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XENOPUS LAEVIS PEROXIREDOXINS ARE EXPRESSED IN DISTINCT TISSUES DURING EARLY EMBRYOGENESIS

(Spine Title: EXPRESSION OF PEROXIREDOXINS IN *XENOPUS LAEVIS* DEVELOPMENT)

(Thesis Format: Monograph)

By

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Graduate Program in

Biology (Developmental Biology)

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science

The School of Graduate and Postdoctoral Studies
The University of Western Ontario

London, Ontario, Canada

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THE UNIVERSITY OF WESTERN ONTARIO

SCHOOL OF GRADUATE AND POSTDOCTORAL STUDIES

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Entitled:

***Xenopus laevis* Peroxiredoxins Are Expressed In Distinct Tissues During Early Embryogenesis**

Is accepted in partial fulfillment of the requirements for the degree of Master of Science

Date

Dr. Robert Dean
Chair of the Examination Board

ABSTRACT

Development in the frog, *Xenopus laevis*, requires the utilization of yolk glyco-lipo-proteins in a temporally- and spatially-dependent manner. The metabolism of the yolk produces H_2O_2, a potent Reactive Oxygen Species (ROS). Peroxiredoxins (Prdxs) are a family of 6 anti-oxidant enzymes that, amongst other roles, reduce H_2O_2. Prdxs reduce H_2O_2 through a thiol-redox reaction at conserved cysteine residues, which results in the creation of disulfide bonds. Recently the thiol-redox reaction of Prdxs has also been implicated in several cell signaling systems. Herein is a report of the cloning and expression patterns during development of 6 peroxiredoxin homologs from the frog *X. laevis*. Sequence analysis has confirmed their identity as well as their evolutionary relationship with peroxiredoxins from several other species. Using RT-PCR and *in situ* hybridization analysis we have shown that there is early, robust and unique expression patterns for all six homologs during development. All six *X. laevis* peroxiredoxins are expressed in neural regions including the brain, and eyes, as well as the somites. Different expression patterns for each peroxiredoxin are also observed in the pronephric region, including the proximal and distal tubules and the Wolffian duct. Expression of several peroxiredoxins was also observed in the blood precursors and the olfactory placode. These results suggest important roles for all six peroxiredoxins during early development. These roles may be restricted to their functions as anti-oxidant enzymes, but may also be related to their emerging roles in redox signaling, which is discussed herein.

KEYWORDS

Peroxiredoxins; redox signaling; *Xenopus laevis*; development; embryogenesis; pronepheros; optic placode; blood islands; thiol redox; in situ; hydrogen peroxide; antioxidant enzyme

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LIST OF ABBREVIATIONS

ATP	Adenosine triphosphate
BI	Blood islands
bp	Base pairs
BP	Blood precursors
BR	Brain
cDNA	Complementary DNA
CN	Cranial nerves
DIG	Digoxigenin
DNA	Deoxyribonucleic acid
DT	Distal tubule
Dvl	Disheveled
ER	Endoplasmic reticulum
EY	Eye
FB	Forebrain
GDE2	Glycerophosphodiester phosphodiesterase 2
GDPD	Glycerophosphodiester phosphodiesterase domain
GER	Gain electrons reduction
H_2O_2	Hydrogen peroxide
HB	Hind brain
HCG	Human chorionic gonadotropin
hpf	Hours post fertilization
JNK	c-Jun N-terminal kinases
LEF	Lymphoid enhancer factor
LEO	Lose electrons oxidize
Li	Liver
MMR	Marks modified ringer
mRNA	Messenger RNA
$NADP^+$/NADPH	Nicotinamide adenine dinucleotide phosphate
NCBI	National Center for Biological Information
NOX	NADPH oxidase
NOS	Nitric oxide synthase
Nrx	Nucleoredoxin
NT	Neural tube
$O_2^{\cdot}$	Superoxide
OA	Oral-aboral axis
Of	Olfactory placode
ODC	Ornithine decarboxylase
OP	Optic placode
PA	Pharyngeal arches
PBS	Phosphate-buffered saline
PT	Proximal tubule
PCR	Polymerase chain reaction
PD	Proctodeum
pK_a	Acid dissociation constant

PN	Pronepheros
Prdx	Peroxiredoxin
Redox	Reduction-oxidation
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RT-PCR	Reverse transcription polymerase chain reaction
SM	Somites
S_PH	Peroxidatic thiol
S_POH	Peroxidatic sulfenic acid
S_PO_2H	Peroxidatic sulfinic acid
S_RH	Resolving thiol
S_ROH	Resolving sulfenic acid
S_RO_2H	Resolving sulfinic acid
Srx	Sulfiredoxin
TCF	Transcription factor
TG	Thyroid gland
Trx	Thioredoxin
TrxR	Thioredoxin reductase
Vc	Vasculature
X Prdx	Xenopus peroxiredoxin
WD	Wolffian duct
WNT	Wingless

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

1.1 Reduction and Oxidation

Redox (reduction and oxidation) are chemical reactions important for many biological and chemical processes. Redox describes the transfer of electrons, or oxidation state, from one molecule to another. Reduction is the *gain* of electrons, and *decrease* in oxidation state and oxidation is the *loss* of electrons, and *increase* in oxidation state. This process of gaining and loosing electrons is extremely important in biology as during many biochemical reactions, most notably cellular respiration, the formation of free radicals can occur (molecules with unpaired valence electrons). Biological free radicals, also known as Reactive Oxygen Species (ROS), are highly reactive and can directly, or through a chain reaction of events, cause oxidative damage to proteins, lipids and nucleotides. The most common biological ROS produced by cellular metabolism is superoxide (O_2^-), which can be formed in the final step of cellular respiration (Boveris and Cadenas, 1975). Superoxide is highly unstable and quickly converted to hydrogen peroxide within cells by the enzyme super oxide dismutase.

1.2 Hydrogen Peroxide

Hydrogen peroxide (H_2O_2) is the most common and well-known ROS and is produced by the dismutation (simultaneous oxidation and reduction of a molecule) of O_2^- and H_2 to H_2O_2 and O_2. H_2O_2 is produced by all known organisms and can easily diffuse through cellular membranes. The highest levels are created in the mitochondria, where cellular respiration occurs, but elevated levels are also found in organelles such as the peroxisomes where beta-oxidation of long chain fatty acids occurs (Kunau et al., 1995). Therefore those tissues and cell types that contain more mitochondria and/or peroxisomes,

such as muscle and actively respiring tissue, will produce and contain higher levels of H_2O_2.

Hydrogen peroxide is an oxidizing agent and can cause the oxidation of a variety of biological elements. This oxidation has, for the most part, been deemed as a negative effect, as it is usually detrimental to the proper function of the component in question (proteins, lipids or nucleic acids). However, growing evidence has shown that H_2O_2 can act as a second messenger within cells to convey signal cascades leading to a variety of outcomes, such as proliferation, differentiation and the growth of cells (Davies, 1999; Rhee et al., 2005; Mandal et al., 2010; Benitez-Alfonso et al., 2011).

1.3 Anti-oxidant elements

Cells contain many enzymes and molecules that act as anti-oxidant elements. Though these elements work through a variety of mechanisms, the outcome of their actions is largely the same. Anti-oxidant elements reduce ROS species (such as H_2O_2), and in the process they become oxidized. Oxidized elements are then either eliminated or continually recycled through further reduction steps. The final step of the cycle involves reduction by an electron donor such as NADPH, returning the system to a reduced state. The main cellular oxidative compound is hydrogen peroxide and most anti-oxidants are specifically designed to eliminate this “threat”. Anti-oxidants are housed in all cellular compartments but are concentrated in areas that produce high levels of ROS, such as the mitochondria, or in areas that require high levels of protection from oxidative damage, such as the nucleus (Jones and Go, 2010).

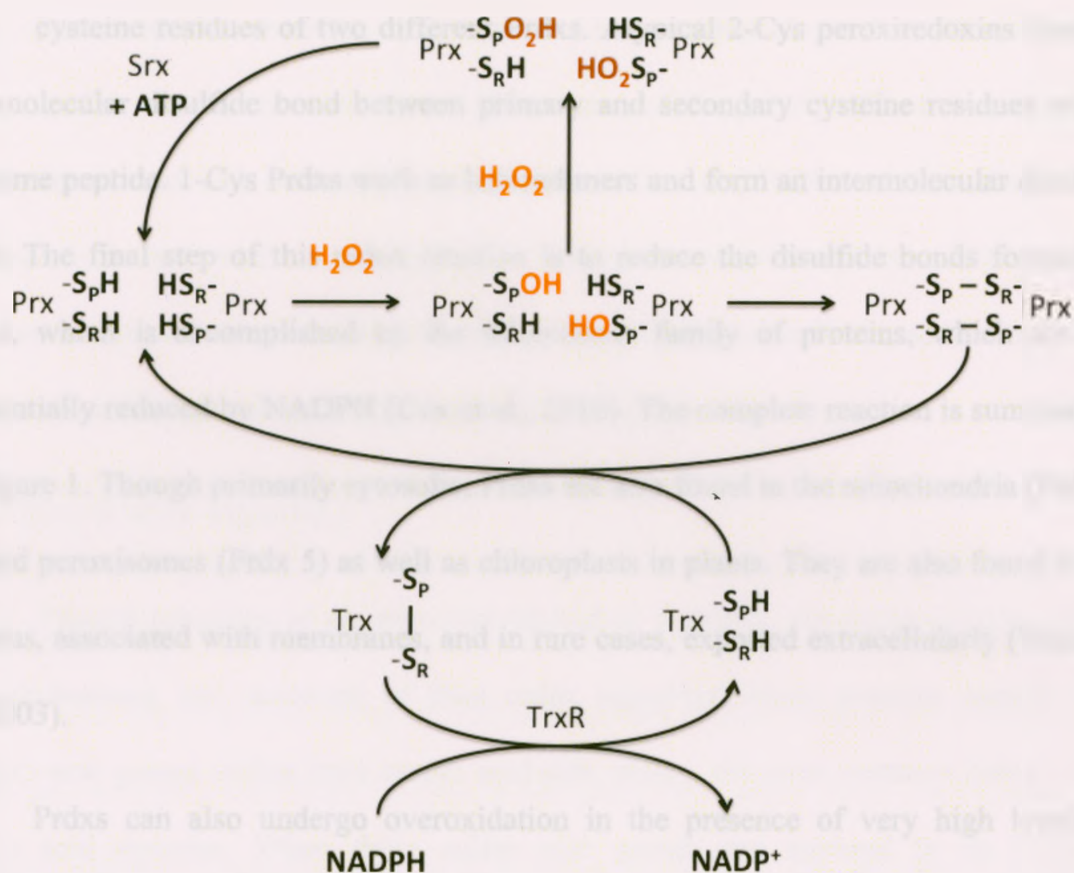
Biological anti-oxidant elements include organic compounds, such as glutathione, vitamins A and E, and most importantly, genetically encoded enzymes. The latter group includes proteins such as catalase, superoxide dismutase and a variety of peroxidases such as glutathione peroxidase, and the peroxiredoxins, which are members of the thioredoxin class of enzymes. These proteins are highly conserved in almost all organisms and are extremely important for the maintenance and survival of cells and tissues.

1.4 Peroxiredoxins

Peroxiredoxins (Prdxs), also known as thioredoxin peroxidases, are a newly described and expanding family of thiol specific anti-oxidant proteins (Shuvaeva et al., 2009). They are highly conserved proteins first identified in yeast, later found in plant and animal cells, and in almost all bacteria and Achaea (Radyuk et al., 2001; Wood et al., 2003; Isermann et al., 2004; Han et al., 2005a; Knoops et al., 2007). In vertebrates there are classically six members, that have been identified in fish, chicken, frogs, mice and humans, amongst others.

The vertebrate Prdxs protein family (Prdx 1-6) can be divided into 3 subgroups based on the number and configuration of conserved cysteine residues (typical 2-Cys, atypical 2-Cys and typical 1-Cys) (Hall et al., 2009). All Prdxs contain either one or two conserved cysteine residues depending on the subgroup. The primary/peroxidatic cysteine, present in all Prdxs, is responsible for reducing H_2O_2 and other free radicals. It is oxidized to a sulfenic acid when exposed to H_2O_2 and subsequently forms a disulfide bond with a secondary/resolving cysteine residue (Cox et al., 2010) (Figure 1). Typical 2-Cys Prdxs form dimers caused by disulfide bond formation between the primary and secondary

Figure 1. Schematic for the mechanism of action of the thioredoxin system of antioxidant enzymes. Each typical- and atypical-2-Cys peroxiredoxin (Prdx) contains a peroxidatic and resolving cysteine residue (S_PH and S_RH, respectively). Typical 1-Cys peroxiredoxins do not contain a resolving cysteine, and are resolved by disulfide formation with a non-peroxiredoxin protein (not shown). The peroxidatic cysteine residue is selectively oxidized by H_2O_2 to form a sulfenic acid intermediate (S_POH) and subsequently forms a disulfide bond with another similarly oxidized peroxiredoxin. The disulfide bond is then reduced by thioredoxin (Trx), which subsequently forms an intermolecular disulfide bond, which is reduced by thioredoxin reductase (TrxR) donating an electron from NADPH, returning the system to a reduced state. Alternatively, if H_2O_2 levels are high or continuous, it can push Prdx-S_POH to an "overoxidized" state and form a sulfinic acid intermediate (S_PO_2H). Sulfinic acids cannot be reduced by Trx, but must be resolved by the enzyme sulfiredoxin (Srx) which only exists in eukaryotes and utilizes ATP to complete the reaction and bring Prdx-S_PO_2H back to its reduced state (Prdx-S_PH). Modified from Rhee et al (2007) and Wood et al (2003).



Prx -S_PO_2H HS_R- Prx
-S_RH HO_2S_P-
Srx
+ ATP
H_2O_2
H_2O_2
Prx -S_PH HS_R- Prx
-S_RH HS_P-
Prx -S_POH HS_R- Prx
-S_RH HOS_P-
Prx -S_P – S_R- Prx
-S_R – S_P-
Trx -S_P
-S_R
Trx -S_PH
-S_RH
TrxR
NADPH
$NADP^+$

cysteine residues of two different Prdxs. Atypical 2-Cys peroxiredoxins form an intramolecular disulfide bond between primary and secondary cysteine residues within the same peptide. 1-Cys Prdxs work as heterodimers and form an intermolecular disulfide bond. The final step of this redox reaction is to reduce the disulfide bonds formed by Prdxs, which is accomplished by the thioredoxin family of proteins, which are sub sequentially reduced by NADPH (Cox et al., 2010). The complete reaction is summarized in Figure 1. Though primarily cytosolic, Prdxs are also found in the mitochondria (Prdx 3, 5), and peroxisomes (Prdx 5) as well as chloroplasts in plants. They are also found in the nucleus, associated with membranes, and in rare cases, exported extracellularly (Wood et al., 2003).

Prdxs can also undergo overoxidation in the presence of very high levels of oxidants. This causes the sulfenic acid on the thiol side group to oxidize to a sulfinic acid (Mitsumoto et al., 2001; Rhee et al., 2005; Cox et al., 2010). In some classes of peroxiredoxins - most notably those from lower eukaryotes, such as yeast and Bacteria - this over-oxidation is permanent. The protein sulfiredoxin, found only in higher eukaryotes and vertebrates, can reverse the sulfinic acid of over-oxidized Prdxs back to a sulfenic acid, which can then be reduced through the normal pathway, recycling the protein (Jonsson and Lowther, 2007; Forman et al., 2010) (Figure 1). This mechanism, which can allow bursts of ROS without permanently damaging the population of anti-oxidant enzymes in a cell, has lead to the development of the floodgate hypothesis, and is only known to exist in vertebrates (Rhee et al., 2007). Some Prdxs can also be negatively regulated by phosphorylation, allowing a similar burst of ROS (Woo et al., 2010).

Peroxiredoxins are often over-expressed in many pathological conditions such as cancer, and in response to stress factors (Neumann and Fang, 2007). These conditions are usually associated with increased levels of metabolism and ROS, and accordingly Prdx expression is increased to compensate (Matteucci and Giampietro, 2010). Using peroxiredoxins as markers for such conditions has recently been discussed, however their roles in the actual pathology of such cases are still unknown.

