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**UROCORTINS IN THE  
ZEBRAFISH BRAIN AND  
REDOX REGULATION OF  
EMBRYONIC  
DEVELOPMENT THROUGH  
GLUTAREDOXINS**

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Stockholm 2012

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**Front cover:** Zebrafish embryo two days after fertilization

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Published by Karolinska Institutet

Printed by LarsEric, Sundbyberg

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ISBN: 978-91-7457-659-7

To Everybody Inside  
and Outside the Lab who was  
Part of the Journey  
*(including the zebrafish)*

## **POPULAR SCIENCE SUMMARY**

Life's greatest miracle is the birth of a new human.

But how can we, with our body's complexity far beyond imagination, arise from a single cell, the egg?

Understanding this miracle is the holy grail of developmental biology, but even with our modern scientific technology, embryonic development remains largely a mystery.

Until recently, scientists believed that the oxygen we breathe, although necessary for our survival, leads to deleterious reactions inside our body, and causes nearly every disease that threatens us. Antioxidant therapy in the form of pills quickly became the answer for a long and healthy life. Astonishingly, researchers just learned that oxygen radicals are in fact a two-edged sword: in large amounts they can have deleterious effects on our body, but in small amounts they are absolutely necessary for our cells to be able to communicate with each other, for example, during the development of a new human being.

This thesis is about proteins that control the signaling function of oxygen radicals during embryonic development.

Glutaredoxins are proteins that modify the amino acid cysteine, and convey by that signals of oxygen radicals.

Here, we describe for the first time that glutaredoxins are necessary for a successful embryonic development. Indeed, they are required for the formation of a healthy brain and a working cardiovascular system. Without glutaredoxins, neither of the systems would develop properly in the embryo.

This improves not only our understanding of how oxygen radicals are employed in embryonic development, but it also offers possibilities for new therapeutic strategies against major health issues like Alzheimer's disease and heart infarcts, dilemmas which are known to be connected to miss-regulated oxygen radical signaling.

Interestingly, the development of an embryo is not only driven by its genes, but the environment of the pregnant mother and the young child is also of utmost importance. Stress during early life, both physical and psychological, can have major influence on the growing child. Therefore we additionally analyzed how specific cells in the brain, which are responsible to regulate how the body reacts on stress, develop. This will add valuable information and improve understanding of this critical period during early life.

## ABSTRACT

The cellular redox state is a central regulator of embryonic development. Redox homeostasis and thiol redox signaling are modulated through glutaredoxins (Grxs), glutathione dependent thiol-disulfide oxidoreductases. Grxs can reduce protein disulfides and protein-glutathione mixed disulfides (de-glutathionylation) via distinct reaction mechanisms. Although it has been shown that Grxs protect cells against oxidative stress induced apoptosis, known interaction partners are rare and physiological functions of this protein family are poorly understood.

This thesis represents the first investigation of dithiol Grxs during vertebrate embryonic development, and we demonstrate that vertebrate specific Grx2 is essential for the formation of a functional brain and cardiovascular system.

We characterized a redox circuit, in which Grx2 modulates the activity of a newly identified interaction partner, collapsin response mediator protein 2 (CRMP2), through the reduction of an intra-molecular disulfide. Thereby, Grx2 regulates axonal outgrowth and neuronal survival. Since CRMP2 and Grx2 have already been implicated in various neurological disorders, the redox circuit based on Grx2 might be a promising target for future therapeutic strategies.

Additionally, we unraveled that development of functional vessels, heart, and erythrocytes in the zebrafish embryo is dependent on the de-glutathionylation activity of Grx2. These results have high clinical relevance, as defects in the cardiovascular system are a major reason for human embryonic mortality.

Moreover, we sought to characterize zebrafish Grx2 (zfGrx2) biochemically and biophysically. Mössbauer spectroscopy as well as size-exclusion chromatography demonstrated the coordination of one  $[2Fe_2S]^{2+}$  cluster per zfGrx2 monomer. Further analysis indicated that two out of four additional cysteines, which are conserved across the infraclass of bony fish, are involved in cluster coordination. As this mode of cluster binding is different to all yet described [FeS] Grxs, zfGrx2 might represent a new class of iron-sulfur Grxs.

Embryonic development is also regulated by environmental factors, and the stress axis is responsible to mediate the body's response to those stimuli. Here we investigated the expression pattern of urocortin (UCN) genes, important regulators of the stress axis, in the embryonic zebrafish brain. The specific expression sites indicate that UCNs might modulate locomotor activity through noradrenergic and serotonergic systems.

## LIST OF PUBLICATIONS

- I. **Bräutigam, L.**, Hillmer, J.M., Söll, I., Hauptmann, G.  
Localized expression of urocortin genes in the developing zebrafish brain.  
*J. Comp. Neurol.* **518**: 2978-2995 (2010)
- II. **Bräutigam, L.**, Schütte, L.D., Godoy, J.R., Prozorovski, T., Gellert, M.,  
Hauptmann, G., Holmgren, A., Lillig, C.H. and Berndt, C.  
Vertebrate specific glutaredoxin is essential for brain development.  
*Proc. Natl. Acad. Sci. USA* **108**(51): 20532-7 (2011)
- III. **Bräutigam, L.**, Johansson, C., Kubsch, B., McDonough, M.A., Bill, E.,  
Holmgren, A., Berndt, C.  
An unusual mode of iron-sulfur cluster coordination in teleost glutaredoxin 2.  
*Manuscript*
- IV. **Bräutigam, L.**, Jensen, L.D.E., Nyström, S., Bannenber, S., Uhlén, P.,  
Holmgren, A., Cao, Y., Berndt, C.  
Glutaredoxin 2 is essential for zebrafish cardiovascular development.  
*Manuscript*

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## LIST OF ABBREVIATIONS

|               |  |
|---------------|--|
| ACTH          | Adrenal corticotrophic hormone   |
| ATP           | Adenosin triphosphate  |
| AP-1          | Activator-protein 1  |
| ASK-1         | Apoptosis signaling kinase 1   |
| AVP           | Arginine-vasopressin (hormone)   |
| CRH           | Corticotropin-releasing hormone  |
| CRMP2         | Collapsin-response-mediator protein 2  |
| Cyp26         | Cytochrome P26   |
| DA            | Dorsal aorta   |
| DLAV          | Dorsal-longitudinal anastomotic vessel   |
| DPF           | Days post-fertilization  |
| Duox          | Dual oxidases  |
| <i>E.coli</i> | Escherichia coli   |
| ENU           | N-ethyl-N-nitrosourea<br><i>Induces point mutations; used in zebrafish genetic screens</i> |
| Ex            | Embryonic day <i>x</i>   |
| FGF           | Fetal growth factor  |
| Fli           | Friend leukemia integration<br><i>Earliest marker expressed in endothelial cells</i>       |
| Grx           | Glutaredoxin   |
| GR            | Glutathione reductase  |
| GPx           | Glutathione peroxidase   |
| GSH           | Glutathione  |
| GSSG          | Glutathione disulfide  |
| GST           | Glutathione transferase  |
| HED           | 2-hydroxyethylidysulfide   |
| HPA           | Hypothalamus pituitary axis  |
| HPF           | Hours post fertilization   |
| IL-4          | Interleukin-4  |
| ISV           | Intersegmental vessel  |
| MICAL         | Molecule interacting with CasL   |



|               |  |
|---------------|--|
| MPTP          | 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine<br><i>A neurotoxin targeting dopaminergic neurons, therefore recapitulating symptoms of Parkinson's disease</i> |
| NAD(P)H       | Nicotinamide adenine dinucleotide (phosphate)  |
| NOS           | Nitric oxide synthetase  |
| NOX           | NADPH oxidase  |
| Otx2          | Orthodenticle homeobox 2   |
| PCV           | Posterior cardinal vein  |
| PFA           | Paraformaldehyde   |
| Prx           | Peroxiredoxin  |
| PTEN          | Phosphatase and tensin homologue   |
| ROS           | Reactive oxygen species  |
| RNR           | Ribonucleotide reductase   |
| RNS           | Reactive nitrogen species  |
| SHH           | Sonic hedgehog   |
| SOD           | Superoxide dismutase   |
| TNF- $\alpha$ | Tumor-necrosis-factor $\alpha$   |
| TrxR          | Thioredoxin reductase  |
| Trx           | Thioredoxin  |
| UCN           | Urocortin  |
| UTS           | Urotensin  |
| Wnt           | Wingless   |



# 1 INTRODUCTION

## 1.1 INTRODUCTION INTO THE FIELD

Where do we come from?

This is one of the most fundamental questions of mankind.

One of the first attempts to scientifically address that enigma, besides religious “explanations” long before, was realized by the Dutch physicist Nicolaas Hartsoeker in the 17<sup>th</sup> century, shortly after the invention of the microscope. He examined human sperm and believed to see small, preformed humans, which he named homunculi. The theory of preformism, in which humans were believed to arise in a Matryoshka doll fashion, was born.

Although developmental biology has blossomed during the last century and made astonishing progress, though homunculi have never been observed again, embryonic development remains an enigma until today.

During the last two decades science has made a great leap forward, as researchers started to appreciate a whole new mode of cellular signaling: reactive oxygen species signaling.

Reactive oxygen species (ROS) are chemical intermediates that arise during incomplete reduction of oxygen. These reactive species have long been known to scientists, but were not regarded as biologically significant until the description of the enzyme superoxide dismutase in 1969<sup>1</sup>.

After this seminal discovery, researchers found that ROS were toxic to the integral components of life: DNA, lipids and proteins. Instantly, ROS became stigmatized and were regarded as elicitor for most pathologies including death.

Although, since 1995 scientists began to appreciate the bright side of ROS: their crucial role in cellular signaling<sup>2</sup>.

One of the most important mediators and modulators of redox signals are glutaredoxins, a protein family, which is in the focus of the present thesis.

The development of any higher organism does not stop when it leaves its eggshell or womb. The interaction of the newborn and the young with their environment is vital, and has a crucial effect on its whole life. If the young organism is growing up experiencing intense adverse events, physical and psychological scars can remain forever and may even be passed on to the next generation.

## 1.2 INTRODUCTION INTO THE PRESENT THESIS

The main part of this thesis focuses on the role of Glutaredoxin 2 (Grx2) during embryonic development.

Grxs are oxidoreductases, hence proteins that carry out oxidation and/or reduction of specific substrates. Changing the redox state of these target proteins can have a profound influence on their structural as well as catalytical properties, and therefore transmit specific signals.

The concept of signal transduction via the oxidation/reduction of substrates, known as redox signaling, is of outmost importance for life.

Historically it was long believed that ROS, the mediators of redox signal transduction, pose a threat to the integrity of the cell and elicit many, if not all, of our major health issues. Only in the last two decades, scientists started to unravel the signaling function of ROS.

Even today, central concepts of redox signaling are under debate and much more work is required to understand the foundation of signaling through reactive oxygen species. Therefore, the introduction of this thesis is devoted to a detailed introduction into the field of reactive oxygen species.

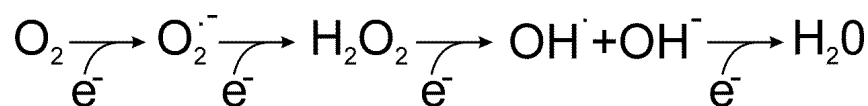
First, I will describe what ROS are, where they come from and why they are toxic to life. I will continue by giving an overview of how cells combat ROS and finally how ROS are employed in redox signaling.

In the second part, I will introduce the protein family of Grxs and give an overview of the current knowledge about their role in health and disease.

