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The role of Pex11-beta in peroxisome biogenesis, intracellular relationship to reactive oxygen species levels and redox-sensitive cell signaling

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Graduate Program in Biology
A thesis submitted in partial fulfillment of the requirements for the degree in Doctor of Philosophy

THE ROLE OF PEX11-BETA IN PEROXISOME BIOGENESIS, INTRACELLULAR RELATIONSHIP TO REACTIVE OXYGEN SPECIES LEVELS AND REDOX-SENSITIVE CELL SIGNALING

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ABSTRACT

Peroxisomes are organelles whose roles in fatty acid metabolism and reactive oxygen species (ROS) elimination have contributed much attention in understanding their origin and biogenesis. Many studies have shown that *de novo* peroxisome biogenesis is an important regulatory process, while yeast studies suggest that total peroxisome numbers are in part regulated by proteins such as Pex11, which can facilitate the division of existing peroxisomes. Although *de novo* biogenesis and divisions are likely important mechanisms to peroxisome functioning, the regulation of peroxisome numbers during embryonic development is poorly understood. Peroxisome number and function are particularly crucial in oviparous animals such as frogs where large embryonic yolk and fatty acid stores must be quickly metabolized, and ROS eliminated. The central role of peroxisomes with respect to ROS is in the generation and scavenging of hydrogen peroxide. Recent studies have revealed their involvement in metabolism of oxygen free radicals that have important functions in cell signaling. Using *Xenopus laevis* as a developmental model, this study demonstrates that overexpression and inhibition of Pex11β directly increases and decreases peroxisome number *in vitro*, and induces an early- or delayed-onset to peroxisome biogenesis *in vivo*, respectively. Knockdown of Pex11β, decreasing peroxisome numbers, induced a bent/double-axis phenotype compared to that of control uninjected embryos. This phenotype has previously been linked to increases in the

redox sensitive-noncanonical Wnt/Planar Cell Polarity (PCP) cell signaling. As a result, this study investigated if changes in peroxisome number could affect intracellular ROS levels, thereby activating redox-sensitive cell signaling pathways such as canonical and noncanonical Wnt signaling. Following inhibition of Pex11β, there were significant increases in ROS levels in *X. laevis* A6 cells. I show for the very first time that changes in cellular ROS levels, as a result of decreases in peroxisome numbers, perturb noncanonical Wnt cell signaling.

KEYWORDS

Peroxisome; Peroxisome division; Peroxisome biogenesis factor; Pex11β; PMP70; Peroxisome proliferator activated receptor; Reactive oxygen species; Catalase; Mitochondria, Hydrogen peroxide; Redox signaling; Wnt/Planar Cell Polarity; Nucleoredoxin; Dishevelled; *Xenopus laevis*

EPIGRAPH

"The only thing that interferes with my learning is my education."

- Albert Einstein

DEDICATION

to those who believe in me

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LIST OF ABBREVIATIONS

AAA-ATPases	ATPases associated with diverse cellular activities
An	animal
ANOVA	analysis of variance
ATG	autophagy related genes
ATP	adenosine triphosphate
ALD	adrenoleukodystrophy
bp	base pair
BCFA	branched chain fatty acid
cDNA	complementary DNA
Cys	cystein
DAPI	4',6-diamidino-2-phenylindole
DLP	dynamin like proteins
DNA	deoxyribonucleic acid
DIG	digoxigenin
Dvl	dishevelled
E1	ubiquitin-activating enzyme
E2	ubiquitin-conjugating enzyme
E3	ubiquitin ligase
EP45	estrogen regulated protein 45 kDa
ER	endoplasmic reticulum
EV	empty vector
FBS	fetal bovine serum

FOV	field of view
Fzd	frizzled
GFP	green fluorescent protein
H_2O_2	hydrogen peroxide
hfp	hours post fertilization
ICC	immunocytochemistry
IHC	immunohistochemistry
ISH	in situ hybridization
IRF	infantile Refsum disease
JNK	c-Jun N-terminal kinase
LCFA	long chain fatty acid
KANL	lysine-alanine-asparagine-leucine
Lef	lymphoid enhancing factor
MBT	mid-blastula transition
MCFA	medium chain fatty acids
MIPA	micropexophagic membrane apparatus
MMR	Mark's modified ringer
mPTS	membrane protein targeting signal
mRNA	messenger ribonucleic acid
NADH	nicotinamide adenine dinucleotide
NADPH	nicotinamide adenine dinucleotide phosphate
Nox	NADPH oxidase
NR	nuclear receptors

Nrx	nucleoredoxin
O_2^-	superoxide anion
PBS	phosphate buffer saline
PCR	polymerase chain reaction
PCP	planar cell polarity
PBD	peroxisome biogenesis disorder
PEX	peroxisome biogenesis factors
pf	post fertilization
PFA	paraformaldehyde
pH	power of hydrogen
PM	plasma membrane
PMP	peroxisome membrane protein
PP	pexophagosome
PPAR	peroxisome proliferator-activated receptor
PPRE	peroxisome proliferator response element
Prdx	peroxiredoxin
PTS	peroxisome targeting signal
RADAR	receptor accumulation and degradation in the absence of recycling
RCDP	rhizomelic chondrodysplasia punctata
Redox	reduction-oxidation
RING	really interesting new genes
RNA	ribonucleic acid
RNS	reactive nitrogen species

ROS	reactive oxygen species
RSSR	disulphide bond
RT	reverse transcriptase
RXR	retinoid-X-receptor
RT-PCR	reverse transcriptase polymerase chain reaction
SDS	sodium dodecyl sulphate
SEM	standard error of mean
SH	thiol
SKL	serine-lysine-leucine
SOD	superoxide dismutase
SOH	sulphenic acid
SO_2H	sulphinic acid
SO_3H	sulphonic acid
SM	sequestration membrane
Tcf	T-cell factor
TF	transcription factor
TRP	tetratricopeptide
Trx	thioredoxin
VLCFA	very long chain fatty acid
VM	vacuolar membrane
Veg	vegetal
ZS	zellweger syndrome
ZSS	zellweger syndrome spectrum

Wnt	wingless

CHAPTER 1

GLOBAL INTRODUCTION AND LITERATURE REVIEW

1.1 Peroxisomes

The myriad of complex functions that are performed by multicellular organisms are carried out by individual cells. Cells in part coordinate their various activities through the presence or regulation of specialized or ubiquitous organelles, which thereby regulate metabolic activities, remove waste, and allow for cell communication and cell migration. Such is the case with peroxisomes, small ubiquitous organelles whose role in lipid metabolism belies their complexity.

1.1.1 Peroxisome overview

Peroxisomes are single membrane-bound subcellular organelles found ubiquitously in virtually all eukaryotic cells. They were first identified by Dr. Christian Du Duve in 1966 as spherical or ovoid cellular structures, ranging in sizes from 0.1 to 1.5 μm in diameter, and containing a fine granular matrix (De Duve and Baudhuin 1966). On average, cells contain roughly 400 peroxisomes occupying 2% of total cell volume. The single membrane of peroxisomes is quite unlike that of other organelles in terms of its permeability properties, mainly because it is more permeable to small molecules such as sucrose and inorganic ions. These unique membrane properties impart onto peroxisomes attributes that are key to their function.

Peroxisomes fulfill a variety of important roles that are essential for normal cells to maintain physiological functions, such as the metabolism of lipids and reactive oxygen (ROS), which are described below. Such vital activities render these organelles essential to the cell, and therefore to overall organismal health. In addition to these ubiquitous activities, peroxisomes have taken on several specialized functions unique to different

species including; glycolysis in protozoa; penicillin biosynthesis in fungi; plasmalogen biosynthesis in mammals, and photorespiration and the glyoxylate cycle in plants (Kurbatova, Dutova et al. 2005). Of particular importance, is the role of peroxisomes in the regulation of ROS. Consequently, they house multiple oxidative and non-oxidative enzymes involved the production and scavenging of ROS, such as catalase, superoxide dismutases (SOD) 1, peroxiredoxin (Prdx) 5, glutathione peroxidase (Gpx) and glutathione (GSH). Their complex antioxidant defense system has identified these organelles as a primary source of oxidative stress, aging and neurodegeneration when they become non-functional, or their numbers are aberrant (Kregel and Zhang 2007).

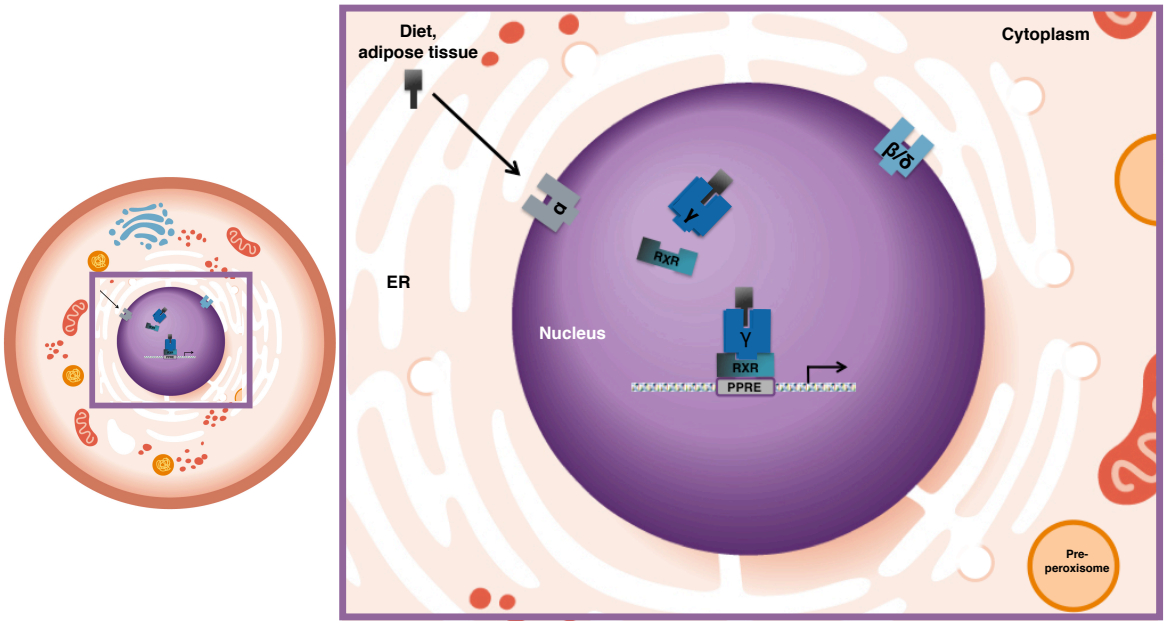
One of the more intriguing aspects of peroxisomes is how cells control the number of these organelles. Eukaryotic cells contain hundreds of peroxisomes under normal cellular conditions, but this number is typically in flux, suggesting there are mechanisms for regulating total peroxisome number. These numbers may change in response to metabolic demands, oxidative stress and extracellular stimuli, indicating the existence of signal transduction pathways that exert additional control over peroxisome numbers (Li and Gould 2002).

1.1.2 Peroxisome proliferator activator receptors (PPARs)

Peroxisome proliferator-activated receptors (PPARs) are a family of nuclear membrane receptors that behave as transcription factors and which upon activation control a variety of cellular and metabolic processes. PPARs were first identified in *Xenopus laevis*, as receptors capable of inducing peroxisome proliferation. PPARs are ligand-activated transcription factors and exist as three isoforms – PPARα, -β/δ and –γ,

all of which have been identified in vertebrates. Upon activation of PPARs, respective receptors form heterotypic dimers with retinoid-X-receptors (RXR) while binding to peroxisome proliferator response elements (PPRE) inducing transcription of genes associated with diverse cellular function (Figure 1.1). The biological functions of PPARα, -β/δ and -δ are distinct, yet all three PPARs individually affect inflammation and homeostasis (Goto, Lee et al. 2011). PPARα is primarily involved in regulating peroxisome proliferation and fatty acid metabolism via transcriptional activation of genes encoding key enzymes involved in metabolism (Finck 2007). PPARβ/δ can trigger adipocyte differentiation by inducing the expression of multiple genes involved in adipogenesis (Goto, Lee et al. 2011). Lastly, PPARδ has received much attention for its ability to regulate fatty acid storage and glucose metabolism, and its relationship to peroxisome number (Goto, Lee et al. 2011). The regulation of PPARs is complex, involving numerous potential ligands that regulate multiple genes (Alvarez-Guardia, Palomer et al. 2011). Overall, PPARs play essential roles in the regulation of cellular differentiation, development, metabolism, tumorigenesis, the number and overall function of peroxisomes and in the regulation of a family of proteins involved in peroxisomes functioning termed peroxisome biogenesis factors.

Figure 1.1. Peroxisome proliferator-activated receptors (PPARs). PPARs are a group of nuclear receptor proteins that function as transcription factors regulating the expression of a wide-variety of genes. PPARs play essential roles in the regulation of cellular differentiation, development and metabolism, peroxisome biogenesis and tumorigenesis. All PPARs heterodimerize with the retinoid X receptor (RXR) and bind to specific regions of DNA sequences termed peroxisome proliferator hormone response elements (PPREs).


Diet,
adipose tissue
Cytoplasm
α
γ
RXR
β/δ
ER
Nucleus
γ
RXR
PPRE
Pre-
peroxisome

1.1.3 Peroxisome biogenesis factors (Pex)

Both peroxisome biogenesis and the regulation of peroxisome number are achieved through the coordinated activity of a family of proteins termed peroxins. Peroxins are nuclear-encoded Pex genes, synthesized on free polyribosomes in the cytosol and post-translationally imported into the organelle (Table 1). Peroxins are found localized in the cytosol, the membrane and the matrix of peroxisomes, and the latter can be imported into the peroxisome matrix in a folded and/or even oligomeric form (Erdmann and Schliebs 2005). Peroxins are essential for function of peroxisomes, and the loss of function of various peroxins abrogates peroxisome formation. Such loss of function conditions are linked to a variety of human diseases termed peroxisome biogenesis disorders (PBD). Consequently, symptoms of these diseases often occur as a result of improper fatty acid metabolism.

Table 1. List of molecular mechanisms and functions of peroxisome biogenesis factors.

GENE	CHARACTERISTICS	BIOGENESIS FUNCTION	MOLECULAR MECHANISM
Pex1	AAA-type ATPase	Matrix protein import	ATP-dependent dislocation of Pex5p
Pex2	RING-finger	Matrix protein import	N/A
Pex3	N/A	PMP-targeting; *de novo* formation	Membrane anchor of Pex19p
Pex4	Ubc	Matrix protein import	Mono-ubiquitination of Pex5p
Pex5	WxxxF-motifs; TPR region, ubiquitinated	Matrix protein import	PTS1-receptor
Pex6	AAA-type ATPase	Matrix protein import	ATP-dependent dislocation of Pex5p
Pex7	WD40 motif	Matrix protein import	PTS2-receptor
Pex8	Coiled-coil domain; leu-zipper	Matrix protein import	Connection of docking- and RING-complex; cargo release (?)
Pex9	N/A	Matrix protein import	ORF of *Yl*PEX9 was misidentified; corresponds to *Hs*PEX26
Pex10	RING-finger	Matrix protein import	N/A
Pex11	N/A	Proliferation	Elongation of peroxisomes
Pex12	RING-finger	Matrix protein import	N/A
Pex13	SH3-domain	Matrix protein import	Member of the docking complex
Pex14	PXXP-motif; phosphorylated	Matrix protein import	Member of the docking complex
Pex15	Phosphorylated	Matrix protein import	Membrane anchor of Pex6p
Pex16	N/A	PMP-targeting; proliferation; *de novo* formation	N/A
Pex17	N/A	Matrix protein import	Member of the docking complex
Pex18	WxxxF-motifs; ubiquitinated	Matrix protein import	PTS2-co-receptor in *Sc*
Pex19	CAAX-box; farnesylated	PMP-targeting; *de novo* formation	PMP-class I receptor and chaperone
Pex20	WxxxF-motifs; ubiquitinated	Matrix protein import	PTS2-co-receptor in most fungi
Pex21	WxxxF-motifs;	Matrix protein	PTS2-co-receptor in *Sc*

	ubiquitinated (?)	import	
Pex22	N/A	Matrix protein import	Membrane anchor of Pex4p
Pex23	DysF	Proliferation	Growth regulation in *Yl*
Pex24	N/A	Proliferation	Separation of peroxisomes in *Yl*
Pex25	N/A	Proliferation	Elongation of peroxisomes
Pex26	N/A	Matrix protein import	Membrane anchor of Pex6p in Hs
Pex27	N/A	Proliferation	Elongation of peroxisomes
Pex28	N/A	Proliferation	Separation of peroxisomes in *Sc*
Pex29	N/A	Proliferation	Separation of peroxisomes in *Sc*
Pex30	DysF	Proliferation	Growth regulation in *Sc*
Pex31	DysF	Proliferation	Growth regulation in *Sc*
Pex32	DysF	Proliferation	Growth regulation in *Sc*

1.2 Physiological Functions of Peroxisomes

1.2.1 Fatty acid β-oxidation

Peroxisomes are the primary site of β-oxidation of very long chain (VLCFA) and long chain (LCFA) fatty acids (Mannaerts and van Veldhoven 1996). The byproducts of β-oxidation in the mitochondrion are fed into the Krebs cycle and electron transport chain to generate ATP, whereas β-oxidation in peroxisomes is not directly coupled to an energy generating system. Instead, it is believed acetyl-CoA, a byproduct of β-oxidation, is utilized as an entry molecule for the Krebs cycle (Mannaerts and van Veldhoven 1996).

Studies have indicated that β-oxidation levels appear to be involved in regulating peroxisome number. For instance, human fibroblast cells deficient in β-oxidation enzymes derived from patients with Zellweger Syndrome (ZS), a PBD, show a decreased number of peroxisomes (Chang, South et al. 1999). In addition to peroxisomal β-oxidation, the compartmentalization of this reaction to peroxisomes may serve to protect cells from high levels of oxidative damage. Repeated β-oxidation events result in the generation of high levels of ROS, which are quickly eradicated by enzymes within the peroxisome, protecting the rest of the cell from these deleterious byproducts.

1.2.2 Peroxisomes and reactive oxygen species (ROS)

Oxygen is consumed in various metabolic reactions in different cellular locations, such as mitochondria, endoplasmic reticulum (ER), and peroxisomes. Unlike mitochondria, β-oxidation in peroxisomes is not coupled to oxidative phosphorylation, and does not lead to the production of ATP. Rather, the high potential electrons are removed from various metabolites to reduce molecular oxygen, yielding hydrogen

peroxide (H_2O_2), superoxide anion ($O_2\cdot^-$) and the hydroxyl radical (·OH) (Antonenkov, Grunau et al. 2010). This supports the notion that peroxisomes play a key role in both the production and subsequent scavenging of ROS. The main physiological function of peroxisomes was at first assumed to be the decomposition of H_2O_2 into water and oxygen via catalase – a prototypical peroxisomal enzyme and marker. However, peroxisomes are involved in a variety of other ROS-related functions. Studies have estimated that about 40% of all H_2O_2 formed in rat liver is derived from peroxisomal oxidases (Boveris, Oshino et al. 1972). To regulate ROS produced in peroxisomes and to maintain the equilibrium between production and scavenging of ROS, peroxisomes harbor multiple antioxidant enzymes in addition to catalase. The detailed mechanisms of ROS metabolism are discussed further in Section 1.8.

1.2.3 Plasmalogen biosynthesis

Plasmalogen is a type of ether-phospholipid that is synthesized in peroxisomes and comprises an estimated 18% of the total phospholipids in mammals (Wanders and Waterham 2006). Plasmalogens are found in numerous human tissues, particularly in the nervous, immune, and cardiovascular systems. In human heart tissue, nearly 30-40% of phospholipids are plasmalogens. Similarly, almost 30% of the phospholipids in the adult human brain are plasmalogens, which compose up to 70% of the myelin sheath (Gorgas, Teigler et al. 2006). Consequently, the absence of plasmalogens results in irregularities in the myelination of nerve cells (along with other physiological defects), and is a large factor that is related to PBDs and their link to severe neurological abnormalities. Plasmalogen biosynthesis was first linked to peroxisomes following the

observations that infants suffering from Zellweger Syndrome displayed severe deficiencies in plasmalogen, caused by defects in peroxisome biogenesis. Although the functions of plasmalogens have not been elucidated, they have been linked to modulators of membrane dynamics (Nagan and Zoeller 2001).

1.3 Peroxisomal matrix protein biogenesis and insertion

1.3.1 Peroxisomal targeting signals and their receptors

Since peroxisomes do not contain DNA, all of their matrix and membrane proteins are nuclear-encoded and synthesized on polyribosomes in the cytosol. Peroxisomal matrix proteins are targeted to the peroxisomal lumen by two distinct peroxisomal targeting signals, PTS1 and PTS2.

PTS1 is a C-terminal tripeptide sequence, serine-lysine-leucine (SKL) and is found on the majority of matrix proteins, while its derivative, lysine-alanine-asparagine-leucine (KANL) is found on distinct peroxisomal matrix proteins such as catalase (Legakis, Koepke et al. 2002). The cytosolic molecular receptor, Pex5, is responsible for the recognition of posttranslational PTS1-containing proteins destined for the peroxisomal matrix. Pex5 contains a highly conserved C-terminal domain, composed of 5-7 tetratricopeptide (TPR) motifs (Williams, Schueller et al. 2011). These TPR domains interact with PTS1 on respective peroxisomal proteins. The Pex5 TPR domains undergo conformational changes, switching from an open to closed conformation following receptor-cargo binding and release, respectively. (Stanley and Wilmanns 2006). The N-terminus of Pex5 is less conserved and interacts with peroxins such as Pex8, Pex13 and

Pex14, which are involved in receptor-cargo docking on the peroxisomal membrane during cargo translocation. However, the extreme N-terminus is essential for Pex5 recycling following matrix protein import, a process that is dependent on either the mono- or polyubiquitination of Pex5, as described in section 1.3.4.

PTS2 is a nona-peptide sequence, R/K-L/V/I/Q-X_2-L/V/I/H/Q-L/S/G/A/K-X-H/Q-L/A/F, found near the N-terminus of a smaller subset of peroxisomal matrix proteins (Petriv, Tang et al. 2004). PTS2 is a less common targeting sequence. Import mechanisms associated with PTS2 are far less understood, but believed to follow a similar pathway as Pex5-PTS1 type cargoes. Delivery of PTS2 proteins to peroxisomes requires the cooperation of the PTS2 cytosolic molecular receptor Pex7.

1.3.2 The peroxisomal importomer

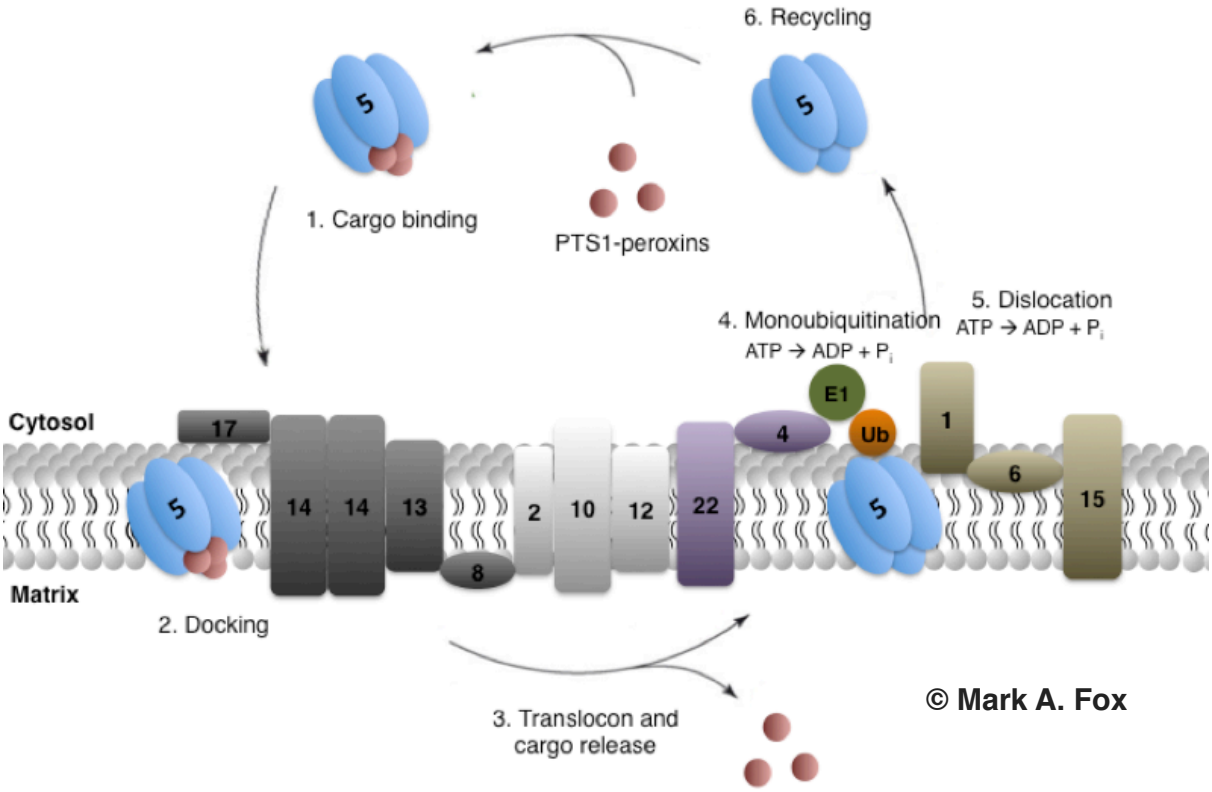
Following binding of peroxisomal proteins to their cytosolic receptors, delivery into the matrix is accomplished by a peroxisomal membrane bound protein complex called the importomer. The importomer is a diverse set of protein complexes located in the peroxisomal membrane that partake in protein translocation and the recycling, or degradation of cytosolic receptors. The importomer is made of two distinct protein complexes, which are bridged by other peroxins.

The first protein subcomplex, made of Pex13, multiple (≥ 2) Pex14s and Pex17, is classified as a docking station for receptors (Figure 1.2) (Rayapuram and Subramani 2006). Pex13 is believed to have a higher affinity for cytosolic receptor-cargo complexes, initiating receptor-cargo docking, while Pex14, is believed to mediate protein translocation into the peroxisomal matrix (Figure 1.2). The second protein subcomplex is

made of three individual ubiquitin ligase E3-like proteins, Pex2, Pex10 and Pex12 (Figure 1.2). These individual proteins contain really interesting new genes (RING) finder domains (Peraza-Reyes, Arnaise et al. 2011), a protein structural domain of the zinc finger type, that mediate PTS receptor recycling and/or degradation. The importomer also includes individual proteins, such as Pex8 and Pex3, which bridge the docking and RING subcomplexes to one another (Figure 1.2) (Meinecke, Cizmowski et al. 2010).

