2017), behavior (Orger and de Polavieja 2017), chronobiology (Froland Steindal and Whitmore 2019), and regeneration (Marques et al. 2019). Main features for this success were the transparency of zebrafish embryos, which allows for *in vivo* imaging helped by a wealth of transgenic lines targeting e.g. cell behavior, neural activity and gene expression patterns (Chow and Vermot 2017; Trinh le and Fraser 2013); and the small size of both adults and embryos, allowing large-scale mutagenesis and chemical compound screens (Bambino and Chu 2017; Lam and Peterson 2019; Patton and Zon 2001) (Figure 1). Importantly, zebrafish are used to model human diseases, both to understand the molecular mechanisms of pathogenesis and to provide a system amenable to rapid preclinical testing of new drugs and treatments (Davis and Katsanis 2017; Lam and Peterson 2019).

The zebrafish as a model system for embryonic development

Embryonic development of the zebrafish is very rapid (Kimmel et al. 1995): gastrulation, which generates the three germ layers and establishes the embryonic axis, is accomplished by 10 h post fertilization (hpf). The subsequent somitogenesis period is characterized by the formation of mesodermal tissue blocks next to the midline. These somites will give rise to muscle tissue and skeletal elements. By 24 hpf, basic features of major organ systems such as the nervous and cardiovascular systems as well as fin folds are formed. Slightly later, the spontaneous movements of the embryo become responsive to touch and light stimuli, indicative of increasing functional integration within the

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Figure 1: Zebrafish expressing genetic sensors serve as a tool guiding targeted manipulation and analysis of redox processes in development and regeneration.

nervous system. Retinal ganglion cells begin to form and their axons grow towards the optic tectum, where they will form functional connections by 72 hpf (Kita et al. 2015). At 48 hpf, embryos begin to hatch from their eggshells, the chorions, and at 120 hpf they start to hunt for food. At around 3–4 weeks, the primordial gonads start to differentiate into testis or ovary, and sexual maturity is reached at the age of 3 months (Orban et al. 2009).

Importantly, many of the genes regulating embryonic patterning and tissue differentiation are conserved between zebrafish and mammals (Schier and Talbot 2005; Solnica-Krezel and Sepich 2012; Yabe and Takada 2016; Zinski et al. 2018), including genes of the Hedgehog (Hh), Bone Morphogenetic Protein (BMP), Nodal, Fibroblast Growth Factor (FGF), Wnt, retinoic acid (RA) and Notch-Delta pathways. Zebrafish studies have provided insight, for example, into functions of the TGF β -type Nodal signaling pathway in germ layer specification and left-right asymmetry (Zinski et al. 2018). In addition to conserved players and interactions, differently organized network features have been revealed as well, which give us glimpses of the functional and evolutionary plasticity of such regulatory networks (Pogoda and Hammerschmidt 2009; Yabe and Takada 2016). Notably, the possibility in zebrafish of observing the development of different embryonic stem cell populations *in vivo* and to follow the differentiation trajectories of their progeny already allows for insights

that may inform the development of stem cell therapies for human patients (Perlin et al. 2017; Sutcu and Ricchetti 2018).

The zebrafish as a model system for regeneration

Resident stem cell populations also play a major role in the regeneration of many adult tissues after injury (Mokalled and Poss 2018; Marques et al. 2019). Due to its strong regenerative capacity upon tissue damage, contrasting with the limited capabilities of mammals, the zebrafish has become a model of choice to study regeneration in a variety of tissues, including kidney, liver, pancreas, brain, spinal cord, heart and fins (Marques et al. 2019). A better understanding of the molecular mechanisms allowing for tissue regeneration in the so-called “lower” vertebrates may give cues on how to improve or even launch regenerative processes in species with limited tissue regeneration capacities, such as ourselves (Iismaa et al. 2018; Marques et al. 2019).

The regeneration process easiest to study in zebrafish is probably fin regeneration, which is initiated for example by clipping of the distal part of the tail fin (Sehring and Weidinger 2020). In an initial wound response, leukocytes are recruited and the wound is closed by an epidermal cap. Then a so-called blastema is formed in the underlying tissue. This mass of dedifferentiated cells proliferates and its cells differentiate into the missing tissue types as regeneration proceeds. Similar processes of wound response and the formation of an underlying blastema-like tissue can also be observed upon regeneration of the larval fin fold (Kawakami et al. 2004; Mateus et al. 2012; Roehl 2018). Dedifferentiation and proliferation followed by re-differentiation equally occur during heart regeneration, as shown by lineage tracing studies that revealed pre-existing cardiomyocytes as the source of the new tissue (Jopling et al. 2010; Kikuchi et al. 2010). Also trans-differentiation processes are possible: for example, pancreatic β -cells can be regenerated from α -cells (Lu et al. 2016). Following a different strategy, regeneration of injured brain tissue, for example upon a stab wound to the telencephalon, requires the activation of neural stem cells, which are radial glia cells of the ventricular zone (Schmidt et al. 2013; Zambusi and Ninkovic 2020).

A few common insights emerge from these studies. All regeneration requires proliferation of precursors and differentiation into the cell types of the destroyed tissue, with differences between tissues mainly in the origin of the

precursors (stem cells or dedifferentiation) and the differentiation trajectories (re- or transdifferentiation). These processes frequently reemploy signaling molecules and networks active during embryonic development (Cardozo et al. 2017; Sehring and Weidinger 2020; Yang and Kang 2019), either in the same organ (e.g. transcription factors involved in anterior-posterior patterning of limb buds (Nachtrab et al. 2013)) or in another context (e.g. *simplex/Fam35B*, a regulator of Wnt signaling, newly expressed during larval and adult fin regeneration (Kizil et al. 2009, 2014)). Interestingly, enhancer elements driving injury induced expression of *leptin b* in zebrafish can still be activated in mice, suggesting a conserved upstream regulatory network that in mammals has been uncoupled from regeneration-inducing processes, but could potentially be employed to stimulate regeneration (Kang et al. 2016; Yang and Kang 2019).