Finally, I will give a short introduction into the vertebrate stress axis, including information to the corticotropin-releasing hormone, and I will finish with an introduction into the model organism zebrafish.

### 1.3 REACTIVE OXYGEN SPECIES – AN OVERVIEW

Oxygen is a paramagnetic, diatomic molecule that has two unpaired electrons in the outer shell. Due to this energetically unfavorable state, the  $O_2/H_2O$  redox couple has a very high redox potential (+816 mV), which makes it attractive for energy metabolism. Complete reduction of oxygen to water requires 4 electrons, two per oxygen atom. According to Pauli's principle of electron spins, these electrons cannot be transferred to oxygen all at once, but must be taken up one after another<sup>3</sup>. Incomplete reduction of oxygen leads to highly reactive intermediates, such as superoxide ( $O_2^{\cdot-}$ ), hydrogen peroxide ( $H_2O_2$ ) and the hydroxyl radical ( $OH^{\cdot}$ ) (see Figure 1).



**Figure 1: Reactive oxygen species arise during incomplete reduction of oxygen.**  $O_2^{\cdot-}$ : superoxide;  $H_2O_2$ : hydrogen peroxide;  $OH^{\cdot}$ : hydroxyl radical;  $OH^-$ : hydroxyl anion

The term reactive oxygen species comprises these chemical species as well as their secondary reaction products, e.g. hypohalous acids. ROS can be subdivided in non-radical, like  $H_2O_2$ , or radical species like superoxide and the hydroxyl radical. All these oxidants have characteristic chemical properties and therefore distinct reaction profiles within the cell.

#### 1.3.1 Toxicity of reactive oxygen species

The fact that our body utilizes ROS in fighting infections emphasizes the toxicity of these chemical species.

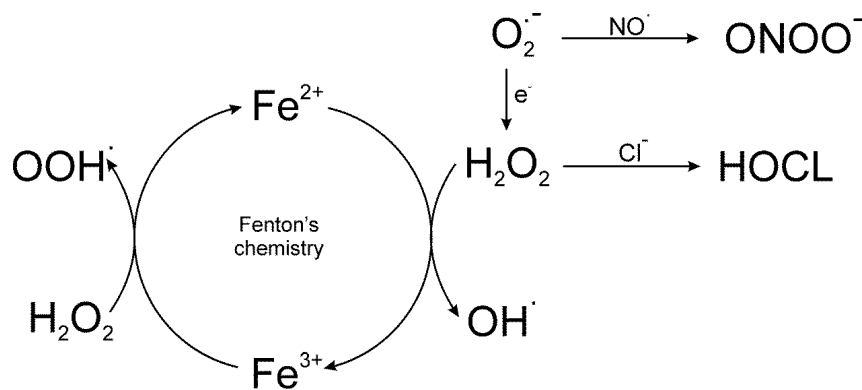
Indeed, depending on their reactivity and concentration, the majority of ROS display deleterious effects to most if not all cellular components. Primary ROS like superoxide and especially hydroxyl radicals indiscriminately oxidize proteins, lipids, and DNA and will induce, if not detoxified, cellular damage and ultimately cell death<sup>4</sup>.

One of the most dramatic reactions is Fenton's chemistry, first described by John Fenton in the late 19<sup>th</sup> century<sup>5</sup>. In presence of redox active bivalent iron ( $Fe^{2+}$ ),  $H_2O_2$  is reduced to hydroxyl radicals that instantly oxidize all biological molecules. This reaction can proceed indefinitely as the by-product  $Fe^{3+}$  can be re-reduced to  $Fe^{2+}$  through  $H_2O_2$ <sup>6</sup> (see Figure 2). Not only primary, but also secondary ROS can harm the cell. For example, in presence of nitric oxide, superoxide can react to peroxynitrite

(ONOO<sup>-</sup>) that can easily cross cell membranes<sup>7</sup>. Peroxynitrite is a strong oxidant and potent trigger of cell death, but has recently received attention as signal transducer<sup>8</sup>.

The dual effect of peroxynitrite, namely inducing cell death as well as being involved in cellular signaling, is not a peculiar characteristic of this particular ROS species. During the last twenty years, researchers have realized that ROS are not only toxic for cellular survival and crucial mediators of many major health issues, but that they also are important transmitters of cellular signaling and therefore indispensable for life.

In the next chapters, I will give an overview of how and where ROS are produced, what systems are involved in their detoxification and how ROS are employed in cellular signaling events.



**Figure 2: Primary and secondary reaction products of ROS.** Superoxide can be degraded to hydrogen peroxide by superoxide dismutase, or it can react, in presence of nitric oxide, to peroxynitrite. Hydrogen peroxide can generate large amounts of hydroxyl radicals in presence of redox active iron due to Fenton's chemistry, but it also can be halogenated to the corresponding hypochlorous acids through myeloperoxidases.

### 1.3.2 Reactive oxygen species - Production

There are multiple aerobic metabolic processes that generate ROS as a waste product, and enzymes like xanthine oxidases<sup>9</sup>, cytochrome P450 systems<sup>10</sup> as well as protein folding processes in the endoplasmatic reticulum constantly leak electrons into their surroundings<sup>4</sup>. But the major sources of ROS within the animal cell are mitochondria. The electron transport chain, which is located in the inner mitochondrial membrane, transfers electrons from reducing equivalents (NADH) to oxygen, and thereby generates an electrochemical proton gradient that is used to synthesize energy in form of ATP. It is estimated that about 1-2 % of all oxygen, which is consumed in the mitochondria, is converted to superoxide by electrons that leak mainly from complex I

and III<sup>11</sup>. The manganese superoxide dismutase (MnSOD), located in the matrix, converts superoxide into H<sub>2</sub>O<sub>2</sub>, which can diffuse freely across membranes or channel via aquaporins<sup>12</sup>.

Although various antioxidant systems are present in mitochondria, the superoxide tension is about 5-10 fold higher compared to the cytosol or nucleus<sup>13</sup>, and significant amounts of H<sub>2</sub>O<sub>2</sub> are constantly produced in mitochondria<sup>14</sup>.

ROS are not only side products of many metabolic processes, they are also actively produced by dedicated enzymatic systems. Already in the 1960s it has been described that neutrophils and macrophages induce a deadly oxidative burst when engulfing microbes<sup>15</sup>, and in the 1980s the respective enzyme has been cloned: NADPH oxidase (Nox)<sup>16,17</sup>. Soon thereafter, other Nox enzymes have been discovered and today the mammalian Nox family comprises seven members: Nox 1-5, which produce superoxide, and dual oxidases (Duoxs) 1 and 2, which produce H<sub>2</sub>O<sub>2</sub>. All family members display distinct expression and localization profiles, and are conserved across species<sup>18</sup>. Interestingly, Nox enzymes are almost exclusively found in multi-cellular organisms<sup>19</sup>.

### 1.3.3 Reactive oxygen species - Elimination

The levels of ROS within the cell are determined by the rate of their production and their elimination. For its survival and propagation, the cell has to establish an optimal level for each individual ROS species. Therefore, a multitude of enzymatic and non-enzymatic systems has evolved that control, individually or in concert, the degradation of ROS<sup>4</sup>.

#### 1.3.3.1 Low molecular weight compounds in antioxidant defense

Vitamin C (ascorbic acid) is an essential nutrient for humans. It has to be taken up with the diet, as humans lack a functional gulonolactone-oxidase gene, which is required for the terminal step of ascorbic acid synthesis. Vitamin C has two hydroxyl groups with one of them being deprotonated at physiological pH. It can undergo one-electron oxidation, e.g. reducing ROS, yielding the ascorbic acid radical. In contrast to all other reactive oxygen radicals, the electron configuration of this radical is stabilized through the aromatic structure, rendering its reduction potential rather low. The ascorbic acid radical can in turn either dismutate or be recycled at the expense of GSH or NADH<sup>20</sup>. Although potentially detoxifying ROS *in vitro*, the antioxidant effect of vitamin C *in vivo* is still under dispute<sup>21</sup>. Another vitamin that might be important in antioxidant defense is vitamin E (tocopherol). Tocopherols exhibit a high rate constant towards

lipid-peroxyl radicals and can protect against lipid peroxidation<sup>22</sup>. But as for vitamin C, the evidence of vitamin E as antioxidant *in vivo* is limited<sup>23</sup>. Besides vitamin C and E, other low molecular weight species like carotenoids and plant phenols have been implicated in antioxidant defense<sup>4</sup>.

### 1.3.3.2 Enzymatic systems in antioxidant defense

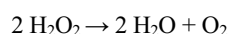
In addition to vitamins and other dietary compounds, a variety of enzymes degenerate reactive oxygen species. As their name indicates, superoxide dismutases (SODs) catalyze the dismutation of superoxide into H<sub>2</sub>O<sub>2</sub> and oxygen:



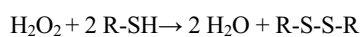
Three SODs with distinct expression profiles have been identified in mammals. SOD1 and SOD3 are copper/zinc enzymes, located in the cytosol or plasma, respectively. SOD2 on the other hand is manganese-dependent and found in the mitochondria<sup>24</sup>.

Hydrogen peroxide, the product of superoxide dismutation, can be removed by two types of enzymes:

- (i) Catalases catalyze the dismutation of H<sub>2</sub>O<sub>2</sub> into water and oxygen:



- (ii) Peroxidases catalyze the reduction of H<sub>2</sub>O<sub>2</sub> to water and couple this reaction to the oxidation of a substrate:



In case of glutathione peroxidases (GPxs), peroxides are reduced at the expense of GSH. Five GPx have been identified in mammals and all are selenocysteine-containing enzymes<sup>25</sup>. GPx4 can, in contrast to the other GPxs, efficiently reduce lipid peroxides<sup>26</sup>. Peroxides can also be reduced by peroxiredoxins (Prxs) which are, unlike GPxs, dependent on reducing equivalents provided by thioredoxins (Trxs)<sup>27</sup> or, in some cases, Grxs<sup>28</sup>. Six Prxs have been described in mammalian systems, all of which with distinct intracellular distribution. Prxs cannot only degrade H<sub>2</sub>O<sub>2</sub> but also organic hydroperoxides and peroxynitrite. Besides their role as antioxidants, Prxs have been implicated in redox-mediated signal transduction<sup>29</sup> (see also 1.4). Another powerful system controlling the cellular redox milieu is comprised of Trxs and Grxs. Those members of the thioredoxin family of proteins will be discussed in detail in chapter 1.5. All enzymatic reactions detoxifying ROS are dependent on reductive equivalents in the form of electrons. Those are provided by NADPH, which is primarily synthesized by



glucose-6-phosphate dehydrogenase during the oxidative phase of the pentose phosphate pathway, but also through the de-amination of glutamate.

### 1.3.3.3 *The glutathione system in antioxidant defense*

Glutathione is an enzymatically synthesized tri-peptide, which is present in millimolar concentrations in the cytosol<sup>30</sup>. Although GSH is probably not the quantitatively dominant thiol<sup>31</sup>, it represents the major cellular antioxidant and determines, together with its oxidized form GSSG, the cellular redox potential<sup>32</sup>. Moreover, it possesses a critical role in cellular redox signaling<sup>33</sup>, which will extensively be discussed in chapter 1.4.1.1.

GSH is synthesized by a two-step process in the cytosol. First, glutamate-cysteine ligase (GCL) synthesizes gamma-glutamyl-cysteine that is subsequently coupled to glycine by glutathione synthetase. The first step of GSH synthesis is feedback regulated through GSH levels.