The importomer is also associated with receptor recycling machinery, which is responsible for tagging receptors either for recycling back to the cytosol for additional rounds of cargo import, or degradation. The recycling machinery consists of Pex4, a homologue of E2 ubiquitin conjugating enzyme, which is anchored to the importer by Pex22, and Pex1 and Pex6, and two ATPases associated with diverse cellular activities (AAA-ATPases), which are anchored to the importomer by Pex15 and Pex26 (Figure 1.1) (Rosenkranz, Birschmann et al. 2006). Through a series of coordinated interactions, receptors deliver their respective cargos to the importomer and are continuously shuttled back to the cytosol for subsequent rounds of import by the receptor-recycling machinery, unless otherwise targeted for degradation (Figure 1.2 versus 1.3). These processes must be tightly regulated to achieve normal peroxisome function. Indeed, recent mammalian studies have shown that a lack of any components of the importomer is characterized by the cytosolic mislocalization of peroxisomal matrix proteins (Purdue and Lazarow 2001).

Figure 1.2. Peroxisomal matrix protein import and receptor recycling pathway. Peroxisomal matrix protein import is divided into six distinct steps. (1) Receptor-cargo binding in the cytosol. Cytosolic receptors recognize PTS containing cargo in the cytosol and are transported to the peroxisomal importomer. (2) Receptor-cargo docking. Receptor-cargo complexes dock on the cytoplasmic face of the peroxisome membrane at the importomer docking station made of Pex17, Pex14 and Pex13. This docking station is proposed to be involved in tethering the receptor to the membrane. (3) Translocation of the receptor-cargo complex. Pex2, Pex10 and Pex12 form a RING finger complex, which work in conjunction with Pex8 to translocate the respective cargo into the peroxisomal matrix. (4) Monoubiquitination and (5) dislocation of the receptor. Monoubiquitination is mediated by the E2-enzyme Pex4, which is membrane-anchored by Pex22. The release of the receptor requires AAA-ATPases Pex1 and Pex6, in an ATP dependent manner, following which (6) the receptor is recycled into the cytosol for additional rounds of matrix protein import.


6. Recycling
5
1. Cargo binding
PTS1-peroxins
5
4. Monoubiquitination
ATP → ADP + P$_i$
5. Dislocation
ATP → ADP + P$_i$
E1
Ub
Cytosol
17
14
14
13
2
10
12
22
4
1
6
15
5
5
8
Matrix
2. Docking
3. Translocon and
cargo release
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1.3.3 Cargo translocation into the peroxisomal matrix

The mechanism regarding translocation of different proteins across the peroxisomal membrane and release of cargo into the matrix remains largely unknown, particularly as large, folded, even oligomeric proteins can cross the peroxisomal membrane. One hypothesis with respect to protein translocation into the matrix involves the receptor Pex5, which is believed to alter peroxisomal membrane topology during the protein import cascade (Nair, Purdue et al. 2004). Although it seems clear that the receptor–cargo complex reaches the luminal side of the membrane, it is still unknown whether only part of Pex5 extends into the lumen, referred to as the shuttle hypothesis, or whether the whole receptor enters the matrix, known as the extended shuttle hypothesis. Following these membrane-associated events, cargo is released and Pex5 is translocated back to the cytosol, where respective cargo is translocated into the peroxisomal lumen.

1.3.4 Cargo release

Various hypotheses have described the mechanism whereby matrix proteins are dissociated from their receptors and released into the peroxisomal lumen, however, these theories remain unverified. One model predicts that a pH gradient is responsible for receptor-cargo dissociation, resulting in cargo release in the peroxisomal matrix. Acidification of the cytosolic face of the importomer via hydrogen pumps is hypothesized to mediate this process (Meinecke, Cizmowski et al. 2010). This model is based on previous findings in yeast, which predicts the oligomeric states of Pex5 can switch from a cargo-bound tetramer at neutral pH (7.2) to a cargo-free monomer at acidic pH (6.0) (Wang, Visser et al. 2003)

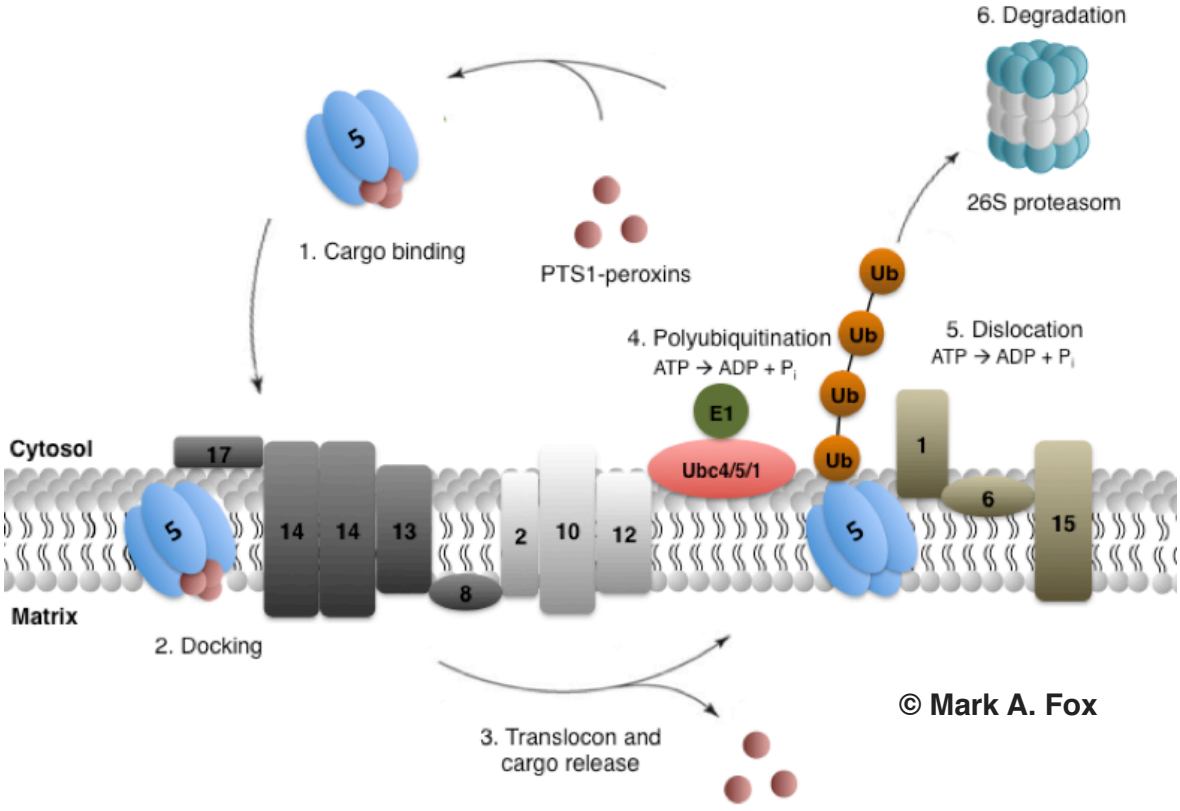
Another component of the importomer, Pex13, was found to have a higher affinity for cargo-free receptors, relative to cargo-bound receptors. Therefore, other hypotheses for cargo release have been proposed that highlight the interactions between the N-terminal region of Pex5 with the docking station of the importomer – Pex14, Pex13 and Pex17 (Platta and Erdmann 2007). These protein interactions could have an effect on the conformation of the TPR domain on Pex5, which may switch from a closed conformation back to an open conformation, resulting in cargo release (Stanley and Wilmanns 2006). However, much of this theory remains speculative and requires further investigation.

1.3.5 PTS receptor recycling and the RADAR pathway

Following cargo translocation and release, cargo-free receptors either shuttle back to the cytosol for additional rounds of protein import, or are degraded via the proteasome – a process referred to as the receptor accumulation and degradation in the absence of recycling (RADAR) pathway (Leon and Subramani 2007) (Figure 1.3). Once cargo is released into the peroxisomal matrix, cytosolic receptors are modified by either monoubiquitination, the linkage of a single ubiquitin molecule, or polyubiquitination, the conjugation of at least four ubiquitin molecules. Mono- and polyubiquitination serve as signals for receptor recycling or proteasomal degradation via RADAR, respectively (Purdue and Lazarow 2001; Platta and Erdmann 2007). Both processes require a ubiquitin-activating enzyme (E1), a ubiquitin-conjugating enzyme (E2), and ubiquitin ligase (E3) to conjugate ubiquitin to cytosolic receptors (Kerscher, Felberbaum et al. 2006). Current opinion is that the RADAR pathway is utilized only under certain

physiological conditions such as the occurrence of dysfunctions in cytosolic receptors (Ma, Agrawal et al. 2011).

Figure 1.3. Peroxisomal matrix protein import and RADAR pathway. Peroxisomal matrix protein import is divided into six distinct steps. (1) Receptor-cargo binding in the cytosol. Cytosolic receptors, such as Pex5, recognize PTS containing cargo in the cytosol and are transported to the peroxisomal importomer. 2) Receptor-cargo docking. Receptor-cargo complexes dock on the cytoplasmic face of the peroxisome membrane at the importomer docking station consisting of Pex17, Pex14 and Pex13. This docking station is proposed to be involved in tethering the receptor to the membrane. (3) Translocation of the receptor-cargo complex. Pex2, Pex10 and Pex12 form a RING finger complex, which works in conjunction with Pex8 to translocate the respective cargo into the peroxisomal matrix. (4) Polyubiquitination and (5) dislocation of the receptor. Polyubiquitination is mediated by enzymes Ubc5, Ubc4 and Ubc1, and occurs at the peroxisomal membrane. The release of the receptor requires AAA-ATPases Pex1 and Pex6, in an ATP dependent manner. (6) Receptor degradation via the proteasome. Numbers on diagram represent individual peroxins.


6. Degradation
26S proteasom
1. Cargo binding
PTS1-peroxins
4. Polyubiquitination
ATP → ADP + Pi
5. Dislocation
ATP → ADP + Pi
Ub
Ub
Ub
Ub
E1
Ubc4/5/1
Cytosol
Matrix
17
14
14
13
8
2
10
12
1
6
15
5
5
2. Docking
3. Translocon and
cargo release
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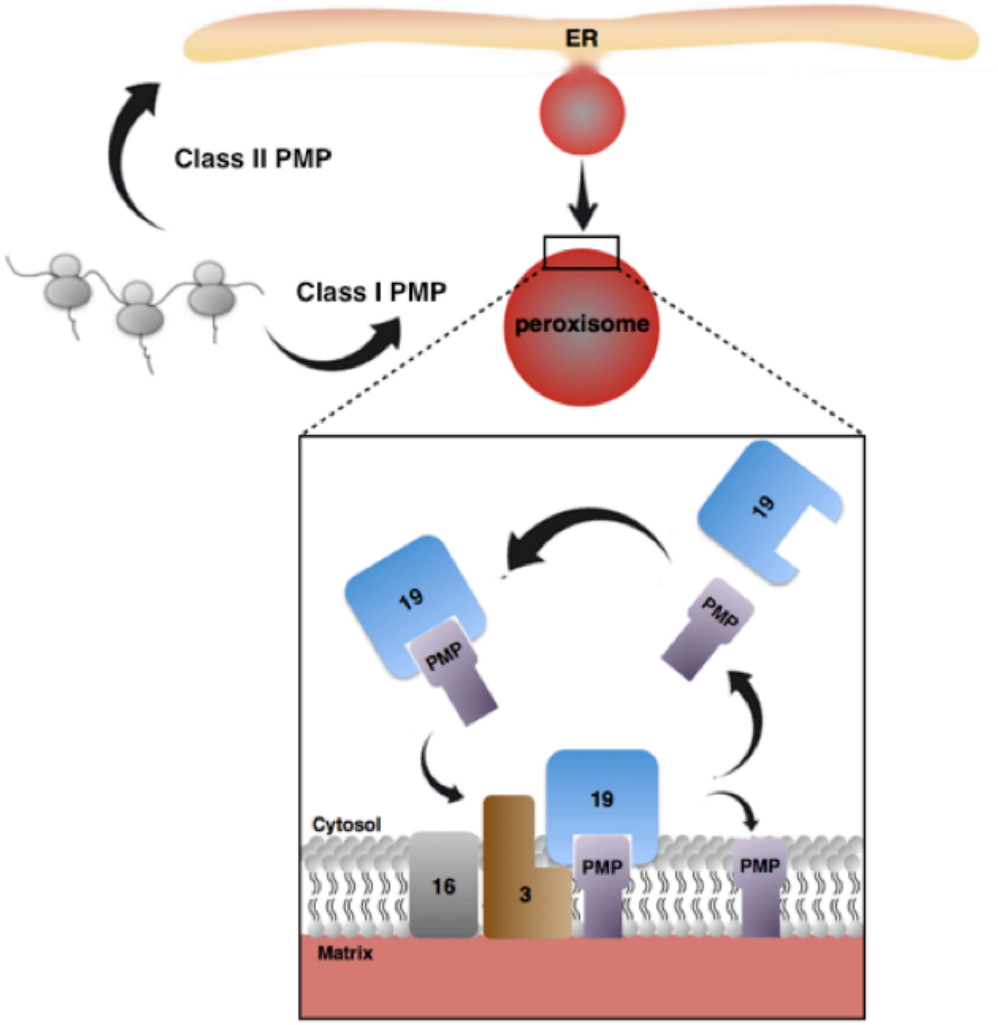
1.4 Peroxisomal membrane protein biogenesis and insertion

1.4.1 The role of mPTS in PMP Biogenesis

As with peroxisomal matrix proteins, peroxisomal membrane proteins (PMPs) are encoded in the nucleus and must be imported into the peroxisome membrane or ER post-translationally. The insertion of PMPs is accomplished by two distinct sorting pathways; Class I and II. Class I PMPs are synthesized on free ribosomes in the cytosol and are subsequently imported into the peroxisomal membrane (Figure 1.4). Insertion depends on internal membrane targeting sequences (mPTS) and requires the peroxins Pex19 and Pex3, and in some organisms Pex16 (Platta and Erdmann 2007). Deletion of either of these proteins results in the absence of detectable peroxisomal membrane structures. It is believed that Pex3 is a membrane recruitment factor for cargo-loaded Pex19 (Fang, Morrell et al. 2004). Pex19 can shuttle between the cytosol and the peroxisomal membrane, ferrying newly synthesized PMPs in a stable conformation for membrane insertion (Jones, Morrell et al. 2004). The functional role of Pex16 is less clear, however it has been suggested that it may function as a tethering factor for Pex3, or as a component of the membrane-insertion machinery for PMPs. In addition to a membrane anchoring sequence, class I PMPs exhibit conserved Pex19-binding sites, which are also required for their membrane import and stability (Vizeacoumar, Vreden et al. 2006). Conversely, the second PMP sorting pathway (Class II) is independent of the functions of Pex19, Pex3 and Pex16, and in fact it is Pex3 and Pex16 alone that are believed to represent Class II PMPs (Fang, Morrell et al. 2004). Unlike class I PMPs, these proteins are not targeted directly to peroxisomes, but instead appear to be sorted to the peroxisome

via the ER (Figure 1.4) (Hoepfner, Schildknegt et al. 2005) a process, which is discussed further in section 1.4.2.

Figure 1.4. Import of Class I and Class II PMPs. Class I PMPs contain a Pex19 binding site, a membrane anchor sequence and an mPTS sequence. These PMPs are synthesized on polysomes and recognized by Pex19, which ferries proteins from the cytosol to the peroxisomal membrane via docking to Pex3 at the membrane. Class II PMPs are targeted to the ER and are believed to behave as membrane recruitment factors in the *de novo* biogenesis of peroxisomes.

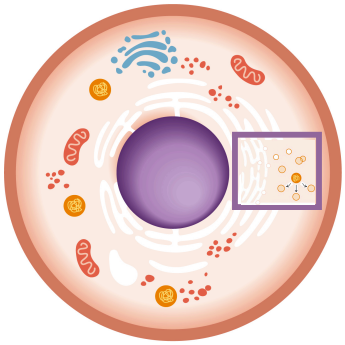

ER
Class II PMP
Class I PMP
peroxisome
19
PMP
19
PMP
19
PMP
PMP
Cytosol
16
3
Matrix

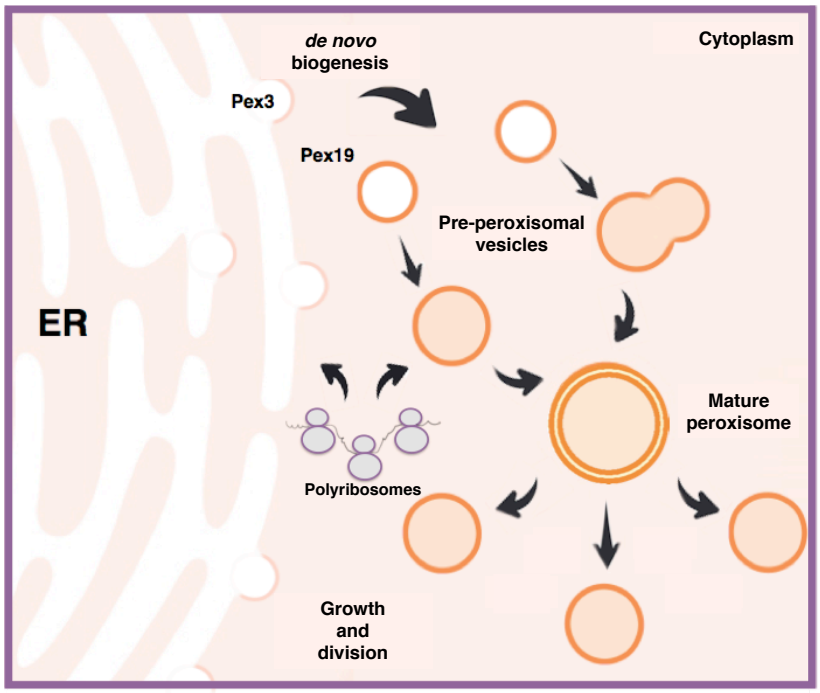
1.4.2 Trafficking of Class II PMPs through the ER

The notion that class II PMPs are trafficked to the peroxisome through the ER is supported by findings that show certain PMPs contain ER-specific modifications. For instance, the integral PMPs Pex2 and Pex16 are N- or O-glycosylated, and the tail anchored peroxin, Pex15, is O-mannosylated (Elgersma, Kwast et al. 1997; Titorenko and Rachubinski 1998).

Furthermore, pulse-chase experiments in mutant yeast lacking functional peroxisomes showed the reappearance of peroxisomes following complementation analyses with Pex3 and Pex19 (Hoepfner, Schildknegt et al. 2005). Pex3 was fluorescently labeled and detected as punctate-like structures in the ER (Fang, Morrell et al. 2004). These structures were found to bud from the ER in a Pex19-dependent fashion, indicating Pex3 and Pex19 are both necessary and sufficient to mediate peroxisomal vesicle budding from the ER. These data suggest that the ER is a membrane template for *de novo* peroxisome biogenesis, and as such, it is referred to as the preperoxisomal reticulum (Figure 1.5). As many of the steps involved in the formation of peroxisomes are still being elucidated, there also remains a lack of understanding regarding the parameters that regulate the number and size of peroxisomes found in a cell.

Figure 1.5. Peroxisome *de novo* biogenesis. Peroxins are synthesized on free polyribosomes in the cytosol and sent to the endoplasmic reticulum (ER) or directly into functional organelles. The *de novo* biogenesis pathway suggests that peroxins, such as Pex3 – an intrinsic membrane protein, enters the ER through the Get1 translocon, and can bud from the ER, utilizing the ER-membrane as a template for *de novo* biogenesis referred to as the preperoxisomal reticulum. With the assistance of Pex19, a molecular chaperone, Pex3 can bud from the ER. Following the formation of preperoxisomes, the organelles themselves can mature via fusion of adjacent organelles or via membrane/matrix protein import.




de novo
biogenesis
Cytoplasm
Pex3
Pex19
Pre-peroxisomal
vesicles
ER
Mature
peroxisome
Polyribosomes
Growth
and
division

1.5 Pexophagy

A variety of cellular and physiological responses partake in governing the number of peroxisomes at any given time. Peroxisomes can change in number by a selective degradation autophagy-related process termed pexophagy. Knowledge of these processes remains limited, however, in recent years the molecular mechanisms are starting to unfold. Pexophagy is a complex process, involving over 30 different proteins in addition to a variety of autophagy related genes (ATGs). Two basic models for selective peroxisome degradation have been described termed macro- and micropexophagy.

1.5.1 Macropexophagy

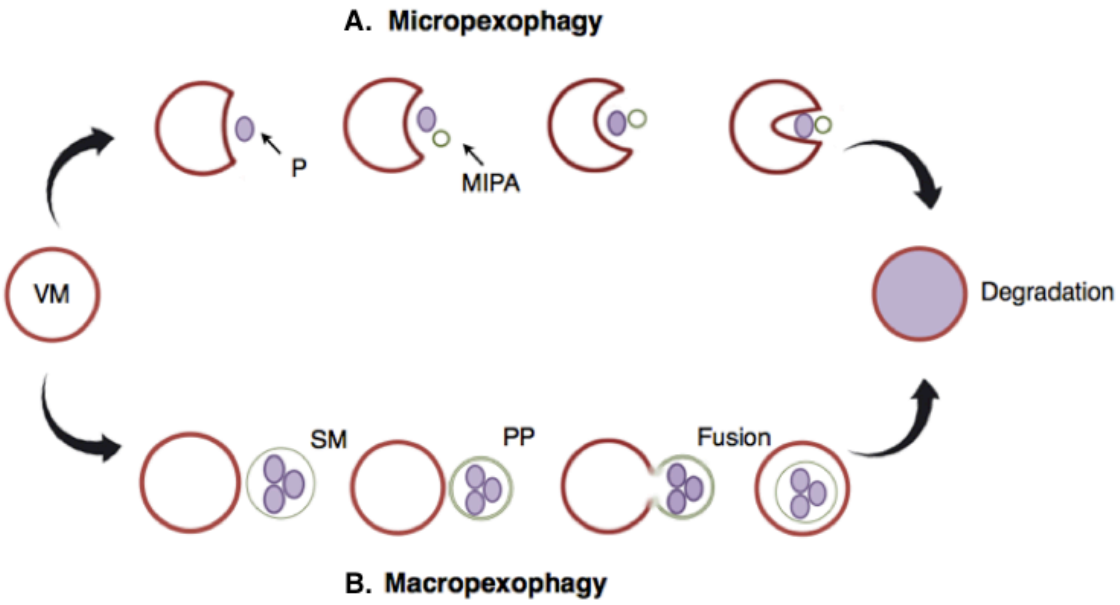
The selectivity of macropexophagy is such that it is strictly related to mature organelles. It is believed that Pex3, an integral membrane protein involved in PMP import machinery, is removed from the peroxisomal membrane, polyubiquitinated and degraded via the proteasome – a process required to initiate macropexophagy (Bellu, Salomons et al. 2002). Pex14, part of the importomer involved in protein docking, functions similarly in macropexophagy as it is required for the docking of ATG11, which initiates the formation of a sequestration membrane (SM). Formation of the SM produces a double membrane pexophagosome (PP), which will fuse with adjacent vacuoles, resulting in eventual degradation by hydrolases (Figure 1.6).

1.5.2. Micropexophagy

The process of micropexophagy requires the function of several proteins involved in macropexophagy and other autophagic pathways. Vacuolar membranes first develop

protrusions adjacent to the surface of target peroxisomes and engulf them sequentially (Farre and Subramani 2004). ATG11 and ATG28 interact with Pex14, which tags peroxisomes for vacuolar engulfment – a process completed by the formation of the micropexophagic membrane apparatus (MIPA) (Mukaiyama, Baba et al. 2004) (Figure 1.6). Peroxisomes are subsequently degraded in the vacuolar lumen by hydrolases.

Figure 1.6. The two models of pexophagy: macro- and micropexophagy. Depicted are morphological intermediates and the corresponding proteins relevant for each step. (**A**) After initiation of micropexophagy, the peroxisomes (purple) become engulfed by invagination of the vacuolar membrane (red). In addition, the micropexophagy apparatus (MIPA; green) is required for complete sequestration of peroxisomes. Finally, the peroxisomes are degraded in the vacuolar lumen by hydrolases. (**B**) Macropexophagy is specific to mature peroxisomes, which are recognized by a sequestration membrane (SM; green single membrane). Completion of sequestration produces the pexophagosome (PP, green double membrane), which represents one peroxisome engulfed by two membranes. The outer membrane of the pexophagosome fuses with the vacuolar membrane, resulting in the release of the peroxisome (which is still surrounded by the inner membrane) into the lumen of the vacuole, and its subsequent degradation.