1.5 Thiol Redox signaling

Though emerging as an important biological regulator, not much is known about the mechanisms and outcomes of thiol redox signaling. Many proteins contain thiol (sulfur) side groups within their amino acid side chains, the most common being on the amino acid cysteine. When these sulfur side groups are exposed to an oxidative insult/attack they are converted to sulfenic acid, or if the insult is high, sulfinic acid. These oxidized residues can then form either intra- or inter-molecular disulfide bonds, affecting the tertiary structure, and perhaps function, of the affected proteins (Klomsiri et al., 2011). These modifications can be compared to the more classically well-known phosphorylation of tyrosine, threonine and serine side groups within proteins. One main difference between these two mechanisms is that oxidation occurs at different rates for different thiols and depends on many factors including side group exposure and adjacent amino acids. Every thiol has its own pK_a, or acid dissociation constant, which governs its reactivity during redox reactions. Therefore most oxidative changes can only occur when specific levels of oxidation/reduction are achieved in the sub-cellular environment (Paulsen and Carroll, 2010; Ferrer-Sueta et al., 2011). These reactions are much more useful in gated mechanisms, such as the sensing of oxidative insult and the subsequent

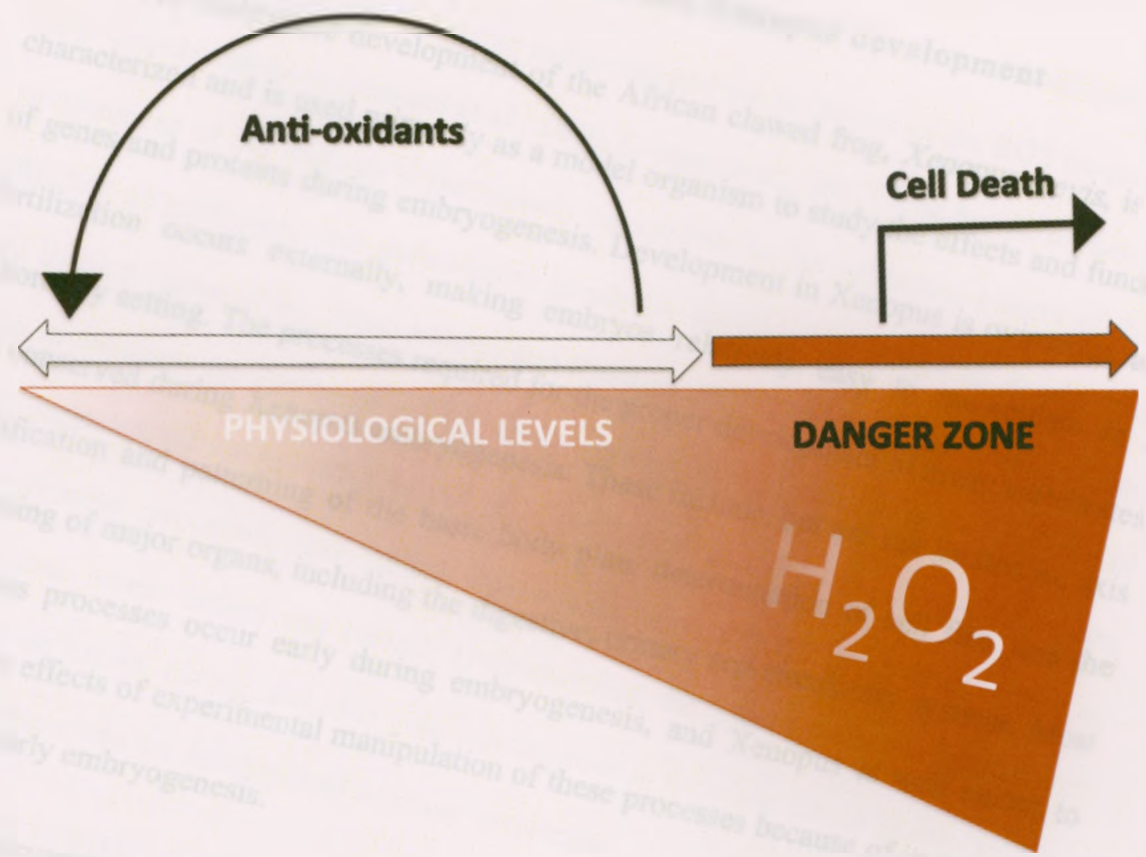
anti-oxidant response which occurs in stress response signaling systems (such as JNK signaling), rather than a cascade, such as that which occurs with phosphorylation (Janssenheininger et al., 2008). More evidence however, is in support of the existence of locally confined changes in redox level within the cytoplasm of cells, caused by ROS produced by plasma membrane oxidases elicited by cytokines and growth factors, or inactivation of anti-oxidant activity (Thannickal et al., 2000; Thannickal and Fanburg, 2000; Rhee, 2003). These changes can effectively transmit local signals through H_2O_2 and thiol redox mechanisms without affecting the global redox levels within a cell, which may otherwise activate a stress response or even apoptosis. This transient H_2O_2 can oxidize cysteine residues within the active sites of transcription factors, kinases and phosphatases, activating or deactivating them through conformational changes (Rhee, 2003; Funato et al., 2006; Winterbourn and Hampton, 2008; Yan et al., 2009). These factors are important for many biological processes including, but not limited to proliferation, differentiation and growth, and are also active during the development of many organisms. Therefore redox signal transduction may be an important and active factor that contributes to the regulatory processes that occur during embryogenesis.

1.6 Interplay between peroxiredoxins, hydrogen peroxide and signaling

It is well known that global cellular changes in redox potential (either over-oxidation or reduction) can cause changes in the oxidative stress response, and levels of anti-oxidants. This is mainly accomplished by a gated mechanism, where once a certain level of oxidation occurs (as the result of insult or respiration), sensor proteins become oxidized triggering the transcription and translation of anti-oxidant enzymes (such as peroxiredoxins), and the production of anti-oxidant molecules to maintain redox

homeostasis within the cellular environment. If the oxidative insult is too high, this can push the system to apoptosis and in some cases necrosis, by the activation of less sensitive (but more crucial) redox sensing proteins and elements within mitochondria (Ott et al., 2007) (Danger Zone, Figure 2). This creates a range of ROS levels where low, physiologically relevant amounts can exist, but as higher levels are reached they are eliminated (Figure 2). The exact levels of ROS, which create these zones, are unknown but are thought to vary between systems and cell types. It is now widely believed that ROS elements are crucial for the proper regulation of many biological events. The cell not only produces hydrogen peroxide within the mitochondria and other organelles as a byproduct, but also deliberately near membranes or in the cytosol by proteins such as the Nitric Oxide Synthases (NOSs), and NADPH oxidases (NOXs) (Thannickal and Fanburg, 2000; Li, 2006; Coant et al., 2010; Dickinson et al., 2010). These deliberate bursts occur in response to specific cytokines and growth factors, are highly regulated, and can cause redox changes in proteins leading to changes in transcription factor activity and gene expression through disulfide exchanges (Klomsiri et al., 2011). An important piece of evidence that supports this is that peroxiredoxins, as the most abundant cytosolic anti-oxidant in most eukaryotic organisms, can be transiently turned off by over-oxidation, allowing these bursts in ROS/H_2O_2 and sub-sequent signaling (Mitsumoto et al., 2001). Maintaining a physiological range and achieving oxidation levels required for thiol redox signaling are accomplished by a delicate balance between antioxidants/peroxiredoxins and ROS/H_20_2 production in a temporally and spatially dependent manner. More research is needed to understand the regulation and full extent of this process in cells, tissues, and during development.

Figure 2. Schematic of the physiological balancing act controlling hydrogen peroxide levels. Hydrogen peroxide is created within cells and levels are controlled by the coordinated action of anti-oxidant enzymes and molecules. A range of fluctuating hydrogen peroxide levels is permitted (Physiological Levels), allowing signaling through a variety of mechanisms. These low levels are maintained through a number of mechanisms, however, once a tipping point is reached, balance is lost, and this may send the cell towards apoptosis and necrosis (Danger Zone).



Anti-oxidants
Cell Death
PHYSIOLOGICAL LEVELS
DANGER ZONE
H2O2

1.7 General overview of metabolism and Xenopus development

The embryonic development of the African clawed frog, *Xenopus laevis*, is well characterized and is used primarily as a model organism to study the effects and function of genes and proteins during embryogenesis. Development in Xenopus is oviparous, and fertilization occurs externally, making embryos relatively easy to manipulate in a laboratory setting. The processes required for the proper development of many vertebrates are conserved during Xenopus embryogenesis. These include, but are not limited to, axis specification and patterning of the basic body plan, determination of cell fate, and the patterning of major organs, including the digestive, urinary and circulatory systems. Most of theses processes occur early during embryogenesis, and Xenopus is well suited to study the effects of experimental manipulation of these processes because of its rapid and external early embryogenesis.

Embryogenesis proceeds rapidly after zygote formation to gastrulation (stage 10.5, 12 hours post fertilization (hpf)), neurulation (stages 16-20, 17-20 hpf), organogenesis (roughly stage 30, 36 hpf), and hatching as a tadpole (stage 37, 54 hpf). The first structures to visibly develop are anterior structures in the head region including the eyes and brain (roughly stage 28, 32 hpf), followed by the somites (muscle) on the dorsal side of the embryo. Organogenesis begins soon after, and includes the differentiation of organs such as heart, lung, kidneys and other embryonic structures (Nieuwkoop and Faber, 1994).

Maternal cues are absent during Xenopus embryogenesis and the majority of energy stores are present as glyco-lipo-proteins in the yolk, a large storage of nutrients located on the ventral side of the embryo (Wallace and Selman, 1985; Jorgensen et al.,

2009). The consumption of yolk stores occurs during specific periods of development and the regulatory signals governing this utilization and the mediation of the ROS by-products are relatively unknown (Armant et al., 1986; Cooper et al., 2007). The fragile intracellular environment of the embryo is particularly susceptible to the damaging effects of ROS, affecting physiological and pathological processes during many stages of development and beyond (Inoue et al., 2004; Menon and Rozman, 2007). Most anti-oxidant enzymes, such as catalase, are expressed at high levels starting at stage 20, when the majority of yolk consumption occurs (Cooper et al., 2007; Fox et al., 2011). However, many developmental processes that utilize metabolism and cellular respiration, such as gastrulation, occur before this period. Understanding the sub-cellular management of metabolism before, during, and after yolk consumption is therefore an intriguing target of investigations into the development of many vertebrates.

1.8 Redox signaling during development

Redox signaling is clearly a real and emerging phenomenon, and is likely important for many biological processes. There has been much speculation about the potential roles of metabolism, mitochondria, ROS, and thiol redox sensitive proteins such as peroxiredoxins and certain transcription factors (REDOX), during the development of many organisms including nematodes, sea urchins, fish, frogs and mammals (Harvey et al., 2002; Coffman and Denegre, 2007; Covarrubias et al., 2008; Van Blerkom, 2009; Hernández-García et al., 2010; Paulsen and Carroll, 2010). REDOX therefore may be involved in the control of axis formation, tissue differentiation, and cell movement and proliferation during development. The following are several examples of such an

occurrence, but more work needs to be done to uncover the full effect REDOX has during embryogenesis.

Originally hypothesized in the early 1940s (Child, 1941) it has recently been shown that distribution of mitochondria within developing embryos affects specification of the embryonic axis. In the sea urchin *Strongylocentrotus purpuratus* the secondary, Oral-Aboral (OA) axis, is organized by localized zygotic expression of Nodal, which is a redox sensitive protein (Coffman et al., 2004; Coffman and Denegre, 2007). It was found that the highly oxidizing influences of mitochondria localized to the oral pole of sea urchin embryos activates Nodal and contributes to the specification of the OA axis.

One of the most canonical developmental signaling mechanisms, WNT signaling, has also recently been linked to REDOX. A member of the thioredoxin family of proteins (which reduce peroxiredoxins), called Nucleoredoxin (Nrx), has been shown to interact with WNT signaling pathways during early embryogenesis (Funato et al., 2006). When in a reduced state, Nrx is bound to the Dishelved protein (Dvl), and blocks downstream WNT signaling. When exposed to oxidative stress, Nrx dissociates from Dvl allowing signal transduction through the WNT pathway and activation of the TCF/LEF transcription factor. It was concluded that Dvl and Nrx interact in a redox sensitive manner utilizing a catalytic cysteine residue within the Nrx protein (Funato et al., 2006). WNT signaling is important for anterior/posterior axis formation in embryos, and knockdown and overexpression of *Nrx* affects axis formation. Knockdown of *Nrx* by morpholino injection caused a lack of head formation in Xenopus embryos, while microinjection of *Nrx* mRNA was also able to rescue the duplicate axis phenotype of injection of *Dvl* mRNA (Funato et al., 2006).

Peroxiredoxins themselves have also been linked directly to developmental signaling via a thiol redox mechanism (Novitch and Butler, 2009). GDE2 is a glycerophosphodiester phosphodiesterase domain (GDPD) containing transmembrane protein (Yan et al., 2009). Retinoic acid up regulates GDE2 in differentiating neurons, and induces neuronal fate specification via its GDPD extracellular domain. GDE2 is held in an inactive state by an intramolecular disulfide bond, which can be removed via reduction by Prdx 1. Since there are many peroxiredoxins, which all have very similar mechanisms of action as well as a multitude of proteins within the GDPD family of enzymes, this sort of interaction may be widespread during development in many organisms (Novitch and Butler, 2009; Yan et al., 2009).

1.9 Hypothesis

Hydrogen peroxide, its modulators the peroxiredoxins, and the interplay between these two factors, have been implicated in intracellular redox signaling and are presumed to have important development effects. Therefore, I hypothesize that; 1) *Xenopus laevis* peroxiredoxins will be highly conserved at both the protein and DNA level to peroxiredoxins from other species of vertebrates and invertebrates; 2) *Xenopus laevis* peroxiredoxins will be expressed differentially both spatially and temporally during development, and; 3) that one or more of the peroxiredoxins has a role during development in defense against high levels of ROS during this delicate process and/or, in intracellular signaling via thiol redox or control of ROS signaling.

I plan on investigating these hypotheses by cloning and sequencing all six *Prdx* isoforms in *Xenopus laevis*, and examining their expression patterns during development

temporally and spatially, by semi-quantitative RT-PCR and whole mount *in situ* hybridization, respectively. Because there are six unique members of this family of enzymes, it is possible that they have unique functions and therefore unique expression patterns during development. This will hopefully shed light on their potential roles during embryogenesis and act as the basis for further studies into their unique functions during this essential time period.

CHAPTER 2

MATERIALS AND METHODS

2.1 Animal care and handling

Rearing and fertilization of embryos were carried out as previously described (Sive et al., 2000). Adult male and female *X. laevis* were obtained from Xenopus One (Dexter, Michigan). Animals were maintained at room temperature on a 12-hour light/dark cycle, and were fed fish food and had their tank water changed 3 days/week. Fertilizations were carried out by injecting adult females with 200-500 units of human chorionic gonadotropin (HCG) 18 hours prior to ovulation to obtain eggs. Testes were isolated from anesthetized adult male *X. laevis* by surgical removal and maintained in 1X Marks Modified Ringers (MMR) [88 mM NaCl, 1 mM KCL, 0.41 mM CaCl2, 0.33 mM $Ca(NO_3)_2$, 0.82 mM $MgSO_4$, 2 mM $NaHCO_3$, 10 mM HEPES, pH 7.4] in a Petri dish. All procedures were in compliance with the animal care standards set by The Canadian Council on Animal Care.

2.2 Fertilization and embryo manipulation

Eggs from ovulating females were squeezed onto macerated *X. laevis* testes in 1 mL of 1X MMR in a Petri dish. After 2 minutes the dish was flooded with 20 mL of 0.1X MMR and left for 20-30 minutes until cortical rotation had occurred. Fertilized eggs were then de-jellied in a 3% cysteine solution (L-cysteine free base in 0.1X MMR, pH 8.0) for no more than 5 min. Eggs were then washed 4x with 30 mL of 0.1X MMR to remove excess cysteine and transferred to a Petri dish. Embryos were maintained in 0.1X MMR until the desired stage was achieved. Dead embryos were removed and 0.1X MMR changed regularly to prevent contamination. Developing embryos were staged as previously described (Nieuwkoop and Faber, 1994).

2.3 Cloning of *X. laevis* Prdx 1-6

Analysis and homology search of the NCBI database (http://blast.ncbi.nlm.nih.gov/) showed provisional sequences for *X. laevis* peroxiredoxins (NM_001092016.1, NM_001091945.1, NM_001092661.1, NM_001092449.1, NM_001092111.1, NM_001089200.1). Based on these sequences, primers were designed against the 5' and 3' end of each molecule (Appendix 1). mRNA was isolated from adult frog liver tissue with an RNeasy® kit (Qiagen), and cDNA was created using qScript^{TM} cDNA Synthesis Kit (Quanta Biosystems) according to manufacturer's specifications. PCR was preformed using KAPA Hi-Fi Taq PCR Kit (KAPA Biosystems) according to the manufacturer's protocol, and using respective forward and reverse primers for each *Prdx* using liver cDNA as template. PCR products were stained with ethidium bromide and visualized by gel electrophoresis on a 1% agarose gel. Bands corresponding to the expected amplicon sizes were excised from the gel and purified using the QIAquick® Gel Extraction Kit (Qiagen) as per manufactures instructions. Purified amplicons were then cloned into pCR®II-TOPO® Vector (Invitrogen), and sequenced to confirm their identity (DNA Sequencing Facility at Robarts Research Institute, London, ON, Canada). After analysis (see below) sequences were subsequently submitted to GenBank (accession numbers: JF820061, JF820062, JF820063, JF820064, JF820065, JF820066) (Appendix 2).

2.4 Phylogenetic analysis of X Prdx 1-6

Multiple sequence alignment of the deduced and curated amino acid sequences of the peroxiredoxins from several organisms was performed using ClustalW2 (Chenna et al., 2003) (Appendix 3), and a phylogenetic tree was created using the neighbor-joining method (Saitou and Nei, 1987) (Figure 3). The sequences used in Figure 3 and Appendix 3 were as follows: *Homo sapiens:* NP_859048.1, NP_005800.3, NP_006784.1, NP_006397.1, NP_036226.1, NP_004896.1, *Mus musculus*: NP_035164.1, NP_035693.2, NP_031478.1, NP_058044.1, NP_036151.1, NP_031479.1, *Gallus gallus*: XP_001233893.1, XP_426543.2, XP_416800.2, NP_001034418.1, *Danio Rerio*: NP_001013489.1, NP_001002468.1, NP_001013478.2, NP_001082894.1, NP_001019577.1, NP_957099.1, *Drosophila melanogaster*: NP_477510.1, NP_524387.1, NP_525002.1, NP_001027191.1, NP_523463.2. Putative signal sequences were identified as previously described (Bendtsen et al., 2004). To confirm results of the phylogenetic analysis, Multiple sequence alignment and phylogenetic tree construction was done using MUSCLE (multiple sequence comparison by log-expectation) and T-Coffee (Notredame et al., 2000; Edgar, 2004).