GSH is not distributed uniformly within the cell, but it rather seems that each compartment has its specific GSH concentration, GSH/GSSG ratio, and therefore redox potential<sup>34</sup>. The pools of GSH in the cytosol, the mitochondrion, and potentially also in the endoplasmic reticulum are independent from each other, establishing different redox potentials in these organelles<sup>35</sup>.

GSH is essential for ROS defense, as it is a cofactor for GPxs, Grxs and glutathione-transferases (GSTs). The latter is a class of enzymes that couple nucleophilic the GSH to electrophilic xenobiotics for detoxification<sup>36</sup>. It is currently under debate if GSH protects directly or indirectly, e.g. as cofactor for detoxification enzymes, against ROS. GSH can react with several ROS species *in vitro* (poorly with superoxide though), reducing them to less toxic species. As GSH is present in millimolar amounts in the cell, these reactions are feasible *in vivo*. On the other hand, *in vivo* reaction kinetics based on  $K_m$  values and concentrations of several antioxidant defense systems indicate that peroxiredoxins are the primary enzymes to break down  $H_2O_2$ <sup>37</sup>. This hypothesis is supported by the fact that in presence of physiological amounts of GSH (and absence of peroxiredoxins)  $H_2O_2$  is able to diffuse about 1.5 mm<sup>37</sup>.

But no matter if GSH protects cells directly or indirectly against ROS, it plays, without doubt, a major role in redox signaling which will be discussed in detail in chapter 1.4.1.1.

#### 1.4 SIGNAL TRANSDUCTION THROUGH REACTIVE OXYGEN SPECIES

About 20 years ago, studies showed that cell proliferation was induced upon exposure to low concentrations of  $\text{H}_2\text{O}_2$ <sup>38</sup>. But a physiological role of  $\text{H}_2\text{O}_2$  in cellular signaling was not considered until the discovery of Nox enzymes in non-phagocytotic cells<sup>39</sup>. Today, the signaling function of ROS in physiology and pathology is accepted and in the focus of intensive research.

It was long suggested that the overall cellular redox potential is the essential force driving redox signaling. The thiol/disulfide couples would be kept in equilibrium through the GSH/GSSG redox couple, and as protein thiols vary in its pKa value, they would show distinct sensitivities towards changes in the overall redox potential. In recent years, this concept was substantially reviewed. Redox signaling is now suggested to be “compartmentalized” and relayed through specific redox reactions without any alterations of the general cellular redox potential<sup>40,41</sup>. A prerequisite for this concept is the reversibility, kinetic feasibility, and specificity of these specific redox modifications.

The term reactive oxygen species encompasses a variety of radical and non-radical species that vary in their chemical reactivity (see also chapter 1.3). Due to these intrinsic chemical properties and the resulting kinetic and thermodynamic reaction profile,  $\text{H}_2\text{O}_2$  is the only ROS that qualifies as second messenger in cellular signaling<sup>42</sup>. Moreover, it is enzymatically produced and degraded at confined locations and time, which is a key for the specificity of signals. The hydroxyl radical is unlikely to be involved in cellular signaling as it reacts indiscriminately with almost any biological molecule at rate constants close to the limit of diffusion<sup>42</sup>. Superoxide might react with some iron-sulfur proteins<sup>43</sup>, but its reaction with protein thiols is too slow in comparison to its brake-down by superoxide-dismutase<sup>44</sup> to convey redox signals. Although, superoxide can, in the presence of NO, generate peroxynitrite, which is a potent mediator of reactive nitrogen species (RNS) signaling. A few hundred proteins are believed to be nitrosylated *in vivo*, and the ratio of ROS/RNS is a pivotal regulator of immune responses<sup>45,46</sup>.

### 1.4.1 Cysteines as major targets of redox signaling

Regulation of enzymatic function *via* thiol-redox reactions, referred to as “thiol redox control”, has first been conceptualized due to discoveries in chloroplasts. In 1977, Bob Buchanan described with his coworkers how plant Trx, which was photochemically reduced by ferredoxin-Trx reductase, regulated central chloroplast enzymes through reduction of disulfide bonds<sup>47</sup>. Since this initial discovery in plants, the field of redox regulation *via* protein thiols has vastly expanded<sup>48</sup>.

Of all amino acids, cysteine represents a unique target for reversible oxidation/reduction, and regulation of cell signaling *via* cysteine modifications is best established. In addition to cysteines, methionine, tyrosine, tryptophan, and selenocysteine residues are also susceptible for redox modifications.

As most ROS react at physiological concentration only with the thiolate anion, a low pKa is a key feature for every redox active cysteine residue. Whereas free cysteine residues have a pKa of about 8 to 9, redox active cysteines may display values below 7 and are therefore present in their thiolate form at physiological pH. The relatively low pKa of redox active cysteine residues is stabilized through their chemical environment including hydrogen bonds and polar amino acid side chains<sup>49,50</sup>. The characteristics of this environment might also add to the specificity of redox signals<sup>44</sup>.

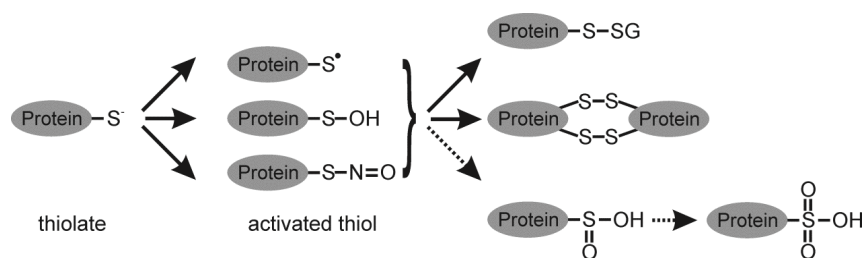
During redox signaling events, the thiolate anion becomes oxidized to sulfenic acid by H<sub>2</sub>O<sub>2</sub>. The sulfenic acid might in turn condense with proximal, intra- or intermolecular SH groups to form disulfide bridges, it might be glutathionylated (see also chapter 1.4.1.1) or further oxidized to sulfinic acid<sup>51</sup>. The latter seems, although reversible in specific cases through sulfiredoxins<sup>52</sup>, only to occur during severe oxidative stress<sup>42</sup>. Further oxidation of sulfinic acid to sulfonic acid is theoretically possible, but questionable in physiological circumstances<sup>42</sup>. Activated cysteine residues can also be nitrosylated, mediating reactive nitrogen species (RNS) signaling. Detailed information of signaling *via* RNS can be found elsewhere<sup>8</sup>.

Importantly, for cellular signaling events to occur and to cease, reversibility of cysteine modifications is essential. The major enzymes catalyzing reversible cysteine modifications, e.g. reduction of disulfide bonds, are Trxs and Grxs (see chapter 1.5).

### 1.4.1.1 Glutathionylation of cysteine residues as mode of signal transduction

Glutathionylation as redox dependent protein modification has attracted much attention during the last years. Initially characterized as protective mechanism for redox active cysteine residues against over-oxidation, it is now established that glutathionylation is, besides a thiol protectant, also a critical post-translational modification conveying redox signals<sup>33</sup>.

Today, it is relatively well understood how glutathione mixed disulfides are reduced. Deglutathionylation, i.e. the removal of the GSH moiety from proteins, is enzymatically catalyzed mainly by Grxs<sup>53</sup> (see also 1.7.1), but potentially also by sulfiredoxins<sup>54</sup> and GSTs<sup>55</sup>. On the other hand, it is much less established how proteins are glutathionylated. A mere thiol-disulfide exchange seems to be very unlikely, as the GSH/GSSG ratio under physiological conditions is unable to convert protein thiols to protein-mixed disulfides. A decline of the GSH/GSSG ratio to 1:1 would be necessary for a 50 % conversion, a scenario which is unlikely even under severe conditions of oxidative stress<sup>56</sup>. It is much more reasonable that activated cysteine residues, like the thiyl radical (R-S<sup>•</sup>), sulfenic acid (R-S-OH) or S-nitrosyls (R-S-NO) react with GSH to form a protein-mixed disulfide (see Figure 3). Glutathionylation of nitrosylated cysteines might also mediate a crosstalk between ROS and RNS signaling. Although, kinetic properties of glutathionylation of activated cysteine residues under conditions mimicking the intracellular milieu have yet to be evaluated<sup>33</sup>. Additionally, there is also evidence that glutathionylation is enzymatically catalyzed by Grxs<sup>57,58</sup>, GST- $\pi$ <sup>59</sup> and sulfhydryl oxidases<sup>56</sup>.



**Figure 3: Cysteines are the major targets of redox signaling.** Redox active cysteine residues are present in the thiolate form at physiological pH. They can be activated to a thiyl radical, to sulfenic acid or to S-nitrosyls. Those activated thiols can condensate with proximate thiols, or they can be glutathionylated, and thereby modify protein function. Activated thiols can also be further oxidized to sulfinic and sulfonic acid, but their *in vivo* function is under discussion.

### 1.4.2 How is specificity created in redox signaling?

A key to the concept of redox signaling is to understand how specificity is achieved. A signal has to be generated, conveyed to a distinct target, and to be ceased after transmission. As described above, H<sub>2</sub>O<sub>2</sub> is likely the only ROS to be employed as second messenger.

But how is specificity of H<sub>2</sub>O<sub>2</sub> signaling ensured?

#### (i) Compartmentalization

Nox enzymes (see 1.3.2) have specific cellular and sub-cellular localization profiles and assembly of functional Noxs is strictly controlled. Localized production of H<sub>2</sub>O<sub>2</sub> would therefore only activate target proteins in close proximity<sup>60</sup>.

#### (ii) Reactivity and accessibility of the target cysteine residues

As described above, the reactivity of the target cysteine residue is a key to its susceptibility for redox modifications. The surrounding amino acids of the cysteine residue determine not only the structure and therefore accessibility of the cysteine, but also the pKa value and therefore the reactivity with H<sub>2</sub>O<sub>2</sub> itself.

#### (iii) Facilitated targeting through sensor proteins

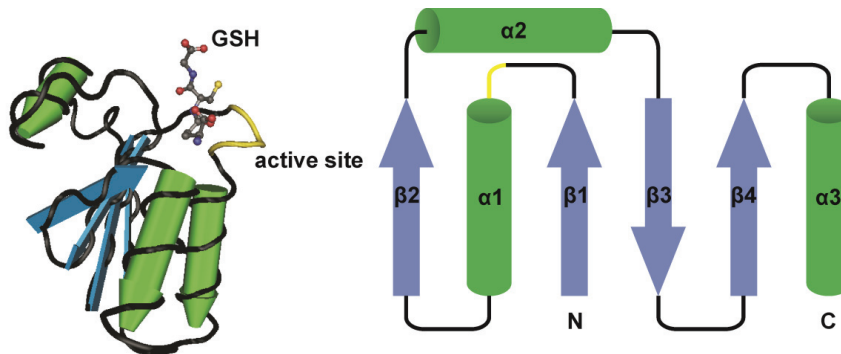
Although H<sub>2</sub>O<sub>2</sub> is a strong oxidant, rate constants with protein thiolates are insignificant (~20 M<sup>-1</sup>s<sup>-1</sup>) due to a high activation energy<sup>44</sup>. Kinetic modeling showed that peroxiredoxins are the only known direct targets of H<sub>2</sub>O<sub>2</sub> at physiological concentrations<sup>44</sup>. This gave rise to the concept of facilitated redox signaling through sensor proteins: intracellular redox signaling would not occur directly, but be mediated through a highly reactive thiol protein that is directly oxidized by H<sub>2</sub>O<sub>2</sub>, e.g. Gpx3 in yeast<sup>61</sup> or peroxiredoxins in mammalian systems<sup>29</sup>.