Two other common themes in regeneration are particularly pertinent to the topic of this review. First, the nervous system plays a crucial role in several regeneration processes, because denervation or inhibition of neural activity can impair regeneration (Mahmoud et al. 2015; Simoes et al. 2014). Second, the immune system and the inflammatory response mounted by it have been shown to play important roles in brain, spinal cord, heart and fin regeneration (Bosak et al. 2018; Martins et al. 2019; Marques et al. 2019; Petrie et al. 2014). Among the mechanisms implicated in these functions is the production and sensing of reactive oxygen species, as we will discuss in further detail below.

Reactive oxygen species and their role in cellular signaling processes

Reactive oxygen species (ROS) comprise several small molecules containing oxygen which easily engage in redox reactions, such as the hydroxyl radical ($\text{OH}\cdot$), the superoxide anion ($\text{O}_2^{\cdot-}$) or hydrogen peroxide (H_2O_2). ROS are produced, for example, in the mitochondria (Wong et al. 2017) or by membrane bound NADPH-dependent oxidases (Nox1-5 and Duox1-2) that generate superoxide anions on the non-cytoplasmic side of the membrane (Panday et al. 2015). Superoxide dismutase enzymes convert superoxide anions to O_2 and H_2O_2 , while catalase degrades H_2O_2 to water and O_2 (Glorieux and Calderon 2017; Winterbourn 2020). High levels of ROS causing DNA damage and oxidation of proteins and lipids classically have been implicated in metabolic diseases and ageing. This somewhat simplistic view of “oxidative stress” has recently been

modified, arguing for “antagonistic pleiotropy” of ROS functions partially based on clinical observations that global antioxidant treatments were not successful in prevention or amelioration of several diseases or even had detrimental effects (Bjelakovic et al. 2007; Ristow et al. 2009; Sies and Jones 2020). Furthermore, recent redox proteomics studies of ageing rather found a remodeling of redox signaling networks than a global increase of thiol oxidation (Menger et al. 2015; Xiao et al. 2020). Consistently, ROS molecules are now known to play important roles in cellular signaling under physiological conditions (Finkel 2011; Schieber and Chandel 2014; Sies et al. 2017; Sies and Jones 2020).

A whole network of proteins serve as ROS scavengers and regulators of the oxidation state of proteins in the cell via the thiol residues of specific cysteines (Cys). Peroxiredoxins (Prxs) can reduce peroxides including H_2O_2 , and they themselves are reduced by interaction with thioredoxins (Trxs) and glutaredoxins (Grxs), which also target other proteins (Hanschmann et al. 2013). Glutaredoxins take their reduction equivalents from glutathione, a peptide composed of cysteine, glycine, and glutamate, the latter bound via its γ -carboxylgroup. Its reduced form (GSH) is abundant in the cell and forms a redox couple with its disulfide form GSSG. Glutathione peroxidase family members use GSH to reduce H_2O_2 or organic hydroperoxides (Margis et al. 2008). Glutathione reductases reduce GSSG, while thioredoxin reductases reduce oxidized forms of thioredoxins (Hanschmann et al. 2013). Both reductase classes derive reduction equivalents from NADPH, generated for example in the pentose phosphate cycle. NADPH thus can serve both to produce (via Nox enzymes) and to scavenge H_2O_2 .

By regulating the oxidation state of key residues in cellular signaling proteins, the components of this system directly influence signaling pathways (Finkel 2011; Holmstrom and Finkel 2014; Sies et al. 2017). For example, the Wnt signaling pathway component Dishevelled is bound by the thioredoxin-like nucleoredoxin under reduced conditions, thereby inhibiting Wnt signaling (Funato et al. 2006). Similarly, the nuclear localization of the transcription factor Nrf2, a component of the oxidative stress response, is regulated by the oxidation state of Keap1. Oxidized Keap1 cannot target Nrf2 for ubiquitination and subsequent degradation, so that it accumulates and localizes to the nucleus (Zhang and Hannink 2003). Signaling pathways can also use H_2O_2 as a second messenger (Holmstrom and Finkel 2014), and recent findings suggest that peroxiredoxins may play a widespread role in relaying this H_2O_2 signal to target proteins (Stocker et al. 2018a; Stocker et al. 2018b).

Genetically encoded sensors for imaging of H₂O₂ levels *in vivo*

To fully understand the roles that H₂O₂, other ROS species and redox state play in cellular signaling and other physiological processes, information on their spatiotemporal dynamics *in vivo* is needed. By now, there exists a plethora of small compounds for imaging of various redox species (for an overview, see Hernandez-Garcia et al. 2010; Winterbourn 2014; Zielonka and Kalyanaraman 2018). While their application appears straightforward, there are a number of caveats that have to be considered especially when applying them *in vivo*. Thus, the penetration capacity of such sensors into tissues or embryos may be limited, they may be subject to sequestration into particular cell compartments or alter cellular functions. Some sensors are restricted by slow reaction kinetics or by auto-oxidation processes creating a high background level. Finally, specificity is often low, such that these molecules can sometimes be used only as indicators for a general presence of ROS, but not for a certain species.

Compared with these drawbacks, genetically encoded sensors can be better targeted to certain tissues, cells or subcellular compartments (Schwarzlander et al. 2016). Their usually well-developed reversibility and reaction kinetics allow for rapid dynamic measurements (which can, however, be a drawback if one is aiming to measure cumulative levels of ROS production in a tissue and cannot continuously monitor the specimen). When combined with specific redox-related proteins, they can achieve high specificity for a particular ROS species. We here discuss the two families of genetically encoded sensors that have so far been used most frequently in zebrafish studies (Table 1).