2.5 RT-PCR analysis of X Prdx 1-6 mRNA during embryogenesis

To obtain RNA for comparison of transcript levels, approximately 10 embryos were homogenized and RNA was extracted from stages 6, 9, 12, 18/19, 21, 23/24, 28, 32 and 37/38 using an RNeasy® kit (Qiagen). PCR was performed using KAPA Taq PCR Kit (KAPA Biosystems) according to the manufacturer's protocol. Primers used for each X *Prdx* are listed in Appendix 1. PCR products were stained with ethidium bromide and visualized by gel electrophoresis on a 1% agarose gel. *Ornithine Decarboxylase* (*ODC*) was used as a control, and levels of X *Prdx* transcripts were normalized to *ODC*

transcripts and photographed using Quantity One version 4.4.0 software (Bio Rad). Data represents an average of three biological repeats and measurements were made during the exponential phase. One-way ANOVA followed by a post-hoc Tukey's test was performed to determine differences between transcript levels at individual stages (SPSS Release 11).

2.6 Creation and Validation of DIG labeled RNA probes

To obtain probes for whole mount *in situ* hybridization Digoxigenin labeled antisense RNA probes were created from *Prdx* cDNA clones in pCR®II-TOPO® Vector using the DIG RNA Labeling Kit (Roche Diagnostics) using either SP6 or T7 polymerase according to manufactures protocol. DIG labeled probes were purified using an RNeasy® kit (Qaigen) and their size was confirmed by gel electrophoresis on a 3% formaldehyde agarose gel.

2.7 Whole mount *in situ* hybridization analysis

Whole mount *in situ* hybridization was performed as previously described (Deimling and Drysdale, 2009) (Appendix 2). Positive and negative controls were used to determine specificity in the experiment. Following *in situ* analysis embryos were maintained in 1X PBS and visualized using a Leica Fluo™ (model MZ FCIII) dissecting scope. Digital images were captured using a Photometrics Cool SNAP™ CF Camera and edited using Adobe Photoshop CS2 version 9.0.2. Images are representative of three experimental repeats.

2.8 Cross-sectioning of embryos

Embryos subjected to whole mount *in situ* hybridization were subsequently sectioned by razor blade to determine the extent of staining. Locations of cross-sections were through the eye, anterior trunk encompassing the pronephric region, and mid-trunk (Figure 9A). Parallel cuts were made at each specified location and were roughly 100 μm apart.

Chapter 3

Results

3.1 Cloning of *X. laevis Prdx* 1-6

The coding regions of the *X. laevis peroxiredoxins* were amplified from adult liver tissues by PCR using primers designed against provisional sequences obtained from the NCBI database (see Materials and Methods for accession numbers and primer sequences). The *Xenopus peroxiredoxin* (X *Prdxs*) sequences were subsequently compared to the *Prdx* family of enzymes from a variety of other organisms. This analysis allowed me to classify the X *Prdxs* as peroxiredoxins 1-6 corresponding to the vertebrate peroxiredoxin gene family members (Table 1). The X *Prdxs* had open reading frames of 600, 609, 756, 804, 570, and 675 bps for X *Prdx* 1-6, respectively, and deduced protein lengths of 199, 202, 251, 267, 189 and 224 amino acids, respectively (Submitted GenBank accession numbers: JF820061, JF820062, JF820063, JF820064, JF820065, JF820066) (Appendix 3). X *Prdxs* showed high similarity with both mice and human peroxiredoxins at both the DNA and protein level (Table 1). The sequences for X *Prdx* 1-4 contain both a primary and a secondary cysteine residue categorizing them as typical 2-Cys peroxiredoxins. X *Prdx* 5 contains two cysteine residues, comparable to the atypical 2-Cys peroxiredoxins from other species. X *Prdx* 6 has only one cysteine, and is thus a typical 1-Cys peroxiredoxin. The *Xenopus* sequences also contained the appropriate signal and trafficking sequences suitable for each *Prdx* (Wood et al., 2003). *Prdx* 3 and 5 both had cleavable mitochondrial signal sequences, where *Prdx* 5 also contained a known peroxisomal signal sequence on the C-terminal (Table 1). *Prdx* 4 in other species is known to be a secretory pathway enzyme, and can be secreted. The X *Prdx* 4 sequence likewise contains a putative secretory signal (Table 1).

Table 1.
Summary of evolutionarily conserved elements within the *Xenopus laevis* peroxiredoxin homologs

		X Prdx 1	X Prdx 2	X Prdx 3	X Prdx 4	X Prdx 5	X Prdx 6
% DNA Identity	Human[1]	70	72	71	74	69	69
	Mouse[2]	73	67	72	71	61	72
% Protein Identity	Human[3]	83	74	73	81	52	79
	Mouse[4]	82	74	75	78	59	76
1° Cysteine Residue		+	+	+	+	+	+
2° Cysteine Residue		+	+	+	+	+	-
Mitochondrial Signal Sequence		-	-	+	-	+	-
Secretion Signal Sequence		-	-	-	+	-	-
Peroxisomal Signal Sequence		-	-	-	-	+	-

Accession numbers: [1]Human: *NM_181697.2, NM_005809.4, NM_006793.2, NM_006406.1, NM_012094.3, NM_004905.2,* [2]Mouse: *NM_011034.4, NM_011563.5, NM_007452.2, NM_016764.4, NM_012021.2, NM_007453.3,* [3]Human: *NP_859048.1, NP_005800.3, NP_006784.1, NP_006397.1, NP_036226.1, NP_004896.1,* [4]Mouse: *NP_035164.1, NP_035693.2, NP_031478.1, NP_058044.1, NP_036151.1, NP_031479.1*

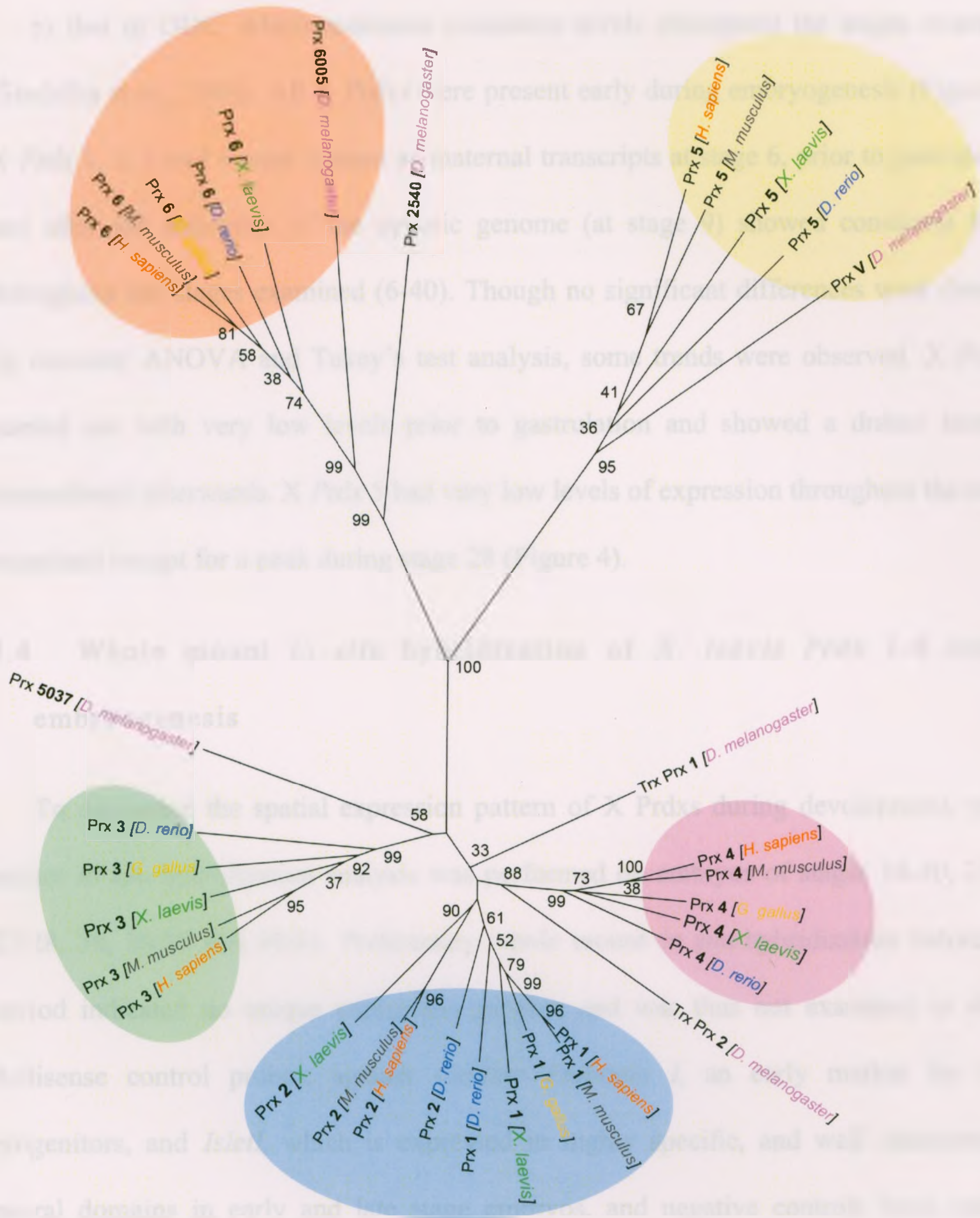
3.2 Phylogenetic analysis of *X. laevis Prdx* 1-6 revealed general conservation with Prdxs from other species

To further identity similarities with peroxiredoxins from other species a phylogenetic tree was constructed of the *Prdx* family members from human, mice, frog, zebrafish, chicken and fly (Figure 3). Based on this data it is clear that the X *Prdx* sequences fit into the classical hierarchy of the vertebrate peroxiredoxins, and this data may also shed light on how other peroxiredoxins from lower invertebrates (specifically *Drosophila*) fit into the global picture of peroxiredoxin evolution. *Prdxs* 1 and 2 are the most closely related in all species examined and show the least divergence in sequence. *Prdx* 3 and 4 are also closely related to 1 and 2, and together these *Prdxs* make up the Typical 2-Cys subgroup. *Prdx* 5 and 6 are highly divergent from the other *Prdxs*, and are structurally different at the protein level. *Prdx* 5 has the atypical 2-Cys architecture, and *Prdx* 6 contains only one active cysteine residue. Based on this phylogenetic analysis, some *Drosophila Prdxs* fit within the classical hierarchy (such as *Prdx* V), whereas others (*Trx Prdx* 1 and 2) have very distinct origins (Figure 3).

3.3 RT-PCR analysis of *X. laevis Prdx* transcripts between stages 6 and 40 of embryogenesis

To determine the temporal expression pattern of the X *Prdxs* during early development, semi quantitative RT-PCR was performed. Developmental stages were selected that preceded and followed the onset of yolk metabolism in most tissues, as well as stages that encompassed significant developmental milestones, including gastrulation, neurulation, cellular differentiation and organogenesis. Levels of X *Prdxs* were compared

Figure 3. Phylogenetic analysis of Prdx amino acid sequences across species. The predicted amino acid sequences of the peroxiredoxins from human, mouse, chicken, zebrafish and fly were compared to predicted amino acid sequence for the 6 Xenopus peroxiredoxins cloned. Sequences were aligned using ClustalW and phylogenetic tree analysis was conducted using the neighbor-joining method. Numbers at branch points represent bootstrap values (n=1000). Accession numbers for source sequences are indicated in materials and methods.



Prx 6005 [D. melanogaster]
Prx 2540 [D. melanogaster]
Prx 6 [X. laevis]
Prx 6 [D. rerio]
Prx 6 [
Prx 6 [M. musculus]
Prx 6 [H. sapiens]
81
58
38
74
99
99
100
Prx 5 [H. sapiens]
Prx 5 [M. musculus]
Prx 5 [X. laevis]
Prx 5 [D. rerio]
Prx V [D. melanogaster]
67
41
36
95
Prx 5037 [D. melanogaster]
Trx Prx 1 [D. melanogaster]
58
33
Prx 3 [D. rerio]
Prx 3 [G. gallus]
Prx 3 [X. laevis]
Prx 3 [M. musculus]
Prx 3 [H. sapiens]
99
92
37
95
Prx 4 [H. sapiens]
Prx 4 [M. musculus]
Prx 4 [G. gallus]
Prx 4 [X. laevis]
Prx 4 [D. rerio]
100
73
38
88
99
Trx Prx 2 [D. melanogaster]
90
61
52
79
99
96
96
Prx 2 [X. laevis]
Prx 2 [M. musculus]
Prx 2 [H. sapiens]
Prx 2 [D. rerio]
Prx 1 [D. rerio]
Prx 1 [X. laevis]
Prx 1 [
Prx 1 [M. musculus]
Prx 1 [H. sapiens]

to that of ODC, which maintains consistent levels throughout the stages examined (Šindelka et al., 2006). All X *Prdxs* were present early during embryogenesis (Figure 4). X *Prdx* 1, 2, 3 and 6 were present as maternal transcripts at stage 6, prior to gastrulation, and after the activation of the zygotic genome (at stage 9) showed consistent levels throughout the stages examined (6-40). Though no significant differences were detected by one-way ANOVA and Tukey's test analysis, some trends were observed. X *Prdx* 4 started out with very low levels prior to gastrulation and showed a drastic increase immediately afterwards. X *Prdx* 5 had very low levels of expression throughout the stages examined except for a peak during stage 28 (Figure 4).

3.4 Whole mount *in situ* hybridization of *X. laevis Prdx* 1-6 during embryogenesis

To determine the spatial expression pattern of X Prdxs during development, whole mount *in situ* hybridization analysis was performed on embryos of stages 18-20, 23/24, 27/28, 30, 36/37 and 40/41. Preliminary whole mount *in situ* hybridization before this period indicated no unique expression patterns and was thus not examined in detail. Antisense control probes, against *Cardiac Troponin I*, an early marker for heart progenitors, and *IsletI*, which is expressed in highly specific, and well characterized, neural domains in early and late stage embryos, and negative controls were used to establish specificity in the experiment (Moreno et al., 2008) (Figure 5).

3.4.1 *X. laevis Prdx* spatial distribution during stages 18-20 of embryogenesis

Figure 4. Temporal expression profiles of *Xenopus laevis* Prdx genes during early development. Levels of Prdx 1 (A), Prdx 2 (B), Prdx 3 (C), Prdx 4 (D), Prdx 5 (E) and Prdx 6 (F) mRNA were examined by RT-PCR analysis. cDNA was created from mRNA extracted from stages 6-40 of embryogenesis according to Nieuwkoop and Faber (1994). Each value has been normalized to the level of ODC mRNA. Error bars represent standard error (n=3). Statistical analysis was performed but no significant differences were detected.

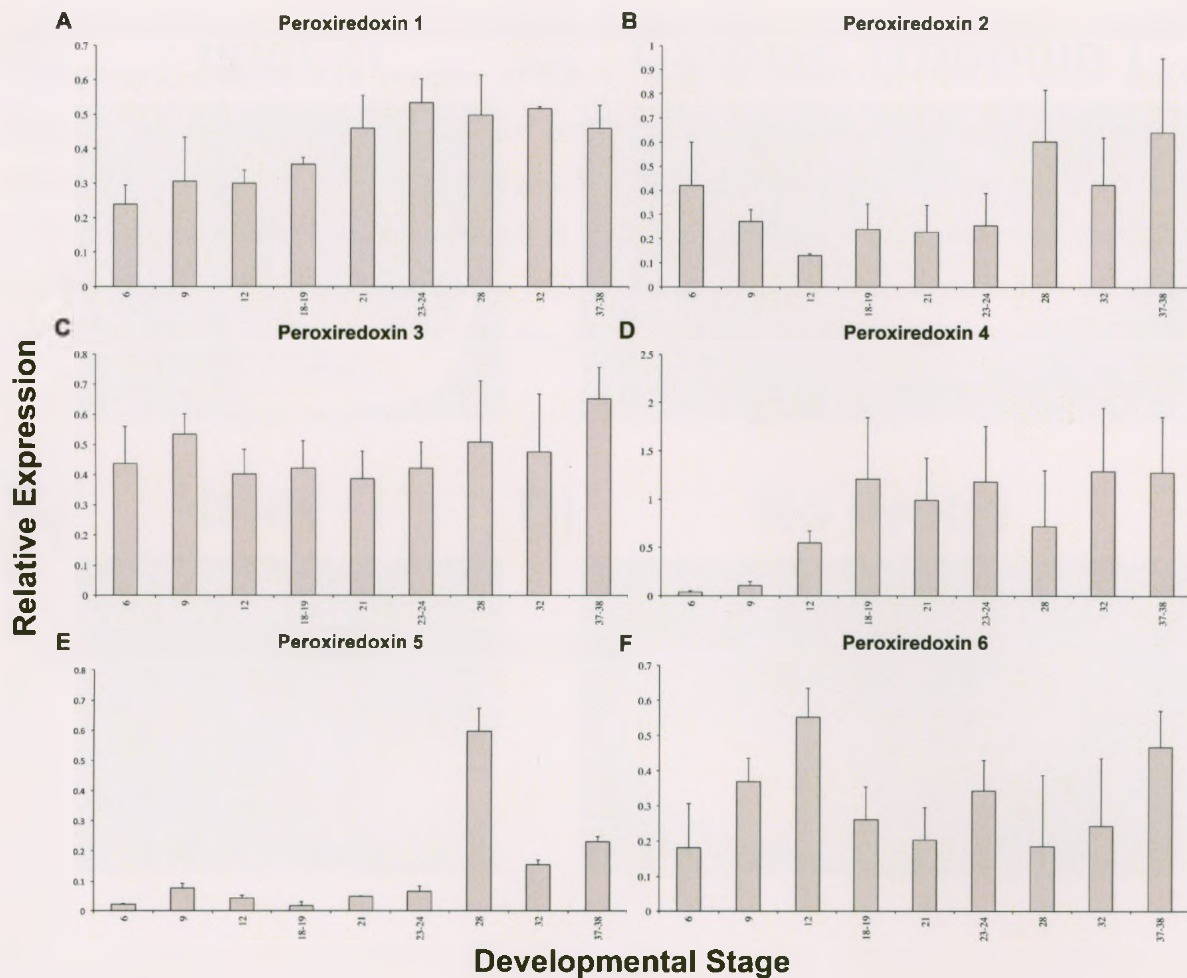
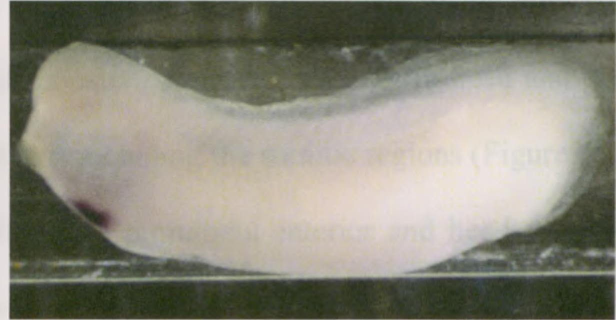

A
Peroxiredoxin 1
B
Peroxiredoxin 2
C
Peroxiredoxin 3
D
Peroxiredoxin 4
E
Peroxiredoxin 5
F
Peroxiredoxin 6
Relative Expression
Developmental Stage

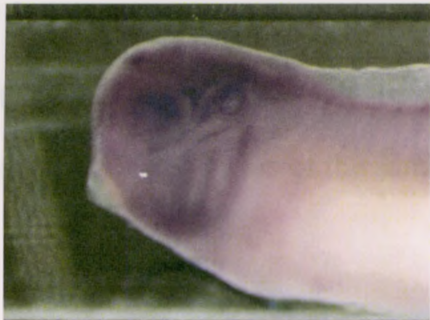
Figure 5. Whole mount *in situ* hybridization of Cardiac Troponin I and Islet-I probes. Whole mount *in situ* hybridization of stage 18 and 30 embryos with antisense Digoxigenin-labeled probe against the coding region for *X. laevis* Islet-I (A, B), and stage 30 embryos with antisense Digoxigenin-labeled probe against the coding region for *X. laevis* Cardiac Troponin I (C), genes was performed to establish specificity in the experiment. The experiment was also performed without a probe as a negative control (D). Anterior is to the left, dorsal is up, (B) shows magnified view of anterior region of stage 30 embryo.