A. Micropexophagy
P
MIPA
VM
Degradation
SM
PP
Fusion
B. Macropexophagy

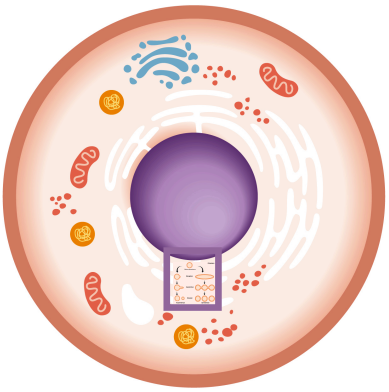
1.6 Regulation of peroxisome size and number

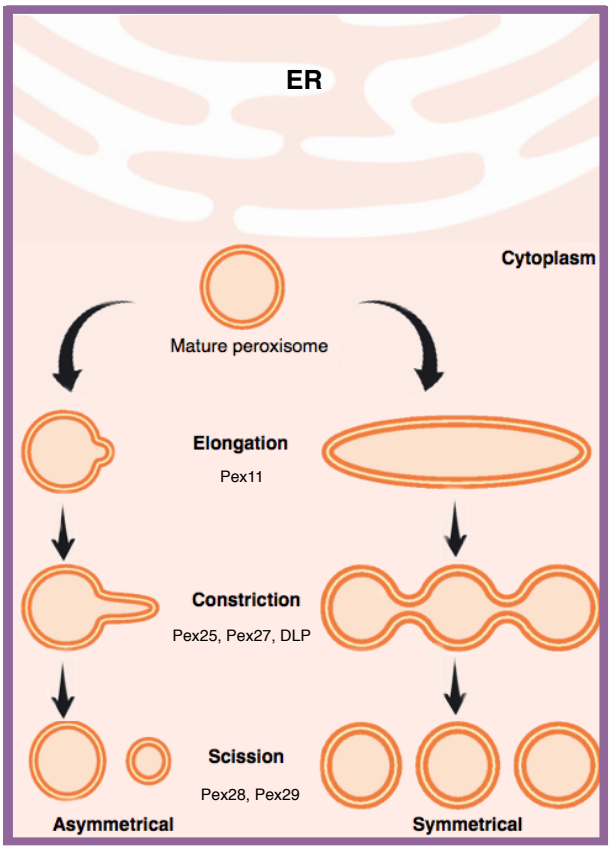
1.6.1 Proteins involved in peroxisome proliferation

The molecular machinery involved in peroxisome proliferation and division is becoming well characterized. The first protein identified as a regulator of peroxisome size and number was Pex11 in *Saccharomyces cerevisiae* (van Roermund, Tabak et al. 2000). Mutation analyses of Pex11 in *S. cerevisiae* indicated that disruption of Pex11 resulted in the formation of few giant peroxisomes per cell, whereas its overexpression induced the formation of multiple small peroxisomes. A similar ability to promote peroxisome proliferation was also reported for human (Abe and Fujiki 1998; Schrader, Reuber et al. 1998; Li and Gould 2002), rodent (Passreiter, Anton et al. 1998), protozoan (Maier, Lorenz et al. 2001), isoforms of Pex11.

There are three isoforms of Pex11, -α, β and γ, which, based on gene knockout and overexpression analyses, differ in expression pattern and phenotypic consequences. The α and γ-isoforms are believed to stimulate peroxisome divisions in response to metabolic cues (Schrader, Reuber et al. 1998). Recently, it was shown that Pex11β participates in peroxisome division by inducing membrane elongation and shape changes in preexisting peroxisomes (Figure 1.7). Elongated membranes on existing peroxisome form small blebs and separate into new peroxisomes with the aid of dynamin-like proteins (DLPs), Pex25 and Pex27 (Delille, Agricola et al. 2010), while separation of the divided, yet clustered peroxisomes is subsequently controlled by Pex28 and Pex29 (Figure 1.7). The role of Pex11β and its pivotal role in regulating peroxisome number is a key element investigated in this thesis.

Figure 1.7. Division cycle of peroxisomes. Peroxisomal membrane proteins Pex11β, Pex25 and Pex27, and dynamin-related protein 1 (DLP1) are involved in peroxisome membrane elongation, constriction and fission. Pex28 and Pex29 mediate separation of the newly divided, yet clustered peroxisomes. Newly synthesized preperoxisomal vesicles mature into functional peroxisomes following further matrix protein import.




ER
Cytoplasm
Mature peroxisome
Elongation
Pex11
Constriction
Pex25, Pex27, DLP
Scission
Pex28, Pex29
Asymmetrical
Symmetrical

1.6.2 Metabolic control of peroxisome abundance

The metabolic regulation of peroxisome abundance is poorly understood. In mammalian cells, defects in peroxisomal fatty acid β-oxidation result in reduced peroxisome abundance (Chang, South et al. 1999). Cellular analyses of peroxisomes from patients with specific deficiencies in acyl-CoA oxidase displays enlarged peroxisomes that are heterogeneous in size (Poll-The, Roels et al. 1988). Additionally, mammalian cell lines mutant for acyl-CoA display a 5-fold reduction in peroxisome abundance compared with normal cells (Chang, South et al. 1999). Another enzyme, thioesterase, which inhibits peroxisomal fatty acid oxidation, was found to reduce peroxisome abundance when overexpressed (Chang, South et al. 1999). These and other findings shed insight on the metabolic role of peroxisomes, and how metabolism relates to peroxisome numbers.

1.6.3 Signals and events leading to peroxisome proliferation

Peroxisomes can rapidly adapt to cellular demands and increase in both size and number. In mammalian cells it is well known that PPARα mediate the induction of peroxisome proliferation (Yu, Cao et al. 2001). However in yeast, the transcription factor alcohol dehydrogenase regulation 1 (Adr1) is involved in peroxisome division and supports a mechanistic model for the divisionary process. Adr1 regulates the expression of acyl-CoA oxidase and interacts with Pex16 found on the matrix face of the peroxisomal membrane (Guo, Kit et al. 2003). These interactions are believed to trigger the formation of a trans-bilayer composed of a distinct set of lipids that constitute a specific platform on the surface of the peroxisome. This platform is used for the

assembly of the division machinery (Guo, Gregg et al. 2007), like Pex11β, which induces the elongation process and increases peroxisome numbers.

Peroxisome numbers may fluctuate in response to changes in metabolic demand, and the number of peroxisomes may have an important influence on ROS levels not only within these organelles, but also within the cytoplasm (del Rio, Sandalio et al. 2006). While elevated levels of ROS are considered deleterious to cellular constituents like proteins, nucleic acids and lipids, low levels are involved in regulating cell signaling cascades and protein modification (Covarrubias, Hernandez-Garcia et al. 2008). Therefore, peroxisome numbers may also serve to regulate ROS such that proper cell signaling can occur. Understanding the relationship between peroxisome number and ROS levels, and the ability for these factors to influence ROS signaling cascades is a key element of this thesis.

1.7 ROS as signaling molecules

At low cellular concentrations, ROS behave as secondary messengers that may partake in a variety of cellular responses via protein redox (reduction-oxidation) modifications. Typically, redox modifications occur at cysteine residues, however, amino acids such as tryptophan, tyrosine and histidine may also undergo similar changes. Cys thiols (SH) are subjected to different degrees of oxidation by various ROS resulting in the generation of sulphenic acid (SOH), sulphinic acid (SO_2H) or sulphonic acid (SO_3H). Additionally, SOH reacts with a second cysteine either in the same or a second protein to yield a disulphide bond. The variety of macromolecules sensitive to redox modifications ranges from phosphatases, kinases, a large number of transcription factors,

and other proteins such as matrix metalloproteinases (MMPs), which are involved in extracellular matrix remodeling (Paulsen and Carroll 2010). ROS, like other secondary messengers, can in addition alter the activity of proteins, mediating various biological responses including gene expression, cell proliferation, angiogenesis, programmed cell death and senescence.

Alternatively, it is also known that increased levels of these short-lived reactive molecules can exert harmful effects by causing oxidative damage to biological macromolecules and disrupting the oxidation state of the cell. To neutralize and protect cellular constituents, cells employ many enzymatic defense mechanisms. Antioxidants partake in the detoxification of ROS, which prevent these deleterious byproducts from causing potential injuries. Antioxidants 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. Biological antioxidants include organic compounds, such as GSH, vitamins A and E, and importantly, genetically encoded enzymes. The latter group includes proteins such as catalase, SOD, and a variety of peroxidases such as Gpx, and Prdx. Some antioxidants are localized to specific subcellular compartments, while others are involved in the global scavenging ROS.

1.7.1 ROS metabolism in peroxisomes

Mitochondria are the primary source of ROS production due to their high metabolic state. However, recent studies have demonstrated the ER and particularly, peroxisomes are key regulators in balancing the redox state of a cell (Schrader and Fahimi 2004). Peroxisomes contain a variety of antioxidants to counteract oxidative stress resulting

from metabolism and other peroxisomal processes, which contributes to redox balance. Imbalance in peroxisomal ROS may damage biomolecules, perturb cellular thiol levels, and deregulate cellular signaling pathways. In recent years, peroxisomal ROS metabolism and signaling have become the focus of a rapidly evolving and multidisciplinary research field (Fransen, Nordgren et al. 2011).

1.7.2 Peroxisomal ROS metabolism and human disease

It is well accepted that alterations in the cellular oxidation state impose a considerable risk for the onset of various diseases and aging. As the intracellular redox state is inherently linked to metabolism, it is becoming increasingly apparent that peroxisomes are involved in human pathologies related to oxidative stress. In this context, it is interesting to note that compromised catalase activity has already been associated with ischemia-reperfusion injury, hypertension, skin pigmentation disorders, retinal disease, degenerative joint disease, heart failure, type 2-diabetes, neurodegenerative disorders, and the initiation and progression of certain cancers (Koepke, Wood et al. 2008).

1.7.3 ROS during animal development

Some developmental processes are almost completely anaerobic, whereas others are ROS-dependent. The spatiotemporal distributions of ROS vary accordingly across different stages of development in various organisms. In mammals, preimplantation and early postimplantation embryogenesis occurs under almost anaerobic conditions, giving insight to the sensitivity of these processes to oxidative stress conditions (Hernandez-

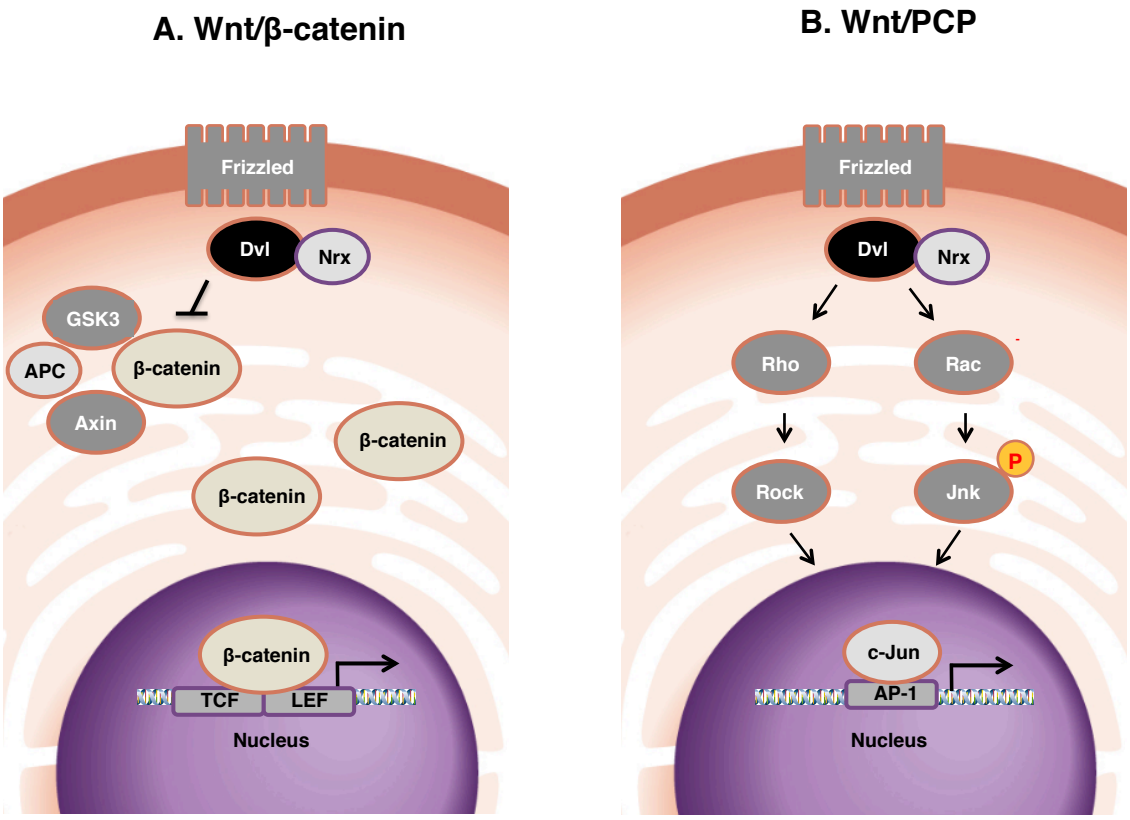
Garcia, Wood et al. 2010). High ROS levels are detrimental for growth of embryos in culture, and administering free radical scavengers improves *in vitro* embryo development (Covarrubias, Hernandez-Garcia et al. 2008). Oxygen toxicity in embryos is well documented, however, direct evidence for the function of ROS in specific developmental processes has only recently become a topic of interest. Developmental processes such as spermatogenesis, oogenesis, fertilization, morphogenesis, angiogenesis and cell migration have all received much attention in terms of their association with ROS (Covarrubias, Hernandez-Garcia et al. 2008). For example, during morphogenesis in mouse embryos, high ROS concentrations are associated with the cell death that occurs in the interdigital regions of the developing limb. ROS levels in the limb appear to be regulated by Gpx4, which the in mouse has a restricted expression pattern only in the limb region (Schnabel, Salas-Vidal et al. 2006). The roles of ROS in development are becoming increasingly well understood. One function of ROS that has received much attention in developmental models such as *X. laevis*, is its role in the redox-regulated mechanisms of Wnt signaling.

1.7.4 Redox regulation of Wnt signaling

Wnt ligands are utilized in many normal developmental processes including differentiation, pattern formation and proliferation (Clevers 2006). The canonical Wnt signaling pathway ultimately results in the accumulation of β-catenin and its translocation to the nucleus, where it activates the transcription factor Lef/Tcf (Figure 1.8A). In order for this to occur, Wnt ligands bind to the Frizzled (Fzd) receptor causing inhibition of the destruction complex, resulting in the cytosolic accumulation of β-catenin

– a process mediated by a protective protein called Dishevelled (Dvl). Recent studies on Wnt signaling in *X. laevis* indicate a redox-sensitive switch for this pathway. It was determined that nucleoredoxin (Nrx), a thioredoxin-related protein, plays a regulatory role in canonical Wnt signaling by directly controlling Dvl activity (Funato, Michiue et al. 2008). Nrx binds to Dvl in its reduced form, which suppresses Wnt signaling. In *X. laevis* embryos, increasing or decreasing Nrx protein level results in embryonic abnormalities, which relate to inhibition or activation of Wnt signaling, respectively. These studies also determined that H_2O_2 oxidizes Nrx, releasing it from Dvl, thus promoting β-catenin accumulation in the absence of Wnt ligand. In addition to canonical Wnt signaling, the noncanonical Wnt/PCP signaling pathway controls cytoskeletal changes through the activation of Rho and c-Jun N-terminal kinase (JNK) signaling cascade (Figure 1.8B). Similarly to the canonical pathway, the Wnt/PCP signaling involves Nrx and Dvl, via redox-sensitive activation, which are involved in regulating *Xenopus* gastrulation movements (Funato, Michiue et al. 2008). From this data, it is predicted that ROS levels are a determinant for the activation of the canonical and noncanonical Wnt signaling pathways, and therefore ROS must be tightly regulated to maintain the balance necessary for normal embryonic development.

Figure 1.8. Schematic representation of Wnt/β-catenin and Wnt/PCP cell signaling pathways. (**A**)Wnt/β-catenin signaling is initiated by association of Wnt with Frizzled at the plasma membrane, leading to the inhibition of β-catenin degradation complex (Axin/APC/GSK-3β), which permits the accumulation of β-catenin and its translocation to the nucleus to activate target gene transcription by associated with Lef/Tcf transcription factors. (**B**) Wnt/planar cell polarity (PCP) is initiated by association of Wnt with Frizzled at the plasma membrane, resulting in the activation of Rho and Rac GTPases. Activation of Rho involves the activation of the Rho-associated kinase, Rock, whereas Rac activation stimulates c-Jun N-terminal kinases (JNK) activity, phosphorylating c-Jun and consequently phosphorylates AP-1 transcription factors.


A. Wnt/β-catenin
B. Wnt/PCP
Frizzled
Dvl
Nrx
GSK3
APC
Axin
β-catenin
β-catenin
β-catenin
β-catenin
TCF
LEF
Nucleus
Frizzled
Dvl
Nrx
Rho
Rac
Rock
Jnk
P
c-Jun
AP-1
Nucleus

1.8 Animal Model

The model system used in these studies was the African clawed frog *Xenopus laevis*. Classified in 1802 by Francois Marie Daudin, embryos of this species have been an important model organism for developmental biologists since the 1800's. Although *X. laevis* does not have the short generation time and genetic simplicity generally desired in model organisms, it is an important organism in developmental biology. *X. laevis* takes 1 to 2 years to reach sexual maturity and, like most of its genus, it is tetraploid. However, embryos are large and easily manipulated, which has given them an important status in the study of developmental biology.

X. laevis has well characterized embryological pathways, cellular movements and detailed fate maps (Appendix B). Controlled ovulation can be induced by injection of commercially available human gonadotropin hormone the evening before morning ovulation. One female can yield hundreds of embryos in a single *in vitro* fertilization, which may be repeated with the same female every four months. Embryos are large and durable, making them suitable for *in vitro* culturing at room temperature in a serum free salt solution. They are also resilient to a variety of invasive experimental techniques such as embryo manipulation and microinjection. Lastly, A6 cells, which are cloned epithelial cells from the X. laevis kidney, have been used as an *in vitro* model. As such, *X. laevis* has been pivotal in aiding our understanding of key early signaling events. For these reasons I have chosen to use *X. laevis* as the model organism to study peroxisome biogenesis and its relationship to ROS and cell signaling events.

1.9 Research Questions

1.9.1 Summary

Peroxisome division is a multistep, highly coordinated serious of events. Proteins termed peroxins, synthesized by peroxisome biogenesis factors (Pex), are responsible for these events. Peroxisomes have many important roles, and their number and function is crucial for normal cellular physiology. The consequences of perturbation or dysfunction of peroxisomes is emphasized in a spectrum of lethal diseases termed peroxisome biogenesis disorders. Recently, it has been established that the Pex11-family of peroxins is involved in the regulation of peroxisome division through membrane elongation (Koch, Pranjic et al. 2010). In this study I first examine the role of Pex11β and its relationship to the regulation of peroxisome division. As the mechanisms of peroxisome division are becoming well understood, very little is known regarding the relationship between peroxisome numbers and the redox (reduction-oxidation) state of cells. Peroxisomes are primarily involved in the β-oxidation of very-long chain fatty acids, producing high levels of reactive oxygen species (ROS) that are eradicated by a dynamic antioxidant defense system. Therefore, I also examine how ROS levels change in response to alterations in peroxisome number. As ROS are known as secondary molecules that contribute to protein modification and activity, I examine how changes in ROS levels, resulting from altering peroxisome numbers, contribute to the redox sensitive mechanisms of Wnt signaling. Taken together, this study sheds light on the relationship between peroxisome number, ROS levels and cell signaling, which could improve our understanding the progression of fatal human peroxisome disorders.

1.9.2 Objectives

The goal of this study is to examine the relationship between Pex11β and peroxisome number, and then, to elucidate how changes in peroxisome numbers affect ROS levels, and in turn how the imbalance in ROS may alter Wnt signaling. This will be achieved by examining and completing the following objectives:

1) Elucidate the relationship of Pex11β to peroxisome number *in vitro* and *in vivo*.
2) Establish how peroxisome number regulates ROS levels *in vitro*.
3) Determine that changes in ROS result in abnormal Wnt signaling *in vitro*.

1.9.3 Hypotheses

1) Overexpression and inhibition of Pex11β will increase or decrease the number of peroxisomes in *X. laevis* A6 cells, respectively. During *X. laevis* embryogenesis, overexpression of Pex11β will induce early-onset of peroxisome biogenesis, while inhibition of Pex11β will result in late-onset of peroxisome biogenesis and the appearance of fully functional peroxisomes.
2) Peroxisome number has a direct effect on the levels of ROS. Modifications to peroxisome number via changes in Pex11β expression will directly affect the levels of H_2O_2 and the levels of global and mitochondrial ROS, *in vitro*.
3) Alterations to the levels of ROS will result in redox modifications to Wnt signaling, *in vitro*.

1.10 References

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CHAPTER 2

PEX11-BETA INDUCES PEROXISOMAL GENE EXPRESSION AND ALTERS PEROXISOME NUMBER DURING EARLY *XENOPUS LAEVIS* DEVELOPMENT.

2.1 Introduction

2.1.1 Overview of peroxisomes

Peroxisomes are single-membrane bound organelles found ubiquitously in eukaryotic cells. They house more than 50 matrix enzymes that participate in a diverse array of metabolic processes including the β-oxidation of very long chain fatty acids (VLCFA) and α-oxidation of long branched-chain fatty acids (Platta and Erdmann 2007). Peroxisomes also contain oxidases that produce the corrosive byproduct hydrogen peroxide (H_2O_2) (Singh 1997). H_2O_2 and other dangerous reactive oxygen species (ROS) are then converted to innocuous products such as water and molecular oxygen by catalase and other enzymes within the peroxisome and in other cellular compartments (Anand, Kwak et al. 2009). Because of their complex roles in both cellular metabolism and ROS elimination, peroxisome function is strongly related to cellular development and eventual cellular senescence when their function begins to fail.

While cellular aging and senescence are well characterized by peroxisomal dysfunction (Legakis, Koepke et al. 2002), little is known about the origin of these organelles, particularly during embryonic development. Important players in the regulation of overall peroxisome numbers are the peroxisome proliferator-activated receptors (PPARs), which were first identified in the early 90s in mice (Issemann and Green 1990). Three types of PPARs have been identified (alpha, gamma, and delta) that function as transcription factors and play critical physiological roles as lipid sensors and regulators of lipid metabolism, as well in the regulation peroxisome numbers (Desvergne, Michalik et al. 2006).

2.1.2 Peroxisome biogenesis

Total peroxisome numbers and peroxisome biogenesis, involves the production of proteins termed peroxins; nuclear encoded by Pex genes, synthesized on free polyribosomes in the cytosol and post-translationally transported into the peroxisomal matrix and membrane (Duhita, Le et al. 2010). Peroxins can facilitate peroxisomal membrane function, biogenesis and division, and the transport of specific cytosolic proteins into the peroxisomal matrix via one of two peroxisomal targeting signals (PTS) (Dodt, Braverman et al. 1995). The PTS2 signal sequence is a complex amino terminal signal composed of N/K-L-X5-Q-H/L, while the PTS1 consists of the C-terminal amino acid sequence SKL and a conserved variant form, KANL (Wolf, Schliebs et al. 2010). Studies have shown proteins with the SKL signal have a higher affinity for peroxisomes than proteins with the relatively weaker KANL PTS1-signal (Koepke, Nakrieko et al. 2007). In addition to various functions within peroxisomes, these cytoplasm-to-peroxisome protein import pathways have been proposed as a necessary mechanism to increase peroxisome numbers from existing peroxisomes (Ma and Subramani 2009). While peroxisome number may be augmented though signal transduction (Li and Gould 2002), the total number of peroxisomes in a cell is regulated by; (i) peroxisome *de novo* biogenesis, (ii) peroxisome proliferation by division and (iii) peroxisome degradation by pexophagy, an autophagy-related process (Platta and Erdmann 2007).

2.1.3 Pex11-family of peroxins

Multiple studies on Pex11 proteins have contributed to understanding their role in peroxisome division, although the specific molecular mechanism that regulates their function are poorly understood (Li and Gould 2002). Expression levels of Pex11 peroxins are directly correlated with peroxisome numbers (Kaur and Hu 2009). For example, a Pex11p knock-down in yeast significantly reduced the number of cellular peroxisomes, whereas, Pex11p overexpression increase their numbers (van Roermund, Tabak et al. 2000). A similar ability to promote peroxisome proliferation was also reported in humans (Li and Gould 2002), rodents and protozoan models (Schrader and Fahimi 2006). All of these studies support a direct role for the Pex11-family in peroxisome division *in vitro*, though little is known about their role during embryogenesis.

2.1.4 Metabolism in vertebrate development

It is unknown whether peroxisomes exist in fertilized eggs, or in early stage vertebrate embryos. While early frog development requires glycogen and lipid reserves to be oxidized, and protein and yolk reserves to be metabolized, surprisingly little is known about the regulation of yolk, vitellogenin and lipid metabolism in oviparous animals such as frogs. Early histological staining studies revealed that yolk and lipid utilization follow gastrulation, but preceded cell differentiation, suggesting the alterative forms of yolk metabolism prior mid-blastula transition (MBT). Selman and Pawsey revealed that frog yolk and lipid utilization took place ventral to the archenteron just prior to stage 20, and within the developing myotomes by stage 23 (Selman and Pawsey 1965).

Other histochemical studies have also shown that yolk and lipid metabolism occurs within the somites as they begin to differentiate between stages 17-24 (Kielbowna 1975). Yolk is then metabolized in most differentiating tissues in the embryo after stage 30 (Kielbowna 1975). This tissue specific utilization of yolk has been more recently confirmed using a variety of approaches including the examination of pH changes, and the involvement of proteases such as cathepsin D, and inhibitors such as EP45/pNiXa/Seryp, a family of serine protease inhibitors (Fagotto 1995). This tissue specific regulation of yolk metabolism during embryogenesis suggests complex underlying developmental controls of these processes.

2.1.5 Hypotheses of overexpressing Pex11β

While peroxisomes are needed for metabolism and ROS regulation, their origins and biogenesis within the embryo are poorly understood. Here I examine the level of expression of peroxisomal genes Pex1, Pex3, Pex5, Pex11β, catalase and PMP70, as well as PPARα, δ, and γ in a *X. laevis* cell line, and during embryonic development. I test the hypothesis that Pex11β has the ability to induce peroxisomal gene expression *in vitro*, and induce early increase in peroxisome number *in vivo*. My results demonstrate that overexpression of Pex11β can increase the number of peroxisomes in A6 cells *in vitro*, and induce an early-onset to peroxisome-like structures during Xenopus embryogenesis *in vivo*. I propose that Pex11β plays a direct role in peroxisome divisions, and additionally, regulating the timing of peroxisome biogenesis during *X. laevis* embryonic development.

2.2 Materials and Methods

2.2.1 Animal care

Adult *X. laevis* were reared in accordance with Canadian Council on Animal Care regulations. Fertilizations were performed according to Wu and Gerhart (Wu and Gerhart 1991), and embryos were staged according to Nieuwkoop and Faber (Nieuwkoop). Embryos to be sectioned were fixed in 4% formaldehyde at desired stages and paraffin-embedded.