HyPer sensors

One broadly used type of genetically encoded sensor for redox state alterations is based on circularly permuted fluorescent proteins (cpFPs) (Kostyuk et al. 2019). In cpFPs, the original protein ends are fused and new termini generated at another place in the sequence while maintaining the overall structure. When now fused to sensor proteins, the more flexible structure of the cpFPs allows the transmission of structural alterations in the sensor moieties to the fluorophore, thereby affecting its spectral properties. The HyPer-type sensors widely used in redox imaging studies consist of a circularly permuted Yellow Fluorescent Protein (cpYFP) that is fused to the regulatory domain of the redox-sensitive bacterial transcription factor OxyR.

Table 1: Overview of genetically encoded thiol redox-sensor variants used in zebrafish studies.

Sensor	Target	Comments	Zebrafish examples
HyPer	H ₂ O ₂	Sensitive to pH	Gauron et al. (2016), Han et al. (2014), Meda et al. (2016), Niethammer et al. (2009), Pase et al. (2012)
HyPer3	H ₂ O ₂	Sensitive to pH, higher amplitude than HyPer	Bilan et al. (2013)
HyPer7	H ₂ O ₂	pH-insensitive, higher sensitivity than other HyPers	Pak et al. (2020)
SypHer	pH	Control for HyPer sensors, redox-insensitive	Ermakova et al. (2018) [<i>SypHer3s</i>]
roGFP2	H ₂ O ₂	Reduced by endogenous Grx	O'Donnell et al. (2013)
roGFP2-Orp1	H ₂ O ₂	Less sensitive than TSA2ΔC _R – based sensors	Panieri et al. (2017), Panieri and Santoro (2017), Weaver et al. (2018)
Grx1-roGFP2	GSH/GSSG		Brautigam et al. (2016), Panieri et al. (2017), Panieri and Santoro (2017), Seiler et al. (2012)

Oxidation of key Cys residues in OxyR in the presence of H₂O₂ leads to conformational changes that alter the excitation properties of the cpYFP, allowing for a ratiometric measurement of OxyR oxidation state. Improved variants of the original HyPer sensor have been created, such as HyPer-3 (Bilan et al. 2013), which shows a higher dynamic range.

Owing to their less rigid structure, cpFPs are more susceptible to environmental changes than their non-permuted counterparts, and this may manifest itself for example in a pH-dependence of the signal (Figure 2). Therefore, a mutated HyPer sensor variant has been developed (SypHer (Poburko et al. 2011) and subsequent variants (Ermakova et al. 2018; Matlashov et al. 2015)), which carries a mutation of the key Cys199 of the OxyR moiety that renders it insensitive to H₂O₂ induced oxidation and reveals the pH-dependent component of the signal, with higher pH leading to higher ratio values. It thereby also doubles as a pH sensor in its own right. Very recently, yet another generation of HyPer based on the *Neisseria meningitidis* OxyR and a mutated cpYFP version with a GFP chromophore sequence has been generated that is more

Figure 2: Protein redox sensors report altered redox state of their reactive cysteines, which depends on their reactivity with specific oxidants and the activity of endogenous thiol reactive redox systems, such thioredoxins (Trx) and glutaredoxins (Grx). Moreover, sensor properties can be sensitive to other parameters such as pH or sensor oligomerization. Thereby, sensor function may be influenced by particular cellular environments. N.m., *Neisseria meningitidis*.

sensitive to H_2O_2 and lacks the pH sensitivity of the original sensors (HyPer7 (Pak et al. 2020)). This sensor represents a promising tool for H_2O_2 monitoring that would avoid the need for parallel acquisition of pH-dependent changes by the SypHer sensors.

roGFP-based sensors

Another type of genetically encoded redox sensors uses a different functional principle. These sensors are based on the redox-sensitive fluorescent proteins roGFP1 and roGFP2, which alter their fluorescent properties dependent on the oxidation or reduction of key Cys residues and thereby enable ratiometric measurement of their oxidation status (Schwarzlander et al. 2016). The roGFPs alone equilibrate with the glutathione redox couple GSH/GSSG, catalyzed by endogenous Grxs. To circumvent this dependence on endogenous proteins, the roGFP moiety can be fused to a redox-sensitive sensor protein moiety which transfers its oxidation state to the Cys residues of roGFP, thereby providing information on the specific redox systems modifying the sensor part. Thus, by directly linking the mammalian Grx1 to roGFP2, the Grx1-roGFP2 sensor measures glutathione redox potential independently of the presence or activity of endogenous Grxs (Gutscher et al.

2008). H_2O_2 was targeted by the fusion of the yeast atypical 2-Cys peroxiredoxin Orp1 to roGFP2 (Delaunay et al. 2002; Gutscher et al. 2009). Sensor redox state of this roGFP2-Orp1 sensor is additionally influenced by endogenous Grx (as for roGFP2 alone) and Trx systems, which allow for reversibility of its oxidation by H_2O_2 under physiological conditions (Figure 2). The yeast peroxiredoxin Tsa2 was employed to generate a more sensitive H_2O_2 sensor version (Morgan et al. 2016). In the Tsa2 ΔC_R mutant used for the sensor, a resolving Cys has been mutated that renders it insensitive to thioredoxin based reduction, thereby increasing its sensitivity to H_2O_2 (Figure 2).