It was suggested before that over-oxidation of peroxiredoxins generates a localized zone with H<sub>2</sub>O<sub>2</sub> available for signal transduction, but this “floodgate” hypothesis is unlikely to be relevant *in vivo*, based on thermodynamic considerations<sup>42,44,62</sup>. Nevertheless, the non-redundancy of Prxs in knock-out models suggests a function beyond pure oxidant defense, and points to a crucial role in transmitting redox signals<sup>63,64</sup>.

## 1.5 THE THIOREDOXIN FAMILY OF PROTEINS

The founding member of the thioredoxin family of proteins was discovered 1964 in *E.coli*. It was characterized as a small heat stable protein providing hydrogen/electrons to ribonucleotide reductase (RNR) for deoxyribonucleotide synthesis, and named thioredoxin<sup>65</sup>. Soon, Trxs were discovered in other organisms and it is now known to be present throughout the kingdom of life<sup>66</sup>.

Today, the thioredoxin family of proteins consists of three main branches, Trxs<sup>67</sup>, Grxs<sup>68</sup>, and Prxs<sup>27</sup>, as well as related proteins including protein-disulfide-isomerases<sup>69</sup>, the bacterial Dsb family of proteins<sup>70</sup>, GPxs<sup>71</sup>, GSTs<sup>72</sup>, members of the intracellular chloride ion channels, and proteins involved in the assembly of cytochrome-C oxidases. Although most members have unique physiological functions, all are characterized by a similar three dimensional structure, called thioredoxin fold<sup>73,74</sup>. This specific architecture has first been described in 1975, when the crystal structure of *E.coli* Trx was published<sup>75</sup>. The thioredoxin fold consists of four to five alpha helices that surround a central core of four to five beta-sheets (see Figure 4). Additionally to the conserved three-dimensional structure, the oxidoreductases Trx and Grx have a highly conserved *cis*-proline that is located closely to the active site motif C-X-X-C (in which X represents any amino acid) in the folded protein<sup>73,76</sup>. Although named thioredoxin fold, the structure of *E.coli* Grx1 represents the most basic form of this protein fold (see Figure 4).



**Figure 4: The thioredoxin fold.** This characteristic protein structure consists of four beta sheets which are surrounded by three alpha helices. The most basic form of the thioredoxin fold is represented by *E.coli* Grx1 (PDB ID: 1GRX). Left: model of the crystal structure in complex with GSH, right: cartoon of the domain organization. Green: helices, blue: beta sheets. Yellow indicates the localization of the active site.

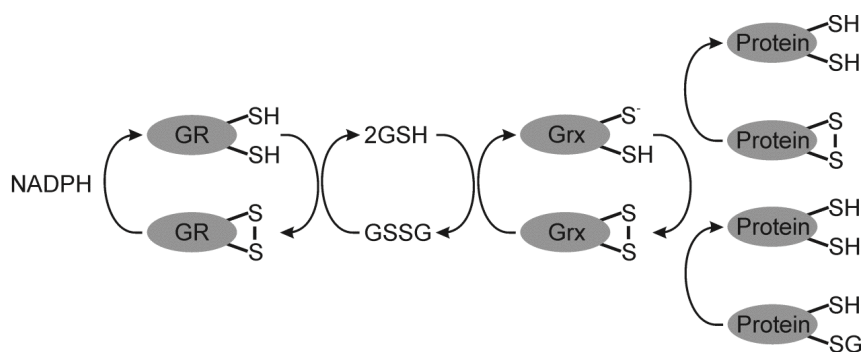
## 1.6 THE THIOREDOXIN SYSTEM

The thioredoxin system is ubiquitously expressed and consists of Trx, thioredoxin-reductase (TrxR) and NADPH as source of reducing equivalents. Besides providing RNR with electrons for DNA synthesis, Trx catalyzes the reduction of methionine sulfoxide reductase<sup>77</sup> and peroxiredoxins<sup>78</sup> (see also 1.3.3.2). Furthermore, Trxs are implicated in oxidative stress defense, and they are regulators of numerous transcription factors and involved in various cellular processes, e.g. gene expression<sup>79</sup>, cell growth, and apoptosis<sup>80,86,87</sup>. Due to the versatile role in cellular physiology, knock-out of single Trx system components is embryonically lethal in rodents<sup>81-84</sup>.

Today, the thioredoxin system is in the focus of cancer research and might represent a promising target for novel therapies. Both Trx and TrxR have shown to be up-regulated in various tumors, and their expression levels were correlated to tumor growth and patient survival<sup>85</sup>.

## 1.7 THE PROTEIN FAMILY OF GLUTAREDOXINS

As described above, Trxs provide electrons for deoxyribonucleotide synthesis which is essential for DNA replication and hence survival of the organism. Therefore, it was surprising that *E.coli* lacking detectable amounts of Trx were still able to grow. Soon, an alternative electron donor for ribonucleotide reductase was found in these mutants, and, since it was dependent on GSH, named glutaredoxin<sup>88-90</sup>. Today, Grxs are defined by their ability to bind and utilize GSH as substrate<sup>76</sup>.

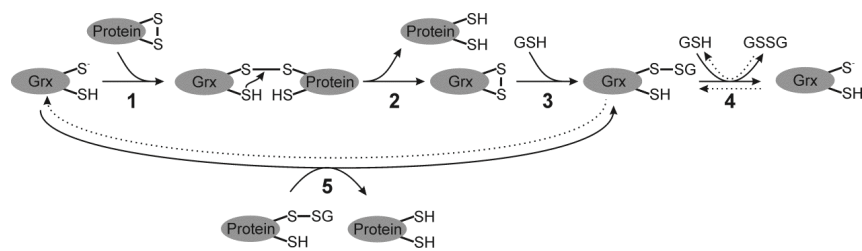


**Figure 5: Flow of electrons in the Grx system.** Grx reduces protein dithiols or protein mixed disulfides and becomes oxidized. Oxidized Grx is re-reduced at the expense of GSH, which is recycled by glutathione reductase and NADPH as final electron source.

### 1.7.1 The glutaredoxin system

Grxs are oxidoreductases which can reduce protein disulfides and catalyze protein de/glutathionylation *via* similar yet distinct mechanisms. Electrons for these reduction processes stem from GSH, which itself is recycled by glutathione reductase (GR) that in turn receives its electrons from NADPH (see Figure 5).

Grxs can reduce protein disulfides using both their N-, and C-terminal active site cysteine residues, the mechanism is therefore called dithiol mechanism. The N-terminal cysteine, which has a low pKa value (3.5 for hGrx1<sup>91</sup>), performs a nucleophilic attack on the target disulfide, yielding an intermediate mixed disulfide (see Figure 6, step 1). This is resolved by the C-terminal cysteine and the reduced protein is released (Figure 6, step 2). The remaining intra-molecular disulfide in Grx is reduced in two subsequent steps. First, a nucleophilic attack of the GSH resolves the disulfide to a mixed disulfide (see Figure 6, step 3), which is in turn reduced by a second molecule of GSH (see Figure 6, step 4). The reduction of glutathione-mixed-disulfides by Grxs requires only the action of the N-terminal cysteine residue, the mechanism is therefore called monothiol mechanism (see Figure 6, step 5)<sup>76</sup>. For both reactions, the nucleophilic attack of the glutathionylated Grx by GSH is the rate determining step (see Figure 6, step 4)<sup>92</sup>. Although deglutathionylation of substrates is preferred by Grxs, during conditions of oxidative stress, hence a low GSH/GSSG ratio, Grxs might also catalyze glutathionylation of substrates, potentially to protect redox active cysteine residues from over-oxidation (see Figure 6, dotted lines)<sup>57,58</sup>. In summary, Grxs can alter both the redox state and posttranslational modification of key cysteine residues. Hence, Grxs are important regulators and mediators of redox signaling.



**Figure 6: Dithiol and monothiol mechanism of Grx.** *solid lines (1-4):* reduction of protein disulfides *via* the dithiol mechanism. *1:* nucleophilic attack on the target disulfide; *2:* release of the reduced substrate; *3,4:* reduction of the intra-molecular disulfide of Grx in two subsequent steps by two molecules of GSH. *5:* deglutathionylation of protein mixed disulfides *via* the monothiol mechanism. Dashed lines indicate the catalysis of protein glutathionylation *via* Grx.



## 1.7.2 Glutaredoxins in vertebrates

The sequenced vertebrate genomes encode two distinct groups of Grxs: two classical dithiol Grxs, Grx1 and Grx2, as well as two monothiol Grxs, Grx3 (PICOT/TXNL-2) and Grx5. Monothiol Grxs are either present with a single Grx domain, or they possess a Trx domain followed by one to three Grx domains<sup>76</sup>. Whereas monothiol Grxs show in general a remarkable conservation from bacteria to mammals<sup>100</sup>, dithiol Grxs display a higher degree of variation among species, although they seem to have originated from a single gene in early evolution<sup>100</sup>. All four vertebrate Grxs have a distinct sub-cellular localization, and execute specific functions that will be discussed in the following chapters.

### 1.7.2.1 *Glutaredoxin 1*

Grx1 is highly conserved among vertebrates, and all orthologues contain the consensus active site motive C-P-T-C. Grx1 is mainly found in the cytosol but, although the hGrx1 gene, GLRX1, does not encode for any sub-cellular localization sequence, also in the nucleus<sup>101,102</sup>, the mitochondrial intermembrane space<sup>103</sup> and in plasma<sup>104</sup>.

The specific sub-cellular localization of Grx1 is the basis for its versatile functions. As described before, Grx1 is an electron donor for ribonucleotide reductase, and its  $K_m$  is lower compared to that of Trx1 (0.6  $\mu\text{M}$  versus 1.8  $\mu\text{M}$  for bovine enzymes)<sup>105,106</sup>. Grx1 has been reported to regulate transcription factors like nuclear factor 1 (NF-1)<sup>107</sup>, nuclear factor  $\kappa\text{B}$  (NF- $\kappa\text{B}$ )<sup>79</sup> and activator-protein 1 (AP-1)<sup>79</sup>. In addition, Grx1 can reactivate protein-tyrosine-phosphatase 1B after it has been glutathionylated and inactivated, e.g. by ROS signaling<sup>108</sup> (see also 1.4.1.1). In the intermembrane space of mitochondria, Grx1 can catalyze reversible glutathionylation of complex I<sup>103</sup>, and in plasma it might be involved in the formation of extracellular disulfides<sup>109</sup>. Moreover, Grx1 can reversibly glutathionylate actin and by that regulate its polymerization<sup>110</sup>.

This modulation of actin polymerization by Grx1 might have profound effects on the shape of the cytoskeleton and hence the migration of cells<sup>111</sup>. Grx1 has also been connected to apoptosis through inhibition of apoptosis-signaling-kinase-1 (ASK-1) as well as regulation of caspase-3 cleavage<sup>112,113</sup>, and it might protect against neurodegenerative diseases like Alzheimer's<sup>114</sup> and Parkinson's disease. In that respect it is interesting to note, that Grx1 levels are regulated by estrogens<sup>115</sup>, which are positively correlated to protection against MPTP-induced neurodegeneration<sup>115</sup>. In line, females have a lower risk to suffer from Parkinson's disease<sup>116,117</sup>.