A) Islet - I

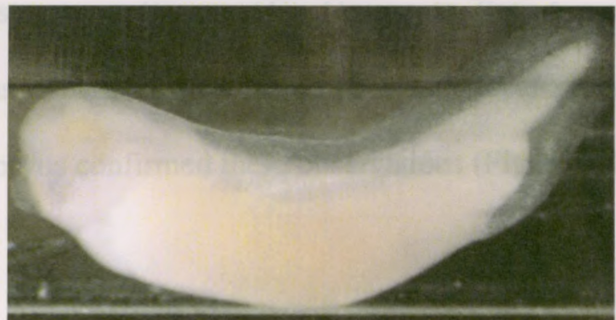
C) Cardiac Troponin I



B) Islet - I



D) No Probe



Stages 18-20 represent the end of neurulation just prior to the beginning of tail bud stages in *X. laevis.* All 6 X *Prdxs* were detected in broad regions of the embryos during these stages (Figure 6). X *Prdx* 1 demonstrated anterior staining in head and eye structures, but lacked staining in the dorsal axis including the somitic regions (Figure 6A). *Prdx* 5 had staining similar to *Prdx* 1 with more prominent anterior and head staining (Figure 6E). *Prdx* 2, 3, 4 and 6 had robust anterior and dorsal staining, where 2, 3 and 4 had broader staining in presumptive neural regions and somites (Figure 6B-D, F). Of note, *Prdx* 3 did not stain in posterior dorsal structures (Figure 6C). Unique to *Prdx* 6 was strong anterior/ventral staining, in regions consistent with blood precursor localization (Figure 6F). Dorsal views of the same embryos confirmed these observations (Figure 6G-L).

3.4.2 *X. laevis Prdx* spatial distribution during stages 23/24 of embryogenesis

Stages 23/24 represent early tailbud stages. Pre-patterning and cell differentiation in the embryo occurs during these stages before the beginning of organogenesis. Expression patterns established during neurulation (Figure 6) continued during these stages (Figure 7). X *Prdx* 1 showed no dorsal staining in the somites, but stained the neural tube (Figure 7A and G). X *Prdx* 1 was strongly present in the anterior neural regions as well as head structures such as the eye, pharyngeal arches and otic placode. Staining for X *Prdx* 2, 3, 4, 5 and 6 at these stages was similar to stages 18-20, with very robust somitic staining (Figure 7B-F). Differences were observed in the subcellular localization of these gene transcripts within the very large somitic cells. The different localization of the mRNA within the somites could be due to the fact that the translated

Figure 6. Expression profiles of *Xenopus laevis* Prdx mRNA during stages 18-20 of embryonic development. Whole mount *in situ* hybridization with antisense Digoxigenin-labeled probes against the coding regions of the *X. laevis* Prdx genes was performed to determine the spatial expression pattern of Prdx 1-6 at stages 18-20. Staining was analyzed laterally (A-F) and dorsally (G-L). Labels indicate specific developing regions of embryos (BP, blood precursors). Anterior is to the left, dorsal is up.

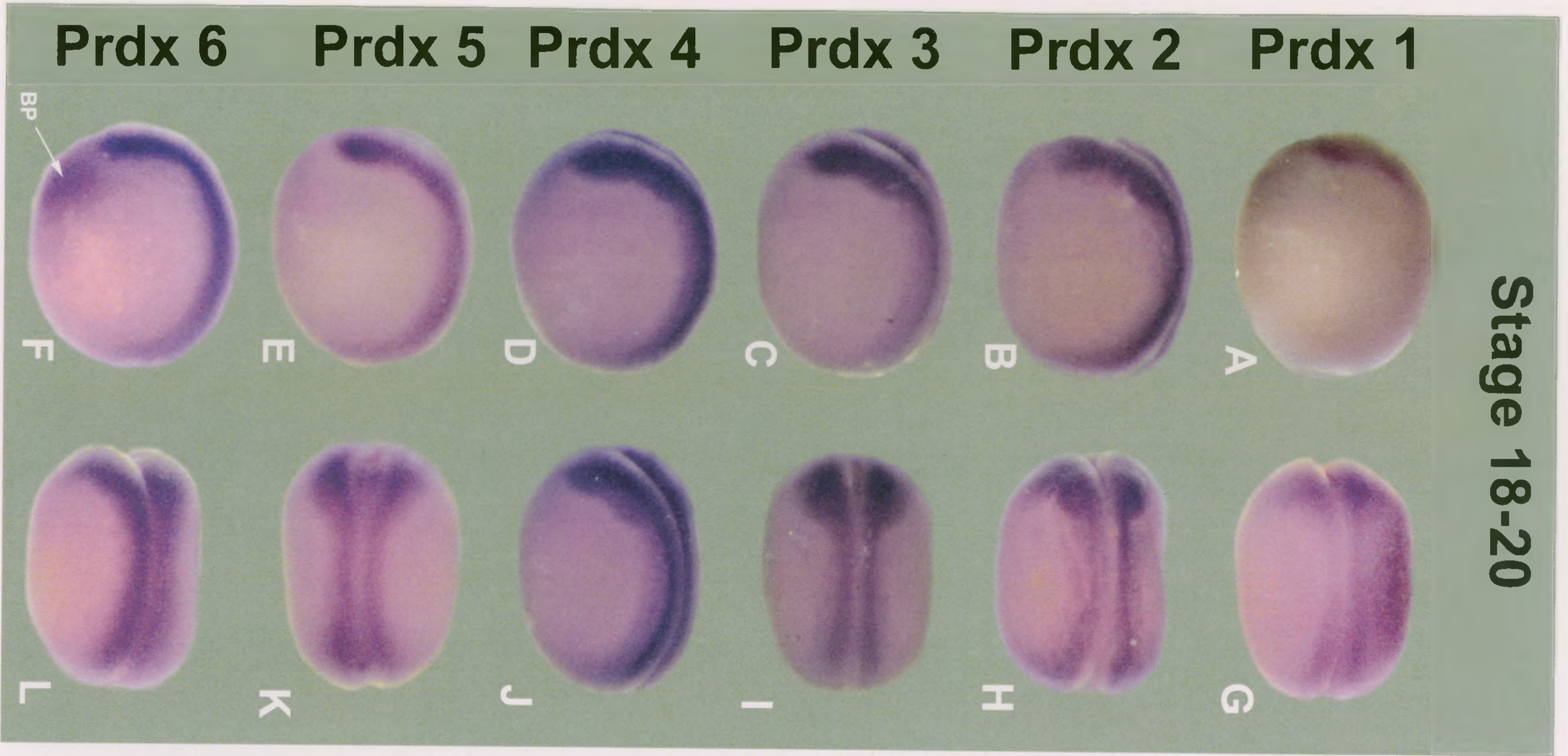
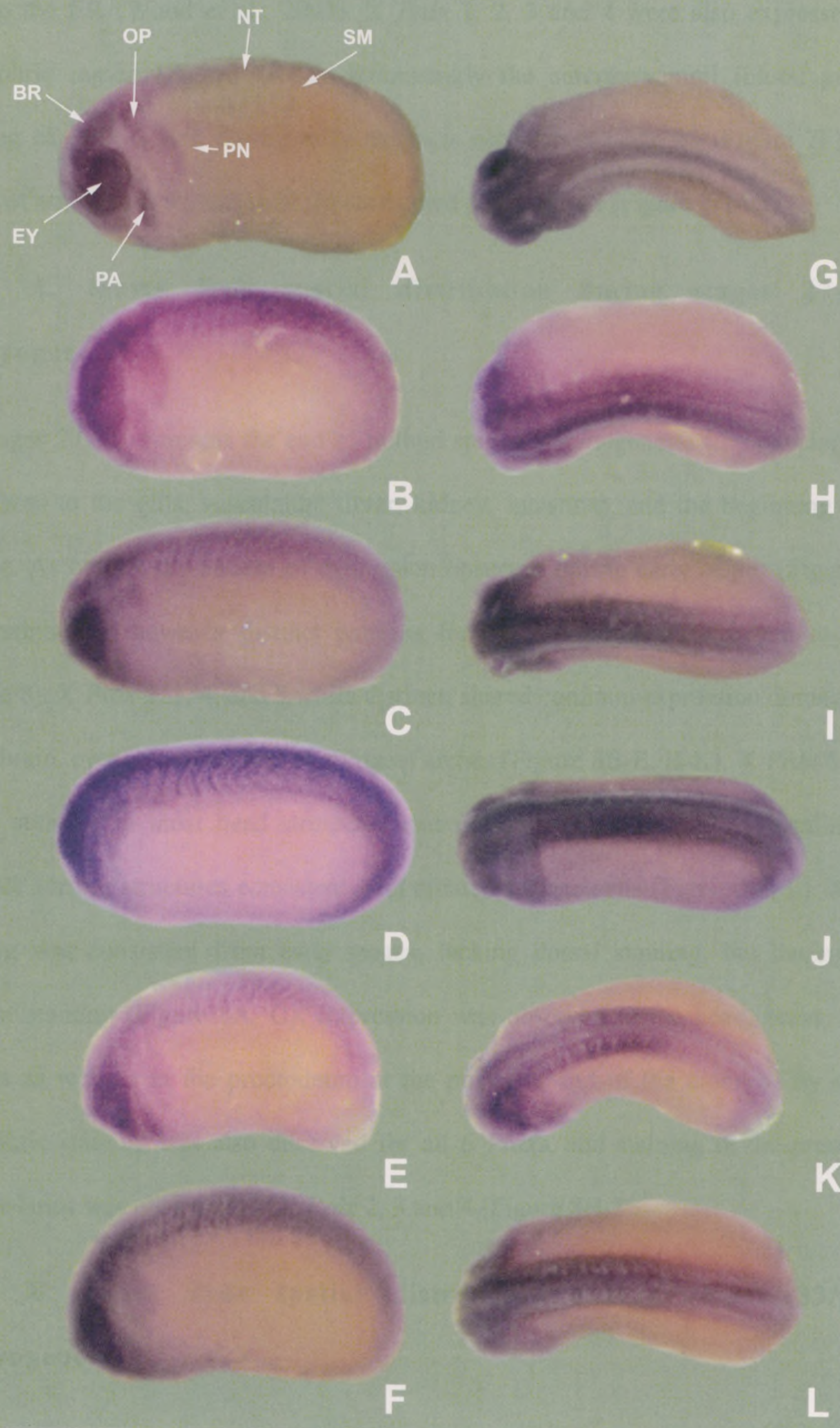

Prdx 6
Prdx 5
Prdx 4
Prdx 3
Prdx 2
Prdx 1
Stage 18-20
BP
A
B
C
D
E
F
G
H
I
J
K
L

Figure 7. Expression profiles of *Xenopus laevis* Prdx mRNA during stages 23/24 of embryonic development. Whole mount *in situ* hybridization with antisense Digoxigenin-labeled probes against the coding regions of the *X. laevis* Prdx genes was performed to determine the spatial expression pattern of Prdx 1-6 at stages 23/24. Staining was analyzed laterally (A-F) and dorsally (G-L). Labels indicate specific developing regions of embryos (BR, brain; EY, eye; OP, optic placode; PN, pronepheros; PA, pharyngeal arches; NT, neural tube; SM, somites). Anterior is to the left, dorsal is up in A-F, dorsal view of same embryos in G-L.



Prdxs

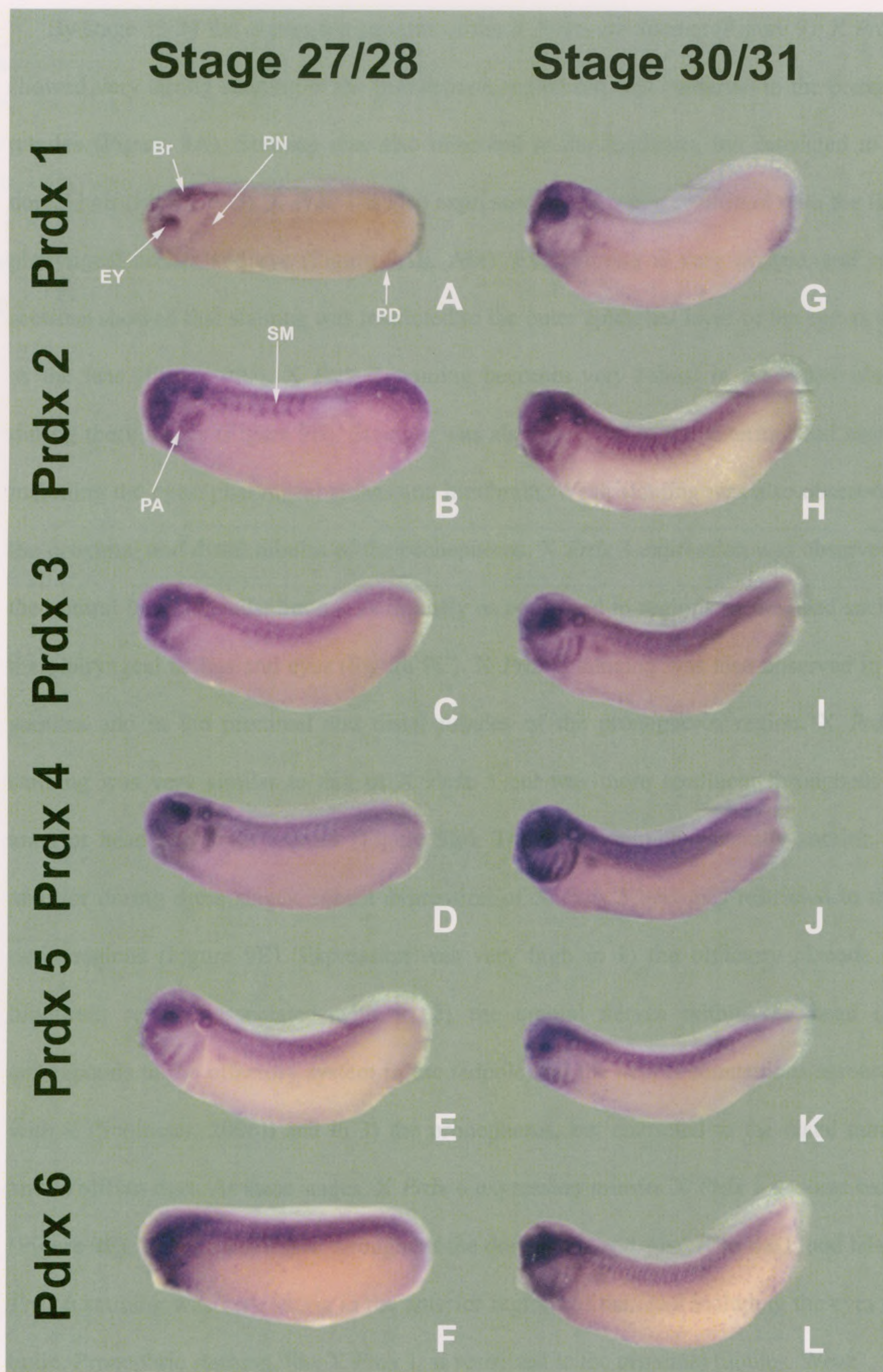
have different subcellular locations – 1, 2 and 6 are cytosolic; 3 and 5 are mitochondrial; 4 is in the ER (Wood et al., 2003). X *Prdx* 1, 2, 3 and 4 were also expressed in the pronephric region (Figure 7A-D). Interestingly the anterior/ventral (blood precursor) staining of *Prdx* 6 seen during early stages is not seen at stage 23 (Figure 7F). Dorsal views of embryos from stages 23/24 confirmed observations (Figure 7G-L).