Compared to cpFP-based sensors, roGFP-type sensors have chromophores which are solvent inaccessible and therefore intrinsically more resistant to environmental conditions such as pH. Consequently, the ratiometric properties of roGFP based sensors are stable within physiological pH ranges (Schwarzlander et al. 2008). However, the interaction with endogenous redox systems may alter sensor properties in different cell types or compartments, depending on the particular GSH/GSSG levels and Grx/Trx system activities present locally, and this feature should be considered when sensor results are interpreted. As the same redox systems also interact with HyPer probes, this caveat applies to them as well (Schwarzlander et al. 2016) (Figure 2). Another unknown for peroxiredoxin-based sensors is whether conditions in different organisms, tissues or cell compartments affect their oligomerization state, which influences oxidation sensitivity, and how this could impact sensor function (Pastor-Flores et al. 2020).

H_2O_2 dynamics and zebrafish development

Redox alterations have long been linked to development. Classical studies by Child used redox sensitive dyes to visualize what he referred to as metabolic gradients in embryos, including the zebrafish (Blackstone 2006; Child 1945). More recently, mitochondria-associated redox activities have been implicated in axis specification in early sea urchin embryos (Coffman and Denegre 2007; Coffman et al. 2009). Interestingly, these processes were linked to Nodal signaling, which plays a role in axis specification and germ layer patterning in vertebrates as well (Zinski et al. 2018). Numerous studies describe the impact of ROS on stem cell behavior, where higher ROS levels are generally associated with differentiation processes, but may also regulate quiescence-proliferation transitions (Bigarella et al. 2014; Prozorovski et al. 2015). Among the

differentiation processes regulated by redox signaling are also axonal growth and neural cell migration (Olguin-Albuerné and Moran 2018; Wilson et al. 2018). Comparatively few studies have examined these aspects of redox biology *in vivo*. In particular, data are needed regarding spatiotemporal dynamics of ROS levels or of local redox potential changes, in order to identify potential ROS sources and to link them to downstream signaling networks. In the past few years, the first studies applying the genetic sensors introduced above to the zebrafish model system have begun to explore such questions.

Gauron et al. (2016) used HyPer driven by a ubiquitous promoter to examine H_2O_2 levels during embryonic development up to 72 hpf. High levels of sensor signal were recorded particularly during somitogenesis and organogenesis stages, which was attributed to decreased H_2O_2 degradation based on activity measurements of catalase from embryonic extracts. Higher signal levels were observed in the nervous system, and treatment with Nox inhibitors affected the size of the projection area of retinal ganglion cell axons in the tectum. The projection deficit could be rescued by treatment with an agonist of the Hedgehog pathway, implicating this signaling pathway as a downstream target of Nox-produced H_2O_2 . Support for a role of Nox enzymes in the regulation of retinal projections also comes from a more recent genetic study by Weaver et al. (2018). Examining chimeric mutants of *nox2/cybb*, in which not all cells are homozygous for the mutation, the authors implicated this Nox in the proper development of the retinal projections to the optic tectum. However, using transient expression of the roGFP-orp1 sensor, they did not observe differences in H_2O_2 levels in homozygous mutants at 48 hpf nor in cultured retinal ganglion cells derived from them, which they interpret as reflecting compensatory regulation by other Nox isoforms. This would fit to the observation that, in contrast to the chimeric mutants, tectal innervation was not decreased in homozygous mutants (although axons were mistargeted within and outside the tectum). Thus, while both studies support a role of Nox enzymes in development of the visual system connections in the brain, it remains unclear how precisely the production and distribution of H_2O_2 regulates the downstream processes. As HyPer sensors are sensitive to pH as well, it will be important to examine if SypHer sensors show overlapping patterns of ratio changes (see for example data obtained with SypHer3s in Ermakova et al. (2018)). roGFP2-orp1 is not affected by pH, but may not have been sensitive enough to pick up the differences between wild-types and *nox2/cybb* mutants. Applying the next generation of sensors, HyPer7 (Pak et al. 2020) and roGFP2-

Tsa2 ΔC_R (Morgan et al. 2016), should help to resolve these issues.

HyPer imaging was also employed to examine retinal progenitor cell differentiation (Albadri et al. 2019). HyPer oxidation signals in the retina were uniformly high at 24 hpf and declined in the central part of the retina by 32 hpf, concomitant with the differentiation of the tissue. These patterns are proposed to be shaped by differential expression of superoxide dismutase (*sod2*) and catalase in the tissue. H_2O_2 may act via lipid peroxidation, notably the production of 9-hydroxystearic acid (9-HSA). Consistently, the small molecule sensor for lipid peroxidation, BODIPY-C11, showed increased oxidation at the site of stem and early progenitor cells, where also increased levels of 4-hydroxynonenal, an end product of lipid peroxidation, were observed. 9-HSA treatment maintained retinal progenitor cells in a proliferative state and prevented differentiation, which appears consistent with the proposed spatiotemporal pattern of H_2O_2 production. By inhibiting histone deacetylase 1 function, 9-HSA seems to activate Wnt and Notch pathways that stimulate proliferation and prevent differentiation, respectively. Interestingly, in the hindbrain, 9-HSA treatment rather reduced the number of proliferating cells, indicating that its effects on quiescence/proliferation/differentiation transitions of stem and progenitor cells may be tissue-specific. As with the studies on the development of retinotectal projections, a precise understanding of the spatiotemporal distribution of H_2O_2 induced redox signaling awaits the application of pH-insensitive sensors to this system, especially because dividing progenitor cells in the embryonic *Xenopus* retina show aerobic glycolysis, a metabolic feature that – like proliferation in general – has been associated with a more alkaline cytoplasmic pH (Agathocleous et al. 2012; Flinck et al. 2018).