2.2.2 Cloning, RNA synthesis, and microinjection

I cloned Xenopus full length Pex11β [GenBank:MGC69071] from total adult liver cDNA using specific primers and SuperScript™ Reverse Transcriptase (Invitrogen) with Platinum® *Taq* DNA Polymerase High Fidelity (Invitrogen) using conditions supplied by the manufacturer. A 5' HA tag was added to Pex11β using specific primers; HA-Pex11β 5'AGA TCT TCA AGC GTA ATC TGG TAC GTC GTA TGG GTA GGG CTT CAG CTT CAG CCA 3' and 5' CGA ACC CAC GAG TCC ATA CTA GT 3'. I also engineered GFP tagged with the PTS1 SKL, using forward 5' AGA TCT ATG GTG AGC AAG GGC GAG 3' and 5' ACT AGT CTA TAA TTT GGA CTT GTA CAG CTC GTC CA 3'. PCR products were cloned into the pCR®II-TOPO vector as per manufacturer's instructions (Invitrogen). Recombinant sequences were confirmed at the Robarts Research Institute DNA Sequencing Facility at the University of Western Ontario. Desired clones were additionally cloned into pcDNA™ TOPO 3.3® TA Cloning `Kit (Invitrogen) for cell culture experiments, and T7TS plasmid and sequenced *in vitro*

RNA production. Capped polyadenylated RNA was synthesized using mMachine mMessage® T7 (Ambion) and visualized on a 1.0% agarose formaldehyde gel to ensure quality and transcription validity. Embryos at the one-cell stage in 4% ficoll in 1X Marks Modified Ringer (MMR) solution were microinjected with approximately 1 ng of desired RNA. Following 4 hours, embryos were transferred to 0.1X MMR for rearing.

2.2.3 Cell lines, transfections and immunocytochemistry

A6 cells derived from *X. laevis* epithelial cells (generous gift from Dr. John Heikkila, University of Waterloo, ON) were grown in Leibowitz-15 media (with 10% FBS and 1% penicillin and streptomycin) at room temperature. All transfections were completed using Lipofectamine Plus LTX Transfection Reagents (Invitrogen) according to manufactures protocol. For immunofluorescence, cells were fixed in 3.7% formaldehyde in Dulbecco's modified PBS (DPBS) (Invitrogen), pH 7.4, for 10 min, and permeabilized in 1% Triton X-100 in DPBS for 10 min. Cells were incubated with either PMP70 (Abcam, ab4965) or catalase (Cedarlane), and/or haemagglutinin (Invitrogen) polyclonal antibodies for 3 hours, washed three times in PBST for 5 minutes each, incubated with fluorescently labeled secondary antibodies for 1 hour, washed again for 5 minutes in DPBS, and mounted on slides using ProGold mounting media (Invitrogen). Samples were visualized with a Zeiss AxioStop 2 Mot. Images were captured with a Retiga 1600 camera (Qimaging) and fluorescence quantifications were completed using Northern Eclipse image capture and analysis software (Empix).

2.2.4 RNA isolations and reverse transcriptase PCR (RT-PCR)

RNA was isolated from cell lysates of all samples two days following transfections. Total RNA was isolated with an RNeasy kit (QIAGEN) from embryos at developmental stages 10, 20, 30, and from A6 cells, was evaluated on a 1.0% agarose formaldehyde gel. Synthesis of cDNA was completed with SuperScript II Reverse Transcriptase (Invitrogen) following manufactures protocol. To analyze RNA expression levels during development, RT-PCR primers were designed against known Xenopus peroxisomal and PPAR genes with the following accession numbers: Pex3 [EMBL:AAH73069.1], Pex5 [NP_001011381], Pex11β [GenBank:MGC69071], PMP70 [EF07060], catalase [BC054964] Pex1 [NM_001091972.1], PPARα [NM_001095362], PPARδ [NM_001087841], and PPARγ [NM_001087843] Mid-log phase RT-PCR products were visualized on a 1% agarose gel and unsaturated band intensities were quantified against control elongation factor-1α [NCBI: NM_001087442] with Quantity One software (Version 4.4.0 Bio-Rad). All quantified PCR reactions were completed in triplicate. The amplicons of peroxisomal genes listed above were cloned with the TOPO-TA Cloning® (Invitrogen) system as described by the manufacturer's protocol and sequenced to ensure gene identities.

2.2.5 Western blot analysis

PMP70 (Abcam, ab4965), catalase (Cedarlane), hemagglutinin and β-actin (Invitrogen) polyclonal antibodies were used to detect protein from both *X. laevis* A6 cell lysates before and after treatments. Bradford protein quantifications were used to ensure that equivalent amounts of protein (10 μg) were loaded for each sample (Bradford 1976).

Primary antibodies were used in a 1 in 1000 dilution and secondary 1 in 10,000 dilution, and blots were developed using an enhanced chemiluminescence kit (Amersham). Band intensities were quantified using Quantity One software (Version 4.4.0 Bio-Rad).

2.2.6 Immunohistochemistry

Paraffin-embedded embryo sections were washed in Xylene and re-hydrated by washing in 100, 90, 80 and 70% ethanol each for 10 minutes twice, followed by 10 min in 0.1% Tween-20 in PBS twice. Histology sections to be immunostained were incubated with a 24-hour primary followed by a two-hour secondary (FITC or Texas Red conjugated) antibody incubation in a 1 in 100 antibody dilution. Embryos were counterstained with DAPI (Invitrogen) according to the manufacturer's protocol. Images were captured and fluorescence quantified with a Zeiss LSM Dou (Live 5 Vario II and 510 Meta) Confocal system using Northern Eclipse image capture and analysis software (Empix).

2.2.7 Statistical analyses

Tests of significance are described within the legend of the figure as required.

2.3 Results

2.3.1 Pex11β altered the RNA levels of peroxisome related genes in X. laevis A6 cells.

We first investigated if Pex11β could alter the RNA levels of the peroxisome related genes Pex1, Pex3, Pex5, Pex11β, catalase, PMP70, PPARα, -δ, and -γ in *X. laevis* A6 kidney epithelial cells, which has not been previously demonstrated in Xenopus before. A6 cells were transfected with plasmids designed to express Xenopus HA-Pex11β, or control full-length GFP. Semi-quantitative RT-PCR analyses revealed a significant increase in Pex11β, PMP70, catalase, Pex5 and PPARα, but a significant decrease in PPARγ mRNA levels, following HA-Pex11β overexpression in A6 cells (Figure 2.1). No significant changes were found in levels of Pex3, Pex1, nor in PPAR δ mRNA (Figure 2.1).

2.3.2 Pex11β increased hallmark peroxisomal protein levels in X. laevis A6 cells.

Since overexpression of HA-Pex11β increased the mRNA levels of catalase and PMP70, we next wanted to determine if there were actual increases in the protein levels of these hallmark peroxisomal proteins. A6 cells were transfected as previously described and protein samples were isolated for Western blots. Using HA specific antibodies, Western blot analysis confirmed bands of expected sizes for HA-Pex11β (63 kDa) in transfected samples (Figure 2.2), confirming the integrity of the HA-tagged construct. Western blot analyses with catalase and PMP70 specific antibodies also revealed bands of expected sizes for both PMP70 (70 kDa) and catalase (55 kDa) (Figure 2.2). A significant increase in catalase and in PMP70 were found following HA-Pex11β overexpression versus control untransfected samples (Figure 2.2A, left three GFP lanes

versus right three HA-Pex11β lanes, and quantified in Figure 2.2B). The use of anti-β-actin demonstrated the relative protein levels in each lane.

Figure 2.1. Overexpressing HA-Pex11β altered peroxisome related gene expression in Xenopus A6 cells. RT-PCR analysis of peroxisomal genes was performed before and after transfection of A6 cells with HA-Pex11β. Two days following transfection 250 ng of reverse-transcribed A6 cell RNAs from control and treatment samples (n=3) were subject to PCR amplification using specific primers for the peroxisome related genes; Pex11β, PMP70, catalase, Pex5, Pex3, Pex1, PPARα, -δ, and -γ. The respective mRNA levels represent measures of mid-log phase RT-PCR product band intensities, relative to levels of EF1α. Genes whose levels were altered significantly as assessed by a paired sample t-test are denoted with an asterisk. Pex11β, PMP70, catalase, Pex5 and PPARα displayed elevated levels of expression following treatment, while PPARγ displayed reduced expression. $P<0.05$, n=3. Values presented are the means ± SE.

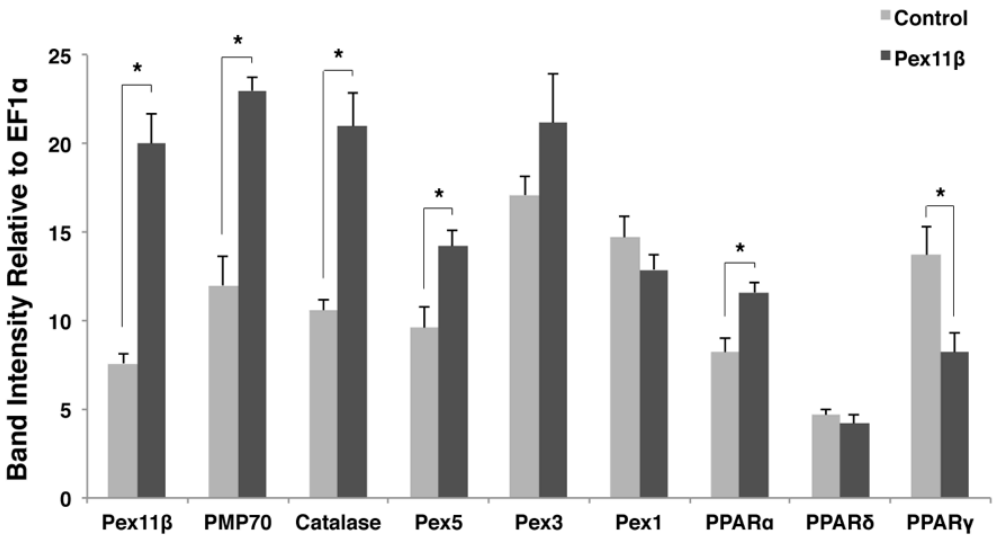
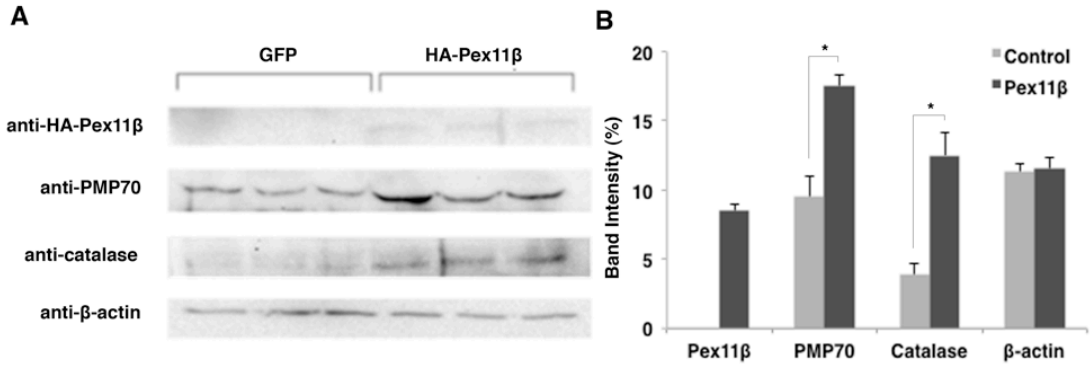

Control
Pex11β
Band Intensity Relative to EF1α
25
20
15
10
5
0
*
Pex11β
PMP70
Catalase
Pex5
Pex3
Pex1
PPARα
PPARδ
PPARγ

Figure 2.2. Overexpression of HA-Pex11β in A6 cells increased catalase and PMP70 protein levels. Western blotting revealed elevated proteins levels of catalase and PMP70 following the transfections of HA-Pex11β, but not GFP, in three samples of A6 cells (A). An HA antibody confirmed the translation and presence of HA-Pex11β in transfected cells (right 3 lanes) versus GFP transfected control cells (left 3 lanes). Catalase and PMP70 antibodies also displayed altered band intensities of each respective protein in HA-Pex11β transfected cells (right 3 lanes) versus GFP transfected cells (left 3 lanes). Protein loading in each lane was confirmed via a β-actin antibody. The Western blot signals were digitized and data were quantified and analyzed to statistically compare protein levels (B). There was a significant increase in the levels of catalase and PMP70 following overexpression of Pex11β, while there was no difference in the levels of β-actin. Statistical relevance of discrepancies between groups (asterisks) was assessed by a paired sample t-test. $P<0.05$, n=3. Values presented are the means ± SE.


A
GFP
HA-Pex11β
anti-HA-Pex11β
anti-PMP70
anti-catalase
anti-β-actin
B
Band Intensity (%)
20
15
10
5
0
Control
Pex11β
*
*
Pex11β
PMP70
Catalase
β-actin

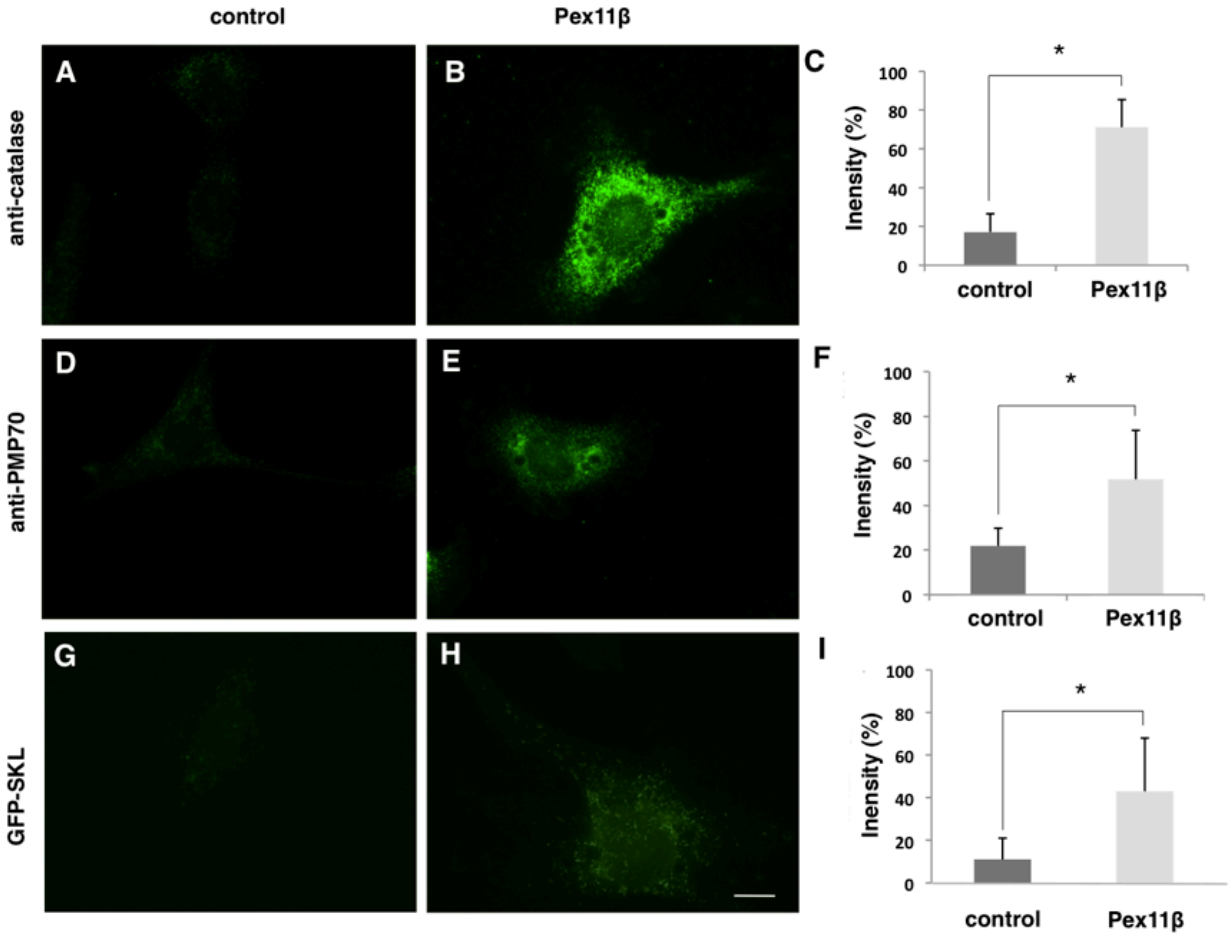
2.3.3 Overexpression of Pex11β increased peroxisome numbers in X. laevis A6 cells.

Although recent studies in several eukaryotic cell lines has revealed that Pex11-proteins can independently increase peroxisome-like structures (Selman and Pawsey 1965), I tested the hypothesis that Pex11β could induce an early onset to peroxisome biogenesis during Xenopus embryogenesis. I first tested whether overexpression of Pex11β could increase peroxisome-like structures and peroxisome number in *X. laevis* A6 cells. Two days following transfection of HA-Pex11β, cells were fixed for immunocytochemistry and probed with PMP70 and catalase antibodies. This allowed us to examine the distribution of PMP70 and catalase protein, and also to quantify their relative protein levels using a fluorescent secondary antibody. Our results indicated that overexpression of HA-Pex11β significantly increased the amounts of both catalase levels (Figure 2.3A versus B) and PMP70 levels (Figure 2.3D versus E) versus control, as determined by quantifying the relative levels of indirect fluorescent; catalase indirect fluorescence levels increased >3 fold (Figure 2.3C), while PMP70 indirect fluorescence levels increased >2.5 fold (Figure 2.3F).

As the increased levels of PMP70 and catalase fluorescent signals may not specifically be related to peroxisome function, I next tested if overexpression of HA-Pex11β could also increase the number of peroxisomes using the peroxisomal marker GFP-SKL as a detection assay. This PTS1 tagged GFP will localize to punctate-like structures in the cytosol when imported into peroxisomes. A6 cells were co-transfected with HA-Pex11β and GFP-SKL, or GFP-SKL alone. Two days following transfection, peroxisome-like structures were assessed by direct immunofluorescence. Our results showed a significant increase in the number GFP-containing bodies (>2-fold, Figure 2.3I)

in cells that expressed HA-Pex11β versus cells transfected with GFP-SKL alone (compare Figure 2.3G and H).

Figure 2.3. Overexpression of HA-Pex11β in A6 cells increased peroxisome numbers. A, D, G are untransfected cells, while B, E, H have been transfected with HA-Pex11β. G and H are additionally transfected with GFP-SKL. Using identical imaging and photography parameters, indirect immunofluorescence using a catalase antibody revealed lower levels of immunofluorescence in untransfected cells (A) versus transfected (B) cells. Similarly, indirect immunofluorescence using a PMP70 antibody revealed lower levels of signal in untransfected cells (D) versus transfected (E). Direct fluorescence for GFP revealed a diffuse signal from GFP in HA-Pex11β untransfected cells, (G) versus the presence of punctate structures in HA-Pex11β transfected cells (H). All images were captured using identical fluorescent settings. The relative fluorescence intensity in 10 regions of twenty randomly imaged cells was quantified using Northern Eclipse software. Graphs on the right represent the average fluorescence intensity of untransfected versus HA-Pex11β transfected cells. Values presented are the means ± SE. Significance at $P<0.05$ was determined using Student's t-test, n=25.


control
Pex11β
anti-catalase
anti-PMP70
GFP-SKL
A
B
C
D
E
F
G
H
I
Inensity (%)
100
80
60
40
20
0
*
control
Pex11β

2.3.4 Pex11β increased peroxisome related gene expression during X. laevis embryogenesis.

I next examined the effects of increased Pex11β *in vivo*, by investigating changes in expression of specific peroxisomal genes, following the microinjection of HA-Pex11β RNA into early Xenopus embryos. To establish a base line, the temporal expression of five peroxisomal genes were first analyzed during the developmental stages of gastrulation (stage 10), neural tube closure (stages 20) and organogenesis (stage 30). In general, with the exception of Pex5, all peroxisomal genes examined in control embryos increased in expression as development progressed, with their lowest expression levels at stage 10, and highest at stage 30 (Figure 2.4, significance between stages denoted by double asterisks). Pex5 expression in control embryos does not vary significantly between stages 10 and 30. The increase in PMP70 RNA level between stages 10 and 20, differs from a previously described decreasing trend between stages 12 and 20 (Marteil, D'Inca et al. 2010). This discrepancy cannot be explained, but may to due to differences in staging. Following microinjection of HA-Pex11β RNA there were significantly increased RNA levels of catalase and PMP70 *at* stages 10, 20 and 30 (Figure 2.4, single asterisk) versus control RNA levels of expression for each gene. Microinjecting HA-Pex11β also resulted in significant increases of Pex3 *at* stages 10 and 30, as well as Pex5 *at* stages 20 and 30. There were no significant changes in Pex3 at stage 20, nor Pex5 *at* stage 10. Changes in Pex11β levels following microinjection of HA-Pex11β reflect the presence of the HA-Pex11β construct.

Further, as ectopic Pex11β significantly decreased PPARγ RNA levels in A6 cells, we investigated whether ectopic Pex11β would similarly alter PPAR levels within

embryos. The injection of Pex11β into embryos significantly increased levels of PPARα, significantly decreased levels of PPARγ, but did not change levels of PPARδ RNA (Figure 2.5), a pattern similar to that seen in the A6 cells.

Figure 2.4. Embryonic overexpression of HA-Pex11β elevated Pex3, catalase and PMP70 levels. The respective mRNA levels represent measures of mid-log phase RT-PCR product band intensities, relative to levels of EF1α. RT-PCR analysis during normal embryogenesis revealed that the levels of all genes examined, with the exception of Pex5, increased as development progressed. First, a repeated measures ANOVA was carried out entering all RNA levels at all 3 stages. When significant, a paired sample t-test was carried out between levels at a given stage in control embryos. This would reveal significant changes in RNA levels of the genes examined during normal development. Significant changes in RNA levels a gene between stages is represented by the double asterisk ** (*P<0.05*). Expression increases with development and there are differences in the levels of Pex3 and PMP70 between all stages, 10vs20, 20vs30 and 10 versus 30. For catalase there are differences between stages 10vs20 and 10vs30 but NOT between 20 versus 30. There are no significant differences in RNA levels between the tested developmental stages for Pex11β or Pex5. As the means were correlated, a MANOVA was carried out on the means of the treatment and control groups at each stage to see whether there were differences in the RNA level of each gene at a given stage, following the Pex11β treatment. As the MANOVA showed a significant effect of condition, a Wilks lambda analysis was used, and the univariate ANOVAs showed that Pex11β treatment resulted in significantly higher levels of gene expression, as represented by the single asterisk * (*P<0.05*). Pex11β, catalase and PMP70 all displayed significant increases in RNA levels all stage 10, stage 20 and stage 30 following treatment. Pex3 displayed elevated expression at only stage 10 and 30, while Pex5 displayed differences only at stages 20 and 30. n=3. Values presented are the means ± SE.

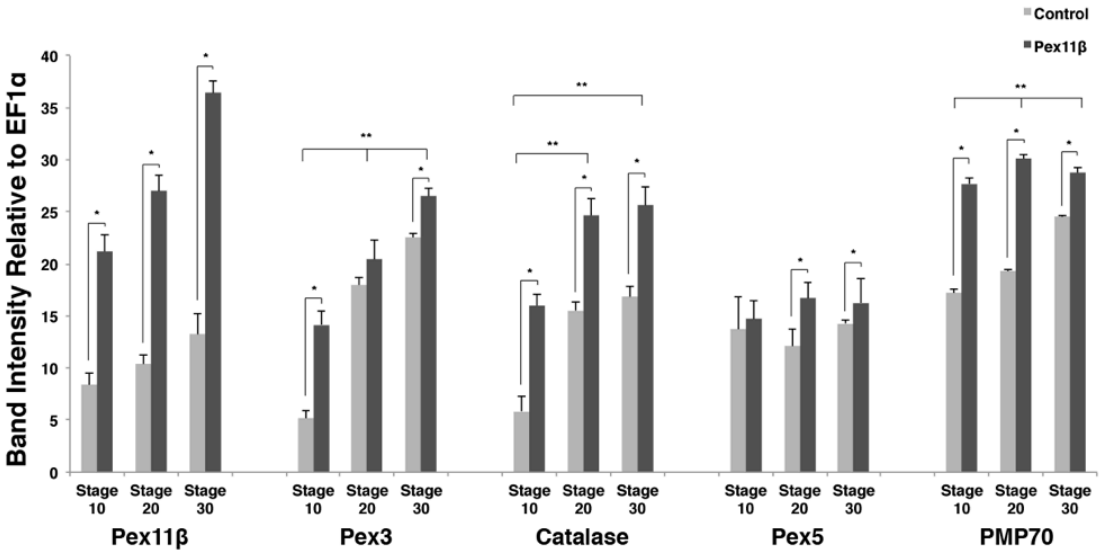
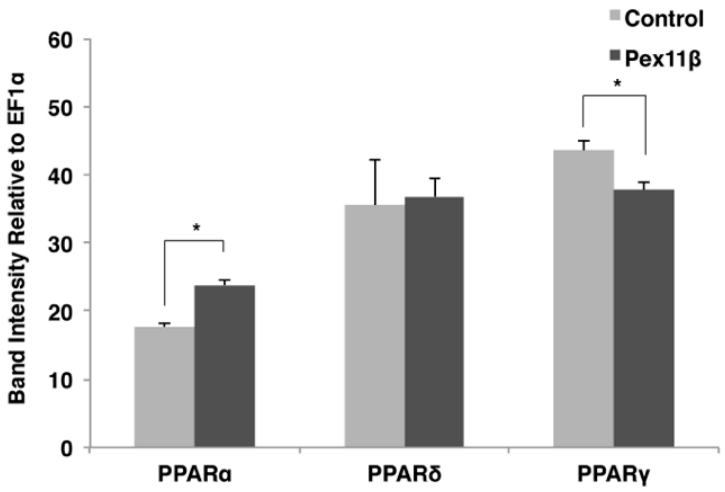

Control
Pex11β
Band Intensity Relative to EF1α
40
35
30
25
20
15
10
5
0
Stage 10
Stage 20
Stage 30
Pex11β
Pex3
Catalase
Pex5
PMP70

Figure 2.5. Overexpression of HA-Pex11β did altered PPARα and γ, but not δ, gene expression during early *X. laevis* embryogenesis. The respective mRNA levels represent measures of mid-log phase RT-PCR product band intensities, relative to levels of EF1α. Using a similar approach and analysis as used in Fig. 4, RT-PCR analysis of RNA isolated from control stage 10 embryos and HA-Pex11β injected embryos revealed significant changes in the expression of PPARα, and PPARγ, but not PPARδ. PPARα levels were elevated by treatment, while PPARγ levels were redueced. $P<0.05$, n=3. Values presented are the means ± SE.


Control
Pex11β
Band Intensity Relative to EF1α
60
50
40
30
20
10
0
*
*
PPARα
PPARδ
PPARγ

2.3.5 Catalase and PMP70 antibodies reveal early punctate organelle structures during X. laevis embryogenesis following Pex11β injections.