3.4.3 *X. laevis Prdx* spatial distribution during stages 27-31 of embryogenesis

Stages 27-31 represent the end of tailbud stages, organogenesis (the development of precursors to the gills, vasculature, liver, kidney, intestine), and the beginning of heart beating. As before, the pattern of expression observed during early stages (Figures 6, 7) was maintained, however distinct patterns for each of the X *Prdx* began to develop (Figure 8). X *Prdx* 2, 3, 4, and 5 while distinct, shared common expression domains in the head (brain, eyes), somites, and pharyngeal arches (Figure 8B-E, H-K). X *Prdx* 6 showed robust staining in most head structures and dorsal somites as well as localization to punctate surface structures consistent with ciliated surface cells (Figure 8F, L). X *Prdx* 1 staining was consistent from early stages, lacking dorsal staining, but having strong anterior staining (Figure 8A, G). Expression was observed in the head, brain, and eye regions as well as in the proctodeum at the posterior end of the embryo. By stage 31 pronephric staining was also observed for all 6 *Prdxs*, and staining in the presumptive blood islands was observed for X *Prdx* 2, 3 and 4 (Figure 8H-J).

3.4.4 *X. laevis Prdx* spatial distribution during stages 33/34 of embryogenesis

Figure 8. Expression profiles of *Xenopus laevis* Prdx mRNA during stages 27/28 and 30/31 of embryonic development. Whole mount *in situ* hybridization with antisense Digoxigenin-labeled probes against the coding regions of the *X. laevis* Prdx genes was performed to determine the spatial expression pattern of Prdx 1-6 at stages 27/28 (A-F) and 30/31 (G-L). Labels indicate specific developing regions of embryos (BR, brain; EY, eye; PN, pronepheros; PD, proctodeum; FB, forebrain; HB, hindbrain; SM, somites; PA, pharyngeal arches). Anterior is to the left, dorsal is up.


Stage 27/28
Stage 30/31
Prdx 1
Prdx 2
Prdx 3
Prdx 4
Prdx 5
Pdrx 6
Br
PN
EY
PD
SM
PA
A
B
C
D
E
F
G
H
I
J
K
L

By stage 33/34 the expression patterns of the X *Prdxs* are distinct (Figure 9). X *Prdx* 1 showed very strong staining in the pronepheric region but was restricted to the proximal tubules (Figure 9A). Staining was also observed in the forebrain, but restricted to the dorsal half (Figure 9Ai). X *Prdx* 1 is also expressed in structures consistent with the liver, pharyngeal arches and eye (Figure 9Aii, Aiii). Eye staining is very unique, and cross sections showed that staining was restricted to the outer epithelial layer of the eye as well as the lens (Figure 9Ai). X *Prdx* 2 staining becomes very robust in the blood islands during these stages (Figure 9B). Staining was also observed in the anterior/head regions including the eyes, pharyngeal arches and hindbrain. Weak staining was also observed in the proximal and distal tubules of the pronepheros. X *Prdx* 3 expression was observed in the ventral blood islands, but not as robustly as compared to regions of the head such as the pharyngeal arches and eyes (Figure 9C). X *Prdx* 3 staining was also observed in the somites and in the proximal and distal tubules of the pronepheros region. X *Prdx* 4 staining was very similar to that of X *Prdx* 3 but was more confluent throughout the anterior head and brain regions (Figure 9D). Though remaining generally somitic and anterior during these stages, robust expression of X *Prdx* 5 becomes restricted to three main regions (Figure 9E). Expression was very high in 1) the olfactory placode and hindbrain regions associated with it, 2) the cranial nerves within the head (this corresponds to the olfactory system in the tadpole and the neural connections associated with it (Schlosser, 2006)) and in 3) the pronepheros, but restricted to the distal tubules and Wolffian duct. At these stages, X *Prdx* 6 expression mirrors X *Prdx* 1 to some extent (Figure 9F). Though expressed throughout the dorsal axis and weakly in the blood islands, *Prdx* 6 staining was very strong in the anterior regions of the head including the eyes and brain. Pronephric staining, like X *Prdx* 1, is restricted to the proximal tubules. Weak

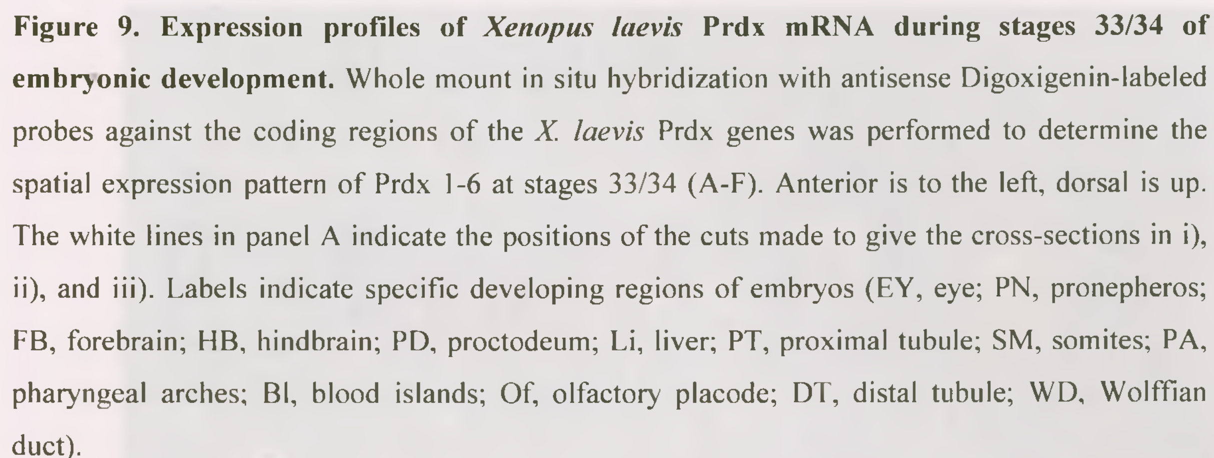
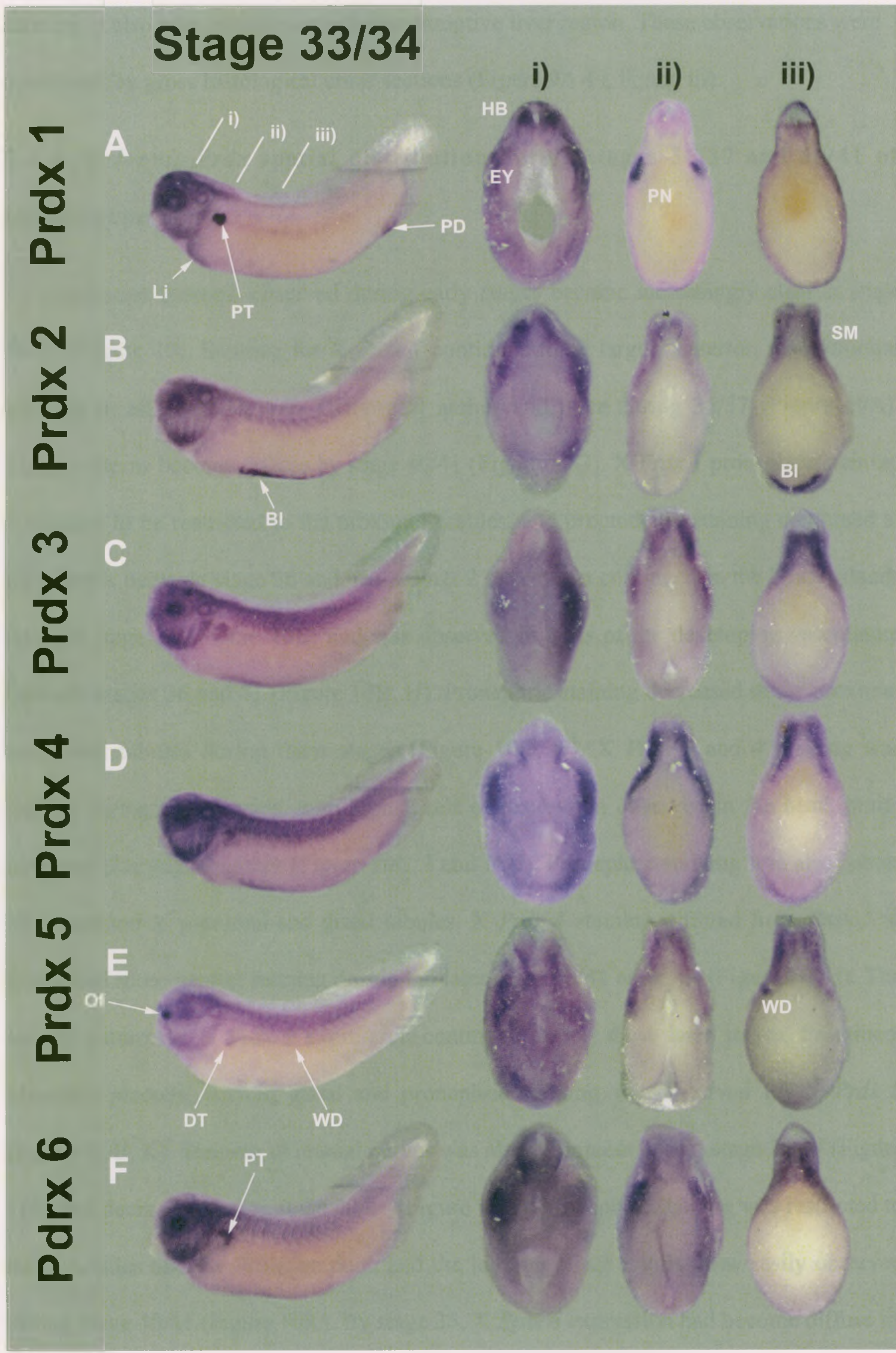


Figure 9. Expression profiles of *Xenopus laevis* Prdx mRNA during stages 33/34 of embryonic development. Whole mount in situ hybridization with antisense Digoxigenin-labeled probes against the coding regions of the *X. laevis* Prdx genes was performed to determine the spatial expression pattern of Prdx 1-6 at stages 33/34 (A-F). Anterior is to the left, dorsal is up. The white lines in panel A indicate the positions of the cuts made to give the cross-sections in i), ii), and iii). Labels indicate specific developing regions of embryos (EY, eye; PN, pronepheros; FB, forebrain; HB, hindbrain; PD, proctodeum; Li, liver; PT, proximal tubule; SM, somites; PA, pharyngeal arches; Bl, blood islands; Of, olfactory placode; DT, distal tubule; WD, Wolffian duct).



Stage 33/34
i)
ii)
iii)
Prdx 1
A
i)
ii)
iii)
HB
EY
PN
PD
Li
PT
Prdx 2
B
SM
BI
BI
Prdx 3
C
Prdx 4
D
Prdx 5
E
Ot
WD
DT
WD
Pdrx 6
F
PT

staining is also seen just dorsal to the presumptive liver region. These observations were confirmed by gross histological cross sections (Figure 9A-Fi, ii, and iii).

3.4.5 *X. laevis Prdx* spatial distribution during stages 36/37 and 40/41 of embryogenesis

Expression patterns observed during early stages become increasingly clear at stage 36/37 (Figure 10). Staining for X *Prdx* 1 continued to be largely anterior, with stronger staining in all brain regions, pharyngeal arches and liver during 36/37 (Figure 10A). These patterns become diffuse by stage 40/41 (Figure 10G). X *Prdx* 1 pronephric staining continued to be restricted to the proximal tubules, and proctodeum staining continued at high levels between stage 36 and 41. X *Prdx* 2 expression continued in the blood islands up until stage 37 (Figure 10B) and was observed in parts of the developing vasculature between stages 36 and 41 (Figure 10B, H). Pronephric staining decreased in the proximal and distal tubules during these stages (Figure 10B, H). X *Prdx* 3 and 4 staining was similar during these stages, with high levels of expression observed in the head, brain, eyes and pharyngeal arches (Figure 10C, I and D, J). Pronephric staining was also robust but restricted to proximal and distal tubules. X *Prdx* 4 staining differed from *Prdx* 3 in that it had more ventral staining during the later stage 40/41 embryos (Figure 10I, J). The unique pattern of X *Prdx* 5 expression continued during these later stages. Prominent olfactory placode, thyroid gland and pronephric staining was observed for X *Prdx* 5 (Figure 10E, K). Staining of cranial nerves was also prominent during stage 36/37 (Figure 10E) but decreased during stage 40/41 (Figure 10K). Pronephric staining was restricted to distal tubules and the Wolffian duct, and the looping of the tubules was easily observed during stage 40/41 (Figure 10K). By stage 36, X *Prdx* 6 expression had become diffuse in

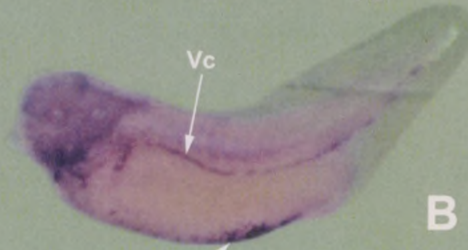
Figure 10. Expression profiles of *Xenopus laevis* Prdx mRNA during stages 36/37 and 40/41 of embryonic development. Whole mount in situ hybridization with antisense Digoxigenin-labeled probes against the coding regions of the *X. laevis* PRDX genes was performed to determine the spatial expression pattern of Prdx 1-6 at stages 36/37 (A-F) and 40/41 (G-L). Labels indicate specific developing regions of embryos (BR, brain; PT, proximal tubule; Li, liver; PD, proctodeum; Ey, eye; Bl, blood islands; Vc, vasculature; PA, pharyngeal arches; PN, pronepheros; SM, somites; Of, olfactory placode; CN, cranial nerves; DT, distal tubule; WD, Wolffian duct; TG, thyroid gland). Anterior is to the left, dorsal is up.

Stage 36/37
Stage 40/41
Prdx 1

Br
PT
Li
PD
A

G

Prdx 2


Vc
Bl
B

H

Prdx 3

Ey
SM
PA
PN
C

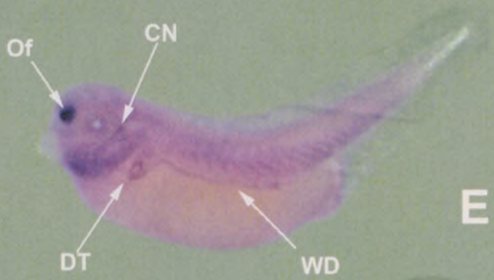
I

Prdx 4

D

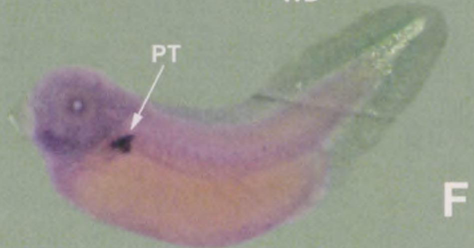
J

Prdx 5


Of
CN
DT
WD
E

TG
K

Prdx 6


PT
F

L

anterior and dorsal regions, except for spots in the pharyngeal arches (Figure 10F). Interestingly, like X *Prdx* 1, pronephric proximal tubule staining continued to be very strong during these later stages of development (Figure 10F, L).

CHAPTER 4

DISCUSSION AND FUTURE PERSPECTIVES

4.1 *Xenopus laevis* peroxiredoxins are highly conserved from other eukaryotes and show classical hierarchy

Proteins that show high levels of conservation of amino acid sequences, structural domains, and mechanisms of action, are usually deemed evolutionarily significant and have important functions (Giudice, 2001). This is especially true of proteins and genes conserved across a large variety of species, and is commonly found within homologous developmental mechanisms (Giudice, 2001). The high sequence similarity of the newly cloned X *Prdxs* may indicate an equivalent similarity in function within different organisms (Table 1, Figure 3). This is further supported by the fact that each of the X *Prdx* genes contains coding regions for cellular trafficking signals required for the proper localization and function of these proteins in humans, mice and other organisms (Wood et al., 2003). As well, the X *Prdx* family fit within the classical hierarchy of vertebrate peroxiredoxins; there are 4 Typical 2-Cys Prdxs (X Prdx 1-4), 1 Typical 1-Cys Prdx (X Prdx 5), and 1 Atypical 2-Cys Prdx (X Prdx 6) in Xenopus (Table 1).

Though there has been phylogenetic analysis of the peroxiredoxin gene family performed in the past, the *Xenopus* Prdxs have yet to be included in such a comparison (Han et al., 2005b; Shuvaeva et al., 2009). Phylogenetic analysis was performed and confirmed using several methods (see materials and methods). X Prdxs fit well into the classical vertebrate peroxiredoxins, which are grouped into 6 distinct members (Figure 3). This encompasses the highly conserved 2-Cys Prdx subgroup (Prdx 1-4) as well as the more evolutionarily distinct 1-Cys and Atypical Prdxs (Prdx 5 and 6), which may consequently have unique functions. Conservation of homologous sequences is usually

indicative of homologous functions and so we can speculate that functions identified for peroxiredoxins in some species would be conserved across vertebrates.

4.2 Analysis of *Xenopus* peroxiredoxin transcript levels indicate all 6 peroxiredoxins are expressed in developing embryos

All of the X *Prdxs* are expressed during embryogenesis in the frog *Xenopus laevis* (Figure 4). All 6 X *Prdxs* are present as maternal transcripts at stage 6 (before activation of the zygotic genome), and show consistent levels of expression throughout the stages examined except for X *Prdx* 5, which shows a strong peak in expression during stage 28, just prior to organogenesis (Figure 4). These expression patterns indicate a role for the *Prdxs* during early embryogenesis, though very little evidence exists to date for such a function. While comprehensive studies have not been performed, there is some evidence of *Prdx* expression during mammalian embryogenesis, as all 6 *Prdxs* are expressed at some level or point during development in mice (Lee, 2003; Donnay and Knoops, 2007; Dammeyer and Arnér, 2011; Godoy et al., 2011). Peroxiredoxin 5 from Drosophila is also expressed during development, and is maternally lethal when genetically knocked out (Radyuk et al., 2009). *Drosophila* Prdx 5 is highly expressed during in very early embryos (2-6 hrs) and again during larval stages 1 and 2 (Gelbart and Emmert, 2010). There is also some evidence of expression of peroxiredoxins during bovine oogenesis and embryogenesis (Leyens, 2004; Leyens et al., 2004). Analysis of whole embryo transcript levels do not necessarily account for domain/tissue specific differences in expression which are better analyzed by *in situ* hybridization of whole embryos.