roGFP2-based sensors have also been used in embryos and larvae for questions more related to disease modeling and drug screening than embryonic development *per se*. Thus, Grx1-roGFP2 sensors have been applied to cancer related questions, namely to examine crosstalk between redox state and the MTH1 enzyme, which detoxifies oxidized nucleotides and has been implicated in the tumor response to oxidative stress (Brautigam et al. 2016), and to study a zebrafish model of tissue invasion, the *meltdown* mutant (Seiler et al. 2012). This model showed interactions between redox signaling in intestinal epithelial cells and smooth muscle cell tension that promote invasion by the epithelial cells, a mechanism potentially relevant in metastasis. Furthermore, to generate new experimental models for examining redox changes in cardiovascular

tissues, Panieri et al. (Panieri and Santoro 2017; Panieri et al. 2017) used Grx1-roGFP2 or roGFP2-Orp1 targeted to the cytoplasm, mitochondria or the nucleus in endothelial cells or cardiomyocytes of zebrafish embryos. As a proof-of-principle for drug screening approaches, the lines could indicate compartment- and tissue-specific effects on the sensor state when treated with compounds targeting glutathione synthesis or the pentose phosphate pathway. However, this required concomitant treatment with H_2O_2 or the redox cycling agent menadione, as no alterations were observed in the absence of these treatments (except for differences in basal oxidation state of both sensor types between the compartments, with sensors most reduced in the nucleus and most oxidized in the mitochondria). Detection of endogenous changes in response to compound treatment may require the application of the more sensitive sensors mentioned above.

H_2O_2 changes during regeneration in zebrafish

The first imaging study using a genetically encoded redox sensor which addressed processes related to regeneration in the zebrafish examined the wound response to a cut in the tail fin of embryos (Niethammer et al. 2009). After an initial immediate tissue contraction, within about 15 min after the cut leukocytes were recruited to the wound site. Immediately upon amputation, a gradient of HyPer ratio values was visible in the surrounding tissue, with most oxidized values at the site of the cut. The signal started to become apparent at about 3 min post wounding at the wound margin and reached a peak at about 20 min, upon which it slowly declined. Pharmacological Nox inhibition and morpholino oligonucleotide mediated knock-down of Duox reduced the sensor signal and prevented leukocyte recruitment. A follow-up study combining HyPer imaging with modelling approaches examined how far H_2O_2 may penetrate from the wound margin into the surrounding tissue before it becomes inactivated by antioxidant activities (Jelcic et al. 2017). Peak (extracellular) levels at the wound margin were estimated to not exceed 5 μM . With modelling indicating that antioxidant reactions should become exhausted above 2 μM , it was estimated that such high concentrations occur up to 30 μm away from the wound margin.

These findings were incorporated into a model of signaling by tissue wounds describing a sequence of events which starts with the immediate release of Damage Associated Molecular Pattern (DAMP) molecules such as ATP

that may both serve as chemoattractants and trigger downstream signaling events related to inflammation. These processes include Ca^{2+} mediated signaling that stimulates production of ROS and polyunsaturated fatty acids (PUFAs), which then potentially serve as relay attractants that are more stable than the rapidly dissipated DAMPs (Niethammer 2016). Thus, H_2O_2 has been proposed to activate the Src family kinase Lyn and thereby recruit neutrophils (Yoo et al. 2011). However, the relatively limited penetration of sufficient high levels of H_2O_2 into the tissue suggested by Jelcic et al. (2017) calls for additional studies examining the importance of such a mechanism compared to other candidates for attractants acting downstream or in parallel of H_2O_2 . Indicating potential common regulatory mechanisms, a link between early ROS production at the wound site and Src kinase signaling has also been proposed in organizing cell polarity for wound closure in early zebrafish embryos (Hunter et al. 2018) and for the regenerative response of epithelial cells in larval tail fin regeneration (via the Src kinase fynb (Yoo et al. 2012)). Additionally, ROS regulate vimentin expression via Nf κ B signaling, which is necessary for collagen-based projections at the wound edge that guide regenerative growth (LeBert et al. 2018).

The attraction of leukocytes to the wound is important for the subsequent regeneration processes. It has been proposed that the leukocytes may exert negative feedback on early wound signals, by lowering H_2O_2 levels via myeloperoxidase in neutrophils (as imaged using a transgenic line driving HyPer in this cell type (Pase et al. 2012)) and by an inhibitory effect of macrophages on expression of the cytokine IL1 β in the wound epithelium (Hasegawa et al. 2017). The precise timing of these activities and their interplay with ROS triggered processes in the wound tissue itself may create an environment that allows regeneration to proceed (Roehl 2018).

The importance of ROS production for regeneration was equally suggested by studies in other regeneration models involving different tissues and species. Thus, HyPer imaging in *Xenopus* tadpole tails revealed similar gradients of sensor excitation ratio upon wounding as those observed in zebrafish (Love et al. 2013). Here, H_2O_2 production was sustained over the next few days while the tail regenerates. Tail fin regeneration was impaired upon lowering of H_2O_2 levels via pharmacological treatments with Nox inhibitors and ROS scavengers for three days post-amputation, as well as upon morpholino-mediated interference with expression of *cyba*, a subunit of Nox complexes (Love et al. 2013). Downstream effects of this H_2O_2 production included the stimulation of proliferation via a Wnt/FGF20 signaling network. Interestingly, H_2O_2 production is also paralleled by increased O_2

consumption in the regenerating tail, leading to stabilization of the hypoxia signaling factor HIF1 α by local hypoxia that influences regeneration in a parallel pathway (Ferreira et al. 2018).