To determine if Pex11β could induce an early onset to peroxisome-like structures during Xenopus development, I used immunohistochemistry to visualize changes in the embryonic distribution of PMP70 and catalase, in response to microinjecting HA-Pex11β RNA. Fertilized embryos were microinjected with HA-Pex11β RNA, fixed at stages 10 and 20, and sectioned for immunohistochemistry. PMP70 and catalase signals were undetected in stage 10 under control conditions (Figure 2.6A and 2.7A) using specific antibodies. Punctate structures were visualized within the somites at stage 20 in control sections, using PMP70 (Figure 2.6B), and catalase (Figure 2.7B) specific antibodies. Following microinjection of HA-Pex11β, I were able to detected punctate PMP70 (Fig. 6C) and catalase (Fig. 2.7C) signals at stage 10 in pre-somitic mesoderm, and increased levels of immunofluorescence for both proteins in stage 20 somites compared to control uninjected embryos (compare Figure 2.6B vs 2.6D, and 2.7B vs 2.7D).

Figure 2.6. Microinjecting HA-Pex11β RNA increased PMP70 immunofluorescence levels during *X. laevis* embryogenesis. Both control (A and B) and HA-Pex11β injected (C and D) embryos at developmental stages 10 (A and C) and 20 (B and D), were fixed then sectioned for immunohistochemical analysis in somites for PMP70. At stage 10 PMP70 protein is undetected in somitic mesoderm under control conditions (A), whereas following microinjection of HA-Pex11β PMP70 protein is detectable in punctate structures (C). At stage 20, PMP70 protein was detected in both control and following microinjecting HA-Pex11β (B and D). An HA antibody was also used to confirm the ectopic presence of HA-Pex11β. DAPI (blue), PMP70 (green), HA-Pex11β (red), colocalization of HA-Pex11β and catalase (yellow). Images were taken at 60x magnification.

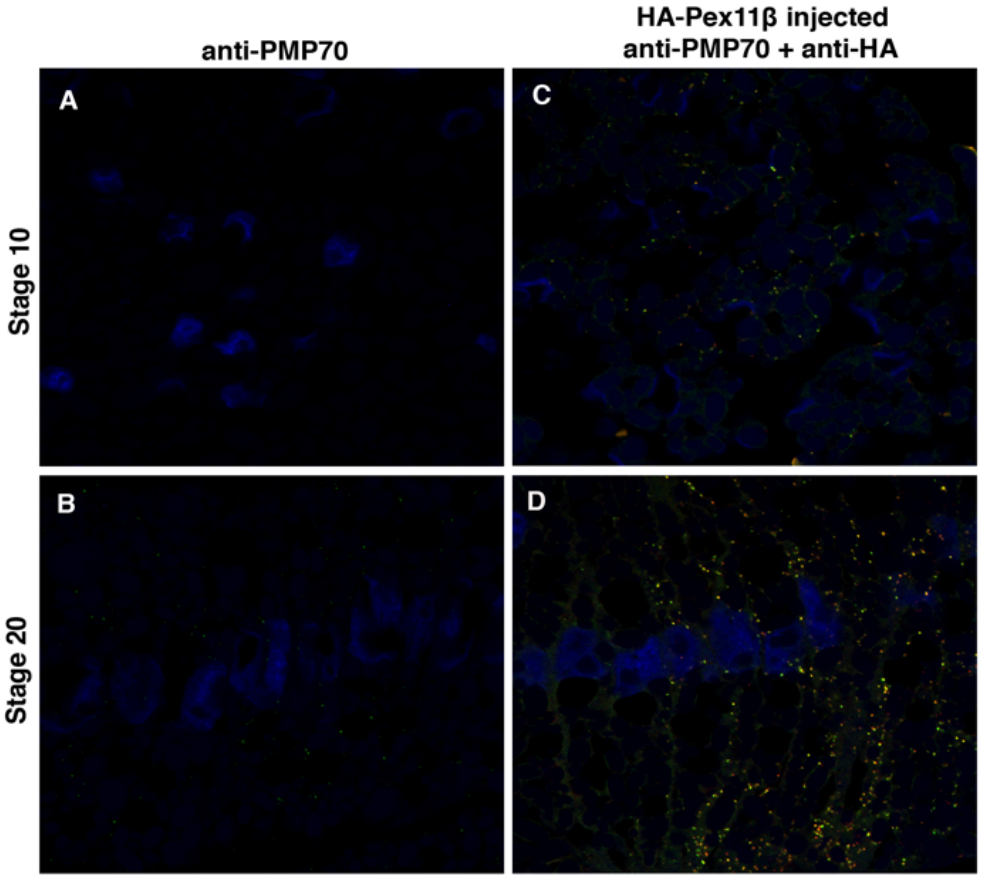
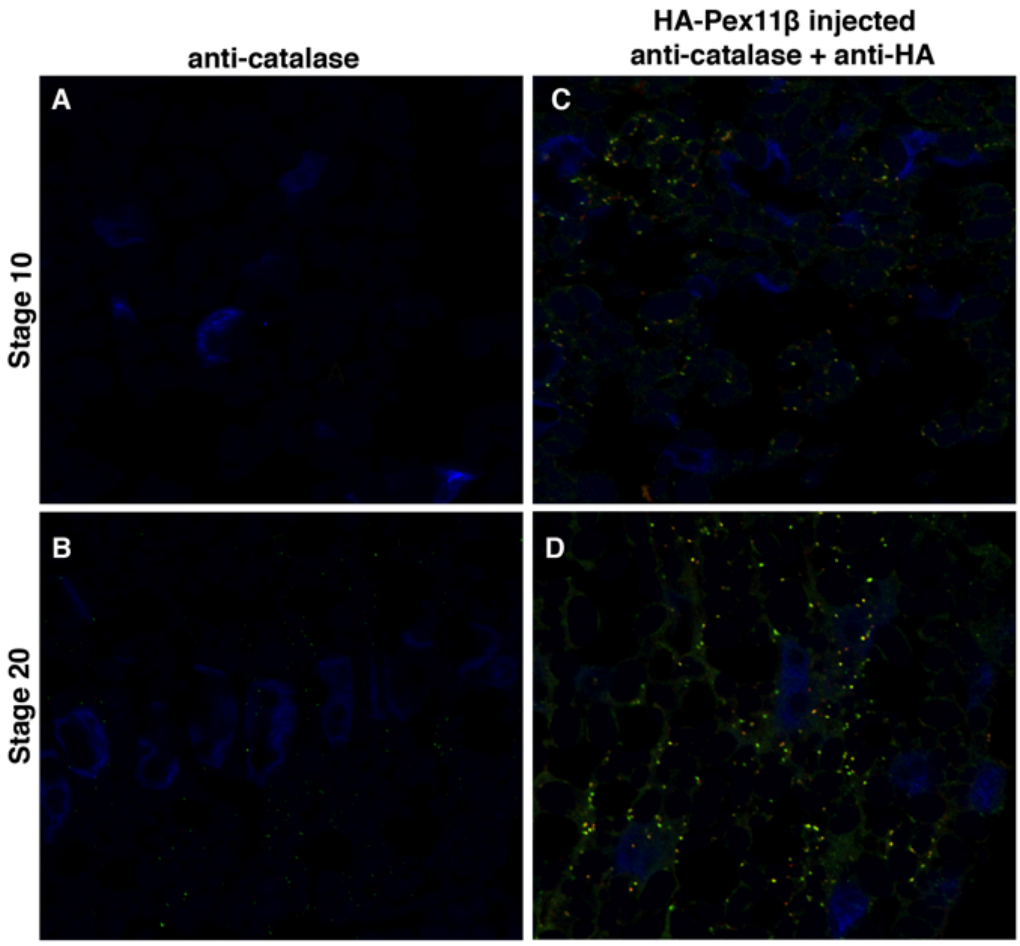

anti-PMP70
HA-Pex11β injected
anti-PMP70 + anti-HA
A
C
B
D
Stage 10
Stage 20

Figure 2.7. Microinjecting HA-Pex11β RNA increased catalase immunofluorescence levels during *X. laevis* embryogenesis. Both control (A and B) and HA-Pex11β injected (C and D) embryos at developmental stages 10 (A and C) and 20 (B and D), were fixed then sectioned for immunohistochemical analysis in somites for catalase. At stage 10 catalase protein is undetected in somitic mesoderm under control conditions (A), whereas following microinjection of HA-Pex11β catalase protein is detectable in punctate structures (C). At stage 20, PMP70 protein was detected in both control and following microinjecting HA-Pex11β (B and D). An HA antibody was also used to confirm the ectopic presence of HA-Pex11β. DAPI (blue), PMP70 (green), HA-Pex11β (red), colocalization of HA-Pex11β and PMP70 (yellow). Images were taken at 60x magnification.

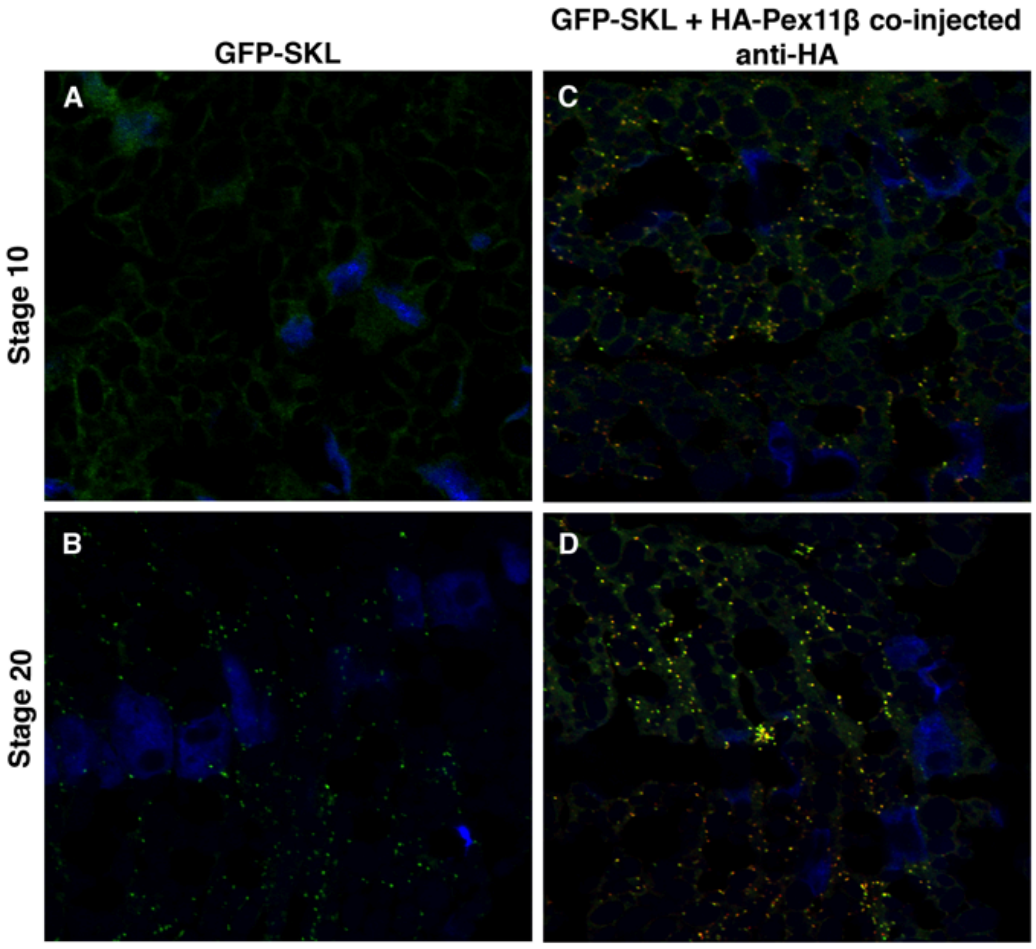

anti-catalase
HA-Pex11β injected
anti-catalase + anti-HA
A
C
B
D
Stage 10
Stage 20

2.3.6 Overexpression of Pex11β triggered an early-onset to peroxisome accumulation during Xenopus embryogenesis.

In order to determine when peroxisomes are first present during embryonic development, focusing on the dorsal mesoderm, I microinjected GFP-SKL into early-fertilized embryos. Histological sections taken of developmental stage 10 embryos revealed that peroxisomes were not visible, as GFP-SKL revealed a diffuse staining pattern that lacked punctate structures (Figure 2.8A), similar to that seen in control GFP injections embryos (data not shown). However, GFP-containing punctate bodies, indicative of peroxisomes, are readily visible in control embryos at stage 20 in the somites (Figure 2.8B).

Next, I wanted to determine if overexpression of Pex11β could induce an accumulation of peroxisomes, similar to how overexpression in A6 cells increased peroxisome numbers. Histological sections showed that ectopic expression of HA-Pex11β in embryos resulted in the presence of punctate GFP-containing bodies at stage 10 (Figure 2.8C), versus the diffuse pattern of GFP-SKL seen at this stage in the dorsal structures of uninjected embryos (Figure 2.8A). Further, there was also a relative increase in the number of punctate structures at stage 20 following HA-Pex11β and GFP-SKL (Figure 2.8D), compared to the microinjection of GFP-SKL alone (Figure 2.8B).

Figure 2.8. Microinjecting HA-Pex11β RNA increased the number of peroxisome-like GFP-SKL structures during *X. laevis* embryogenesis. Both control (A and B) and HA-Pex11β injected (C and D) embryos at developmental stages 10 (A and C) and 20 (B and D), were fixed then sectioned for immunohistochemical analysis in somites for GFP-SKL. At stage 10, GFP fluorescence is present as a faint diffuse stain under control conditions (A), whereas following microinjection of HA-Pex11β punctate GFP structures could be seen at this early stage (C). At stage 20, punctate GFP structures were detected in both control and following microinjecting HA-Pex11β, where numbers were increased in the injected samples. An HA antibody was also used to confirm the ectopic presence of HA-Pex11β. DAPI (blue), GFP-SKL (green), HA-Pex11β (red), colocalization of HA-Pex11β and GFP-SKL (yellow). Images were taken at 60x magnification.


GFP-SKL
GFP-SKL + HA-Pex11β co-injected
anti-HA
Stage 10
Stage 20
A
B
C
D

2.4 Discussion

2.4.1 Role of Pex11β in peroxisome biogenesis in vitro

Pex11 proteins were first identified in yeast as peroxisomal membrane proteins that could increase peroxisome number when overexpressed and significantly reduce peroxisome number when interrupted (Karnati and Baumgart-Vogt 2009). Early studies suggested that Pex11 proteins acted primarily on medium-chain fatty acid oxidation, affecting peroxisome divisions indirectly (van Roermund, Tabak et al. 2000). Schrader and colleagues were the first to show in human fibroblasts that overexpression of human Pex11β was sufficient to induce peroxisome proliferation (Schrader, Reuber et al. 1998). Recently, it has been shown that Pex11β participates in peroxisome divisions through membrane elongation and shape changes of existing peroxisomes. Elongated membranes fill with imported matrix proteins, form into small blebs and separate into new peroxisomes with the aid of a dynamin-like protein (Delille, Agricola et al. 2010). While yeast studies have shown that peroxisomes only arise through division (Motley, Ward et al. 2008), and mammalian cell studies have suggested that they arise from both *de novo* and division mechanisms (Kim, Mullen et al. 2006), little is known about peroxisome biogenesis during embryonic development. The question of peroxisome inheritance remains largely unresolved, particularly as I have shown that peroxisomes are absent in early frog embryos, and arise only later due to embryonic and or metabolic cues (Cooper, Walsh et al. 2007).

I put to test whether overexpression of Pex11β could induce an early-onset to peroxisome biogenesis or accumulation during early Xenopus embryogenesis. This is particularly intriguing, as stage 10 embryos have no detectable peroxisomes. Thus, as

Pex11β participates in peroxisome division, and no detectable peroxisomes are present in early embryos, Xenopus represents a novel model where the role of Pex11β in peroxisome number can be examined. The utility of microinjection and relative ease of expression and localization assays enables specific questions related to Pex11β to be addressed. First, I sought to show that Pex11β is sufficient to regulate peroxisome related protein and RNA levels, and increase the number of peroxisomes in *X. laevis* A6 cells. My RT-PCR analysis indicated significant increases in RNA levels for both catalase and PMP70, amongst other genes, following overexpression of Pex11β. Using Western blot analysis I confirmed that HA-Pex11β increased catalase and PMP70 proteins levels, and immunohistochemistry confirmed that HA-Pex11β increased the number of both catalase and PMP70 positive punctate structures in A6 cells. Additionally, as GFP-SKL can be transported into peroxisomes, co-transfection of HA-Pex11β and GFP-SKL revealed an increase in the number of peroxisome-like structures. These results strongly support the idea that Pex11β can independently promote increases to the number of peroxisomes in Xenopus A6 cells.

2.4.2 Role of Pex11β in peroxisome biogenesis in vivo

The primary focus of this study was to elucidate the role of Pex11β *in vivo*. Very little is known about what cellular mechanisms regulate the *de novo* biogenesis of peroxisome during Xenopus development. Using a different GFP-KANL reporter, I had previously reported their detection at stage 30 in the ectoderm (Cooper, Walsh et al. 2007). Histochemical studies in frog have suggested that yolk protein and lipid metabolism occurs at different stages in different tissues (Mes-Hartree and Armstrong

1980). Interestingly, early yolk metabolism is seen in the newly formed muscles – the somites, but not in the large yolk-filled endodermal cells that are present on the ventral side of the embryo (Mes-Hartree and Armstrong 1980). Here, using HA-Pex11β and other specific assays, I demonstrate that peroxisomes are detectable in somites at stage 20, but not at stage 10.

In agreement with the presence of peroxisome by stage 20, the RNA levels of most peroxisomal genes examined changed temporally during early development. Pex11β Pex3, Catalase, and PMP70 showed increasing trends in expression as development proceeded, peaking stage 30, with cytosolic-bound peroxisomal receptor Pex5 not varying during these stages. This suggested that transcripts are present and increasing towards the eventual onset of peroxisome biogenesis and/or their subsequent proliferation. These changes in Pex3 and Pex11β RNA levels relate well with previous studies that have demonstrated their roles in division (Pinto, Grou et al. 2009). If Pex11β, did play a key regulatory role, I next determined how microinjecting HA-Pex11β mRNA would affect the relative levels of key peroxisomal genes. Changes of Pex11β RNA levels simply reflect and confirm the presence on the transfected construct. The Pex11β resulted in the significant increases in RNA levels for catalase and PMP70 at all stages tested (10, 20 and 30). There were also increases in the levels of Pex3 and Pex5 at two of the three stages examined, however, these changes were not as dramatic. From this data, I conclude that Pex11β can play a role in the early induction of these peroxisomal genes. Interestingly, as was examined with Pex11β in A6 cells, PPARα RNA levels increased, PPARγ decreased, and PPARδ was unchanged by ectopic Pex11β in embryos. Given that PPARα has roles in the β-oxidation of fatty acids, PPARγ a role

in lipid catabolism and adipocyte differentiation, and that while expressed ubiquitously, PPARδ functions remain unclear, the significance of our findings are not known. Furthermore, the relationship between PPARs, other metabolic regulators, yolk utilization and peroxisome numbers certainly bears further investigation.

I focused on the distribution of catalase and PMP70 protein within the somites and found that catalase and PMP70 proteins are first localized as punctate structures suggestive of peroxisomes at stage 20, with no detectable signal at stage 10. To corroborate this immunological finding I microinjected GFP-SKL RNA, whose product could be transported into peroxisomes. Our stage 10 histology sections revealed diffuse signals from GFP, indicating that peroxisomes are not yet present, as the SKL-tagged GFP was not localized. However, we were able to show that GFP-SKL localized to punctate-like structures in the somites at stage 20, indicating that peroxisomes are present at this stage.

2.4.3 Pex11β induces an early-onset to the accumulation of peroxisomes during embryogenesis

With these results in mind, I next tested whether microinjecting HA-Pex11β RNA could induce an early accumulation to the number of peroxisomes. While peroxisomes are present at stage 20, perhaps all needed precursors are present earlier in the embryo and waiting a developmental or metabolic cue to form functional peroxisomes. Following the microinjection of HA-Pex11β, I were able to visualize peroxisome-like structures using GFP-SKL at stage 10. This suggested that needed peroxisomal precursors, including matrix proteins and other division proteins, such as dynamin-like

proteins are present. Interestingly, together with the data that showed that HA-Pex11β injections increased the transcription of peroxisomal genes, this suggests that Pex11β is a key regulator of peroxisome onset and proliferation during Xenopus development. For the very first time, I are able to show that Pex11β can independently induce an early onset to peroxisome accumulation *in vivo*.

From my data I conclude that Xenopus Pex11β is essential for regulating peroxisome number both in A6 cells *in vitro* and *in vivo* in embryos. Ectopic expression *in vivo* demonstrated for the very first time Pex11β's ability to induce peroxisome related gene expression, and additionally to promote the early formation of peroxisome-like structures in embryos.

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CHAPTER 3

MORPHOLINO-INDUCED KNOCKDOWN OF *XENOPUS LAEVIS* PEX11B REVEALS ITS PIVOTAL ROLE IN PEROXISOME BIOGENESIS DURING EMBRYONIC DEVELOPMENT.

3.1 Introduction

3.1.1 Peroxisome overview

Peroxisomes are multifunctional single-membrane enclosed organelles that are present in all eukaryotic cells. Peroxisomes house over 50 different matrix enzymes that are linked to a diverse spectrum of metabolic activities, which can vary among different species, developmental stages, and cell types (Delille, Dodt et al. 2011). The fundamental processes mediated by peroxisomes include reactions involved in lipid metabolism; such as the synthesis of ether lipids, β-oxidation of very long chain fatty acids (VLCFA), 2-methyl branched fatty acids, polyunsaturated fatty acids, and defense systems for the *in situ* scavenging and elimination of peroxides, free radicals and other reactive oxygen species (ROS) (Schrader and Fahimi 2006).

Peroxisomes are highly dynamic and capable of adapting to a variety of environmental and developmental cues by altering their morphology, number and enzyme content (Ma, Agrawal et al. 2011). The number and overall level of action of peroxisomes is one of the mechanisms that regulate ROS levels in cells. High levels of ROS levels are deemed detrimental to normal cellular functioning; whereas low ROS levels may contribute to cell signaling, suggesting that peroxisome numbers and functions must be tightly regulated. The biogenesis of peroxisomes is accomplished by the coordinated activity of over 30 different peroxisomal matrix and peroxisomal membrane proteins (PMPs) termed peroxins. Peroxins are nuclear encoded by Pex genes, synthesized on free cytosolic polysomes and transported directly to peroxisomes or the endoplasmic reticulum (ER) (Ma and Subramani 2009).

3.1.2 Peroxisome biogenesis

Most PMPs are imported into the ER and then sorted into pre-peroxisomal compartments in preparation for ER vesicle budding – a Pex3/Pex19 dependent process (Ma, Agrawal et al. 2011). This *de novo* pathway is dependent on the ER, behaving as a template for peroxisome biogenesis commonly referred to as the pre-peroxisomal reticulum (Ma, Agrawal et al. 2011). These vesicles can then fuse with neighboring vesicles forming mature functional peroxisomes. The alternate maturation pathway is reliant on duplication of preexisting peroxisomes by fission and subsequent growth by matrix protein import, a process mediated by cytosolic receptors Pex5 and Pex7 (Fujiki 2000). Matrix protein import is dependent on the recognition of two distinct peroxisome-targeting signals (PTS). PTS-l is a conserved sequence of three amino acids (serine-lysine-leucine) at the extreme C-terminus of most matrix proteins that are targeted to peroxisomes by the Pex5 receptor. PTS-2 is a broad consensus nonapeptide sequence (R/K-L-X_5-Q/H/-L) found on matrix proteins at or near their N-terminus that are recognized by the cytosolic Pex7 receptor. Both Pex5 and Pex7 deliver their respective cargo to peroxisomes by docking with the peroxisomal importomer, a dynamic peroxisomal translocon whose function is reliant on over 12 different peroxins, which includes Pex1, an AAA-ATPase (Shiozawa, Maita et al. 2004). Functional peroxisomes also contain PMP70 proteins, which are involved in the transport of long chain acyl-CoA across the peroxisome membrane (Imanaka, Aihara et al. 2000).

3.1.3 Peroxisome biogenesis disorders

In addition to their roles in metabolism and elimination of free radicals, peroxisomal functioning is crucial to human development, as evident by the many peroxisome biogenesis disorders (PDB). PDB's such as the Zellweger syndrome spectrum (ZSS), X-linked Adrenoleukodystrophy (ALD) and rhizomelic chondrodysplasia punctata, are characterized by dysfunctional or lack of total peroxisomes. Mutations in the Pex7 gene are responsible for the latter disorder, whereas mutations in any one of many other Pex genes cause the ZSS and ALD disorders (Krause, Rosewich et al. 2006).

3.1.4 Pex11-family of peroxins

The Pex11-family has received much interest with respect to ZSS due to their ability to independently induce peroxisome proliferation. Pex11 proteins are unique PMPs that directly participate in peroxisome divisions in yeasts (Li and Gould 2003), plants (Orth, Reumann et al. 2007), mammals (Thoms and Erdmann 2005) and recently amphibians (Fox, Walsh et al. 2011). In particular, Pex11β can induces peroxisome proliferation via elongation of the existing peroxisomal membrane, followed by constriction and final division via fission into peroxisomes (Li and Gould 2003). While studies support a direct role for the Pex11β in peroxisome division *in vitro*, little is known about its role during embryogenesis.

Most oviparous and many viviparous embryos depend on the rapid metabolism of stored fuels early in their development to provide the energy for early embryonic events. In addition to the metabolism of fatty acids, the metabolic activities of early embryos

generate a variety of ROS elements whose levels must be tightly regulated such that they are not detrimental, but still available to play signaling roles. ROS levels have been shown to be important in regulating various cellular processes and signaling cascades. Nucleoredoxin, a thioredoxin related protein, was shown to inhibit Wnt-β-catenin signaling through disheveled in *Xenopus laevis* (Funato and Miki 2010). *X. laevis* development — whose early cell signaling cascades are well understood, and utilization of yolk stores require peroxisome function with respect to both the breakdown of fatty acids and the regulation of ROS levels — provides an excellent model with which to examine the developmental roles of peroxisomes.