Since Grx1 seems to fulfill essential functions in the cell, it is surprising that knock-out mice with targeted disruption of the GLRX1 gene are viable. Even mice homozygous for the loss of Grx1 do not display any morphological differences compared to wildtype littermates. On the other hand, it seems that knock-out of Grx1 sensitizes mice to ischemia/reperfusion<sup>118</sup> and some allergic airway disease<sup>119</sup>, although contradictory data have been published<sup>120</sup>. However, it would be interesting to establish if other oxidoreductases, e.g. Grx2, can compensate for the loss of Grx1.

#### 1.7.2.2 *Glutaredoxin 2*

A second mammalian dithiol Grx was discovered in 2001 and named Grx2<sup>121,122</sup>. The corresponding gene in humans, GLRX2, gives rise to three different isoforms due to alternating start codons as well as alternative splicing. Whereas hGrx2a possesses a mitochondrial translocation sequence, hGrx2b and hGrx2c are localized in the cytosol<sup>123</sup>. Expression of the two latter was specifically confined to testis and various cancer cell lines<sup>123</sup>. In contrast to the Grx1 consensus active site motive C-P-T-C, the active site of Grx2 harbors a serine instead of the proline residue. This exchange increases the affinity of Grx2 for glutathionylated proteins compared to Grx1<sup>124</sup>, and it was described as the main prerequisite for the coordination of a [FeS] cluster (see 1.7.3). Moreover, the active site serine enables Grx2 to receive electrons not only from GSH, but also from thioredoxin reductase<sup>124</sup>. Although the physiological relevance has recently been questioned<sup>31</sup>, this might be an important backup pathway under conditions of a low GSH/GSSG ratio, i.e. oxidative stress<sup>124</sup>. Indeed, Grx2 has been implicated in oxidative stress induced apoptosis. Knock-down of Grx2 sensitized HeLa cells against oxidative stress induced cell death, whereas over-expression protected against apoptotic stimuli<sup>125,126</sup>. The protective role was connected to prevention of cardiolipin release<sup>126</sup>, and to the ability of Grx2 to redox-regulate complex I of the electron transport chain<sup>58</sup>. Moreover, Grx2 has been implicated in the protection against ischemia/reperfusion induced oxidative stress. Over-expression of Grx2 in a mouse model improved the GSH/GSSG ratio upon cardiac and kidney ischemia/reperfusion, and prevented loss of cardiolipin, release of cytochrome-c, as well as activation of caspases<sup>127,128</sup>. Moreover, Grx2 has also been implicated in the etiology of neurodegenerative disorders like Parkinson's disease<sup>129,130</sup>.

Interestingly, specific structural characteristics including a common structural disulfide bond have evolved exclusively in vertebrate Grx2s<sup>131</sup>. This might indicate that Grx2s have evolved crucial functions for the development of vertebrate specific structures like

brain or vascular system. Indeed, during mouse embryonic development, expression of Grx2 was observed from embryonic day (E) 7 onwards, the same time when the nervous system commences developing<sup>132</sup> and in the adult brain of rodents and humans, Grx2 was mostly confined to neurons<sup>129,133,134</sup>.

### 1.7.2.3 Monothiol Glutaredoxins

Monothiol Grxs can be, as described above, characterized as single-domain and multi-domain proteins and both forms have the functional equipment to catalyze the reduction of mixed disulfides between proteins and GSH.

Grx3 is a multi-domain monothiol Grx and localized in the cytosol. It has been reported that Grx3 translocates into the nucleus during conditions of oxidative stress, and modulates AP-1 and NF- $\kappa$ B signaling pathways<sup>135</sup>. Furthermore, Grx3 is connected to the immune response as it acts as positive regulator of IL-4 and TNF- $\alpha$  signaling<sup>136</sup>. Moreover, it has been proposed that the activity of Grx3 is regulated by its iron-sulfur cofactor<sup>137</sup>, and in yeast it was demonstrated that Grx3 is essential for intracellular iron-trafficking<sup>138</sup>.

In contrast to Grx1 knock-out animals, Grx3<sup>-/-</sup> mice are not viable and die between E12.5 and E14.5. Lethality might potentially be the result of defects in cell cycle progression and cardiac malformations, as Grx3 regulates serum response factor, an important transcription factor involved in heart development<sup>139,140</sup>.

Grx5 is a mitochondrial located single-domain Grx. Although being a monothiol Grx and therefore lacking the C-terminal active site cysteine residue, yeast Grx5 is still able to reduce protein disulfides. This could be due to a conserved additional cysteine outside the active site, which might resolve the mixed disulfide that occurs during the dithiol mechanism<sup>141</sup>.

A chromosomal walk has identified a mutation in Grx5 as the cause for the hypochromatic anemia in the zebrafish mutants called shiraz<sup>142</sup>. This and other observations from yeast strongly suggest that Grx5 is an essential component of the iron-sulfur cluster assembly machinery, most probably assisting to transfer the [FeS] cluster to the target protein<sup>143-147</sup>. Furthermore, there is evidence that Grx5 participates in redox regulation of PTEN<sup>148</sup>, and osteoblast apoptosis<sup>149</sup>.

### 1.7.3 Iron-sulfur cluster coordinating glutaredoxins

Iron-sulfur clusters are essential for many critical enzymes, but the components they are built of, namely iron and sulfur, are toxic for the cell<sup>6,150</sup>. Hence, a complex machinery is dedicated to their homeostasis as well as to the biosynthesis of iron-sulfur cofactors. In eukaryotes, two distinct yet connected mechanisms are responsible for iron-sulfur cofactor synthesis<sup>151</sup>. Both, the cytosolic iron-sulfur protein assembly machinery (CIA), and the mitochondrial iron-sulfur cluster assembly system (ISC system) perform synthesis of [FeS] clusters in two distinct reaction steps: (i) transient assembly of the [FeS] cofactor on scaffold proteins and (ii) transfer of the pre-assembled cluster to the target apo-proteins<sup>152</sup>.

HGrx2a was the first member of the thioredoxin family of proteins being characterized as iron-sulfur cluster protein. The cluster bridges two monomers *via* the N-terminal active site cysteine and non-covalently bound GSH, with the latter being in constant exchange with the free GSH pool<sup>153</sup>. As the holo protein is inactive, the metal centre is proposed to serve as redox sensor, activating the protein under conditions of decreased GSH/GSSG ratio, i.e. oxidative stress<sup>154</sup>.

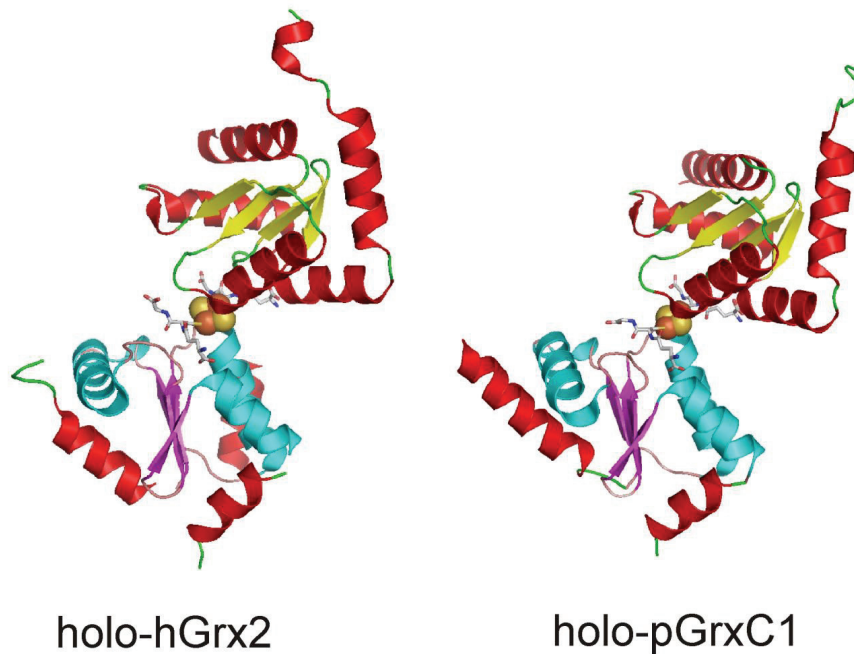
After this initial description, more members of the Trx family have been characterized as [FeS] proteins. Today, those include monothiol and dithiol Grxs from different species, like human Grx3<sup>137</sup> and Grx5<sup>155</sup>, yeast Grx3, Grx4 and Grx6<sup>138,156</sup>, *Trypanosoma* 1-CGrx1<sup>157</sup> as well as poplar GrxC1<sup>158</sup> and *Arabidopsis* GrxC5<sup>96</sup>.

In all these Grxs, the cluster is coordinated by the N-terminal cysteine residue of the active site motive as well as a non-covalently bound GSH molecule, as it was originally described for hGrx2<sup>155,158-161</sup> (see Figure 7). The ability of the C-X-X-C motive to bind a metal cofactor is not restricted to members of the thioredoxin family of proteins, but it extends also to other proteins such as metallothioneins<sup>162</sup> and zinc-finger transcription factors<sup>163</sup>.

Interestingly, the presence of a proline residue within the dithiol motive seems to be critical for the ability of Trx family members to coordinate a [FeS] cluster. Mutants of *E.coli* Trx1, in which an active site proline residue was introduced or replaced, lost or gained the ability to coordinate an iron-sulfur centre<sup>164</sup>. Likewise, hGrx1, in which the active site proline was exchanged, was also able to bind a [FeS] cluster<sup>153</sup>. Although recently, the first iron-sulfur cluster Grx with the active site motive C-P-T-C has been described<sup>165</sup>. Another key feature for cluster coordination is the conserved *cis*-proline

residue of Trx family members, as its exchange enabled mutants of hTrx1 to bind a [FeS] cluster<sup>166</sup>.

Currently it seems that the iron-sulfur cluster of Trx family proteins might serve three distinct functions. It may act as a redox sensor regulating the activity of the protein, as proposed for hGrx2<sup>154</sup>, hGrx3<sup>137</sup> and pGrxC1<sup>158</sup>, it might be involved in the iron-sulfur cluster assembly machinery as suggested for zfGrx5<sup>142</sup> and hGrx5<sup>146,147</sup>, or it might be involved in intracellular iron sensing, and trafficking as demonstrated for yeast Grxs 3 and 4<sup>138,167</sup>.



**Figure 7: Crystal structures of holo human Grx2 and holo poplar GrxC1.** The [FeS] cluster in both hGrx2 and pGrxC1 is coordinated by the N-terminal active site cysteine residue of two protomers and non-covalently bound GSH.

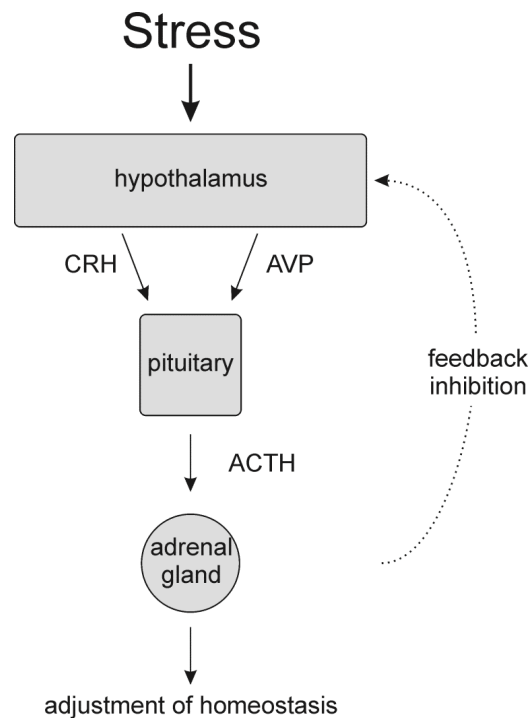
## 1.8 THE STRESS AXIS IN PHYSIOLOGY AND PATHOPHYSIOLOGY

### 1.8.1 The vertebrate stress axis

If life in its dynamic equilibrium is challenged by extrinsic or intrinsic stress (e.g. predators or inflammation) a system beyond the control of the individual becomes activated: the stress response.