Prolonged ROS production during regeneration was equally observed in adult tail fin regeneration in zebrafish employing small molecule sensor (DCF) imaging (Gauron et al. 2013). In this model, based on pharmacological Nox inhibition, ROS production was reported to be involved in apoptosis and JNK kinase regulation that are both required for compensatory cell proliferation. Also in the regenerative response of adult epithelial cells to superficial exfoliation lesions, ROS (as measured by the cell permeable fluorescent probe CellROX Green and likely derived from Noxs) were elevated for at least 24 h and required for the regeneration response, partially by regulating cell hypertrophy (Chen et al. 2016). Similarly, CellROX Orange indicated higher ROS levels in a model of chemical liver injury from 24 h after start of the treatment until at least 48 h, concomitant with leukocyte recruitment (Stoddard et al. 2019). Furthermore, HyPer imaging in the adult zebrafish heart showed sustained increased sensor oxidation levels at 3, 7 and 14 days post injury (but not 1 h after the injury) in the epicardium and adjacent myocardium at the resection site (Han et al. 2014). These observations indicate that ROS may play distinct roles in various phases of different regeneration processes.

Identifying direct targets modified by H₂O₂ or other ROS at early and later stages during the wound response and regeneration should enable a better understanding of the signaling networks connecting the two processes. In zebrafish heart regeneration, H₂O₂ production by Duox was suggested to regulate MAPK signaling via a derepression mechanism involving the phosphatase Dusp6 primarily in the epicardium (Han et al. 2014). Interestingly, a redox proteomics study of adult fin regeneration identified another phosphatase, the protein tyrosine phosphatase Shp2, which is oxidized and thereby probably inactivated during regeneration (Wu et al. 2017). Surprisingly, it is also required for regeneration of the larval tail fin, as mutant embryos lacking Shp2 function do not regenerate. These findings highlight the complexity of the redox sensitive signaling networks regulating regeneration processes.

H₂O₂ was also implicated in axon regeneration into the larval fin (Rieger and Sagasti 2011). Axon activity (i.e., growth and retraction) of severed axons was transiently increased post tail fin amputation, but no further than 50 μ m from the injury, indicating a short range of a wound derived growth promoting signal. Additional experiments (including small molecule sensor imaging with

PFBS-F) showed that Duox-mediated H₂O₂ production in wound keratinocytes was required for axon regeneration, with H₂O₂ effects on macrophage recruitment to the wound and axon growth being independent of each other. Interestingly, the degeneration of the injured axon (Wallerian degeneration) shortly after the cut may require ROS production as well, namely in the mitochondria of the neurons, as assessed in zebrafish embryos transiently expressing roGFP2 targeted to this compartment (O'Donnell et al. 2013). Mitochondria in the severed distal axon part showed an increase in roGFP2 oxidation prior to the synchronous fragmentation of the disconnected axon part. Overexpression of the mitochondrial biogenesis regulator PGC-1 α correlated with reduced oxidation of roGFP2 upon axotomy and stabilized the severed axon part. Thus, distinct subcellular and cellular ROS sources appear to be involved in the wound and regeneration response of axons in zebrafish larvae.

Re-innervation not only is a regenerative response to amputation influenced by H₂O₂, nerves also are required for limb regeneration (Meda et al. 2018). In zebrafish adult fin regeneration, this seems to involve an increase of H₂O₂ production (measured with the small molecule sensor DCF and HyPer) in the fin tissue, apparently triggered via Hedgehog signaling from Schwann cells (Meda et al. 2016). The re-innervating axons then would suppress H₂O₂ levels in the fin again. Potentially indicating a common signaling mechanism upstream of H₂O₂ production, a link between Hedgehog signaling and H₂O₂ was recently also described for regeneration upon larval tail excision. In this model distinct from larval fin fold regeneration, the amputation is performed closer to the trunk, including not only the fin fold tissue but also touching the notochord and surrounding tissues (Romero et al. 2018). Here, H₂O₂ production (measured with the small molecule sensor PFBS-F and blocked with the Nox inhibitor DPI) in cells at the edge of the stump appears to regulate a pathway in which cells derived from the notochord sheath form a bead-like cluster at the cut site. This “notochord bead” releases Hedgehog signals which stimulate regeneration upstream of Wnt, FGF and RA signaling. On the other hand, in embryonic development Hedgehog signaling has been proposed to act downstream of Nox-derived H₂O₂ in the formation of retinotectal projections (Gauron et al. 2016). It will certainly be interesting to further examine these links by charting precise tissue and cell-type dynamics using both reporters for signaling pathways (Moro et al. 2013) and the more specific and sensitive next generation of genetically encoded redox sensors.

Current limitations of the genetically encoded sensors

As evident from the previous paragraphs, the application of genetically encoded redox sensors has allowed us to address questions on subcellular, cellular and tissue distribution of H_2O_2 production in various developmental and regeneration processes; prompting also mechanistic insights into downstream pathways. However, many of the methods used for functional interference with ROS signaling or other molecular mechanisms and for the visualization of ROS have limitations that should be taken into consideration when interpreting the data.

- (1) Small molecule sensors are often used to detect alterations in ROS levels, involving the limitations of specificity, stability and distribution *in vivo* discussed above. Along a similar line, enzyme activity is regularly inhibited using small molecules, which equally may be limited in their specificity and target other enzymes of the same family or unrelated proteins (Sorce et al. 2017).
- (2) Morpholino oligonucleotides have been widely used to interfere with gene expression, generating “morphant” embryos. Experiments involving morpholinos are currently being re-evaluated because of frequently observed off-target effects and p53 mediated toxicity (Blum et al. 2015; Schulte-Merker and Stainier 2014). A recent study indicated that such off-target effects include increased expression of interferon stimulated genes and of components of cellular stress pathways, which might interfere with immune cell function, important in the context of regeneration (Lai et al. 2019). Thus, it is necessary to employ appropriate controls when using morpholinos (Stainier et al. 2017). Ideally, morphant phenotypes should be confirmed by other techniques, notably by generating mutants carrying suitable loss-of-function alleles. This has become possible more easily by the adaptation of CRISPR-Cas9 based systems to the zebrafish (Liu et al. 2019). However, mutants have their drawbacks as well, since genetic compensation mechanisms may mask the function(s) of the targeted gene, and mutants even may appear as wild-type (Blum et al. 2015; El-Brolosy et al. 2019; Rossi et al. 2015). In such cases, it should be confirmed that the mutant allele is indeed a loss-of-function allele and that mutants are resistant to morpholino-induced phenotypes.
- (3) As mentioned above, the genetically encoded H_2O_2 sensors have their own set of limitations that are

intrinsic to their properties and involve interactions with the cellular environment. We will now discuss these issues in more detail for the tail wound assay.