4.3 Whole mount *in situ* hybridization of Xenopus peroxiredoxin mRNA transcripts indicate unique expression profiles for all 6 genes

There are many similarities (and also differences) in the expression patterns of the 6 Xenopus *Prdx* family members (summarized in Table 2). This reflects both the similarity between the members (Table 1, Figure 3), and the very distinct differences in their protein structures and sub-cellular localizations (Wood et al., 2003). *Prdx* 2, 3 and 4 are the most similar in terms of sequence, and show the most similarities in expression both temporally and spatially (Table 2). Given their wide expression domains, their known roles as anti-oxidant enzymes, and the susceptibility of embryos to oxidative damage – it may reflect that these proteins are acting in a protective manner throughout embryogenesis. Knockout studies for peroxiredoxins 1, 2, 3, 4 and 6 in mice have shown their general function in anti-oxidant defense (Lee, 2003; Neumann et al., 2003; Wang, 2003; Li et al., 2008; Iuchi et al., 2009). Mice lacking *Prdx* 1, 2, 3, 4 and 6 are more susceptible to oxidative damage and show higher levels of ROS within their cells. Additionally the knockout study for peroxiredoxin 2 also showed abnormalities in blood progenitors and red blood cells caused by increased ROS levels in those cell types (Lee, 2003). This is consistent with our findings that *Prdx* 2 is highly expressed in the *Xenopus* blood islands, which are blood precursors (Hartenstein, 2006). *Prdx* 3 is specifically localized to mitochondria, and as such is important for mediating the effects of ROS produced through cellular respiration (Wood et al., 2003). X *Prdx* 3 is expressed in most tissues throughout embryogenesis (Figure 4) and has a large spatial expression domain, which may be related to its function as a housekeeping protein within mitochondria, which are present in all cell and tissue types (Figure 6, 7, 8, 9 and 10). Prdx 4 can be secreted, and has been

shown to be important for the antioxidant defense within germ line cells. X *Prdx* 4 with its wide expression domain and very high levels of expression during the stages examined may have a broader protective role during embryogenesis (Iuchi et al., 2009).

The most interesting observation is the variance in expression observed in the different parts of the developing pronephric region (Table 2, Figure 9, and 10). The developing nephron in *Xenopus* is remarkably similar to that of its mammalian counterparts, and is composed of 3 main regions; the proximal tubules, the distal tubules and the connecting ducts (Raciti et al., 2008). Each region serves a unique purpose, the tubules are involved in filtration of the blood whereas the ducts are involved in acid concentration and volume of urine, and each are further subdivided into multiple segments. X *Prdx* 1 and 6 are highly expressed in the proximal tubule, X *Prdx* 5 is expressed in the distal tubule and Wolffian duct, and X *Prdxs* 2, 3 and 4 are expressed throughout the pronepheros. The development of this region, which is the precursor to the kidney, is well characterized (Raciti et al., 2008) and I have demonstrated that peroxiredoxin expression is restricted to specific substructures during different time points of development (Table 2). Other studies have shown that genes with similar expression patterns are involved in defining the boundaries of cell types and structures in the developing kidney (Cho et al., 2011). Perhaps peroxiredoxins, through their modulation of redox states, are also involved in this boundary defining process, or in the differentiation of the actual tissue types themselves, given the clear differences in their proximal versus distal tubule localization.

Also of note is the unique expression pattern for *Prdx* 5. *Prdx* 5, along with *Prdx* 6, is the most evolutionarily diverse family member (Figure 3). Studies in flies (of which *Prx* 5 is the most similar to its vertebrate homologs) have shown that it is required by at least

one of the parents, though not zygotically lethal and important for development (Radyuk et al., 2009). The fact that it is the only member of the Atypical 2-Cys subgroup of peroxiredoxin implies that it might have unique functions. *Prdx* 5 may play a role during the development of specific regions of the embryo (namely olfactory precursors, as well as distal tubules/Wolffian duct), and its potentially unique functions may have evolved to suit these roles.

Though no overt adult phenotypes were observed in mice *Prdx* 1, 2, 3, 4 and 6 knockouts, those studies analyzed only viable offspring, which may not account for more subtle or extreme (early zygotic lethal) developmental defects (Covarrubias et al., 2008). It may be that these experiments are unable to show the developmental effects of the *Prdxs*. To this point, analysis of *Prdx* 1 in chicken and mice has shown its involvement in the differentiation of a specific neural cell lineage through thiol-redox-dependent activation of a member of the GDE2 family of proteins (Yan et al., 2009). GDE2 and other members of its family are expressed throughout neural tissues as well as the heart, gut, lung and bone where they are involved in cellular differentiation events (Yanaka et al., 2003; Rao and Sockanathan, 2005; Yanaka, 2007). The present study has shown that the peroxiredoxin family members are expressed in a wide range of tissues/organs during development; therefore the potential exists for the some sort of interaction with members of the GDE2 family or other similar proteins in a variety of tissues during embryogenesis. GDPD family members may be responsible for the differentiation of the tissues described in this study, including the pronephric regions, or the optic or olfactory placodes, and a similar thiol-redox interaction between those proteins and the peroxiredoxins expressed in those regions may exist (*Prdx* 1, 5 or 6 for example).

Table 2.

Summary of general significant expression of peroxiredoxins 1, 2, 3, 4, 5 and 6 mRNA transcripts in select tissues during Xenopus development

	Prdx 1	Prdx 2	Prdx 3	Prdx 4	Prdx 5	Prdx 6
Eye	+	-	+	+	-	+
Otic Vesicle	-	-	+	+	-	-
Forebrain	+	-	+	+	-	+
Hindbrain	++	+	+	+	-	+
Pharyngeal Arches	+	+	+	+	-	+
Thyroid Gland	-	-	+	+	++	-
Liver	+	-	-	+	-	-
Proximal Tubule	+++	+	++	++	-	+++
Distal Tubule	-	+	++	++	++	-
Wolffian Duct	-	+	+	+	++	-
Proctodeum	++	+	-	+	-	-
Blood Islands	-	++	-	+	-	-
Vasculature	-	++	+	+	-	-
Otic Placode	-	-	-	+	+++	-

"+ - +++": Gene transcript moderately to strongly expressed in corresponding tissue
"-" : Gene transcript detected in very low levels or not at all in corresponding tissue

4.4 Conclusions

1) *Xenopus laevis* peroxiredoxins are highly conserved at both the protein and DNA level to peroxiredoxins from other species of vertebrates.

2) *Xenopus laevis* peroxiredoxins are expressed throughout the stages examined and show distinct spatial expression domains from stage 20 of embryogenesis and beyond.

3) Because of their unique expression patterns, Xenopus laevis peroxiredoxins may have unique roles during development in addition to their roles as anti-oxidants.

4.5 General Summary and future perspectives

Given the expression patterns of the X *Prdxs*, and known roles for these proteins in signaling, it can be suggested that they have unique functions during embryological development. *Xenopus* is a well characterized model organism for developmental biology, and therefore the data presented here could be used for comparison to other developmental systems where the Prdxs may also have unique functions. These unique functions could be elucidated by overexpression and knockdown experiments. Microinjecting mRNA coding for the X *Prdx* genes into fertilized single cell embryos will cause the overexpression of the X Prdx proteins. Additionally, knockdown of X *Prdx* expression can be obtained by the microinjection of antisense oligonucleotides or morpholinos. These alterations can cause disruptions to any signaling mechanisms affected by Prdxs and/or REDOX and are relatively easy to perform with *Xenopus* embryos. It would be interesting to examine the effect of overexpression of X *Prdx* 1, 5 and 6, as these show the most unique expression patterns and are the most evolutionarily diverse, and therefore have the most potential to be involved in developmental signaling.

An alternative way of knocking down levels of active X Prdx proteins is through the expression of a dominant negative isoform. If both of the conserved cysteine residues of a Typical 2-Cys *Prdx* were mutated to serine residues, the dominant negative Prdx (DN Prdx) would form catalytically inactive dimers with other peroxiredoxins of the same subgroup (Kang et al., 2004). Injecting mRNA encoding for a DN Prdx could subtract a large portion of Prdxs from the pool of active Prdxs within a developing embryo and remove their effect on redox signaling.

To analyze the effects of X *Prdx* overexpression and knockdown, whole mount *in situ* hybridization and RT-PCR could be preformed to analyze the levels and expression domains of marker genes for the embryological organs and tissues of interest, including, but not limited to, those of the pronephric region and anterior structures such as the olfactory and otic placodes.

Another interesting avenue to explore would be the level of ROS/H_2O_2 within developing embryos, both spatially and temporally, and to compare this to the levels and expression patterns of the peroxiredoxins. This has been discussed and attempted before, but with little success (Coffman and Denegre, 2007; Covarrubias et al., 2008; Janssenheininger et al., 2008; Hernández-García et al., 2010). Genetically encoded ROS sensors have recently been created, and show great promise (Belousov et al., 2006). mRNA could be created coding for such a sensor and microinjected into embryos to examine ROS production and elimination in real time throughout embryogenesis, and its interaction with endogenous peroxiredoxins, and exogenously expressed forms of peroxiredoxins (normal and DN Prdxs).

It is clear that there is a potential role for peroxiredoxins, REDOX and hydrogen peroxide within the field of developmental biology. Hopefully this study will provide the foundation for continued work in this ever-expanding area of research.

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Appendix 1

Primer Sequences

Amplicon*		Sequence
X Prdx 1	**For**	ATGTCTGTCGGAAATGCAAA
	Rev	TTATTTCTGCTTGCTGAAGT
X Prdx 2	**For**	ATGGCGTGTCCTGTGCGTGC
	Rev	CGTTAGTATTCTTTAGAGAAGA
X Prdx 3	**For**	ATGGCGGCGTCCTGTGGAAG
	Rev	TTAGTGCACTTTCTCAAAGT
X Prdx 4	**For**	ATGGCTCTTCAGCTCCGGCG
	Rev	AGCGTAATCTGGAACATCGT
X Prdx 5	**For**	ATGGCTCTTCGTATCCCGGT
	Rev	CTTAAAGCTGAGATATTATGT
X Prdx 6	**For**	ATGCCTGGAATCCTGCTAGG
	Rev	TTATTGTGGCTGTGCAGTGTA
ODC	**For**	AATGGATTTCAGAGACCA
	Rev	CCAAGGCTAAAGTTGCAG

* For and Rev, forward and reverse primers, respectively

Polymerase Chain Reaction Conditions

	Temperature (°C)	Time (seconds)	Cycles*
Initial Denaturing	94	120	
Denaturing	94	30	X 32/20
Primer Annealing	53	30	
Elongation Period	68/72**	75	
Final Elongation	68/72**	600	
Hold	4	∞	

* Indicates the number of cycles performed for the stages indicated in dark shading
** 68 °C was used for KAPA HiFi Taq Polymerase, 72 °C was used for KAPA Taq Polymerase

Appendix 2

Open Reading Frames of Cloned *Xenopus laevis* Peroxiredoxin Sequences

X Prdx 1:

5' ATGTCTGTCGGAAATGCAAAAATTGGACAGCCTGCACCAGACTTCACAGCTC
AGGCAGTGATGCCAGATGGACAATTCAAAAATCTTAAAATTTCAGACTACAA
AGGAAAGTATGTTGTATTCTTCTTTTACCCTCTGGATTTCACATTTGTGTGCCC
CACTGAGATCATAGCTTTTAGTGACCGGGTTGAGGAATTCAAGAAGATTAAC
TGTGAGGTTATTGGTGCATCAGGGGACTCTCATTACTGTCATTTAGCCTGGAT
TAACCAACCCCGCAAGGAAGGTGGTTTGGGCCAAATGAAAATACCACTTGTG
GCAGATGTTCAGCATACTATCGCTAAGGACTATGGTGTTTTCAAAGAGGATG
ATGGAGTTTCATTTAGAGGTCTCTTTATTATCGATGAGAAAGGCATACTGAGA
CAGATAACAATTAATGACTTACCTGTTGGGCGCTCTGTTGATGAGACTCTGAG
ACTTGTACAGGCTTTCCAGTTCACAGACAAATATGGAGAAGTCTGCCCTGCA
GGATGGCAGCCAGGAAGTGACACCATCAAGCCAGATGTCCAAAAGAGCAAA
GAGTACTTCAGCAAGCAGAAATAA 3'

X Prdx 2:

5' ATGGCGTGTCCTGTGCGTGCAGTGAAAACTCACATTGGCAAGCCATCCCCAG
CCTTTCAGGCCACCGCTTTGGTAAATGGAGAATTTAAAGAGATCCAGCTGTCG
GACTATTTAGGTAAATACGTGGTTTTATTTTTCTACCCGTTGGACTTTACCTTT
GTCTGCCCCACGGAGATCATTGCCTTCAGTAACCACGCTGAAGACTTCAAGA
AGATCAACTGCCAGCTGATTGCCGTCTCTGTAGATTCCCAATTTACCCACCTT
GCATGGACCAAAGTGCCCCGTAAAGAAGGAGGTTTGGGGCCTGTTAATATAC
CCCTGGTGTCTGACCTGACCCATTCTATTGCAAAGGATTACGGTGTCCTGAAA
GAAGAGGATGGAGTTGCATACAGAGGCCTCTTCATTATTGATGGAAAGGGA
TCCTTCGTCAGATCACTATTAACGACCTTCCTGTTGGTCGCTCTGTGGATGAG
ACATTGCGTCTGGTGCAGGCGTTCCAATACACCGATGTACATGGAGAGGTGT
GTCCAGCAGGATGGAAGCCCGGCAGCAGCATCATTAAGCCCAATGTAAAAGA
CAGCAAGGAGTTCTTCTCTAAAGAATACTAA 3'

X Prdx 3:

5'ATGGCGGCGTCCTGTGGAAGGTTATTGGCATCGTGGGTGGGTCGCACAGGCC
GTCTTACTGGGCAACTCCCCCGGGTCGCTGGGTCTTCTGTGCCGAGGAACACG
ACTGCCGTTACACCTTCTATTTGTGCCGCTCAGAAACTCCAGTTCAGCACCAG
TTCAGGGCGGTTCCTTCCTGCCGTTACCCAGCATGCTCCACAATTTAAAGGCA
CGGCTGTAGTCAATGGGGAGTTTAAAGAGCTGAGCCTGGAAGACTTCAAGGG
GAAATATTTGGTTCTCTTCTTTTACCCTCTGGACTTCACATTCGTCTGCCCCAC
GGAGATCGTGGCTTTCAGTAACGAGGCCAATGAATTTCATGATGTCAACTGT
GAGGTCGTGGCAGTGTCTGTGGATTCTCACTTCTGTCACCTTGCATGGACCAA
CACACCTAGAAAGAGTGGAGGACTGGGTCAAATGAACATCCCCCTGCTCTCT
GACCTGAATAAACAAATCTCCCGGGATTATGGGGTTCTGCTGGAGACTGCCG
GCATCGCTCTTAGGGGGCTTTTTATTATTGATCCTAATGGAATAATCAAGCAC
ATGAGTGTGAATGACCTTCCCGTCGGAAGGAGCGTGGAGGAGACCCTGCGAC
TAGTAAAGGCTTTTCAGTTTGTGGAGACGCACGGGGAAGTCTGTCCAGCCAA
CTGGACTCCTGACTCGCCAACAATCAAACCCAGTCCCGAAGGATCCAAGGAC
TACTTTGAGAAAGTGCACTAA3'

X Prdx 4:

5'ATGGCTCTTCAGCTCCGGCGTTATCTCAGGGGCAGCCCAGCTGTCACCCTCT
GTCTCCTCTTGCTGTCAGCCGCCGCTGTCACGTGTGAGGAAGAGCAGTCTCAA
GCCCGGCCTGGCCGCACTGCCCCGGATGGAGAATGTCACTTCTACGCAGGGG
GGCAGGTGTACCCCGGGGAGGCGAGTCGTGTGCCAGTGTCCGATCATTCTCT
GCATCTCAGCAAAGCTAAAATTTCCAAGCCGGCTCCATACTGGGAAGGAACA
GCAGTAATAAATGGAGAGTTCAAAGAGTTGAAACTTACAGATTATAAAGGGA
AATACCTGGTGTTCTTCTTTTATCCTCTTGATTTTACATTTGTCTGCCCAACTG
AAATCATCGCGTTTGGAGACAGAATTGAAGAATTTAGATCCATAAACACCGA
AGTTGTCGCCTGCTCTGTGGATTCTCAGTTCACACACTTGGCATGGATAAATA
CACCACGCAAACAAGGAGGACTTGGGCCCATGAAAATTCCTCTGCTCTCTGA
TTTGACACATCAGATTTCAAAGGATTATGGAGTATATTTAGAAGACCAAGGC
CACACACTAAGAGGACTTTTCATTATTGATGACAAAGGAGTCCTTCGTCAGAT
TACAATGAATGATCTTCCTGTTGGGAGGTCTGTAGATGAAACACTACGTCTAG

TTCAGGCATTCCAGTATACAGACAAACACGGAGAAGTTTGTCCTGCTGGTTG
GAAACCTGGGAGTGAAACTATCATTCCTGACCCAGCCGGAAAACTGAAATAT
TTTGATAAACAACACTGA$^{3'}$

X Prdx 5:

$^{5'}$ATGGCTCTTCGTATCCCGGTATCATTCCTCCTCCTGTCACCTCTGCGAGCAGT
TGCCAGTCCCGCGAGCAGAACAAGAGCCATGTCCATAAAGGTTGGAGATCAA
CTTCCTAATGTGCAGGTGTATGAGGGGGGCCTGGAACAAGGTCAACATCA
GAGATTTGTTCACTAACAAGAAGGGGGTGCTGTTTGGTGTCCCTGGGGCATTC
ACTCCTGGCTGCTCTAAGACACATCTGCCGGGGTATGTAGCTCAGGCAGCAG
AGCTGAAATCCCGCGGAGCAGCAGTTGTTGCTTGCATCTCTGTTAATGACGTG
TTTGTAGTGAGCGAATGGGGAAAGGTGCATGAAGCAGAAGGGAAGGTTTGC
ATGCTGGCAGATCCCTGTGGAGAATTTGCCAAGGCGTGTGGCTTCTTTTGGA
CAAAAAGGAATTATCAGAATTATTTGGAAATCAGCGTTGCAAAAGATTTTCT
ATGGTGGTTGAAGATGGAAAAATTAAAGCCATTAATGTAGAAGAAGACGGG
ACTGGTCTGACCTGCAGCCTGGCAGGAAACATAATATCTCAGCTTTAA$^{3'}$

X Prdx 6:

$^{5'}$ATGCCTGGAATCCTGCTAGGAGACGTCTTTCCTAACTTCGAGGCGGACACCA
CCATTGGCAGAATCAAGTTTCACGATTTCCTCGGGAATTCATGGGGTGTCCTT
TTCTCACACCCACGGGATTATACCCCTGTCTGCACCACTGAGCTTGGACGTTG
TGTAAAGCTGGCTCCGGAGTTCAAAAAACGCAATGTTTCCATGATCGCCCTGT
CAATAGACTCTGTGGAGGATCATCTCGGCTGGAGCAAGGACATCAACTCTTA
TAACTGTGATGAGCCCACAGAGACACTACCCTTTCCTATTATTGCTGATCCCA
AAAGGGAACTGGCTGTACAACTTGGTATGCTTGACCCTGATGAGAAGGACAT
GCAGGGGATGCCAGTGACTGCAAGATGTGTTTTCATCATTGGCCCTGATAAG
AAAATGAAGCTTTCTATTTTGTATCCAGCCACTACTGGAAGAAATTTTGATGA
AATTTTGAGAGTTGTGGATTCTCTTCAACTGACTGCAGTTCATAATGTTGCAA
CTCCGGTGGATTGGAAGCCGGGTGATCGAGTCATGGTACCCCCAAATGTTCCT
GAAGAAGAAGCAAGTAAAATATTTACATGTGGCGTCTTCACCAAAGAGCTCC
CTTCTGGAAAGAAATACCTGAGATACACTGCACAGCCACAATAA$^{3'}$

Appendix 3

Multiple sequence alignment using ClustalW2 of the Prdxs 1-6 from various species*.

**Homo sapiens* (Human), *Mus musculus* (Mouse), *Xenopus laevis* (Xenopus), *Gallus gallus* (Chicken), *Danio rerio* (Zebrafish), *Drosophila melanogaster* (Fly).

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Prx3Human          MAAAVG-RLLR-----------------ASVARHVSAIPW---GISATAALWPAACGRTS 39
Prx3Mouse          MAAAAG-RLLW-----------------SSVARHASAISR---SISASTVLRPVASRRTC 39
Prx3Xenopus        MAASCG-RLLA-----------------SWVGRTGRLT-----GQLPRVAGSSVPRNTTA 37
Prx3Chicken        MTPSFLPRPLRDPPPLVLPPGDGSEPSTALVPPPVPCIPSPPPGPACCAALKMAAALRGL 60
Prx3Zebrafish      MAATIG-RLLG------------------ASARGAAVC-----GLKTLVPRNGASVIRAP 36
Prx1Human          ------------------------------------------------------------
Prx1Mouse          ------------------------------------------------------------
Prx1Chicken        ------------------------------------------------------------
Prx2Chicken        ------------------------------------------------------------
Prx1Xenopus        ------------------------------------------------------------
Prx1Zebrafish      ------------------------------------------------------------
Prx2Zebrafish      ------------------------------------------------------------
Prx2Human          ------------------------------------------------------------
Prx2Mouse          ------------------------------------------------------------
Prx2Xenopus        ------------------------------------------------------------
Prx4Human          MEALP-LLAATTPDHGRHRR--LLLLPLLLFLLPAGAVQGWETEERPRTREEECHFYAGG 57
Prx4Mouse          MEARSKLLDGTTASRRWTRKLVLLLPPLLLFLLRTESLQGLESDERFRTRENECHFYAGG 60
Prx4Chicken        MEAARRGPRAGVGSR------ALLLALLLHGAMGAEAEEP--RPPRQRG-DEQCHYYAGG 51
Prx4Xenopus        MALQLRRYLRGSPAV------TLCLLLLSAAAVTCEEEQSQARPGRTAP-DGECHFYAGG 53
Prx4Zebrafish      MDVSRCVKTPREWLG------VLCALFLLSESVVCDGANG--------KREQECYNYAGG 46
TrxPrx2Fly         MSKY------------------LSVLLLSAALVGAAKPED----------NESCYSFAGG 32
TrxPrx1Fly         ------------------------------------------------------------
Prx5037Fly         ---------------------------MSFVAR-------------SLIRNVPLMGKAIL 20
TrxPrx3Fly         ---------------------------MSFVAR-------------SLIRNVPLMGKAIL 20
Prx6Human          ------------------------------------------------------------
Prx6Mouse          ------------------------------------------------------------
Prx6Chicken        ------------------------------------------------------------
Prx6Xenopus        ------------------------------------------------------------
Prx6Zebrafish      ------------------------------------------------------------
Prx6005            ------------------------------------------------------------
Prx2540Fly         ------------------------------------------------------------
Prx5Human          ------------------------------------------------------------
Prx5Mouse          -------------------------------------MLQLGLRVLGCKASSVLR--AST 21
Prx5Zebrafish      ------------------------------------------------------------
Prx5Fly            ---------------------------------------------MRV-LSCKFLGRVVN 14
Prx5Xenopus        -------------------------------------------MALRIPVSFLLLSPLRA 17

Prx3Human          LTNLLCSGS--------SQAK-LFSTSSSCHAPAVTQHAPYFKGTAVV-NGEFKDLSLDD 89
Prx3Mouse          LTDILWSAS--------AQGKSAFSTSSSFHTPAVTQHAPYFKGTAVV-NGEFKELSLDD 90
Prx3Xenopus        VTPSICAA-----------QKLQFSTSSGRFLPAVTQHAPQFKGTAVV-NGEFKELSLED 85
Prx3Chicken        LRRAVPAAGRTLTAQPLLCARRRLTLGASRLAPAVTQHAPFFKGTAVV-NGEFKELTLDD 119
Prx3Zebrafish      QPLACIAA-----------QKACFSISAARWAPAVTQAAPHFKGTAVI-NGEFKEISLGD 84
Prx1Human          -----------------------MSS----GNAKIGHPAPNFKATAVMPDGQFKDISLSD 33
Prx1Mouse          -----------------------MSS----GNAKIGYPAPNFKATAVMPDGQFKDISLSE 33
Prx1Chicken        -----------------------MSS----GKAFIGKPAPDFTATAVMPDGQFKDIKLSD 33
Prx2Chicken        -----------------------MSS----GKAFIGKPAPDFTATAVMPDGQFKDIKLSD 33
Prx1Xenopus        -----------------------MSV----GNAKIGQPAPDFTAQAVMPDGQFKNLKISD 33
Prx1Zebrafish      -----------------------MAA----GNAHIGKPAPDFTAKAVMPDGQFGDVRLSD 33
Prx2Zebrafish      -----------------------MSA----GNAKIGQPAPQFKATAVV-DGQFKDIQLSD 32
Prx2Human          -----------------------MAS----GNARIGKPAPDFKATAVV-DGAFKEVKLSD 32
Prx2Mouse          -----------------------MAS----GNAQIGKSAPDFTATAVV-DGAFKEIKLSD 32
Prx2Xenopus        -----------------------MACPVRAVKTHIGKPSPAFQATALV-NGEFKEIQLSD 36
Prx4Human          QVYPGEASR-----------VSVADHSLHLSKAKISKPAPYWEGTAVI-DGEFKELKLTD 105
Prx4Mouse          QVYPGEASR-----------VSVADHSLHLSKAKISKPAPYWEGTAVI-NGEFKELKLTD 108
Prx4Chicken        QVYPGEAAR-----------VPVTDHSLHLSQAKISKPAPYWEGTAVI-NGEFKELKLTD 99
Prx4Xenopus        QVYPGEASR-----------VPVSDHSLHLSKAKISKPAPYWEGTAVI-NGEFKELKLTD 101
Prx4Zebrafish      HVYPGEAFR-----------VPVSDHSLHLSKAKISKPAPHWEGTAVI-NGEFKELKLSD 94
TrxPrx2Fly         SVYPDQPK---------------GDHQLQYTKAVISKPAPQFEGTAVV-NKEIVKLSLSQ 76
TrxPrx1Fly         -------------------------------MPQLQKPAPAFAGTAVV-NGVFKDIKLSD 28
Prx5037Fly         SQQKQIAAR-------------LLHQTAPLAAVRVQQPAPDFKGLAVV-DNSFQEVKLED 66
TrxPrx3Fly         SQQKQIAAR-------------LLHQTAPLAAVRVQQPAPDFKGLAVV-DNSFQEVKLED 66
Prx6Human          ---------------------------MPGG-LLLGDVAPNFEANTTV-----GRIRFHD 27
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```
Prx6Mouse        ---------------------------MPGG-LLLGDEAPNFEANTTI-----GRIRFHD 27
Prx6Chicken      ---------------------------MPG--LLLGDEAPNFEADTTQ-----GGIRFHD 26
Prx6Xenopus      ---------------------------MPG--ILLGDVFPNFEADTTI-----GRIKFHD 26
Prx6Zebrafish    ---------------------------MPG--ILLGDVFPNFEADTTI-----GKIKFHE 26
Prx6005          ---------------------------MSGKALNIGDQFPNFTAETSE-----GRIDFYD 28
Prx2540Fly       --------------------------------MRLGQTVPNFEADTTK-----GPIKFHE 23
Prx5Human        --------------------------MGLAGVCALRRSAGYILVGGAGGQSAAAAARRCS 34
Prx5Mouse        CLAGRAGR--KEAGWECGGARSFSSSAVTMAPIKVGDAIPSVEVFEGE---PGKKVNLAE 76
Prx5Zebrafish    --MP----------------------------IKVGQRLPAVEVQEED---PGNSLSMAE 27
Prx5Fly          SALPQQII------------SLRSLSKTSAAMVKVGDSLPSVDLFEDS---PANKINTGD 59
Prx5Xenopus      VASP-------------------ASRTRAMSIKVGDQLPNVQVYEGG---PGNKVNIRD 54

Prx3Human        FKG-KYLVLFFYPLDFTFVCP-TEIVAFSDKANEFHDV--NCEVVAVSVDSHFSHLAWIN 145
Prx3Mouse        FKG-KYLVLFFYPLDFTFVCP-TEIVAFSDKANEFHDV--NCEVVAVSVDSHFSHLAWIN 146
Prx3Xenopus      FKG-KYLVLFFYPLDFTFVCP-TEIVAFSNEANEFHDV--NCEVVAVSVDSHFCHLAWTN 141
Prx3Chicken      FKG-KYLVLFFYPLDFTFVCP-TEIVAFSNKANEFHDV--NCEVVAVSVDSHFCHLAWIN 175
Prx3Zebrafish    FKG-KYLVLFFYPLDFTFVCP-TEIVAFSDKANEFHDV--NCAVVGVSVDSHFTHLAWTN 140
Prx1Human        YKG-KYVVFFFYPLDFTFVCP-TEIIAFSDRAEEFKKL--NCQVIGASVDSHFCHLAWVN 89
Prx1Mouse        YKG-KYVVFFFYPLDFTFVCP-TEIIAFSDRADEFKKL--NCQVIGASVDSHFCHLAWIN 89
Prx1Chicken      YRG-KYVVFFFYPLDFTFVCP-TEIIAYSDRADEFKKI--NCEIIGASVDSHFCHLAWIN 89
Prx2Chicken      YRG-KYVVFFFYPLDFTFVCP-TEIIAYSDRADEFKKI--NCEIIGASVDSHFCHLAWIN 89
Prx1Xenopus      YKG-KYVVFFFYPLDFTFVCP-TEIIAFSDRVEEFKKI--NCEVIGASGDSHYCHLAWIN 89
Prx1Zebrafish    YKG-KYVVLFFYPLDFTFVCP-TEIIAFSDAAEGFRKI--NCEIIGASVDSHFCHLAWTK 89
Prx2Zebrafish    YRG-KYVVLFFYPLDFTFVCP-TEIIAFSERAAEFRKI--GVELIAASTDSHFSHLAWIN 88
Prx2Human        YKG-KYVVLFFYPLDFTFVCP-TEIIAFSNRAEDFRKL--GCEVLGVSVDSQFTHLAWIN 88
Prx2Mouse        YRG-KYVVLFFYPLDFTFVCP-TEIIAFSDHAEDFRKL--GCEVLGVSVDSQFTHLAWIN 88
Prx2Xenopus      YLG-KYVVLFFYPLDFTFVCP-TEIIAFSNHAEDFKKI--NCQLIAVSVDSQFTHLAWTK 92
Prx4Human        YRG-KYLVFFFYPLDFTFVCP-TEIIAFGDRLEEFRSI--NTEVVACSVDSQFTHLAWIN 161
Prx4Mouse        YRG-KYLVFFFYPLDFTFVCP-TEIIAFGDRIEEFKSI--NTEVVACSVDSQFTHLAWIN 164
Prx4Chicken      YEG-KYLVFFFYPLDFTFVCP-TEIIAFSDRIEEFRAI--NTEVVACSVDSKFTHLAWIN 155
Prx4Xenopus      YKG-KYLVFFFYPLDFTFVCP-TEIIAFGDRIEEFRSI--NTEVVACSVDSQFTHLAWIN 157
Prx4Zebrafish    YKG-KYLVFFFYPLDFTFVCP-TEIIAFSDRVHEFQAI--NAEVVACSVDSQFTHLAWIN 150
TrxPrx2Fly       YLG-KYVVLLFYPLDFTFVCP-TEIIAFSDRIAEFKKI--KTEVIGVSVDSHFTHLAWIN 132
TrxPrx1Fly       YKG-KYLVLFFYPLDFTFVCP-TEIIAFSESAAEFRKI--NCEVIGCSTDSQFTHLAWIN 84
Prx5037Fly       YRG-KYLVLFFYPLDFTFVCP-TEIVAFSERIKEFHDI--NTEVLGVSVDSHFSHLTWCN 122
TrxPrx3Fly       YRG-KYLVLFFYPLDFTFVCP-TEIVAFSERIKEFHDI--NTEVLGVSVDSHFSHLTWCN 122
Prx6Human        FLGDSWGILFSHPRDFTPVCT-TELGRAAKLAPEFAKR--NVKLIALSIDSVEDHLAWSK 84
Prx6Mouse        FLGDSWGILFSHPRDFTPVCT-TELGRAAKLAPEFAKR--NVKLIALSIDSVEDHLAWSK 84
Prx6Chicken      FLGDSWGILFSHPRDFTPVCT-TELGRAAKLAPEFSKR--NVKMIALSIDSVPDHLAWSK 83
Prx6Xenopus      FLGNSWGVLFSHPRDYTPVCT-TELGRCVKLAPEFKKR--NVSMIALSIDSVEDHLGWSK 83
Prx6Zebrafish    FLGNSWGILFSHPRDFTPVCT-TELARAAKLHEEFKKR--DVKMIALSIDSVEDHRKWSE 83
Prx6005          WMQDSWAILFSHPADFTPVCT-TELSRVAALIPEFQKR--GVKPIALSCDTVESHKGWIE 85
Prx2540Fly       WQGNSWVVLFSHPADFTPVCT-TELGRIAVHQPEFAKR--NTKCLAHSVDALNSHVDWVN 80
Prx5Human        EGEWASGGVRSFSRAAAAMAP-IKVGDAIPAVEVFEGE--PGNKVNLAELFKGKKGVLFG 91
Prx5Mouse        LFKGKKGVLFGVPGAFTPGCSKTHLPGFVEQAGALKAK-GAQVVACLSVNDVFVIEEWGR 135
Prx5Zebrafish    LFSCKRGVLFGVPGAFTPGCSKTHLPGFIQMAGELRAK-GVDEVACISVNDVFVMSAWGK 86
Prx5Fly          LVNGKKVIIFGVPGAFTPGCSKTHLPGYVSSADELKSKQGVDEIVCVSVNDPFVMSAWGK 119
Prx5Xenopus      LFTNKKGVLFGVPGAFTPGCSKTHLPGYVAQAAELKSR-GAAVVACISVNDVFVVSEWGK 113