Upon activation, the stress response coordinates physiology and behavior, adjusts homeostasis and increases the organism's chances of survival.

The main mechanism that elicits the stress response is the hypothalamic-pituitary-adrenal (HPA) axis. Upon stimulation, the hypothalamus releases corticotrophin-releasing-hormone (CRH) together with arginine-vasopressin (AVP). Both hormones stimulate the excretion of adrenocorticotrophic hormone (ACTH) from the anterior lobe of the pituitary into the blood stream. This in turn triggers the secretion of glucocorticoids from the adrenal cortex, which adjusts homeostasis, activates target genes, and hence adapts the organism to the stressor<sup>168</sup> (see Figure 8).



**Figure 8: The vertebrate stress axis.** The brain perceives internal or external stress and activates the hypothalamus. This leads to the release of CRH and AVP, which triggers the pituitary to secrete ACTH. Eventually, the adrenal gland releases glucocorticoids, which adjust the body's homeostasis.

However, excessive stress and prolonged activity of the stress axis can lead to the “general adaptive syndrome”, which includes emotional and somatic disorders and in the final stage exhaustion and death<sup>169</sup>.

However, not only elevated stress and increased activity of the HPA axis contribute to stress axis related-diseases, also embryonic development is an essential component as the base level of the HPA axis is programmed during prenatal and early postnatal development<sup>170</sup>. Indeed it has long been observed that parental and environmental factors during development and postnatal periods have a fundamental impact on the physiology of the offspring, which can persist throughout life and facilitate major health issues<sup>170</sup>. This concept is called “developmental origin of health and disease”. Therefore, it is essential to understand the ontogeny and development of the HPA axis components.

### **1.8.2 The family of corticotropin-releasing-hormones**

The main trigger of the HPA axis is CRH, a peptide hormone that was first described in 1981<sup>171</sup>. Since this initial discovery, three mammalian paralogs have been identified: urocortin 1 (UCN1)<sup>172</sup>, urocortin 2 (UCN2)<sup>173</sup>, and urocortin 3 (UCN3)<sup>174</sup>. The members of the CRH protein family signal *via* two distinct receptors: CRH-receptor 1 (CRH-R1) and CRH-receptor 2 (CRH-R2). Whereas CRH and UCN1 display high affinity for CRH-R1, UCN2 and UCN3 bind exclusively to CRH-R2.

Additionally to the two membrane bound receptors, the soluble glycoprotein CRH-binding protein (CRH-BP) binds both CRH and UCN1. It has been postulated that this might either sequester the ligands, or shield them from enzymatic degradation, but a signaling function of CRH-BP itself is also discussed<sup>175</sup>.

Each CRH related peptide has a unique anatomical distribution profile and its expression is under the control of different genes. Besides expression of UCN1 in hypothalamic areas including the supraoptic nucleus, it is mostly confined to caudal brain areas including the Edinger-Westphal nucleus<sup>176</sup>, but also to brainstem and spinal cord motor neuron nuclei. UCN3 is found mainly in the rostral brain, e.g. in the hypothalamic area and the dorsal-medial amygdala. A substantial amount of CRH peptides is also found in the periphery including heart, intestine, kidney, skin etc. More detailed information about UCN expression can be found elsewhere<sup>177</sup>.

The physiological and behavioral effects of UCN's are closely related to their expression sites. Although it is unlikely that UCN genes directly regulate the secretion of ACTH from the hypothalamus, modulation of the HPA axis in paracrine or autocrine

fashion is likely. Besides a potential role in regulation of the HPA axis, CRH-related peptides have been implicated in cardiovascular function, energy balance, immune function, reproduction and behavior<sup>177</sup>.

## 1.9 THE ZEBRAFISH AS A MODEL ORGANISM IN RESEARCH

The zebrafish (*Danio rerio*) belongs to the group of ray finned fish (*actinopterygii*) and is native to the streams of the Himalayan regions. The adult measures three to five centimeters and weighs about 0.5 grams. For decades, zebrafish have been used as model organisms in developmental biology, taking advantage of the many benefits this fish offers. Among these are their high fecundity, easy and cost-effective handling, transparency of the embryos, and their fast and ex-utero development.

During the mid 1990ties, the use and value of the zebrafish model rapidly increased as two large ENU screens were carried out, resulting in the identification of thousands of developmental mutants<sup>178,179</sup>, and new techniques to access and manipulate the genome of zebrafish have been developed. Among the most important are the morpholino technique, which allows transient knock-down of any gene of interest (see chapter 1.9.1), and the ability to generate transgenic lines, in which cell or tissue specific promoters are fused to fluorescent proteins<sup>180,181</sup>. These and other techniques accelerated dramatically the understanding of zebrafish embryonic development and revealed a remarkable conservation of signaling pathways between zebrafish and humans. Today, the zebrafish is not only used in developmental biology, but it is also a valuable tool in pre-clinical and medical research<sup>182</sup>.

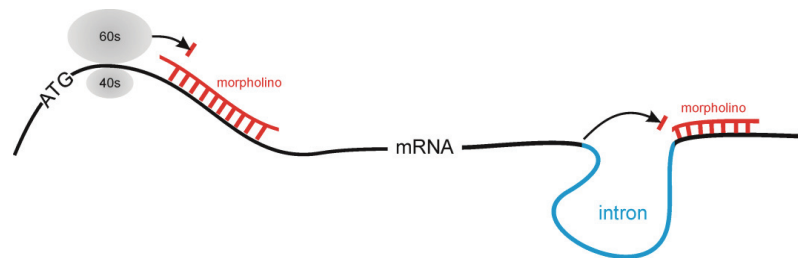
Additionally, the zebrafish has become a novel animal model to study stress and neuropsychiatric diseases<sup>183,184</sup>. It complements the existing rodent models, and allows to study the molecular mechanism of stress compromised brain development due to the accessibility of embryos. Furthermore, zebrafish is an extremely useful model for high-throughput drug discovery screens. For more detailed information on the zebrafish model organism, the readers are referred to some excellent reviews<sup>185-187</sup>.

### 1.9.1 The morpholino technique

Antisense morpholinos are commonly used to knock-down gene expression in the developing zebrafish embryo. These oligonucleotides are targeted *via* complementary base pairing to the RNA of interest, and they are resistant to any known enzymatic degradation due to their neutrally charged phosphorodiamidate backbone. Morpholinos can inhibit either mRNA translation by blocking ribosome assembly/movement, or they can hinder proper transcript processing through splice site blocking (see Figure 9).



Morpholinos are commonly injected into the zygote, where they rapidly diffuse, and ubiquitously knock-down their target gene in the developing embryo. Most morpholino induced phenotypes can only be observed until three to five days post fertilization (dpf), since the antisense oligonucleotides become diluted as the embryo grows.



**Figure 9: Antisense morpholinos can inhibit translation or correct splicing.** Antisense morpholinos are either targeted against the translation-initiation codon or against a splice site. This blocks ribosome assembly and movement or correct pre-mRNA splicing of the target gene.

Evaluating the morpholino activity and specificity of the induced phenotype is a key to all knock-down experiments. If an antibody is available, activity of morpholinos can easily be monitored by immunohistochemistry (see paper II in the present thesis), or, in the case of splice blocking morpholinos, arbitrary splice products can be detected by PCR. The strategy of choice to verify the specificity of the morpholino induced phenotype is a RNA rescue experiment. Simultaneously to the morpholino, artificially synthesized capped mRNA of the target gene is injected, which should, if the observed phenotype is specific, rescue it. Further information about morpholino oligonucleotides and their use in zebrafish can be found elsewhere<sup>188</sup>.

### 1.9.2 Overview of the embryonic development of zebrafish

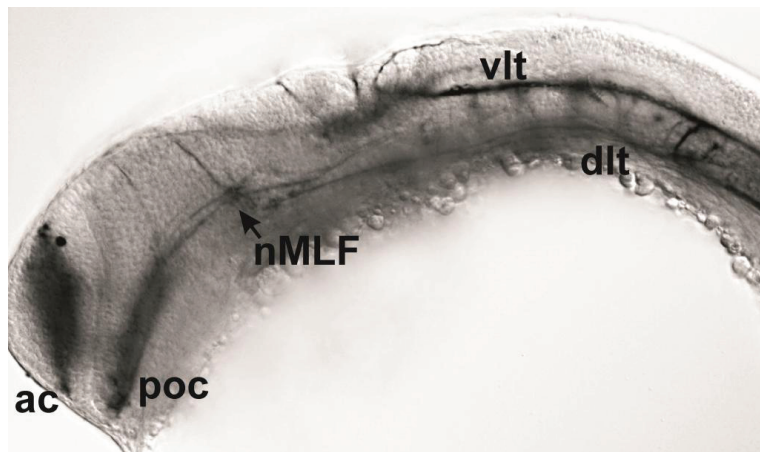
The embryonic development of zebrafish has been characterized in detail and described in the seminal paper by Kimmel and coworkers<sup>189</sup>. The molecular genetics underlying axis formation in zebrafish can be found elsewhere<sup>190</sup>.

Briefly, the fertilized zebrafish egg undergoes a series of cell divisions and meroblastic cleavages until, at about 4 hours post fertilization (hpf), the cells start to migrate and form the three germ layers namely endoderm, mesoderm, and ectoderm<sup>191</sup>. During the following gastrulation and segmentation period, the zebrafish body plan is established, and already at 24 hpf all organ primordia are present and the embryo starts moving. Shortly thereafter, the heart beats for the first time. The zebrafish larvae hatches and swims freely at 48 hpf, and it can feed from 5 dpf.

As described above, the development of the zebrafish is extremely fast, and due to the transparency and ex-utero development of the embryos, all cellular rearrangements can be directly visualized. Interested readers are referred to some beautiful time laps videos of the zebrafish embryonic development<sup>192</sup>.

#### 1.9.2.1 Patterning of the zebrafish central nervous system

The cells building up the vertebrate central nervous system (CNS) originate from the ectoderm, which is specified during gastrulation. Development of the CNS commences during the zebrafish segmentation period, as the neural plate, a flat sheet of ectodermal cells, transforms into a hollow tube that develops distinct morphological compartments along the anterior-posterior axis<sup>193</sup>. As segmentation proceeds, the neural tube gets further subdivided, and by 16 hpf ten neuromeres are visible. The anterior three neuromeres are the progenitors of the telencephalon, diencephalon and midbrain, whereas the caudal rhombomeres will give rise to the hindbrain compartments<sup>189</sup>. During early segmentation, primary neurogenesis is initiated and at 16 hpf the first post-mitotic neurons can be identified in the embryonic brain. These neural clusters extend axons and form a primitive, stereotypic scaffold of axon tracts and commissures<sup>194</sup> (see Figure 10).



**Figure 10: The primitive axon scaffold of the zebrafish embryo.** At 24 hpf, the zebrafish embryo has developed its stereotypic axon scaffold including clusters in the brain and major axon tracts descending to the spinal cord and trunk. Ac: anterior commissure; dlt: dorsal-longitudinal tract; nMLF: nucleus of the medial-longitudinal fascicle; poc: posterior commissure; vlt: ventral-longitudinal tract.