The pH sensitivity of the HyPer sensor makes it necessary to employ controls that allow one to correct for this property. The original study employing HyPer to visualize the wound response tested for pH alterations using YFP alone and a small molecule sensor (Niethammer et al. 2009), but these are not ideal controls due to different sensitivities and the general limitations of small molecule sensors. One way of confirming that redox and not only pH changes occur is to look for downstream effects that can be attributed to increased ROS presence. For example, in the embryonic retina, the observed sensor patterns were compared with signals for lipid peroxidation (Albadri et al. 2019). However, one still needs a control that allows an evaluation of the precise spatiotemporal patterns observed with HyPer. Ideally, one should always show a corresponding SypHer probe side by side to compare patterns of ratio changes. The ratiometric nature of the sensors is an advantage, as the ratio should not be subject to expression level variations that may occur in different transgenic lines or upon transient expression.

In the *Xenopus* tadpole tail wounding assay, pH alterations can be measured with SypHer2 that seem to occur in two phases, first a rapid acidification near the wound and then a more prolonged alkalinization at later stages (Matlashov et al. 2015). These observations indicate that there is not only a change in redox state, but also changes in intracellular pH levels that occur upon wounding. Interestingly, earlier work in *Xenopus* has implicated a proton pump, the V-ATPase, as a player in tail regeneration (Adams et al. 2007). V-ATPase pumps protons out of the cell, which can increase intracellular pH (Hinton et al. 2009). Consistently, the pH increase seen in SypHer2 expressing *Xenopus* tails was blocked by V-ATPase antagonists (but not the initial decrease (Matlashov et al. 2015)). V-ATPase activity is linked to changes in transmembrane voltage that are also important for regeneration. Additionally, alteration of bioelectric properties in tailbud regeneration has been described as occurring downstream of Nox activity (Ferreira et al. 2016), and also Nox/Duox enzymes can alter proton distributions (Ezerina et al. 2014). Thus, both pH and bioelectric changes may contribute to create a regeneration-specific state in the tail. Among the processes proposed to be triggered by bioelectric changes are axonal growth, proliferation and differentiation (Ferreira et al. 2016; Ferreira et al. 2018), and also relocation of myeloid cells and an increase in their antibacterial activity was observed in response to the depolarization at the site of

the tail cut in *Xenopus* (Pare et al. 2017). In the zebrafish, V-ATPase has been implicated in regeneration of the adult fin, where it is required for blastema cell proliferation and expression of RA and FGF signaling pathway components (Monteiro et al. 2014). By contrast, larvae homozygous for a mutant allele of the *atp6v1e1b* gene encoding a V-ATPase subunit showed no difference in fin fold regeneration from wild-types, which might indicate genetic compensation mechanisms or functional redundancy of other proton pumps in this system. Thus, it will be interesting to further dissect the relative contributions and connections of ROS, pH and bioelectric changes during regeneration processes using optical sensors and microelectrode based systems (including for example the scanning ion selective electrode technique [SIET] to measure extracellular pH (Lin et al. 2006)), and to examine their links with other signaling pathways involved in wound response and regeneration.

An intriguing observation is the presence of sensor ratio gradients upon tail fin wounding seen with the new HyPer7 sensor (Pak et al. 2020), which is more sensitive than previous HyPer versions and lacks pH dependency of the signal. This indicates that indeed a gradient of H_2O_2 may be present, but that it involves H_2O_2 levels about 30 times lower than originally estimated. The extracellular concentration of $5 \mu M$ H_2O_2 near the wound margin estimated based on HyPer data (Jelcic et al. 2017) would then translate to $0.2 \mu M$ or less based on HyPer7 data. Would this concentration be sufficient for the attraction of leukocytes to the wound? Klyubin et al. (Klyubin et al. 1996) observed neutrophil chemotaxis toward a source of $(1\text{--}10 \mu M)$ H_2O_2 placed at a distance of a few millimeters *in vitro*; thus, given the shorter distances in the zebrafish tail fin, $0.2 \mu M$ at the cut might still be high enough. However, it would be of interest to recalculate the local H_2O_2 concentrations needed to overcome the antioxidant barriers mentioned above (Jelcic et al. 2017) based on data obtained with the new sensor generation. The gradient of lower concentrations indicated by HyPer7 lends further support to the idea that relay mechanisms are needed for leukocyte attraction to the source of H_2O_2 . Also, because H_2O_2 as well has been reported to inhibit neutrophil migration (Wang et al. 2016), its production at the wound site might help to trap the migrating leukocytes where they are needed, and to avoid overshooting migration across the tissue border.

Conclusions and perspectives

Our overview indicates that genetically encoded redox-sensors have been used successfully to study processes of embryonic development and regeneration, and some

applications have also targeted disease models. However, explaining the distinct spatiotemporal dynamics of signals generated by the different sensor types still requires further studies, ideally in the presence and absence of artificially generated *in vivo* gradients of ROS, pH and bioelectricity. An important unknown is to what extent the different sensor types interact differently with antioxidant and redox relay systems present in different tissues and cellular compartments. For example, the redox switch of OxyR has been reported to be less efficiently reduced by cellular reductants such as Grxs than it is oxidized, allowing prolonged induction of transcription upon H_2O_2 sensing (Aslund et al. 1999; Choi et al. 2001; Cremers and Jakob 2013), while roGFP2-Tsa2 ΔC_R is insensitive to reduction by Trxs (Morgan et al. 2016). Thus, there is a need for sensors systems that are able to separately and specifically monitor these enzymatic activities.