Prx3Human        TPRKNGG---LGHMNIALLSDLTKQISRDYGVLLEGS------GLALRGLFIIDPNGVIK 196
Prx3Mouse        TPRKNGG---LGHMNITLLSDITKQISRDYGVLLESA------GIALRGLFIIDPNGVVK 197
Prx3Xenopus      TPRKSGG---LGQMNIPLLSDLNKQISRDYGVLLETA------GIALRGLFIIDPNGIIK 192
Prx3Chicken      TPRKSGG---LGKMNIPVLSDLTKQISRDYGVLLEGP------GIALRGLFIIDPNGIIK 226
Prx3Zebrafish    TPRKSGG---LGKIQIPLLADLTKQVSRDYGVLLEGP------GIALRGLFIIDPNGIVR 191
Prx1Human        TPKKQGG---LGPMNIPLVSDPKRTIAQDYGVLKADE------GISFRGLFIIDDKGILR 140
Prx1Mouse        TPKKQGG---LGPMNIPLISDPKRTIAQDYGVLKADE------GISFRGLFIIDDKGILR 140
Prx1Chicken      TPKKQGG---LGTMKIPLVSDTKRVIAKDYGVLKEDE------GIAYRGLFIIDEKGILR 140
Prx2Chicken      TPKKQGG---LGTMKIPLVSDTKRVIAKDYGVLKEDE------GIAYRGLFIIDEKGILR 140
Prx1Xenopus      QPRKEGG---LGQMKIPLVADVQHTIAKDYGVFKEDD------GVSFRGLFIIDEKGILR 140
Prx1Zebrafish    TPRKQGG---LGPMNVPLVADTLRSISKDYGVLKEDE------GIAYRGLFIIDDKGILR 140
Prx2Zebrafish    TPRKQGG---LGSMNIPLVADLTQSISRDYGVLKEDE------GIAYRGLFVIDDKGILR 139
Prx2Human        TPRKEGG---LGPLNIPLLADVTRRLSEDYGVLKTDE------GIAYRGLFIIDGKGVLR 139
Prx2Mouse        TPRKEGG---LGPLNIPLLADVTKSLSQNYGVLKNDE------GIAYRGLFIIDAKGVLR 139
Prx2Xenopus      VPRKEGG---LGPVNIPLVSDLTHSIAKDYGVLKEED------GVAYRGLFIIDGKGILR 143
Prx4Human        TPRRQGG---LGPIRIPLLSDLTHQISKDYGVYLEDS------GHTLRGLFIIDDKGILR 212
Prx4Mouse        TPRRQGG---LGPIRIPLLSDLNHQISKDYGVYLEDS------GHTLRGLFIIDDKGVLR 215
Prx4Chicken      TPRKQGG---LGPMKIPLLSDLTHQISKDYGVYLEDQ------GHALRGLFIIDDKRILR 206
Prx4Xenopus      TPRKQGG---LGPMKIPLLSDLTHQISKDYGVYLEDQ------GHTLRGLFIIDDKGVLR 208
Prx4Zebrafish    TPRKQGG---LGPMKIPLLSDLTHQISKDYGVFLEDQ------GHTLRGLFIIDDKGVLR 201
TrxPrx2Fly       TPRKEGG---LGDVKIPLLSDLTHKISKDYGVYLESS------GHALRGLFIIDQTGVLR 183
TrxPrx1Fly       TPRKQGG---LGSMDIPLLADKSMKVARDYGVLDEET------GIPFRGLFIIDDKQNLR 135
Prx5037Fly       VDRKNGG---VGQLKYPLLSDLTKKISADYDVLLDKE------GISLRGTFIIDPNGILR 173
TrxPrx3Fly       VDRKNGG---VGQLKYPLLSDLTKKISADYDVLLDKE------GISLRGTFIIDPNGILR 173
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Prx6Human        DINAYNCEEPTEKLPFPIIDDRNRELAILLGMLDPAEKDEKGMPVTARVVFVFGPDKKLK 144
Prx6Mouse        DINAYNGETPTEKLPFPIIDDKGRDLAILLGMLDPVEKDANNMPVTARVVFIFGPDKKLK 144
Prx6Chicken      DINAYNGDQPVEKLPFPIIADKDRELAVKLGMLDPDERDKDGMPLTARVVFIFGPDKKLK 143
Prx6Xenopus      DINSYNCDEPTETLPFPIIADPKRELAVQLGMLDPDEKDMQGMPVTARCVFIIGPDKKMK 143
Prx6Zebrafish    DILAFNQDKACCPMPFPIIADDKRELSVLLGMLDPDERDKDGMPLTARCVFVVGPDKRLK 143
Prx6005          DIKSFG---KLSSFDYPIIADDKRELALKFNMLDKDEINAEGIPLTCRAVFVVDDKKKLR 142
Prx2540Fly       DIKSYCLD-IPGDFPYPIIADPTRDLAVSLGMLDEEQKKDPEVGKTIRALFIISPDHKVR 139
Prx5Human        VPGAFTP----GCSKVRLLADPTGAFGKETDLLLDDS-----------LVSIFGNRRLKR 136
Prx5Mouse        AHQAEG--------KVRLLADPTGAFGKATDLLLDDS-----------LVSLFGNRRLKR 176
Prx5Zebrafish    QNGADG--------KVRMLADPTGAFTKAVDLVLNNAQ----------LIPVLGNLRSQR 128
Prx5Fly          EHGAAG--------KVRLLADPAGGFTKALDVTID--------------LPPLGGVRSKR 157
Prx5Xenopus      VHEAEG--------KVCMLADPCGEFAKACGLLLDKKE----------LSELFGNQRCKR 155

Prx3Human        HLSVNDLPVGRSVEETLRLVKAFQYVE-THGEVCPANWTPDSP--TIKPSP--AASKEYF 251
Prx3Mouse        HLSVNDLPVGRSVEETLRLVKAFQFVE-THGEVCPANWTPESP--TIKPSP--TASKEYF 252
Prx3Xenopus      HMSVNDLPVGRSVEETLRLVKAFQFVE-THGEVCPANWTPDSP--TIKPSP--EGSKDYF 247
Prx3Chicken      HLSINDLPVGRSVEETLRLVKAFQYVE-THGEVCPANWTPDSP--TIKPSP--EASKEYF 281
Prx3Zebrafish    HMSVNDLPVGRSVEETLRLVKAFQFVE-THGEVCPASWTPKSP--TIKPTP--DGSKEYF 246
Prx1Human        QITVNDLPVGRSVDETLRLVQAFQFTD-KHGEVCPAGWKPGSD--TIKPDV--QKSKEYF 195
Prx1Mouse        QITINDLPVGRSVDEIIRLVQAFQFTD-KHGEVCPAGWKPGSD--TIKPDV--NKSKEYF 195
Prx1Chicken      QITINDLPVGRSVDETLRLVQAFQFTD-KHGEVCPAGWKPGSD--TIKPDV--QKSKEYF 195
Prx2Chicken      QITINDLPVGRSVDETLRLVQAFQFTD-KHGEVCPAGWKPGSD--TIKPDV--QKSKEYF 195
Prx1Xenopus      QITINDLPVGRSVDETLRLVQAFQFTD-KYGEVCPAGWQPGSD--TIKPDV--QKSKEYF 195
Prx1Zebrafish    QITINDLPVGRSIDETLRLVQAFQFTD-KHGEVCPAGWKPGKD--TIKPDV--NQSKDFF 195
Prx2Zebrafish    QITINDLPVGRSVDETLRLVQAFQHTD-KYGEVCPAGWKPGSD--TIVPDV--QKSKEFF 194
Prx2Human        QITVNDLPVGRSVDEALRLVQAFQYTD-EHGEVCPAGWKPGSD--TIKPNV--DDSKEYF 194
Prx2Mouse        QITVNDLPVGRSVDEALRLVQAFQYTD-EHGEVCPAGWKPGSD--TIKPNV--DDSKEYF 194
Prx2Xenopus      QITINDLPVGRSVDETLRLVQAFQYTD-VHGEVCPAGWKPGSS--IIKPNV--KDSKEFF 198
Prx4Human        QITLNDLPVGRSVDETLRLVQAFQYTD-KHGEVCPAGWKPGSE--TIIPDP--AGKLKYF 267
Prx4Mouse        QITLNDLPVGRSVDETLRLVQAFQYTD-KHGEVCPAGWKPGSE--TIIPDP--AGKLKYF 270
Prx4Chicken      QITMNDLPVGRSVDETLRLVQAFQYTD-KHGEVCPAGWKPGSE--TIIPDP--AGKLKYF 261
Prx4Xenopus      QITMNDLPVGRSVDETLRLVQAFQYTD-KHGEVCPAGWKPGSE--TIIPDP--AGKLKYF 263
Prx4Zebrafish    QITMNDLPVGRSVDETLRLVQAFQYTD-KHGEVCPAGWKPGSD--TIIPDP--AGKLKYF 256
TrxPrx2Fly       QITMNDLPVGRSVDETIRLVQAFQYTD-THGEVCPAGWRPGAD--TIVPNP--EEKTKYF 238
TrxPrx1Fly       QITVNDLPVGRSVEETLRLVQAFQYTD-KYGEVCPANWKPGQK--TMVADP--TKSKEYF 190
Prx5037Fly       QYSINDLPVGRSVDEVLRLIKAFQFVE-QHGEVCPANWNPNSNPATIKPDV--EESKKYF 230
TrxPrx3Fly       QYSINDLPVGRSVDEVLRLIKAFQFVE-QHGEVCPANWNPNSNPATIKPDV--EESKKYF 230
Prx6Human        LSILYPATTGRNFDEILRVVISLQLTA-EKRVATPVDWKDGDS-VMVLPTIPEEEAKKLF 202
Prx6Mouse        LSILYPATTGRNFDEILRVVDSLQLTG-TKPVATPVDWKKGES-VMVVPTLSEEEAKQCF 202
Prx6Chicken      LSILYPATTGRNFDEILRVVDSLQLTA-YKKVATPVDWKCGDS-VMVVPTLPDEEAKKLF 201
Prx6Xenopus      LSILYPATTGRNFDEILRVVDSLQLTA-VHNVATPVDWKPGDR-VMVPPNVPEEEASKIF 201
Prx6Zebrafish    LSILYPATTGRNFDEILRVVDSLQLTA-TKKVATPVDWKPGQE-VMVIPSLSDEEANKLF 201
Prx6005          LSILYPATTGRNFDEILRVIDSLQLTQ-TKSVATPADWKQGGK-CMVLPTVKAEDVPKLF 200
Prx2540Fly       LSMFYPMSTGRNVDEILRTIDSLQLTDRLKVVATPANWTPGTK-VMILPTVTDEEAHKLF 198
Prx5Human        FSMVVQDGI----------VKALNVEPDGTGLTCSLAPNIISQL---------------- 170
Prx5Mouse        FSMVIDNGI----------VKALNVEPDGTGLTCSLAPNILSQL---------------- 210
Prx5Zebrafish    YAMLIENGV----------VTKLSVEPDGTGLTCSLASNFLAEV---------------- 162
Prx5Fly          YSLVVENGK----------VTELNVEPDGTGLSCSLANNIGKK----------------- 190
Prx5Xenopus      FSMVVEDGK----------IKAINVEEDGTGLTCSLAGNIISQL---------------- 189

Prx3Human        QKVNQ------------------ 256
Prx3Mouse        EKVHQ------------------ 257
Prx3Xenopus      EKVH------------------- 251
Prx3Chicken      EKVHT------------------ 286
Prx3Zebrafish    EKVN------------------- 250
Prx1Human        SKQK------------------- 199
Prx1Mouse        SKQK------------------- 199
Prx1Chicken      SKQK------------------- 199
Prx2Chicken      SKQK------------------- 199
Prx1Xenopus      SKQK------------------- 199
Prx1Zebrafish    SKQN------------------- 199
Prx2Zebrafish    SKQ-------------------- 197
Prx2Human        SKHN------------------- 198
Prx2Mouse        SKHN------------------- 198
Prx2Xenopus      SKEY------------------- 202
Prx4Human        DKLN------------------- 271
Prx4Mouse        DKLN------------------- 274
Prx4Chicken      DKLN------------------- 265
Prx4Xenopus      DKQH------------------- 267
Prx4Zebrafish    DKLN------------------- 260
TrxPrx2Fly       AKNN------------------- 242
TrxPrx1Fly       ETTS------------------- 194
Prx5037Fly       SKHG------------------- 234
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TrxPrx3Fly          SKHG------------------- 234
Prx6Human           PKGVFTKELPSGKKYLRYTPQP- 224
Prx6Mouse           PKGVFTKELPSGKKYLRYTPQP- 224
Prx6Chicken         PKGVFTKDLPSGKKYLRYTPQPE 224
Prx6Xenopus         TCGVFTKELPSGKKYLRYTAQPQ 224
Prx6Zebrafish       PAGFTLKEVPSGKKYIRYT-KP- 222
Prx6005             PDGIETIELPSGKSYLRITPQP- 222
Prx2540Fly          PKGFDKVSMPSGVNYVRTTDNY- 220
Prx5Human           -----------------------
Prx5Mouse           -----------------------
Prx5Zebrafish       -----------------------
Prx5Fly             -----------------------
Prx5Xenopus         -----------------------
```

Appendix 4

Whole mount *in situ* hybridization protocol

Day 1: Probe Hybridization

From embryos fixed in Mempfa and stored in 100% Methanol @ -20
(**all steps are done at room temp unless otherwise noted**)

1 x 5 min	75% Methanol : 25% Ttw	
1 x 5 min	50% Methanol : 50% Ttw	
1 x 5 min	25% Methanol : 25% Ttw	
2 x 5 min	100% Ttw	
2 x 5 min	Triethanolamine	(0.74g in 40mls dH20, pH 7.8) Add Acetic Anhydride to 2nd wash
2 x 5 min	5 µl Acetic Anhydride in Triethanolamine	Do not go too long with this step or embryos will degrade and disintegrate on second day
5 x 5 min	Ttw	
1 x 10 min	50% RNA hybridization buffer; 50% Ttw	
1 x 10 min	RNA hybridization buffer	
1 x 1 **hour**	RNA hybridization buffer	**@ 65 °** If you have time you tend to get lower background if you go a little bit more than an hour (ie. 1.5-2hours)
Overnight	Probe in hybridization buffer	@ 65 °

Day 2: Antibody

Remove Probe and return to tubes (*Probe can be reused many times-for whole mounts anyway*)

1 x 10 min	100% RNA hybridization buffer	@ 65 °

1 x 10 min	50% RNA hybridization buffer : 50% 2X SSC	@ 65 °
1 x 10 min	25% RNA hybridization buffer : 75% 2X SSC	@ 65 °
2 x 30 min	100% 2X SSC	**@ 37 °**
3 x 45 min	0.2X SSC	**@ 65 °**
1 x 10 min	Ttw	RT
1 x 10 min	TBT	RT
1 x 1 **hour**	20% Sheep Serum in TBT	Blocking Heat Treat Serum
Overnight	Anti-DIG antibody	Antibody 1 μl antibody per 5 mls Blocking Solution as above @ 4 °

Day 3: Colour Reaction

12 x 30 min	TBT	RT
1 x 10 min	Alkaline Phosphatase Buffer	
------	Precipitating Substrate	(BM Purple or NBT/BCIP in AP Buffer) Leave until desired intensity is reached

Day 4: Fixing and Bleaching

When desired intensity of strain is reached:

1 x 5 min	25% Methanol : 75%
1 x 5 min	50% Methanol : 50%
1 x 5 min	75% Methanol : 25%
1 x 5 min	100% Methanol

1 x 5 min	75% Methanol : 25%	
1 x 5 min	50% Methanol : 50%	
1 x 5 min	25% Methanol : 75%	
1 x 20 min	MEMPFA	
3 x 5 min	Ttw	
1 x 2 hours	Bleaching Solution	2.5 mls Formamide, 1.25 mls 20% SSC, 0.5 mls H202, dH2O to 50 mls Bleach for 2 hours or until acceptable contrast between embryo and stain has been reached.
3 x 5 min	Ttw	
------	1 X PBS	For imaging. Embryos can be stored for a few days in 1 x PBS @ 4 ° before imaging, but longer term storage dehydrate into 100% methanol and store @ -20 °

Whole mount *in situ* hybridization solutions

Hybridization Buffer

All concentrations equal final concentration in 100 mL

50% Formamide	50 mL of Formamide
5 x SSC	25 mL of 20 x SSC
1 mg/mL Torula RNA	2 mL of 50 mg/mL Torula RNA
1 x Denhart's	1 mL of 100 x Denhart's
100 ug/mL Heparin	10 mg Heparin
0.1% Tween 20	0.1 mL Tween 20
5 mM EDTA	5 mL 0.2 M EDTA

20 x SSC (1 L)

175.3g NaCl
88.2g Na Citrate
pH to 7.0

1 x TBS (1 L)

6.1g TRIS
9g NaCl
pH to 8.4

TTw is TBS + 0.1% Tween 20

TBT is 0.1% Tween 20, 1% BSA in TBS

Alkaline Phosphatase Buffer

100 mM Tris (pH 9.5)

50 mM $MgCl_2$

100 mM NaCl

0.1% Tween 20

2 mM levamisol

Keep aliquots frozen at –20 with levamisol.

Researcher: Dr. S. Damjanovski

Biosafety Approval Number: BIO-UWO-0141

Expiry Date: March 28, 2011

July 21, 2008

Dear Dr. Damjanovski:

Please note your biosafety approval number listed above. This number is very useful to you as a researcher working with biohazards. It is a requirement for your research grants, purchasing of biohazardous materials and Level 2 inspections.

Research Grants:
- This number is required information for any research grants involving biohazards. Please provide this number to Research Services when requested.

Purchasing Materials:
- This number must be included on purchase orders for Level 1 or Level 2 biohazards. When you order biohazardous material, use the on-line purchase ordering system (www.uwo.ca/finance/people/). In the "Comments to Purchasing" tab, include your name as the Researcher and your biosafety approval number.

Annual Inspections:
- If you have a Level 2 laboratory on campus, you are inspected every year. This is your permit number to allow you to work with Level 2 biohazards.

To maintain your Biosafety Approval, you need to:

- Ensure that you update your Biohazardous Agents Registry Form at least every three years, or when there are changes to the biohazards you are working with.
- Ensure that the people working in your laboratory are trained in Biosafety.
- Ensure that your laboratory follows the University of Western Ontario Biosafety Guidelines and Procedures Manual for Containment Level 1 & 2 Laboratories.
- For more information, please see: www.uwo.ca/humanresources/biosafety

Please let me know if you have questions or comments.

Regards,

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Fax: 519-661-3420