3.1.5 Hypotheses of Pex11β knockdown

I have previously shown that overexpression of Pex11β can increase the number of peroxisomes in A6 cells *in vitro*, and induce an early-onset to peroxisome-like structures during *X. laevis* embryogenesis *in vivo* (Fox, Walsh et al. 2011). In an effort to improve our understanding of Pex11β, I have generated a Pex11β-morpholino (MO) to test the hypothesis that knock down of Pex11β has the ability to decrease peroxisome related gene and protein expression, and peroxisome numbers in both A6 cells and during *X. laevis* embryogenesis. Our results demonstrated that knocking down Pex11β decreased the number of peroxisomes in A6 cells *in vitro*, and resulted in modifications to the size and distribution of peroxisomes during embryogenesis *in vivo*. Our data suggests, in compilation with our previous findings, that Pex11β has a direct role in regulating peroxisome biogenesis during *X. laevis* embryogenesis.

3.2 Materials and Methods

3.2.1 Embryo Work

Adult *X. laevis* were reared in accordance with Canadian Council on Animal Care regulations. Fertilizations were performed according to Wu and Gerhart (Wu and Gerhart 1991), and embryos were staged according to Nieuwkoop and Faber (Nieuwkoop 1973). Embryos to be sectioned were fixed in 3% formaldehyde at developmental stages 15 and 30. Embryos were dehydrated, and infiltrated with paraffin wax for tissue embedding and sectioning at Robarts Research Molecular Pathology Core Facility (London, ON., Canada).

3.2.2 Microinjection and Morpholino Design

Morpholino oligos were injected using a Nanoject II (Drummond Scientific Company). *X. leavis* embryos at the one-cell stage were injected with 2.3 nl of 400 μM morpholino (MO) oligos. Design and synthesis of morpholinos was performed by Gene Tools (Gene Tools, Philomath, USA). Morpholinos were engineered as translation-blocking targets against *X. laevis* Pex11β [GenBank:MGC69071], [antisense CGCTGAACCGAACCCACGAGTCCAT]. Additionally, a carboxyfluoresceinated morpholino oligos targeted to *X. laevis* β-catenin gene were purchased as a prepared control oligo from Gene Tools [antisense TTTCAACCGTTTCCAAGAACCAGG]. Each morpholino was used in at least three independent experiments and injected each time at three different concentrations (400, 600 and 800 μM).

3.2.3 Cloning, RNA Synthesis, and Microinjection

We cloned Xenopus full length Pex11β [GenBank:MGC69071] from total adult liver cDNA using specific primers using and SuperScript™ Reverse Transcriptase (Invitrogen) with Platinum® *Taq* DNA Polymerase High Fidelity (Invitrogen) using conditions supplied by the manufacturer. A 5' HA tag was added to Pex11β using specific primers; HA-Pex11β 5'AGA TCT TCA AGC GTA ATC TGG TAC GTC GTA TGG GTA GGG CTT CAG CTT CAG CCA 3' and 5' CGA ACC CAC GAG TCC ATA CTA GT 3'. We also engineered GFP tagged with the PTS1 SKL, using forward 5' AGA TCT ATG GTG AGC AAG GGC GAG 3' and 5' ACT AGT CTA TAA TTT GGA CTT GTA CAG CTC GTC CA 3'. PCR products were cloned into the pCR®II-TOPO vector as per manufacturer's instructions (Invitrogen). Recombinant sequences were confirmed at the Robarts Research Institute DNA Sequencing Facility at the University of Western Ontario. Desired clones were additionally cloned into pcDNA™ TOPO 3.3® TA Cloning Kit (Invitrogen) for cell culture experiments, and T7TS plasmid and sequenced *in vitro* RNA production. Capped polyadenylated RNA was synthesized using mMachine mMessage® T7 (Ambion) and visualized on a 1.0% agarose formaldehyde gel to ensure quality and transcription validity. Embryos at the one-cell stage in 4% ficoll in 1X Marks Modified Ringer (MMR) solution were microinjected with approximately 1 ng of desired RNA. Following 4 hours, embryos were transferred to 0.1X MMR for rearing.

3.2.4 Cell lines, Transfections and Immunocytochemistry

A6 cells derived from *X. laevis* epithelial cells (generous gift from Dr. John Heikkila, University of Waterloo, ON) were grown in Leibowitz-15 media (with 10%

FBS and 1% penicillin and streptomycin) at room temperature. All transfections were completed using Neon Transfection System (Invitrogen) performed according to the manufacturer's protocol with two pulses of 1250 V and 20 ms. For immunofluorescence, cells were fixed in 3.7% formaldehyde in Dulbecco's modified PBS (DPBS) (Invitrogen), pH 7.2, for 15 min, and permeabilized in 1.5% Triton X-100 in DPBS for 10 min. Cells were incubated with either PMP70 (Abcam, ab4965) or Catalase (Cedarlane), polyclonal antibodies for 5 hours, washed three times in PBST for 10 minutes each, incubated with fluorescently labeled secondary antibodies for 1 hour, washed again for 5 minutes in DPBS, and mounted on slides using ProGold Mounting Media (Invitrogen). Samples were visualized with a Zeiss AxioStop 2 Mot. Images were captured with a Retiga 1600 camera (Qimaging) and fluorescence quantifications were completed using Northern Eclipse image capture and analysis software (Empix).

3.2.5 RNA Isolations and Reverse Transcriptase PCR (RT-PCR)

RNA was isolated from A6 cell lysates two days following transfections, and total RNA was isolated from embryos at developmental stages 15, 30, 45, with an RNeasy kit (QIAGEN), both of which were evaluated on a 1.0% agarose formaldehyde gel. Synthesis of cDNA was completed with SuperScript II Reverse Transcriptase (Invitrogen) following manufactures protocol. To analyze RNA expression levels during development, RT-PCR primers were designed against known *X. laevis* peroxisomal and PPAR genes with the following accession numbers: Pex3 [EMBL:AAH73069.1], Pex5 [NP_001011381], Pex11β [GenBank:MGC69071], PMP70 [EF07060], Catalase [BC054964] Pex1 [NM_001091972.1], PPARα [NM_001095362], PPARδ

[NM_001087841], and PPARγ [NM_001087843]. Mid-log phase RT-PCR products were visualized on a 0.8% agarose gel and unsaturated band intensities were quantified against control elongation factor-1α (EF1α) [NCBI: NM_001087442] using Quantity One software (Version 4.4.0 Bio-Rad). All quantified PCR reactions were completed in triplicate. The amplicons of peroxisomal genes listed above were cloned with the TOPO-TA Cloning® (Invitrogen) system as described by the manufacturer's protocol and sequenced to ensure gene identities.

3.2.6 Western Blot Analysis

Pex11β (Abcam, ab74507), β-catenin (Invitrogen), PMP70 (Abcam, ab4965), Catalase (Cedarlane), and β-actin (Invitrogen) polyclonal antibodies were used to detect protein from both *X. laevis* A6 cell and embryonic lysates before and after treatments. Bradford protein quantifications were used to ensure that equivalent amounts of protein (13 mg) were loaded for each sample [34]. Primary antibodies were used in a 1 in 5000 dilution and secondary 1 in 8,000 dilution, and blots were developed using an enhanced chemiluminescence kit (Amersham). Band intensities were quantified using Quantity One software (Version 4.4.0 Bio-Rad).

3.2.7 Immunohistochemistry

Paraffin-embedded embryo sections were washed in xylene and re-hydrated by washing in 100, 90, 80, and 65% ethanol each for 10 minutes twice, followed by 10 min in 0.1% Tween-20 in PBS three times. Histology sections to be immunostained were incubated with a 24-hour primary, followed by a two-hour secondary (FITC conjugated)

antibody incubation in a 1 in 400 antibody dilution. Embryos were counterstained with DAPI (Invitrogen) according to the manufacturer's protocol. Images were captured and fluorescence quantified with a Zeiss LSM Dou (Live 5 Vario II and 510 Meta) Confocal system using Northern Eclipse image capture and analysis software (Empix).

3.2.8 Statistical Analyses

Tests of significance are described within the legend of the figure as required.

3.3 Results

3.3.1 Knockdown of Pex11β altered peroxisome related gene expression in X. laevis A6 cells.

To study the effects of reduced levels of Pex11β during *X. laevis* embryogenesis we first investigated if knockdown of Pex11β could alter the RNA levels of the peroxisome related genes Pex1, -3, -5, -11β, Catalase, PMP70, PPARα, -δ, and -γ in *X. laevis* A6 kidney epithelial cells. A6 cells were transfected with a translation start-site blocking morpholino oligonucleotides designed to knockdown Pex11β protein levels. Cells were transfected with full-length GFP DNA as a control. Semi-quantitative RT-PCR analyses revealed a significant decrease in PMP70 and Pex1 mRNA levels, and a significant increase in PPARγ mRNA levels, following transfection of Pex11β-MO (Figure 3.1). No significant changes were found in levels of Pex11β, Catalase, Pex3, -5, PPARα nor in PPARδ mRNA. Levels are displayed relative to EF1α expression (Figure 3.1).

3.3.2 Knockdown of Pex11β in X. laevis A6 cells decreased PMP70 protein levels.

Since knockdown of Pex11β decreased mRNA for PMP70, we wanted to determine if there were actual decreases in the relative protein levels for this hallmark peroxisomal protein, that is found in functional peroxisomes. A6 cells were transfected with morpholino oligonucleotides designed to knockdown either Pex11β or β-catenin protein levels, or cells were transfected with full-length GFP DNA as a control. Pex11β and β-catenin specific antibodies confirmed bands of expected sizes for both Pex11β (63 kDa) and β-catenin (94 kDa), and confirmed the efficacy of each morpholino (Figure

3.2A) as they reduced their respective protein levels. Western blot analysis for Catalase revealed bands of expected sizes (55 kDa), but Catalase levels were unchanged following knockdown of Pex11β (Figure 3.2A). However, Western blot analysis for PMP70, which revealed bands of expected sizes for PMP70 (70 kDa), did display a significant decrease in PMP70 protein levels with Pex11β-MO versus both β-catenin and GFP controls (Figure 3.2A, left three GFP lanes versus middle three Pex11β-MO lanes versus right three β-catenin lanes, and quantified in Figure 3.2B. The use of β-actin (41 kDa) specific antibodies confirmed the relative protein levels in each lane (Figure 3.2A).

Figure 3.1. Pex11β morpholino altered peroxisome related gene expression in *X. laevis* A6 cells. RT-PCR analysis was performed using RNA isolated from A6 cells transfected with Pex11β-MO or GFP (control) DNA. Two days following transfection reverse-transcribed cDNAs were subject to PCR amplification using primers specific to peroxisomal genes; Pex11β, PMP70, Catalase, Pex5, Pex3, Pex1, PPARα, -δ, and -γ. The respective transcript levels represent measures of mid-log phase RT-PCR product band intensities, relative to levels of EF1α. Genes whose levels were altered significantly as assessed by a paired sample t-test are denoted with an asterisk (*). PMP70 and Pex1 displayed reduced levels of expression following treatment, while PPARγ displayed increased expression. $P<0.05$, n=3. Values presented are the means ± SE.

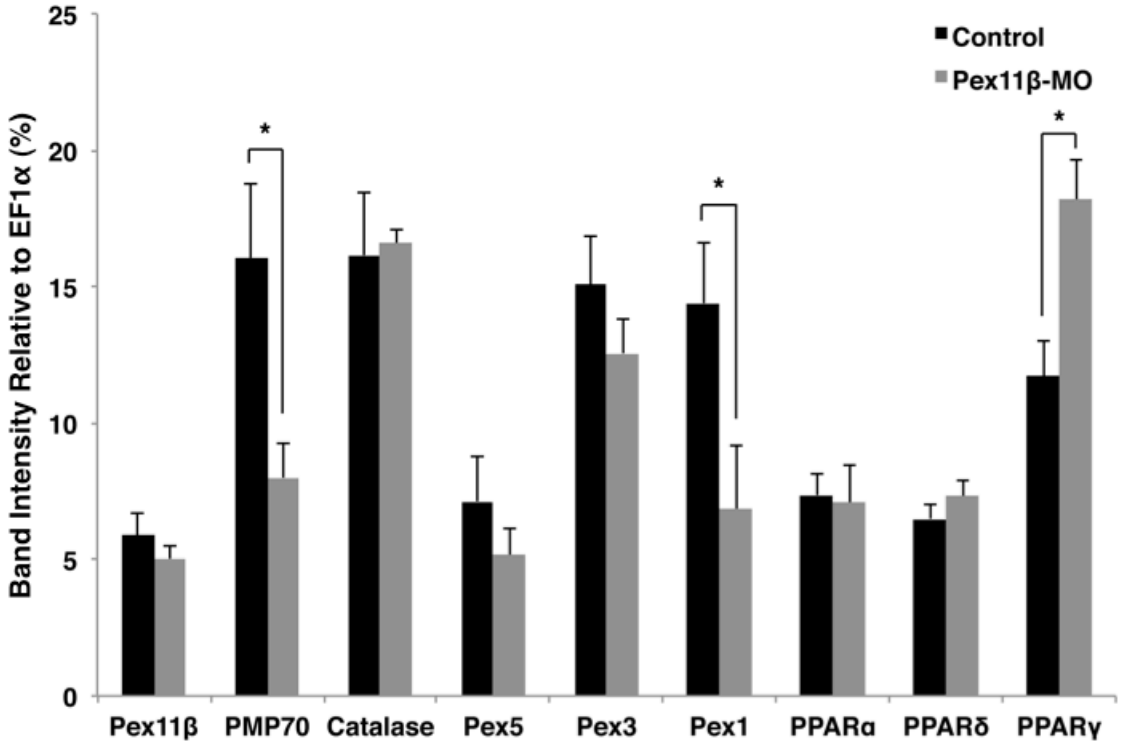
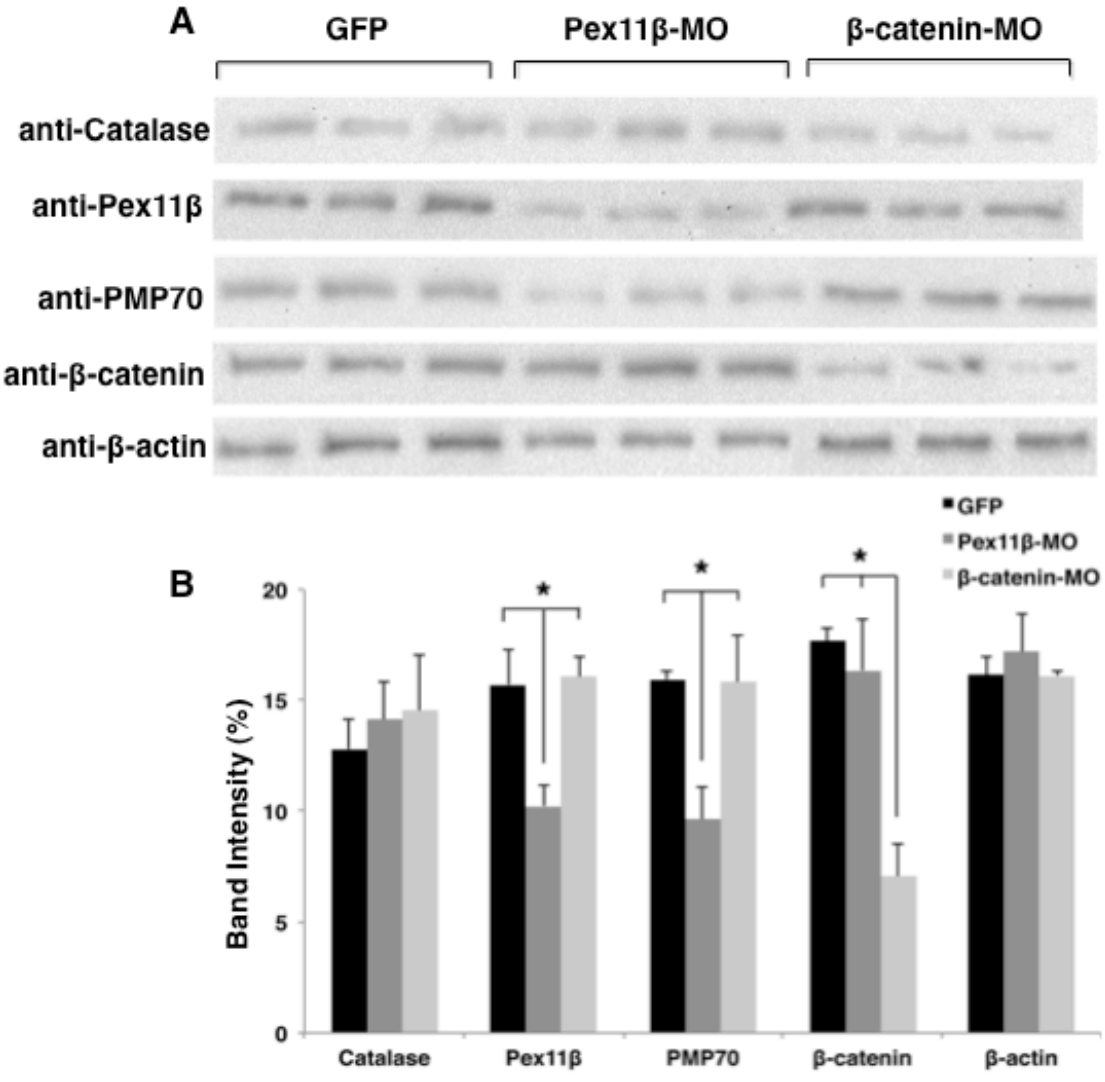

Band Intensity Relative to EF1α (%)
25
20
15
10
5
0
Control
Pex11β-MO
*
*
*
Pex11β
PMP70
Catalase
Pex5
Pex3
Pex1
PPARα
PPARδ
PPARγ

Figure 3.2. Pex11β morpholino decreased PMP70 protein levels in *X. leavis* A6 cells. **(A)** Western blot analysis revealed decreased proteins levels of PMP70 and Pex11β following transfection of Pex11β-MO. Levels of these proteins remained unchanged in cells transfected with GFP DNA or β-catenin MO (A). Pex11β and β-catenin antibodies confirmed the efficiency of both the Pex11β-MO and β-catenin-MO versus GFP DNA control transfections. Catalase protein levels were unaffected by neither Pex11β-MO nor β-catenin-MO. Protein loading in each lane was confirmed and standardized via a β-actin antibody. **(B)** Western blot signals were digitized and data were quantified and analyzed to statistically compare protein levels. There was a significant decrease in the levels of Pex11β and PMP70 protein following transfection of Pex11β-MO, but not with β-catenin-MO or GFP DNA. Additionally, there was a significant decrease in the levels of β-catenin following transfection of β-catenin-MO versus Pex11β-MO and GFP control cells. There was no significant change in the levels of Catalase. Statistical relevance of discrepancies between groups (asterisks) was assessed by a paired sample t-test. $P<0.05$, n=3. Values presented are the means ± SE.

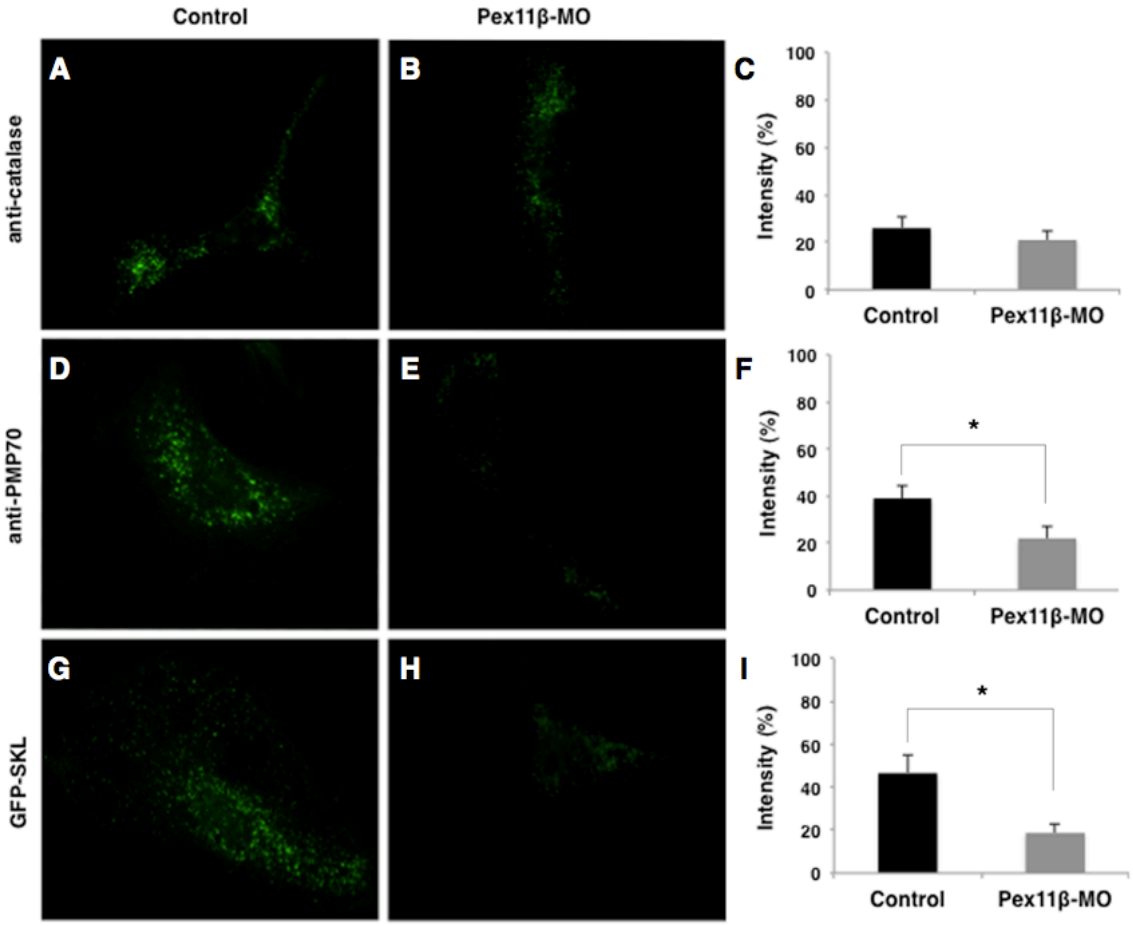

A
GFP
Pex11β-MO
β-catenin-MO
anti-Catalase
anti-Pex11β
anti-PMP70
anti-β-catenin
anti-β-actin
B
GFP
Pex11β-MO
β-catenin-MO
Band Intensity (%)
20
15
10
5
0
Catalase
Pex11β
PMP70
β-catenin
β-actin

3.3.3 Knockdown of Pex11β in X. laevis A6 cells decreased the number of PMP70-positive peroxisome-like structures.

To determine whether knockdown of Pex11β has an effect on peroxisome-like structures and numbers we examined the effects of knocking down Pex11β in *X. laevis* A6 cells using immunocytochemistry. One day following transfection, A6 cells were fixed and probed with Catalase and PMP70 specific antibodies. Immunocytochemistry allowed us to examine the distribution of both Catalase and PMP70 protein, and to quantify their relative protein levels using a fluorescently conjugated secondary antibody. Consistent with our Western blot analysis our results indicated that knockdown of Pex11β significantly decreased the number of PMP70-positive punctate spots (Figure 3.3D versus E). Quantifying the relative levels of indirect fluorescent for PMP70 demonstrated that levels decreased >1.5 fold (Figure 3.3F). The Pex11β-MO had no effect on the number Catalase-positive punctate spots (Figure 3.3A versus B).

To ensure that decreases in PMP70 levels were in fact representative of peroxisomes, we tested whether knockdown of Pex11β could also alter the distribution of the peroxisome maker GFP-SKL. We have previously shown that GFP-SKL in *X. laevis* will localize as punctate-like structures indicative of peroxisomes (Fox, Walsh et al. 2011). A6 cells were co-transfected with Pex11β-MO and GFP-SKL, or GFP-SKL alone. One day following transfections, punctate-like structures were visualized by direct immunofluorescence. Our results indicate that the number of GFP-SKL containing bodies decreased in cells that were transfected with Pex11β-MO versus GFP-SLK alone (Figure 3.3G versus H). These results were quantified and shown to decrease >2.5 fold (Figure 3.3I).

Figure 3.3. Pex11β morpholino altered PMP70 distribution in *X. laevis* A6 cells. A, D, and G are untransfected cells, while B, E, and H are transfected with Pex11β-MO. G and H were additionally co-transfected with GFP-SKL. Using identical imaging and photography parameters, indirect immunofluorescence using a Catalase antibody revealed no difference of signal in untransfected cells (A) versus those containing the Pex11β-MO (B). However, indirect immunofluorescence using a PMP70 antibody revealed a lower number of immunofluorescent signals in transfected cells (E) versus untransfected (D) cells. Direct fluorescence for GFP revealed a punctate signal from GFP-SKL in untransfected cells, (G) versus a more diffuse GFP staining patterns in Pex11β-MO transfected cells (H). The relative fluorescence intensity in 10 regions of twenty-five randomly imaged cells was quantified using Northern Eclipse software. Graphs on the right represent the average fluorescence intensity of non-transfected versus Pex11β-MO transfected cells. Values presented are the means ± SE. Significance at $P<0.05$ was determined using Student's t-test, n=25.


Control
Pex11β-MO
anti-catalase
anti-PMP70
GFP-SKL
A
B
C
D
E
F
G
H
I
Intensity (%)
100
80
60
40
20
0
Control
Pex11β-MO
*

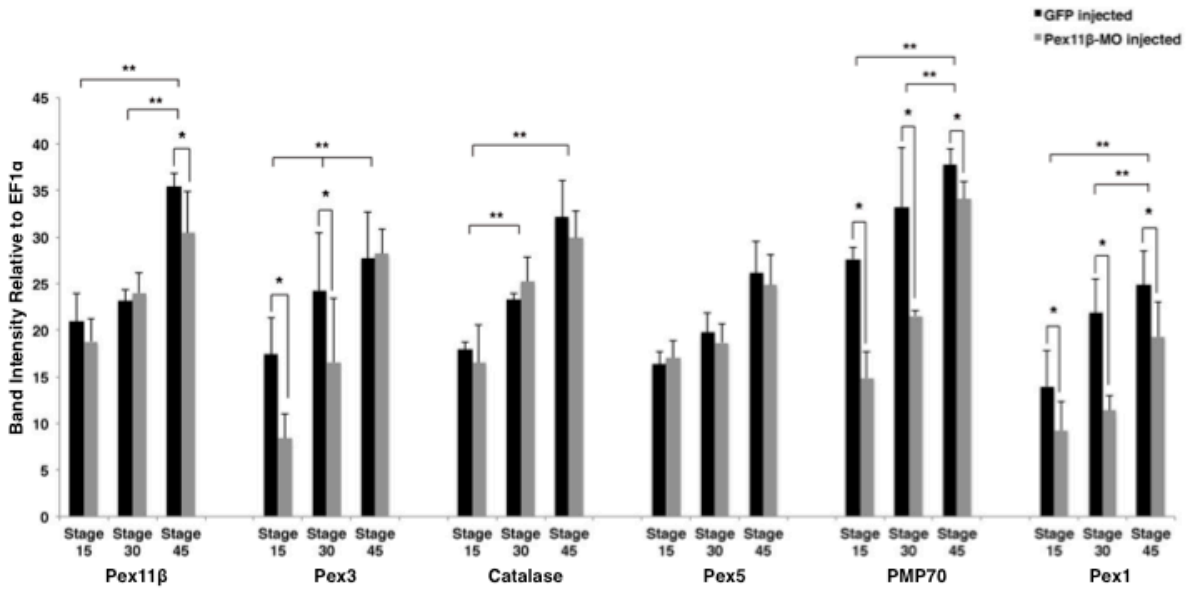
3.3.4 Pex11β decreased peroxisome related gene expression during X. laevis embryogenesis.