Among the many zebrafish models of human diseases in which ROS alterations have been implicated in pathogenesis are also those of endocrine diseases like diabetes (Di Marco et al. 2015; Gut et al. 2017). It has been shown in zebrafish larvae that different H_2O_2 levels can stimulate either proliferation or neogenesis of pancreatic β -cells, and it will be interesting to link these data with measurements of endogenous H_2O_2 levels under different nutritional challenges to further explore roles of H_2O_2 signaling in the regulation of β -cell mass (Ahmed Alfar et al. 2017). Sensor imaging might also help to further dissect the recently observed discrepancies in redox-regulated gene expression between zebrafish models of primary and secondary adrenal insufficiencies, diseases of glucocorticoid deficiency (Weger et al. 2018). Given the strong links between the circadian clock and ROS regulation (Putker and O'Neill 2016) (also apparent in the adrenal gland itself (Kil et al. 2012; Rhee and Kil 2016)), it will be important to consider time-of-day dependent effects when working with such older larval models (Weger et al. 2013, 2016).

Besides spatiotemporal monitoring, also spatiotemporal manipulation of ROS production will be important for a profound understanding of redox signaling in development, disease and regeneration. Tissue specific catalase overexpression to scavenge H_2O_2 has been used to examine heart regeneration and the formation of β -cells (Ahmed Alfar et al. 2017; Han et al. 2014). In a complementary approach, the D-amino acid oxidase (DAAO) enzyme that can be expressed tissue-specifically and targeted to particular cellular compartments allows localized H_2O_2 production upon addition of a D-amino acid, thereby enabling also temporal control (Bogdanova et al. 2017; Pak et al. 2020). Further developments here may target the regulation of specific ROS species.

An important task will be the identification of effector molecules that translate H_2O_2 alterations into a biological response. Redox proteomics is a powerful tool that can identify direct targets of ROS species (Leichert et al. 2008; Lennicke et al. 2016; Topf et al. 2018), and can be combined with transcriptomics, proteomics and metabolomics approaches that comprehensively map downstream effects. The genetically encoded sensors can provide detailed information on spatiotemporal aspects of H_2O_2 signaling that can guide the choice of time points or tissues to which such methods are applied. Spatiotemporal mapping of transcriptome changes with cellular resolution by single cell RNA-seq approaches has been successfully used in the context of both development and regeneration in the zebrafish (Cosacak et al. 2019; Farrell et al. 2018; Lush et al. 2019; Spanjaard et al. 2018; Wagner et al. 2018). Combining such data with highly resolved single cell imaging of whole embryos into a digital embryo will enable to model cell movements and differentiation processes in unprecedented detail (Keller et al. 2008; Sladitschek et al. 2020), and such models could also incorporate redox sensor derived data. Isolating cells with specific redox signaling states for further studies could be accomplished using fluorescence activated cell sorting (FACS) based on sensor oxidation state, analogous to previous studies in zebrafish embryos targeting cells labelled according to signaling pathway activity, tissue identity or cell cycle phase (Dickmeis et al. 2001; Dickmeis et al. 2004; Wragg et al. 2020). However, FACS may alter the redox state of cells (Llufrio et al. 2018), so results should be compared with data obtained by laser capture microdissection (Veldman et al. 2007; Zoidl et al. 2004) or other nano-sampling techniques (Dickmeis et al. 2019). Such single-cell based analyses should allow to produce a clearer picture of which H_2O_2 effects in which tissues and cells are important for which developmental and regenerative processes.

It is still incompletely understood how H_2O_2 signals propagate, both extracellularly and intracellularly. While the intracellular aspects could be targeted by sensor imaging approaches under targeted manipulation of intracellular H_2O_2 sources (Pak et al. 2020), more information is needed on the spreading of H_2O_2 in the extracellular space. This task will require the development of optical sensors with specific properties adapted to the extracellular environment, or the application of redox sensitive microelectrode methods (Bozem et al. 2018). Also potential roles of mechanisms alternative to diffusion should be clarified, including the recently suggested distribution of ROS producing enzymes by microvesicles (Hervera et al. 2019) or perhaps the cytoneme-based spreading that has been proposed for morphogen transport (Kornberg 2014).

The zebrafish, with its unique potential to combine whole organism with (sub-)cellular imaging and molecular genetic studies, is likely to continue to further our understanding of redox processes in development and regeneration. A prerequisite for this is a detailed mechanistic understanding of the tools applied for visualization of ROS *in vivo*, and the application of multiple strategies to cross-validate findings and to examine their upstream regulation and downstream consequences.

Acknowledgments: We thank Philipp Gut and Andrea Schink for a critical reading of the manuscript. We are grateful to Miriam Otto for help with figure preparation. We apologize to those colleagues whose work could not be cited due to space constraints and refer the reader to the cited review articles for further information on the primary literature.

Author contribution: All the authors have accepted responsibility for the entire content of this submitted manuscript and approved submission.

Research funding: Research in the Dickmeis lab is funded by the Schwerpunktprogramm SPP1710 “Dynamics of Thiol-based Redox Switches in Cellular Physiology” (DI913/6–1) and the Graduiertenkolleg GRK2039 “Molecular Architectures for Fluorescent Imaging of Cells” of the Deutsche Forschungsgemeinschaft, by the “Effect-Net” research network in water research of the Ministerium für Wissenschaft, Forschung und Kunst Baden-Württemberg, and by the Helmholtz Programme BioInterfaces in Technology and Medicine (BIFTM).

Conflict of interest statement: The authors declare no conflicts of interest regarding this article.

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