As described above, the vertebrate brain is subdivided in multiple regions along the dorsal-ventral and anterior-posterior axis. These axes are established by concentration

gradients of key transcription factors. For example, in the posterior most region of the developing zebrafish embryo, FGF and Wnt genes are expressed. Those inhibit on the anterior genes like *otx2* and *cyp26*. The latter hydrolyzes retinoic acid (RA), which normally induces posterior neural ectoderm. Wnt, RA and FGF also regulate the activity of homeobox genes that specify distinct regions of the neural tube along the anterior-posterior axis. Also the dorsal-ventral axis of the neural tube is specified through gradients of transcription factors. Whereas BMPs are expressed at the roof of the neural tube, the floor is exposed to SHH signaling. The gradients of those two transcripts induce downstream transcription factors that in turn specify neuronal identity along the dorsal-ventral axis.

Interested readers are referred to Gilbert Scott's seminal book "Developmental Biology" for further information<sup>195</sup>.

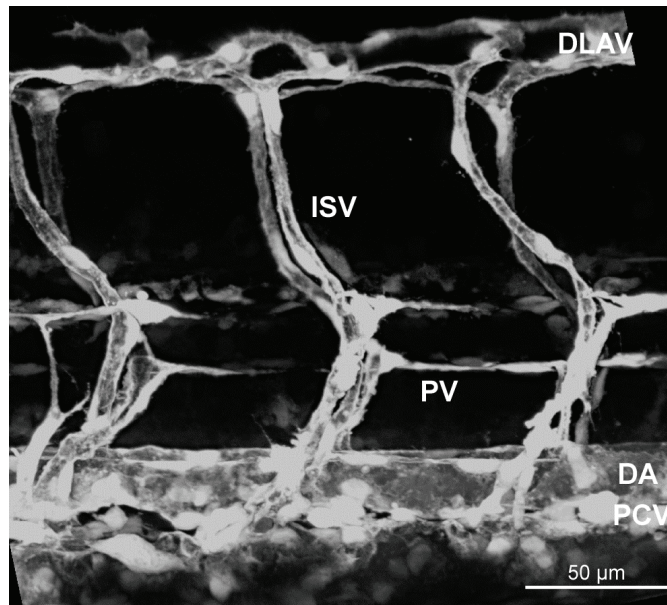
#### 1.9.2.2 Embryonic development of the vasculature

Especially in the field of cardio-vascular research, the zebrafish offers great advantages compared to classical rodent or bird model organisms. Amongst others, transgenic zebrafish lines, most importantly the *fli:EGFP* transgenic line, have facilitated a great leap forward in our understanding how the vascular system develops<sup>196</sup>.

In general, vascular development can be divided in two distinct processes: vasculogenesis and angiogenesis. The first describes the formation of blood vessels through migration and *de novo* coalescence of endothelial cells, and the latter the process of refining the existing vascular network.

The common progenitors of endothelial cells and angioblasts, called hemangioblasts, differentiate in the ventral mesoderm during zebrafish gastrulation. As development continues, two distinct waves of hemangioblasts migrate to the midline, where they coalesce and form the main axial vessels: the dorsal artery and the common cardinal vein. Beginning at 24 hpf, primary and secondary intersegmental vessels sprout and finally build up the vascular network in the zebrafish embryo<sup>197,198</sup>. At the edge of each sprout sits the tip-cell that explores its environment with numerous filopodial protrusions and senses repelling and attracting cues in the surrounding tissue. Those chemical road signs guide the nascent vessels on specific paths to their targets, regulate branching and ensure the formation of a stereotypic vasculature (see Figure 11). Interestingly, hypoxia does not seem to play a role in zebrafish embryonic angiogenesis, and larvae can develop several days without a functional cardiovascular system, as they gain sufficient oxygenation through diffusion<sup>199,200</sup>. Among vascular

guidance cues are receptor-ligand pairs such as plexins/neurophilins, semaphorins, netrins, ephrins and others<sup>201-203</sup>. Strikingly, those systems are also guiding growing axons to their targets, and during the last years studies have highlighted the structural and anatomical similarities of blood vessels and axons<sup>204,205</sup>.



**Figure 11: The trunk vasculature in zebrafish embryos at 48 hpf.** DA: dorsal aorta; DLAV: dorsal-longitudinal anastomotic vessel; ISV: intersegmental vessel; PV: parachordal vascular sprouts.

## 2 PRESENT INVESTIGATION

### 2.1 AIMS OF THIS STUDY

Vertebrate development remains, even today, an enigma.

During the last decade, signaling through reactive oxygen species has been implicated for the first time in embryonic development, but specific redox dependent processes have rarely been described so far. The main targets of ROS signaling are cysteine residues, and Grxs catalyze reversible redox modifications of those redox switches. Thereby, Grxs might be important regulators of redox signaling pathways that direct embryonic development.

So far, our understanding of the role of Grxs in development is mostly restricted to plants<sup>97,98</sup>, and only monothiol Grxs were described as being essential in embryonic development<sup>139,142</sup>.

The development of any higher organism is not exclusively encoded by its DNA and driven by posttranslational modifications, but the environment in which the embryo and the young develop is crucial as well. Both positive as well as negative stimuli have far reaching consequences for later life and can even be passed on to future generations.

The specific questions we wanted to answer within this thesis were the following:

- Are vertebrate specific glutaredoxins important regulators of embryogenesis?
- What specific processes are they regulating?
- What are the biochemical and biophysical characteristics of the vertebrate specific glutaredoxin homologue in our model organism, the zebrafish?
- What other regulatory systems determine embryonic development in zebrafish, focusing on the stress axis?

## 2.2 RESULTS AND CONCLUSIONS

### 2.2.1 Paper I

#### *Localized expression of urocortin genes in the developing zebrafish brain*

Urocortins/urotensins have been identified in the 1990s and are highly conserved homologues of the corticotropin-releasing hormone. Their major role is the modulation of the hypothalamus-pituitary-adrenal (HPA) axis activity, but they are also implicated in a variety of other physiological processes including locomotor activity and cardiovascular function.

Although embryonic programming of the HPA axis is of utmost importance for behavior, physiology, and pathophysiology for the whole life span, surprisingly little is known about the development of the HPA system.

In this paper, we characterized the expression pattern of urocortin (UCN) / urotensin (UTS) genes in the developing zebrafish brain. This characterization is the first step to identify genetic and molecular pathways controlling UCN1 and UCN3 lineage specification.

Indeed, by semi-quantitative PCR we detected UTS1 and UCN3 transcripts in the developing zebrafish embryo.

The main site of UTS1 transcription was found to be the nucleus of the medial longitudinal fascicle (nMLF), which corresponds to the Edinger-Westphal nucleus in mammals. Moreover, we could confine UCN1 transcription to various regions in the caudal brain, including the locus coeruleus, specific hindbrain nuclei, the Mauthner neuron, and primary motor neurons. The major site of UCN3 expression was localized in the region of the raphe nucleus and the brain stem.

The specific expression sites of both UTS1 and UCN3 indicate that these CRH homologues might modulate locomotor activity through the noradrenergic and serotonergic systems in response to stressors.

### 2.2.3 Paper II

#### *Vertebrate Glutaredoxin 2 is essential for brain development*

Analysis of the available genomes revealed that vertebrates possess a specific subgroup of Grx2s, which is characterized by two additional cysteine residues, which establish an intra-molecular disulfide bond. We speculated that this particular group of Grx2s might be involved in the development of vertebrate specific structures like their sophisticated brains.

Using the zebrafish as model organism, we were able to show that antisense morpholino induced knock-down of zebrafish Grx2 (zfGrx2) leads to impaired formation of the axonal network, as well as death of developing neurons. By several lines of evidence, we could exclude oxidative stress as a cause. In line with the impaired axonal development in zebrafish lacking zfGrx2, we were able to correlate over-expression of Grx2 in a human cellular model to an increase of neurite length and branching points. Pull-down assays identified collapsin-response-mediator-protein 2 (CRMP2) as interaction partner of Grx2. CRMP2 is the central transmitter of semaphorin signaling, regulating axonal guidance and neuronal survival. We could establish a functional relationship between Grx2 and CRMP2 *in vivo*, and show that over-expression of CRMP2 is capable to rescue the phenotype induced by loss of Grx2. It was published before that oxidation of CRMP2 is required for semaphorin signal transmission. Using *in vivo* techniques together with a newly developed biochemical approach, we revealed that the redox state of CRMP2 is correlated to the level of Grx2 protein. Moreover, oxidized CRMP2 was directly reduced by the Grx system, and changes of the CRMP2's redox state were accompanied by alterations of its three dimensional structure.

Based on these data, we hypothesized that the cellular pool of CRMP2, which gets oxidized upon activation of semaphorin signaling, is reduced and thereby recycled by the Grx system. Loss of a functional Grx system will result in an over-oxidized pool of CRMP2, which impairs the development of a functional brain.

#### 2.2.4 Paper III

##### *An unusual mode of iron-sulfur cluster coordination in teleost glutaredoxin 2*

The group of vertebrate Grx2s is characterized by two extra cysteine residues outside the active site that establish an intra-molecular disulfide bond. Grx2s of the bony fish infraclass, including zebrafish Grx2 (zfGrx2), contain four additional cysteines of so far unknown function.

In this paper, we sought to characterize through site directed mutagenesis the role of those additional cysteine residues in zfGrx2 concerning protein activity, stability, and their ability to coordinate an iron-sulfur cluster.

The crystal structure of apo zfGrx2, determined at a resolution of 2.6 Å, showed a typical Trx fold, highly similar to that of hGrx2. The teleost specific cysteines, which were located on the protein surface, were not essential for enzymatic catalysis, but rather required for structural stability of the protein.

ZfGrx2 coordinates one [2Fe2S]<sup>2+</sup> cluster per monomer as revealed by Mössbauer spectroscopy and size exclusion chromatography, and the holo protein was found to be enzymatically active.

These results indicated a different cluster coordination in zfGrx2 compared to all yet described [FeS] Grxs. All previously characterized [FeS] Grx coordinate the cluster *via* the N-terminal active site cysteines of two protomers (forming a bridged dimer) and non-covalently bound GSH, which renders the holo enzyme inactive.

Indeed, coordination and stability of the [FeS] cluster in zfGrx2 was neither dependent on the N-terminal active site cysteine nor on GSH. Site-directed mutagenesis demonstrated that the teleost-specific cysteines C116 and C117, localized at the C-terminus, were required for [FeS] cluster coordination.

The crystal structure of zfGrx2 indicates that this cysteine pair is near to the vertebrate specific structural disulfide between C28 and C113. Together, these four cysteine residues form an environment, which allows the coordination of one [FeS] cluster per zfGrx2 monomer.

Based on these observations, we propose that zfGrx2 and potentially Grx2s from other teleosts, may be capable to coordinate a [FeS] cluster in the monomer, using the four cysteine residues C28, C113, C116 and C117.



## 2.2.5 Paper IV

### *Glutaredoxin 2 is essential for zebrafish cardiovascular development*

The nervous and vascular system have striking anatomical similarities, and many signaling pathways that have been implicated in axonal guidance also direct nascent vessels to their targets.

Therefore, we speculated that Grx2 might not only be important for brain development, but also for the maturation of the cardiovascular system.

Indeed, antisense morpholino-mediated knock-down revealed that zfGrx2 is essential for the formation of the vascular system, a functional heart and maturation of erythrocytes in developing zebrafish embryos.

Although initial formation of the main axial vessels was not affected by the lack of zfGrx2, laser-scanning microscopy and two-photon laser-scanning time-laps microscopy revealed that inter-segmental vessels grew without regard to somitic boundaries, bifurcated ectopically, and hence failed to establish a functional vascular network.