To test our hypothesis that knockdown of Pex11β has a direct role in regulating peroxisome biogenesis during embryogenesis, we next examined the effects of decreased Pex11β *in vivo*, by investigating changes in expression of peroxisome related genes following the microinjection Pex11β-MO into early *X. laevis* embryos. The temporal expression of five peroxisomal genes were first analyzed during developmental stages 15, 30 and 45.

With the exception of Pex5, all peroxisomal genes examined in control embryos increased in expression as development progressed with their lowest expression levels at stage 15 followed by increasing levels from stages 30 to 45. (Figure 3.4, significance between stages denoted by double asterisks). Pex11β, PMP70 and Pex1 were found to significantly increase between developmental stages 15 versus 45 and 30 versus 45 (Figure 3.4, double asterisks). Pex3 was found to significantly increase between all three stages examined (Figure 3.4, double asterisks) and Catalase was found to significantly increase between developmental stages 15 versus 30 and 30 versus 45 (Figure 3.4, double asterisks).

Following microinjection of Pex11β-MO there were significantly decreased RNA levels of PMP70 and Pex1 at stages 15, 30 and 45 (Figure 3.4, single asterisk) versus the control RNA levels for each gene. Microinjecting Pex11β-MO also resulted in significant decreases of Pex3 at stages 15 and 30, as well as Pex11β at stage 45. There were no significant changes in Pex11β at stages 15 or 30 nor Catalase at stages 15, 30 and 45 following microinjecting Pex11β-MO.

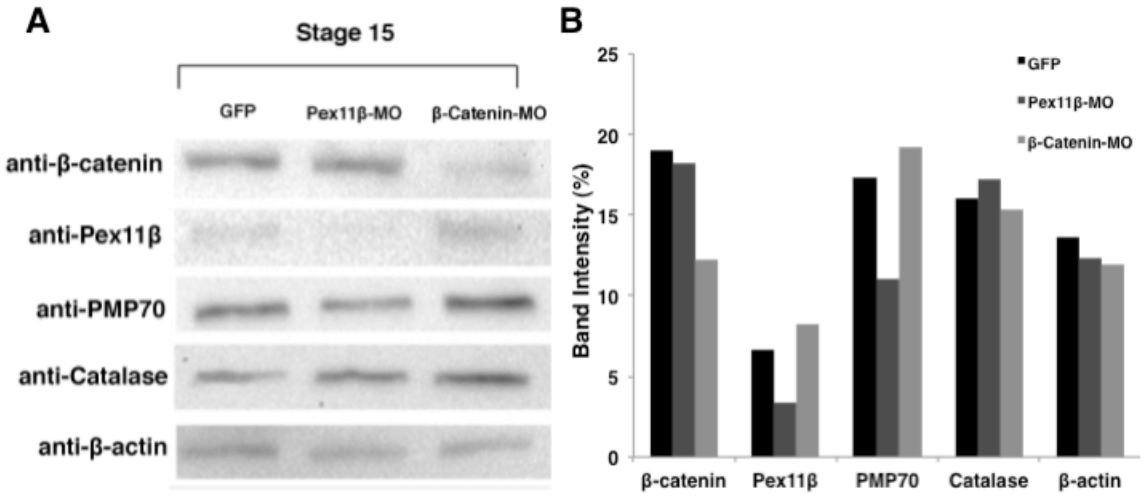
Figure 3.4. Pex11β morpholino reduced Pex11β, Pex3, and PMP70 RNA levels in *X. laevis* embryos. The respective bar heights represent measures of mid-log phase RT-PCR product band intensities, relative to levels of EF1α. RT-PCR analysis during normal embryogenesis revealed that the levels of all genes examined, with the exception of Pex5, increased as development progressed from stage 15 to 30 to 45. A repeated measures ANOVA was carried out comparing RNA levels at all 3 stages of control embryos. When significant, a paired sample t-test was carried out between levels at a given stage in control embryos. Significant changes in RNA levels of a gene between stages is represented by the double asterisk ** ($P<0.05$). Pex3 expression levels differed between all stages, 15 versus 30, 30 versus 45 and 15 versus 45. Catalase levels differed at stages 15 versus 30 and 15 versus 45 but NOT between 30 versus 45. For Pex11β and Pex1 there are differences between stages 30 versus 45 and 15 versus 45 but NOT between 15 versus 30. As the means were correlated, a MANOVA was carried out on the means of the treatment and control groups at each stage to see whether there were differences in the RNA level of each gene at a given stage, in the presence of the Pex11β morpholino. As the MANOVA showed a significant effect of condition, a Wilks lambda analysis was used, and the univariate ANOVAs showed the Pex11β morpholino resulted in significantly lower levels of gene expression, as represented by the single asterisk * ($P<0.05$). PMP70 and Pex1 displayed significant decreased RNA levels stage 15, stage 30 and stage 45. Pex3 displayed decreased levels at stages 15 and 45, while Pex11β displayed decreased expression only at stage 45. n=3. Values presented are the means ± SE.


GFP injected
Pex11β-MO injected
Band Intensity Relative to EF1α
0
5
10
15
20
25
30
35
40
45
Stage 15
Stage 30
Stage 45
Pex11β
Pex3
Catalase
Pex5
PMP70
Pex1

3.3.5 Embryonic knockdown of Pex11β decreased PMP70 protein levels at developmental stage 15.

We next wanted to determine if knockdown of Pex11β had an effect on our hallmark peroxisome related protein PMP7, *in vivo*, similar to our *in vitro* data. Fertilized embryos were microinjected with Pex11β-MO, β-catenin-MO or full length GFP mRNAl. Protein extracts were taken at developmental stage 15 and purified for Western blot analysis. Pex11β and β-catenin specific antibodies confirmed bands of expected sizes for both Pex11β (63 kDa) and β-catenin (94 kDa), and confirmed the efficacy of each morpholino *in vivo* (Figure 3.5A) as they reduced their respective protein levels. Catalase (55 kDa) levels were found unchanged following microinjection of Pex11β-MO, β-catenin-MO or GFP (Figure 3.5A). However, Western blot analysis for PMP70 (70 kDa) revealed a decrease in PMP70 protein levels with of Pex11β-MO relative to both β-catenin-MO and GFP (Figure 3.5A, left GFP lane versus middle Pex11β-MO lane versus right β-catenin lane, and quantified in Figure 3.5B). This decrease in PMP70 levels was also seen when the Western blot data was digitized and examined graphically. The use of β-actin (41 kDa) specific antibodies confirmed the relative protein levels in each lane (Figure 3.5A). A representative blot is shown, though repeated experiments showed consistent results.

Figure 3.5. Pex11β morpholino decreased PMP70 protein levels in *X. laevis* embryos. **(A)** Following the microinjection of a Pex11β-MO at the one cell stage, Western blotting revealed decreased proteins levels of Pex11β and PMP70 proteins in stage 15 embryos. No change Pex11β and PMP70 protein levels was seen in control GFP DNA or β-catenin-MO injected embryos. Pex11β and β-catenin antibodies confirmed the efficiency of both Pex11β-MO and β-catenin-MO reagents. Catalase protein levels were unaffected by Pex11β-MO, β-catenin-MO and GFP injections. Protein loading in each lane was confirmed via a β-actin antibody. **(B)** Western blot signals were digitized and data were quantified relative to β-actin levels. A representative blot is shown, though repeated experiments showed consistent results.


A
Stage 15
GFP
Pex11β-MO
β-Catenin-MO
anti-β-catenin
anti-Pex11β
anti-PMP70
anti-Catalase
anti-β-actin
B
Band Intensity (%)
25
20
15
10
5
0
GFP
Pex11β-MO
β-Catenin-MO
β-catenin
Pex11β
PMP70
Catalase
β-actin

3.3.6 PMP70 antibodies revealed a later accumulation of punctate structures during X. laevis embryogenesis following injection of Pex11β-MO.

In order to determine if knockdown of Pex11β could reduce peroxisome-like structures during development, we used immunohistochemistry to visualize changes in the embryonic distribution of Catalase and PMP70, in response to microinjecting Pex11β-MO. Fertilized embryos were microinjected with Pex11β-MO and fixed at stages 15 and 30, and sectioned for immunohistochemical analysis. Catalase and PMP70 signals were present at very low levels at stage 15 under control conditions (Figure 3.6A and 3.7A). Punctate structures were visible within the somites of control embryos at stage 30 using Catalase (Figure 3.6B), and PMP70 (Figure 3.7B) specific antibodies. The linear organization of the DAPI stained nuclei is a typical feature seen during of *X. laevis* somite differentiation. Following microinjection of Pex11β-MO, Catalase signals remained largely unchanged, with very few punctate signals seen at stage 15 increasing in number by stage 30, similar to the control embryos (Figure 3.6A versus C and 3.6B versus D).

However, following the microinjection of Pex11β-MO, PMP70 signals were altered. While the relatively few PMP70 specific signals at stage 15 were not different between the control and Pex11β-MO injected embryos (Figure 3.7A versus C) there is a difference in the number of punctate signals at stage 30 with few being present in embryos that have been injected with Pex11β-MO.

Figure 3.6. Pex11β morpholino had no affect on Catalase immunofluorescence in stage 15 and 30 somites in *X. laevis* embryos. Both control (A and B) and Pex11β-MO injected (C and D) embryos at developmental stages 15 (A and C) and 30 (B and D), were fixed then sectioned for immunohistochemical analysis in somties. Using a Catalase antibody, punctate structures were detected at stage 15 and 30 under control conditions (A and B) and following microinjection of Pex11β-MO (C and D). There was no difference in the number or pattern of the signal between control and morpholino injected embryos. Identical imaging and photography parameters were used for all images. DAPI (blue), PMP70 (green). The linear arrangement of the stage 30 somitic nuclei is typical in *X. laevis*. Images were taken at 60x.

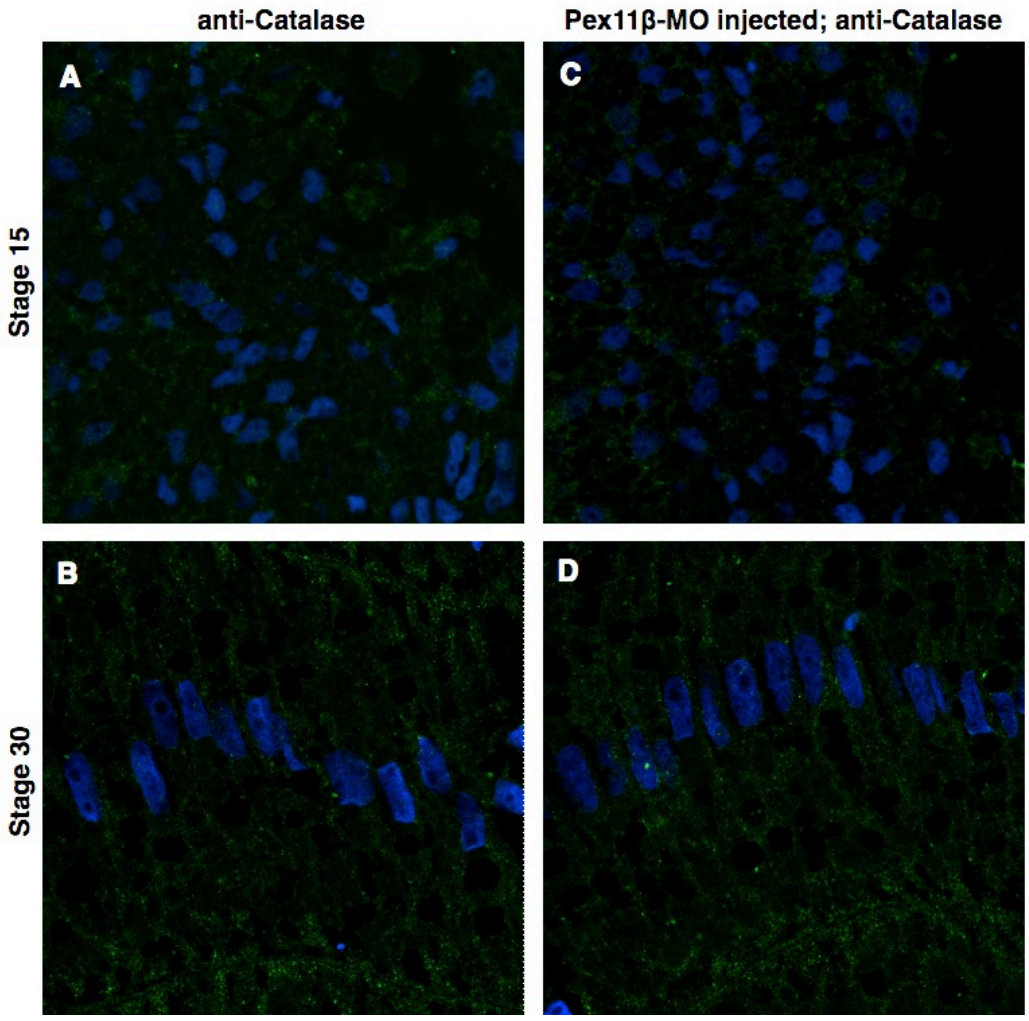
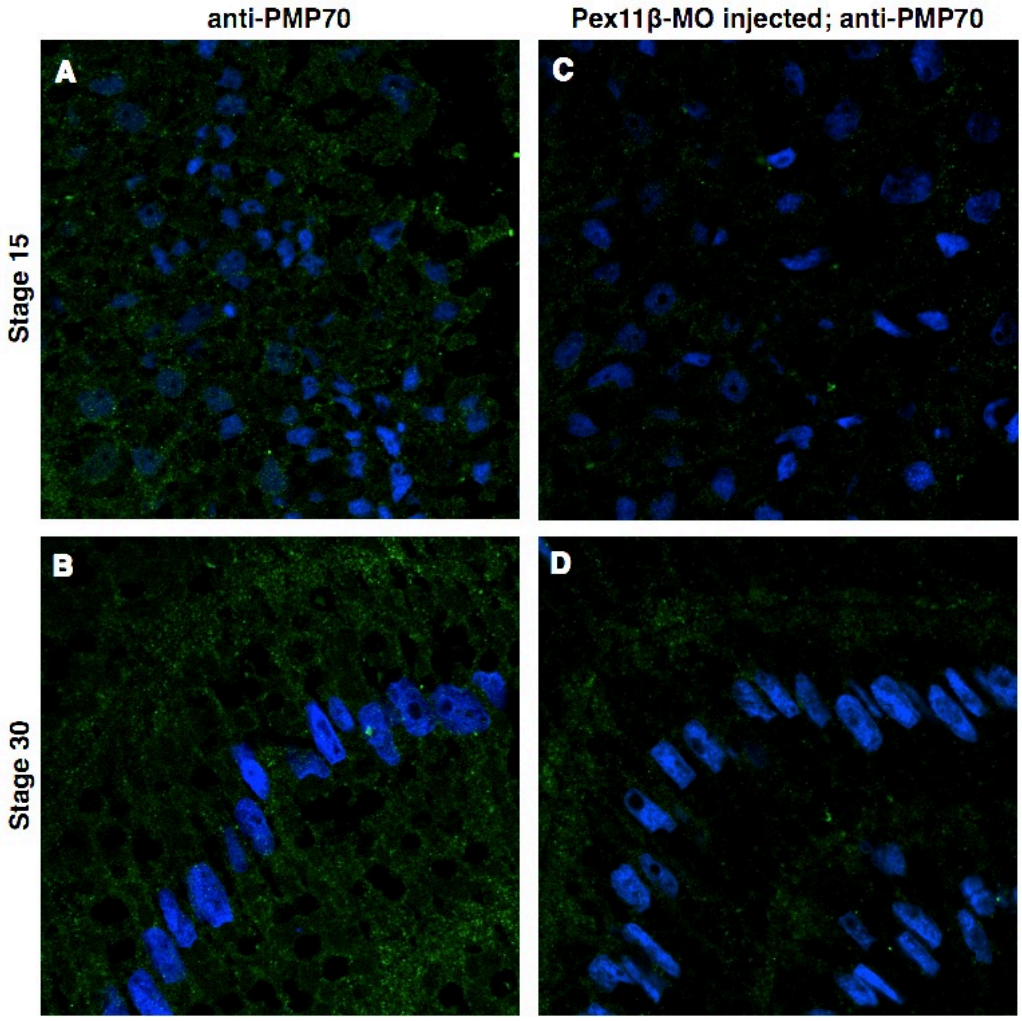

anti-Catalase
Pex11β-MO injected; anti-Catalase
A
C
B
D
Stage 15
Stage 30

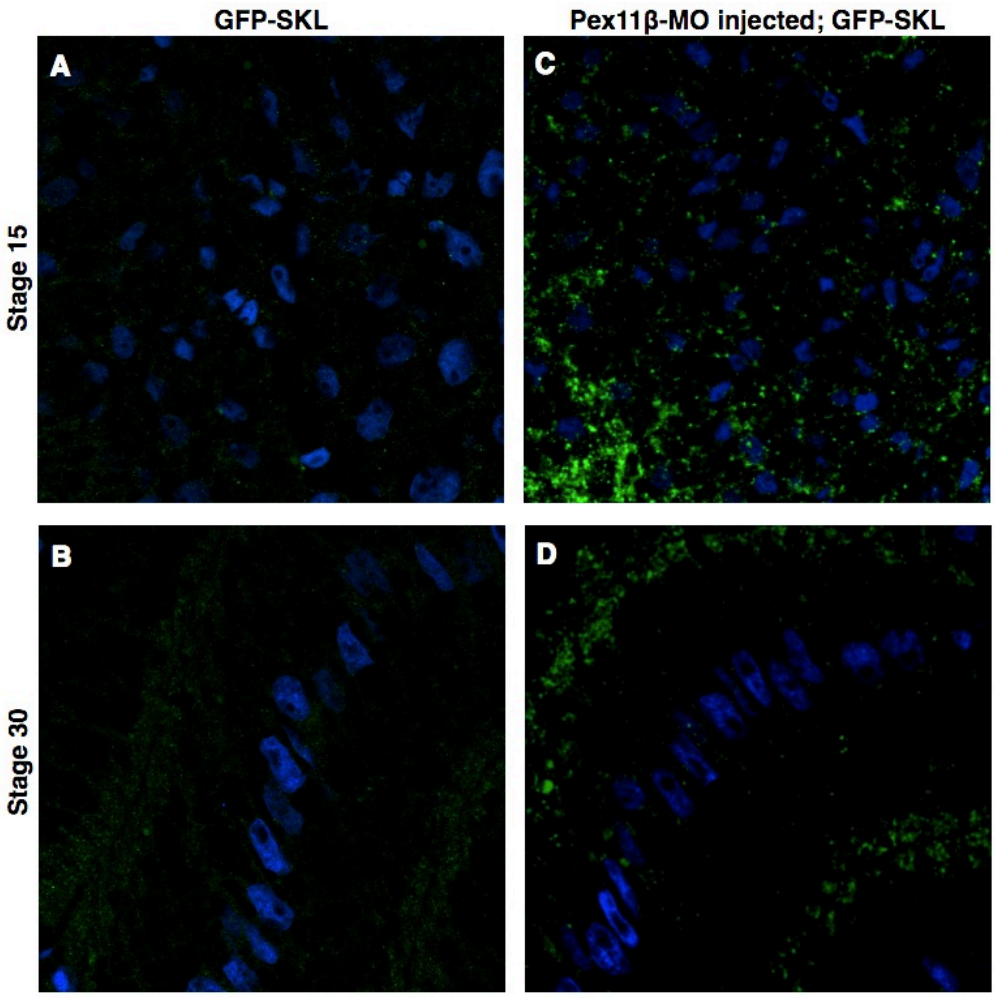
Figure 3.7. Pex11β morpholino decreased PMP70 immunofluorescence in stage 15 and 30 somites in *X. laevis* embryos. Both control (A and B) and Pex11β-MO injected (C and D) embryos at developmental stages 15 (A and C) and 30 (B and D), were fixed then sectioned for immunohistochemical analysis in somites. Using a PMP70 antibody punctate structures are detected at stage 15 and 30 in somitic mesoderm of uninjected embryos (A and B). In embryos that had been injected at the one cell stage with Pex11β-MO, the PMP70 antibody signal decreased at stage 15 and 30 (C and D) compared to the control. Identical imaging and photography parameters were used for all images. DAPI (blue), PMP70 (green). The linear arrangement of the stage 30 somitic nuclei is typical in *X. laevis*. Images were taken at 60x.


anti-PMP70
Pex11β-MO injected; anti-PMP70
A
C
B
D
Stage 15
Stage 30

3.3.7 Knockdown of Pex11β induced a change in peroxisome distribution and size during X. laevis somite maturation.

Next, we wanted to determine if knockdown of Pex11β had an effect on peroxisome number or peroxisome biogenesis. Fertilized embryos were microinjected with GFP-SKL alone, or co-injected with Pex11β-MO and GFP-SKL, were fixed at stages 15 and 30, and sectioned for immunohistochemical analysis. We used direct immunofluorescence to visualize the affects of Pex11β-MO on the localization patterns of GFP-SKL. In the absence Pex11β-MO GFP-SKL resulted in few punctate signals at stage 15 (Figure 3.8A), but the number of distinct punctate signals increased at stage 30 (Figure 3.8B), and were reminiscent of those seem at this stage by Catalase (Figure 3.6B) and PMP70 (Figure 3.7B). However, Pex11β-MO resulted in the presence of large diffuse GFP-SKL-containing bodies at stage 15 and 30 (Fig 3.8A and B versus 3.8C and D). These structures were more prevalent and more abundant at stage 15 versus 30 (Figure 3.8C versus D).

Figure 3.8. Pex11β morpholino altered the size and distribution of GFP-SKL-positive structures in stage 15 and 30 somites in *X. laevis* embryos. Embryos injected at the one cell stage with GFP-SKL alone (A and B) or with GFP-SKL and Pex11β-MO (C and D) were both reared to developmental stages 15 (A and C) and 30 (B and D), and then fixed and sectioned for immunohistochemical analysis. Using a GFP antibody punctate structures are detected in control embryos at stage 15 and 30 (A and B). In embryos that had been injected at the one cell stage with Pex11β-MO the GFP antibody signal was stronger and more widely distributed (A vs C). By stage 30 the GFP antibody signal had decreased compared to stage 15, but was still more prevalent and widely distributed than in embryos that have not be injected with the Pex11β-MO (B vs D) punctate GFP structures numbers decreased with an increase in diffuse GFP staining. Identical imaging and photography parameters were used for all images. DAPI (blue), PMP70 (green). The linear arrangement of the stage 30 somitic nuclei is typical in *X. leavis*. Images were taken at 60x.


GFP-SKL
Pex11β-MO injected; GFP-SKL
A
C
B
D
Stage 15
Stage 30

3.4 Discussion

3.4.1 Role of Pex11 in peroxisome biogenesis

Our recent understanding that ROS moieties can play important roles in cell signalling has brought new attention to organelles such as peroxisomes as they could have important developmental roles. Peroxisomes can arise from the ER as pre-peroxisomal compartments, which bud off in a Pex3/Pex19 dependent manner, utilizing the ER as a template for *de novo* biogenesis. These vesicles then form mature peroxisomes by fusion with neighboring peroxisomes, or by matrix protein import mediated by cytosolic chaperones Pex5 and Pex7. In addition to ER *de novo* biogenesis, there is also evidence that peroxisomes can arise from the budding of pre-existing peroxisomes (South and Gould 1999). Duplication of preexisting peroxisomes by fission, growth and maturation is the major pathway for proliferation facilitated by Pex11 and a specific set of dynamin related proteins (DRPs) (Thoms and Erdmann 2005). In addition, other molecules and mechanisms have been shown to regulate peroxisome abundance such as PPARγ, shown to play important roles in peroxisome biogenesis. PPARγ also regulates a variety of other peroxisome-independent metabolic processes, where as Pex11β functions seem limited to peroxisome biogenesis.

The key hypotheses tested here is that undifferentiated embryonic cells have no or low levels of peroxisomes which are needed as cells differentiate, and that Pex11β plays a key role in regulating their embryonic abundance. This would be particularly true in differentiating cells that are highly metabolic, such as developing *X. laevis* somites. While there are few reports that specifically describe the lack of peroxisomes in undifferentiated or stem cells, several studies do describe an increase in peroxisome

numbers as cells differentiate. Studies using hair follicle stem cells (Karnik, Tekeste et al. 2009), epidermal side population with stem cell-like characteristics (Carr, Oberley-Deegan et al. 2011), embryonic stem cells (Kuai, Cong et al. 2006; Ostadsharif, Ghaedi et al. 2011), intestinal immature stem cells (Phipps, Connock et al. 2000), all demonstrate that peroxisomal numbers increase as these stem cells differentiate. Further, using a GFP-tagged peroxisomal marker, we recently reported that peroxisomes are not detectable in early *X. laevis* somites (Fox, Walsh et al. 2011). This data is supported by other embryological work that shows that peroxisomes are detected at low levels in human and rat trophoblast and other extraembryonic tissues where their numbers increased with gestation (Phipps, Connock et al. 2000).

3.4.2 Reduced protein levels of Pex11β in vitro reduced peroxisomal structures

As we have previously shown that ectopic expression of Pex11β during frog embryogenesis resulted in the early presence of peroxisomes, we sought to examine the effects of decreasing embryonic Pex11β levels. A *X. laevis* translation blocking morpholino was shown through Western blot analysis to reduce Pex11β protein levels, along with the use of a control β-catenin-MO, when used in both A6 cells and embryos.