Furthermore, heart field formation and heart looping was impaired in embryos lacking zfGrx2, causing severe physiological disturbances like a decreased heart pumping efficiency and reduced blood flow in the axial vessels as demonstrated by angiograms. Besides, erythrocytes were not maturing, as they lacked functional hemoglobin in zfGrx2 knock-down embryos.

Rescue experiments demonstrated that all phenotypes were specific for the loss of zfGrx2. Moreover, injection of zfGrx2 mRNA variants revealed that only the N-terminal active site cysteine was required for a phenotypic rescue, which strongly argues for the Grxs specific function of (de)-glutathionylation as mechanistic basis for the phenotypes.

The here demonstrated importance of redox regulation *via* Grx2 for cardiovascular system formation has not been appreciated before. Since one of the main reasons for human embryonic mortality are defects in the developing cardiovascular system, the results from our study promise clinical significance.

### 3 DISCUSSION AND FUTURE PERSPECTIVE

Glutaredoxins are pivotal regulators and transmitters of redox signals.

With the papers included in the present thesis, we have for the first time unraveled the importance of dithiol Grxs in embryogenesis. We demonstrated crucial functions of Grx2 in the development of a functional brain and cardiovascular system, and characterized by that the first thiol-redox regulated pathway in the important model organism zebrafish.

Both in zebrafish and a human cellular model, we were able to show that Grx2 redox regulates CRMP2, the central mediator of semaphorin signaling, and thereby controls axonal growth and neuronal survival.

The newly discovered redox circuit based on Grx2 might have crucial implications beyond embryonic brain development. Numerous neurological disorders like epilepsy and multiple sclerosis, as well as various neurodegenerative conditions including Alzheimer's and Parkinson's disease have been connected to either Grx2, semaphorin signaling or, in some cases, to both<sup>114,130,206-208</sup>. Therefore, we strongly believe that this redox circuit may represent a new and promising target for future therapeutic strategies in the area of neurological disorders.

Furthermore, we presented data in this thesis that clearly implies pivotal functions of Grx2 in cardiovascular development. Lack of Grx2 did not only disturb the formation of a functional vascular system but also disrupted heart development and maturation of erythrocytes. We are confident that identification of interaction partners mediating the function of Grx2 in cardiovascular development would be of great clinical importance, as one of the major reason for human embryonic mortality are congenital cardiac diseases<sup>209</sup>.

Moreover, development of new vessels is not only important for embryonic survival, but also for tumor growth, progression and metastasis<sup>210,211</sup>. Therefore, it would be of utmost importance to study if Grx2 influences malignant angiogenesis as well. Additionally to being able to knock-down Grx2 levels in the zebrafish, we have already developed zebrafish strains that ubiquitously over-express Grx2. These systems could be used in combination with a powerful new zebrafish model that has been developed to study tumor metastasis and pathological angiogenesis<sup>212</sup>.

Human Grx2 was the first member of the thioredoxin family of proteins being characterized as [FeS] protein. The cluster bridges two monomers and is coordinated by

the N-terminal active site cysteine residues and non-covalently bound GSH. This mode of cluster coordination is conserved in all yet described [FeS] Grxs.

We were able to identify a  $[2Fe_2S]^{2+}$  centre in zfGrx2, but its coordination seems to be different compared to all iron-sulfur Grxs identified so far. Our data suggests that the iron-sulfur cofactor is bound *via* teleost specific cysteine residues in the monomer. We are currently trying to decipher the *in vivo* function of this [FeS] cluster. A role as redox sensor, as proposed for the [FeS] centre in hGrx2, seems to be unlikely, as the cluster in zfGrx2 has no influence on enzymatic activity.

Due to the presence of the [FeS] cofactor, hGrx2 is supposed to be inactive during resting conditions<sup>153,154</sup>, which raises the question how Grx2 can execute its function in brain and cardiovascular development. An overall increase of the cellular redox potential to release the iron-sulfur cofactor seems unlikely, as this would interfere with numerous other redox signaling pathways. A more plausible scenario would be the local generation of H<sub>2</sub>O<sub>2</sub> to induce [FeS] cluster dissociation. A spatially defined oxidizing environment can be generated by Nox enzymes<sup>18</sup>, MICAL monooxygenases and others. Indeed, MICAL was reported to oxidize CRMP2 *via* local H<sub>2</sub>O<sub>2</sub> generation and thereby to facilitate semaphorin signal transduction<sup>213</sup>.

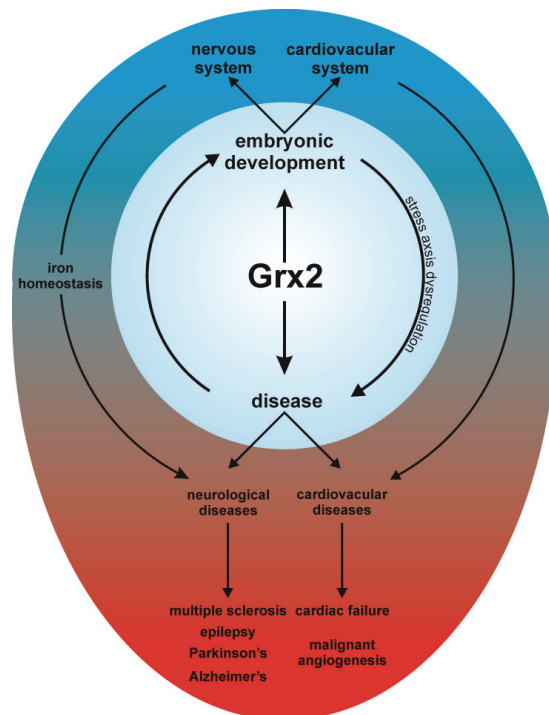
Cell differentiation, especially differentiation of stem cells, is known to be associated with a change of the cellular redox potential, but the role of redox signaling during differentiation, development and disease is in general not well understood. Therefore, new tools and different models are urgently required.

Establishing the zebrafish as model also in this field would offer valuable advantages. This vertebrate system closes the gap between cell cultures and mice, and it even surpasses rodents in many aspects, e.g. with its transparency, rapidly accessible genome and fast *ex utero* embryonic development.

Currently, we are developing transgenic zebrafish strains (zebROS) based on previously published Grx1-roGFP and Orp1-roGFP fusion constructs<sup>214,215</sup>. These strains would make it possible to monitor changes of the cellular redox potential *in vivo* and real time, for example during stem cell differentiation. Moreover, those lines could be crossed with established zebrafish models of human diseases e.g. Alzheimer's<sup>216</sup> and Parkinson's disease<sup>217</sup>, multiple sclerosis<sup>218</sup> and cancer<sup>219</sup>. All these major health issues have already been connected to oxidative stress, but our new tool would allow, for the first time, to study the impact of the cellular redox environment in disease onset, progression and treatment in real time in living organisms. Moreover, it could help to answer if oxidative stress is cause or consequence in these diseases.

The vertebrate stress axis adjusts the body's physiology to the environment. Although its initial programming is crucial and has lifelong consequences, little is known about embryonic development of the stress axis. Here, we characterized the expression pattern of urocortin genes, homologues to the main stress peptide CRH, in the developing zebrafish brain. This knowledge can now be used to decipher the genetic pathways responsible for lineage specification, and help to further establish the zebrafish as model in this research field.

In this thesis, we have analyzed a variety of systems that guarantee successful development of a new life. In particular Grx2s seem to be vital beyond embryonic development and we are just about to understand the importance of Grx2s in health and disease (see Figure 12).



**Figure 12: Grx2 and a functional stress axis are pivotal for health and disease.** Grx2 regulates essential pathways that are crucial for embryonic development and disease. Due to its central role, Grx2 represents a promising target for new therapeutic strategies against neurological and cardiovascular diseases including malignant angiogenesis. Impaired stress axis development can lead to various diseases in the young and adult.

## 4 ACKNOWLEDGMENTS

The work for this thesis has been carried out at the Department of Biosciences and Nutrition and at the Department of Medical Biochemistry and Biophysics.

The years that I have been working with this thesis were exiting, enlightening and challenging. During the journey of my PhD studies, I have had the great privilege to meet fantastic and inspiring people inside and outside the laboratory.

Many of those have become friends for life.

Here I want to express my deepest gratitude to some of those:

**Professor Arne Holmgren**, my main supervisor. You are the most fascinating cornucopia of scientific knowledge. There is literally nothing you don't know. Thank you so much for accepting me (back) as graduate student in your group, all the inspiring discussions, for your trust and for supporting me in endless ways.

My co-supervisor **Dr. Carsten Berndt**. You have truly been an extraordinary supervisor, an amazing supporter, and a fantastic and creative teacher. Indeed, you were (and are) the most helpful and dedicated supervisor ever. You are the main reason that I am where I am. Thank you so much for your support, guidance and trust! Brain prize 2014! ☺

**Dr. Giselbert Hauptmann**, my co-supervisor. Thank you so much for introducing me to the zebrafish model. Everything I know about it I learned through you and **Iris**. Without you, my scientific experience would not be the same.

**Professor Elias Arnér**. Your door is *always* open, and you always had time for my questions and concerns. Thank you for all your support!

My gratitude also goes to **all the collaborators and coauthors** I have had the privilege to work with! You were fantastic and inspiring colleagues and I learned so much from you!

A big thank to **Lena**, for being so incredibly helpful with all administrative matters! **Jack**, thank you for all the bread you brought me, for endless ordering and helping wherever you can!

A special thanks goes to **Diana** for supplying me and the whole lab with huge Easter, Christmas and Nikolaus chocolate surprises!

**Chris**, you were the main reason that I enjoyed life in Stockholm so much and decided to stay here. It is not the same anymore since you moved back to Germany. I will never forget the great times we had in and outside the lab. Next year: Greenland transverse!

**Marcus**, for being a great hiking partner in winter and summer, although we always have to carry extra amounts of food for you. PauPau! **Eva**, keine Kerzen!! You made Stockholm to the best time of my life! It is such a pity that you moved back to Germany! **Maria**, for the good old happy times! **Will**, for not being the first American who drowned above the polar circle with his ice-claws packed in his backpack. This year's winter hiking with you was truly awesome! **Sofi**, you became such good friend inside and outside the lab, and you were a great Swedish teacher! I absolutely agree that "diskpropp" and "durkslag" are the first Swedish words everybody should learn! **Katarina** and **Irina**, I am so glad that you both came to our lab, you truly make it a great place to work! And our coffee breaks early in the morning are a perfect starter for another busy lab day! **Tomas**, you are *the* protein expression&purification expert! Thank you for all the good advises! **Olle**, you are an amazing cook! More please! We will visit you in the archipelago, I promise!

**Jianqiang**, probably the friendliest persons I have ever met. Thank you for keeping my tomatoes alive and the lab a better place! **Gilbert** and **Nasim**, you have been great colleagues and friends! **Pegah**, I am so glad that we became close friends despite that I made you cry the first time we met!

I am very thankful to all the present and past members of the lab! You created the best working atmosphere I could have wished for. I enjoyed so much working together with all of you!

**Bastian**, **Sarah**, **Janine** and **Sabine**, I am glad that you survived my first supervising attempts! You indeed helped, and taught me a lot!

I want to express my gratitude to all the animal caretakers in the **zebrafish core facility**! You make life so much easier and keep my fish happy!

*Last but not least the most important people in my life:*

Mama, Papa und Anke. Danke fuer alles was ihr mir mit auf den Weg gegeben habt.

Berit, du bist der Mittelpunkt in meinem Leben.

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