We demonstrated a decrease in PMP70 and Pex1 RNA levels, followed by an increase in PPARγ RNA levels after transfection of Pex11β-MO in A6 cells. The decrease in PMP70, and unchanged Catalase levels, was confirmed at the protein level by Western blot analysis, and visualized using immunocytochemistry. Together, these data suggest that reduced Pex11β levels decreased the PMP70 protein, and the number of PMP70-positive vesicles, but not the levels of Catalase. This reduction in the number of

PMP70-positive vesicles could be due to the reduction of fission, due to reduced Pex11β levels, from preexisting peroxisomes. The level of Catalase, which also functions independent of peroxisomes in the cytoplasm, was unaffected. Increases in PPARγ and Pex1 RNA levels further suggest that cells are compensating for reduced peroxisomal numbers by increasing their rate of metabolism, through PPARγ, and increasing their peroxisome maturation, through Pex1 (Gould and Valle 2000).

3.4.3 In vitro changes in protein levels of Pex11β are also seen in vivo

As knockdown of Pex11β was able to reduce the number of PMP70-positive vesicles in differentiated epithelial A6 cells, we next sought to examine the effects of reduced Pex11β levels in early stage *X. leavis* embryos where cells are differentiating. As with our previous study we focus on the developing somites – cells that are metabolically active and easy to identify. Knockdown of Pex11β in embryos, as in A6 cells, reduced Pex1 and PMP70 RNA levels, while Pex5 and Catalase RNA levels remained unchanged. Pex3 RNA levels are reduced significantly in embryos, though this reduction was not significant in A6 cells. At the protein level Pex11β and PMP70 protein levels decreased, with no changes in Catalase, consistent with our cell culture data. These relative changes in protein levels were also visible at stage 15, and stage 30 somites using immunohistochemistry. A Catalase specific antibody showed no difference in the number of Catalase-positive punctate structures at stage 15, nor stage 30 somites in Pex11β morpholino injected embryos. However, Pex11β knockdown resulted in a decreased number of punctate PMP70-specific structures at both stages 15 and 30. As this altered distribution of PMP70 structures suggested a decrease in the number of

peroxisomes, a GFP-SKL reporter that is capable of being imported into peroxisomes was used. The import of GFP-SKL is likely to involve Pex3 and -19, and not PMP70. The GFP-SKL reporter revealed the presence of large organelle structures following Pex11β morpholino injections, particularly at stage 15. These organelles were distinct from the ones seen with the PMP70 antibody. The decreased number of PMP70 positive organelles in response to lower Pex11β levels could be due to incomplete fission of existing peroxisomes, which would require Pex11β. Thus the larger organelles represent immature peroxisomes that await fission and import of other peroxisomal components, such as PMP70. As development continued from stage 15 to 30 budding of peroxisomes occurred at a slower rate, and thus more PMP70 punctate signals are present at stage 30 than 15. Catalase, which is known to have functions independent of peroxisomes, is not affected by this change in peroxisome numbers.

These results showed that Pex11β played an important role during embryogenesis to regulate peroxisome numbers, key metabolic processes, overall ROS levels and thus cell signalling pathways. Pex11β deficiency in mice is lethal (Li, Baumgart et al. 2002), where the deletion of both alleles caused a 30% reduction in peroxisome number in brain and the deletion of only one allele resulted in altered SOD2 levels, but not Catalase levels (Ahlemeyer, Gottwald et al. 2011). Thus Pex11β can control embryonic peroxisome numbers, and can regulate specific metabolic genes, but not Catalase.

We have previously demonstrated that an increase in Pex11β resulted in increases in both Catalase and PMP70. With Pex11β acting as a regulator of peroxisome numbers, as numbers increased there was an embryonic response to also increase Catalase and PMP70 protein levels (along with other peroxisomal proteins). However in this study,

when Pex11β levels decreased, cellular responses are different, as seen by the distinctive localization of GFP-SKL, and the delay in the accumulation of mature PMP70 containing organelles. This suggests that cellular responses to such perturbations are specific and vital as these embryonic cells attempt to maintain crucial levels of metabolism and ROS signalling to allow their proper differentiation.

3.4.5 Concluding remarks

In conclusions, this study suggests that *X. laevis* Pex11β has a pivotal role in the regulation of peroxisome biogenesis in both A6 cells *in vitro*, and during embryogenesis *in vivo*. Morpholino-induced knockdown of Pex11β demonstrated that Pex11β a key regulator in biogenesis contributes to the regulation of peroxisome number during *X. laevis* embryogenesis.

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CHAPTER 4

PEROXISOME NUMBERS DIRECTLY AFFECT LEVELS OF ROS AND THE REDOX-SENSITIVE MECHANISM OF WNT/PCP CELL SIGNALING

4.1. Introduction

4.1.1 Peroxisome overview

Peroxisomes are membrane-bound organelles that are primarily involved in the oxidation of fatty acids and the biosynthesis of plasmalogens, an ether-linked phospholipids involved in the myelination of nerve cells. As a byproduct of metabolism, these organelles generate a variety of reactive oxygen species (ROS), which can be harmful to various biological processes. To compensate for such high levels of ROS, peroxisomes contain an array of antioxidant defense enzymes, which are involved in the detoxification of these deleterious molecules. Imbalance in ROS levels can be damaging to cellular constituents and potentially deregulate redox-sensitive cell signaling pathways implicated in a variety of cell and developmental processes (Antonenkov, Grunau et al. 2010).

The purpose of this chapter is to determine whether or not changes in peroxisome numbers can modify ROS levels, thereby resulting in altercations to redox-sensitive cell signaling. Specifically, we are examining if altered ROS levels have an effect on Wnt signaling in *Xenopus laevis* A6 cells. It has been previously demonstrated that the redox mechanisms of Wnt signaling are sensitive to ROS during *X. laevis* development (Funato, Michiue et al. 2008). This research, however, examines for the first time the relationship between peroxisome numbers and cellular ROS levels as a factor effecting redox-sensitive Wnt signaling.

4.1.2 Peroxisomal ROS and its relation to the cellular redox state

Little attention has been directed to the importance of peroxisomes in the maintenance of cellular ROS levels. Recently, peroxisomal ROS metabolism and its relationship to cell signaling has become an exciting and rapidly evolving multidisciplinary research field, with important implications for both development and disease (Schrader and Fahimi 2006; Bonekamp, Volkl et al. 2009). For instance, human patients suffering from an inherited deficiency in catalase (the hallmark peroxisomal antioxidant) have an increased risk of developing age-related diseases including diabetes, atherosclerosis, and cancer (Goth and Eaton 2000). Additionally, the absence of functional peroxisomes caused increased apoptosis in the developing mouse cerebellum, which was predicted to be a result of increased levels of ROS (Krysko, Hulshagen et al. 2007). These observations and others shed light on the relationship between peroxisome function and cellular ROS levels.

Of significant interest is the relationship between peroxisome number and the cellular oxidation state. Cellular aging compromises the import of peroxisome targeting signal 1 (PTS1) matrix proteins, affecting in particular, import of the antioxidant catalase (Legakis, Koepke et al. 2002). In addition, the overall number of peroxisomes was shown to drastically increase during cellular senescence, (Legakis, Koepke et al. 2002), a process associated with increased systemic oxidative stress (Muller 2009).

It was suggested that cellular senescence may correspond to the regulation of peroxisome size and number. As mammalian cells senesce, peroxisomes were found to increase in size, suggesting a relationship between aberrant peroxisome function, aging and oxidative stress (Legakis, Koepke et al. 2002). There is also substantial evidence that

peroxisome number and morphology can drastically change upon exposure of cells to various conditions of oxidative stress. For example, depletion of cellular glutathione (GSH), a non-enzymatic antioxidant, results in peroxisome elongation in mammalian cells (Schrader and Fahimi 2006). These data suggest that changes in peroxisome shape, size and number respond to changes in ROS and are involved regulating cellular ROS levels.

4.1.3 Hydrogen peroxide as a signaling molecule

Peroxisomes are organelles that both produce and break down hydrogen peroxide (H_2O_2). The latter is accomplished by catalase, which catalyzes the decomposition of H_2O_2 into molecular oxygen and water. While catalase has been extensively characterized as a molecular guardian of H_2O_2, many studies have revealed the role of H_2O_2 as a signaling molecule. Among the various ROS produced, H_2O_2 is the most abundant as it is present at an *in vivo* concentration of 10^{-7} M, and has the longest half-life ($t_{½} = 10^{-5}$s) (Paulsen and Carroll 2010). The relative stability and uncharged nature of H_2O_2 permits enhanced diffusion across long distances and membranes. H_2O_2 has been shown to act as a paracrine signal in plant cell differentiation (Bienert, Schjoerring et al. 2006), and more recently, in the recruitment of immune cells to wound sites in zebrafish larvae (Niethammer, Grabher et al. 2009). H_2O_2 is quickly generated in peroxisomes and the cytosol, and these and other aforementioned properties make it an ideal mediator of signal transduction processes.

4.1.4 Redox signaling through protein thiol oxidation

It is well known that the reversible phosphorylation of kinases, phosphatases and transcription factors functions as a switch to modulate protein activity. ROS are well known regulators of a variety of cell signaling pathways through their ability to oxidize and modulate protein activity. An important cellular sensor of ROS is the thiol (SH) functional group of the amino acid cysteine (Cys). SH groups have a number of oxidation states resulting in the generation of sulphenic acid (SOH), sulphinic acid (SO_2H), sulphonic acid (SO_3H) or the formation of disulphide bonds (R-S-S-R) within a protein itself or with a neighboring protein. Similar to phosphorylation, these various degrees of oxidation function as a switch for modulating protein activity.

A variety of important macromolecules are sensitive to redox modifications by ROS including extracellular matrix molecules, phosphatases, kinases and a number of transcription factors. As a result, many cell signaling pathways have been distinguished as redox sensitive. Of the well documented examples, the Wnt signaling pathway was first identified as redox sensitive in *X. laevis* embryos (Funato, Michiue et al. 2008).

4.1.5 Redox regulated Wnt signaling

Wnts are a family of ligands that are utilized in normal development, and contribute to cellular processes such as differentiation, proliferation and pattern formation (Clevers 2006). Biochemical analyses have revealed that Wnt signaling can occur via several branches of Wnt related pathways. The first identified Wnt pathway, referred to as the canonical Wnt or Wnt/β-catenin pathway, results in the cytosolic accumulation and subsequent nuclear localization of β-catenin, and the transcription

of specific genes with the aid of T-cell factor/lymphoid enhancing factor (Tcf/Lef). Specifically, Wnts bind to the cell surface receptor, Frizzled (Fzd), which activates Dishevelled (Dvl), to inhibit a group of proteins termed the destruction complex, thereby resulting in the cytoplasmic accumulation of β-catenin. Recent studies on Wnts in *X. laevis* indicate a redox sensitive switch for this pathway. It was determined that nucleoredoxin (Nrx), a thioredoxin (Trx) related protein, may play a regulatory role in canonical Wnt signaling by directly controlling Dvl activity (Funato, Terabayashi et al. 2010). Nrx binds to Dvl in its reduced form suppressing Wnt signaling. However, in response to altered ROS levels, H_2O_2 can oxidize Nrx, releasing it from Dvl, and promoting β-catenin accumulation and subsequent gene activation in the absence of a Wnt ligand (Funato, Terabayashi et al. 2010).

Another well-characterized branch of the Wnt signaling pathway, the Wnt/planar cell polarity (PCP) pathway, refers to the polarization of a field of cells within the plane of a cell sheet. This form of polarization is required for diverse cellular processes in vertebrates including convergent extension. Studies showed that activation or loss-of-function of PCP pathway components affects various processes in many organisms, such as ommatidia polarity in the *Drosophila* compound eye and neuronal polarity in mammalian neurons (Smith, Conlon et al. 2000). In vertebrates such as *X. laevis,* this pathway is involved in regulating gastrulation movements and proper body axis formation during embryogenesis (Wallingford and Harland 2001).

In the Wnt/PCP pathway, Wnt binds to Fzd thereby activating Dvl, which is involved in the activation and subsequent phosphorylation of proteins such as Jnk and Rho, leading to enhanced cell movement. Notably, Dvl is also an important component

of the Wnt/PCP pathway and is considered a mediator of signaling activation between these two branches of the Wnt signaling (Axelrod, Miller et al. 1998). Additionally, Nrx plays a crucial role in the Wnt/PCP pathway through regulation of Dvl (Funato, Michiue et al. 2008). Overexpression and inhibition of Nrx also disrupts convergent extension movements that underlie normal gastrulation in *X. laevis* (Funato, Michiue et al. 2008). From this data, it is predicted that ROS is a determinant for the activation of the both the canonical and noncanonical Wnt signaling pathways, and therefore the balance in the levels of ROS must be tightly regulated during normal embryonic development.

4.1.6 Hypotheses

We have previously shown that overexpression and inhibition of Pex11β is sufficient and necessary for regulating peroxisome number both in *Xenopus laevis* A6 kidney epithelial cells and during embryogenesis (Fox, Walsh et al. 2011). As peroxisomes may partake in eradication of cellular ROS, we test the hypothesis that changes to peroxisome number will alter ROS levels. Additionally, we investigate the effect of changes in ROS levels caused by altered peroxisome number on redox sensitive Wnt signaling pathways. As we have elucidated an analogous role of Pex11β both *in vivo* and *in vitro*, we first seek to determine how ROS levels change and investigate the redox-sensitive mechanism of Wnt/PCP in *X. laevis* A6 cell line, due to the robust molecular tools available for *in vitro* research. Our results demonstrated that a decreases in peroxisome number resulted in increased levels of cellular ROS. This increase in cellular ROS was associated with changes in noncanonical Wnt/PCP cell signaling. We therefore propose that peroxisomes are key regulators of cellular ROS levels, highlighting

their importance to intracellular oxidative balance, which can otherwise perturb the redox-sensitive Wnt/PCP cell signaling.

4.2 Materials and Methods

4.2.1 Animal care

Adult *X. laevis* were reared in accordance with the Canadian Council on Animal Care regulations. Fertilizations were performed according to Wu and Gerhart (Wu and Gerhart 1991), and embryos were staged according to Nieuwkoop and Faber (Nieuwkoop). Embryos to be sectioned were fixed in 4% formaldehyde at desired stages and paraffin-embedded.

4.2.2 Plasmids and reagents

pRL-TK and pAP1-Luciferase was provided by Dr. Greg Kelly (University of Western Ontario) and MitoTracker Red (Invitrogen) was generously supplied by Dr. Robert Cumming (University of Western Ontario). Anti-Dvl, IgG-mouse and anti-Nrx antibodies were provided by Dr. Greg Kelly (University of Western Ontario).

4.2.3 PCP/pAP1-Luciferase reporter assay

Cells were electroporated with pAP1-Luciferase and pRL-TK to normalize luciferase levels, plus one of the following as controls and treatments; pcDNA3.1-empty vector (EV), HA-Pex11β and Pex11β-MO in equal amounts and were prepared 24 hours post-transfection using the Dual Luciferase Assay Kit as per manufacturer's instructions (Promega). Luciferase expression was quantified using the GloMax Multi Detection System (Promega).

4.2.4 Microinjection

We previously cloned and sequenced *Xenopus* full length Pex11β (Fox, Walsh et al. 2011), and engineered morpholino oligos with Gene Tools (Gene Tools, Philomath, USA) (Fox, 2012). *X. leavis* embryos at the one-cell stage were injected with 2.3 nl of 400 μM morpholino (MO) oligos. Each morpholino and Pex11β RNA was used in at least three independent experiments and injected each time at three different concentrations (400, 600 and 800 μM).

4.2.5 Transfection and electroporation

A6 cells derived from *X. laevis* epithelial cells (generous gift from Dr. John Heikkila, University of Waterloo, ON) were grown in Leibowitz-15 media (with 10% FBS and 1% penicillin and streptomycin) at room temperature. All transfections were completed using Neon Transfection System (Invitrogen) performed according to the manufacturer's protocol with two pulses of 1250 V and 20 ms. GFP was used as a positive control for transfection efficiency. Samples were visualized with a Zeiss AxioStop 2 Mot at the Biotron Institute for Experimental Climate Change Research at the University of Western Ontario, Canada.

4.2.6 Coimmunoprecipitation

Following treatments, cells were lysed in RIPA buffer and lysates were subject to coimmunoprecipitation and immunoblotting to determine the interaction between Dvl and NRX. Cellular lysates (350 ug) were immunoprecipitated with 2.5 ug of anti-Dvl

antibody (Santa Cruz) overnight at 4°C followed by immunoblot analysis using anti-NRX (Santa Cruz) as described below.

4.2.7 Immunoblot analysis

JNK (Abcam), phospho-JNK (Abcam), Nrx (Santa Cruz), Dvl (Santa Cruz) and β-actin (Invitrogen) polyclonal antibodies were used to detect protein from *X. laevis* A6 cell lysates before and after treatments. Bradford protein quantifications were used to ensure that equivalent amounts of protein (10 μg) were loaded for each sample (Bradford 1976). Primary antibodies were used in a 1 in 1000 dilution and secondary 1 in 5,000 dilution, and blots were developed using an enhanced chemiluminescence kit (Amersham). Band intensities were quantified using Quantity One software (Version 4.4.0 Bio-Rad).

4.2.8 Amplex Red Hydrogen Peroxide/Peroxidase Assay

For the measurement of hydrogen peroxide (H_2O_2) and peroxidase activity we used the Amplex Red Hydrogen Peroxide/Peroxidase Assay according to the manufacturer's instructions (Invitrogen). In the presence of peroxidase, the Amplex Red reagent reacts with H_2O_2 in a 1:1 stoichiometry and produces the red-fluorescent oxidation product, resorufin. Following the respective treatments, A6 cell homogenates were diluted in reaction buffer and added into 96 well plates. For each well, 50 μL of working solution of 100 μM Amplex Red reagent and 0.2 U/mL HRP was added and fluorescence measured after incubation. For H_2O_2 Assay, a standard curve was generated from 0 μM to 5 μM and H_2O_2 concentrations of samples were deduced from the standard

curve. Resorufin fluorescence was measured with excitation at 530-560 nm and emission at 590 nm. The data were analyzed by ANOVA, and means were compared by using Student's *t* test ($P < 0.05$).

4.2.9 MitoTracker Red Assay

To measure the degree of mitochondrial ROS production, A6 cells were transfected with plasmids encoding HA-Pex11β, Pex11β-MO and an empty vector. Cells were seeded in triplicate, at 3.0 X 10^5 cells/well in 6 well plates. After 24 hours, media was replaced with phenol red free L-15 with 200 nm MitoTracker® Red CM-H_2XROS (Invitrogen) and incubated for 20 minutes. After incubation, cells were then washed twice with PBS, and visualized in phenol red-free media using a fluorescent microscope (Zeiss AxioObserver, 20X objective). Ten images from randomly selected regions were taken from each well using a QImaging camera and QCapture Pro Software. The fluorescent intensity of each image was quantified using Image J software.

4.2.10 Intracellular ROS Assay (DCFDA)

To measure the degree of global ROS production, A6 cells were transfected with plasmids encoding HA-Pex11β, Pex11β-MO and an empty vector. Cells were seeded in triplicate, at 3.0 X 10^5 cells/well in 6 well plates. After 24 hours, media was replaced with phenol red free L-15 with 400 nm of 5-(and-6)-Carboxy-2',7-Dichlorofluorescein Diacetate (DCM-H_2DCFDA) (Invitrogen) and incubated for 30 minutes. After incubation, cells were then washed twice with PBS and visualized with a fluorescent microscope (Zeiss AxioObserver, 20X objective). Ten images from randomly selected

regions were taken from each well using a QImaging camera and QCapture Pro Software. The fluorescent intensity of each image was quantified using Image J software.

4.2.11 Statistical Analyses

Data from all experiments were representative of three independent biological replicates performed on separate occasions. Analysis of data between control and treated or transfected groups was performed using a Student's t-Test assuming unequal variances (Excel, Microsoft Corp., Redmond, WA). P values were one-sided and considered statistically significant at the 0.05 level. Statistical data is presented as the mean ± S.E.

4.3 Results

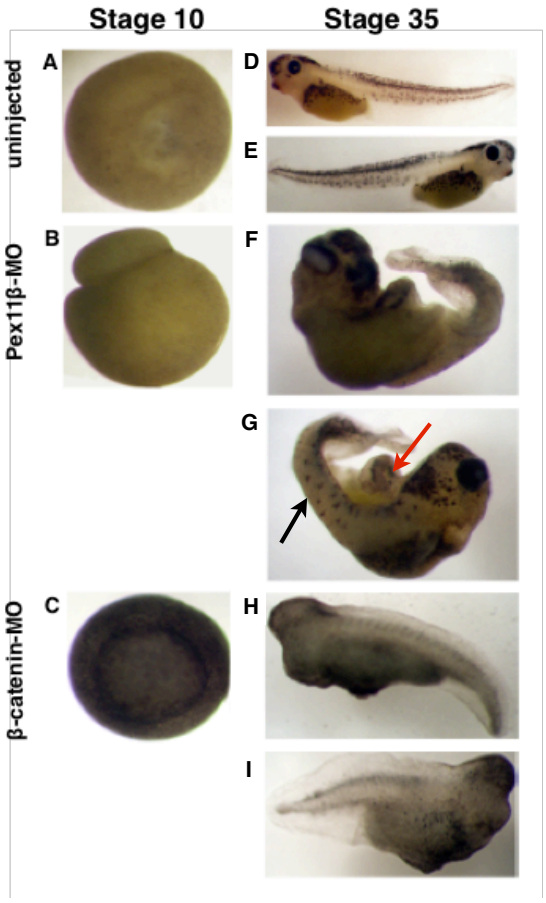
4.3.1 Knockdown of Pex11β yields a bent-axis phenotype in X. laevis developing embryos.

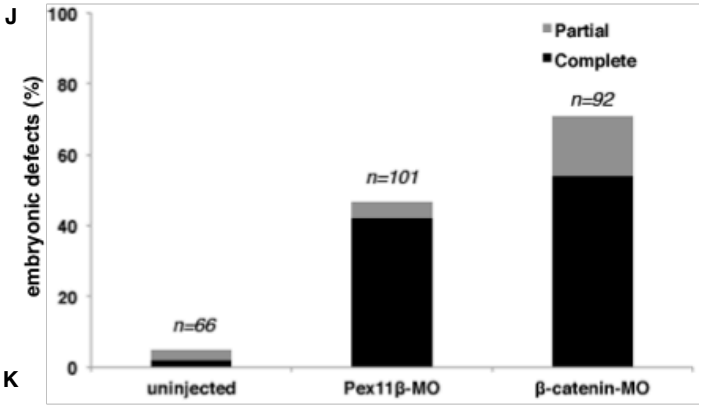
We carried out loss of function analysis with MO against *X. laevis* Pex11β. Previously, we were able to show that Pex11β-MO resulted in decreased protein levels of Pex11β, PMP70, and GFP-SKL containing structures, confirming the integrity of the MO and, the relationship of Pex11β to peroxisome number. Microinjection of Pex11β-MO resulted in distinct developmental abnormalities to early gastrulating embryos compared to uninjected control embryos (Figure 4.1 A versus B). As development proceeds to stage 35, results yield a distinct bent (black arrow) and double axis (red arrow) phenotype (Figure 4.1 D and E versus F and G). As a positive control β-catenin-MO were microinjected that resulted ventralization of embryos, which are apparent at developmental stages 35 relative to uninjected embryos (Figure 4.1 D and E versus H and I).

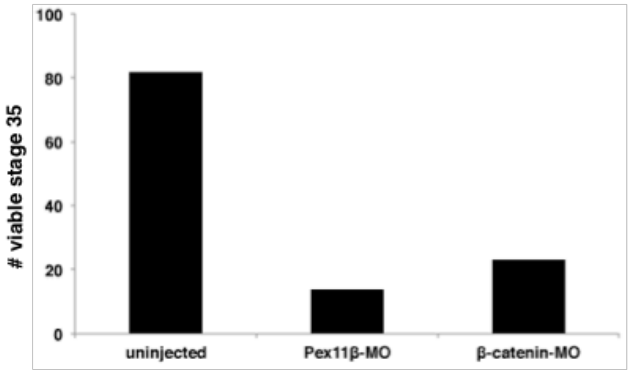
The percentages of embryonic defects were counted in three separate rounds of microinjections. Embryos were counted based on partial or complete phenotypic defects, complete referring to a bent/double axis phenotype and partial defined as slight developmental abnormality compared normal development fate maps by Nieuwkoop and Farber (Nieuwkoop 1972). Uninjected embryos revealed that of 66 embryos counted, less than 8% were defective (Figure 4.1J). Following microinjection of Pex11β-MO, of 101 injected embryos 56% were defective with 50% representing a complete defect (Figure 4.1J). Lastly, microinjection of β-catenin-MO in 92 embryos resulted in 78% defective and 59% with complete defects (Figure 4.1K). The percentage of viable

uninjected and injected embryos were counted at stage 35. Embryos were considered dead if development did not progress following stage 35. 80% of uninjected embryos, <20% Pex11β-MO injected and <23% β-catenin-MO remained alive at stage 35 (Figure 4.1K).

Figure 4.1. Embryonic knockdown of Pex11β yields a double/bent-axis phenotype in *X. laevis* developing embryos. Microinjection of Pex11β-Morpholino (MO caused defects to gastrulation at stage 10 (A versus B) and resulted in a double (red arrow) bent axis (black arrow) to developing embryos at stage 35 (D and E versus F and G). β-catenin-MOs were used as a positive control for our MO study. Microinjecting β-catenin-MOs resulted in ventralization of embryos as anticipated (C, H and I). Defects to developing embryos were quantified at stage 35. Embryos were counted based on partial or complete phenotypic defects, complete referring to a bent/double axis phenotype and partial defined as slight developmental abnormality compared normal development fate maps by Nieuwkoop and Farber (Nieuwkoop 1972). Of the 66 uninjected embryos <8% were defective (J). Following knockdown of Pex11β, 56% of 101 embryos were defective, with 50% representing a complete defect (J). Microinjecting β-catenin-MO in 92 embryos resulted in 78% defective and 59% with complete defects (J). Embryo viability was counted at stage 35 for all treatments. Over 80% of uninjected embryos, <20% Pex11β-MO injected and <23% β-catenin-MO remained alive at stage 35 (K).


Stage 10
Stage 35
uninjected
Pex11β-MO
β-catenin-MO
A
B
C
D
E
F
G
H
I

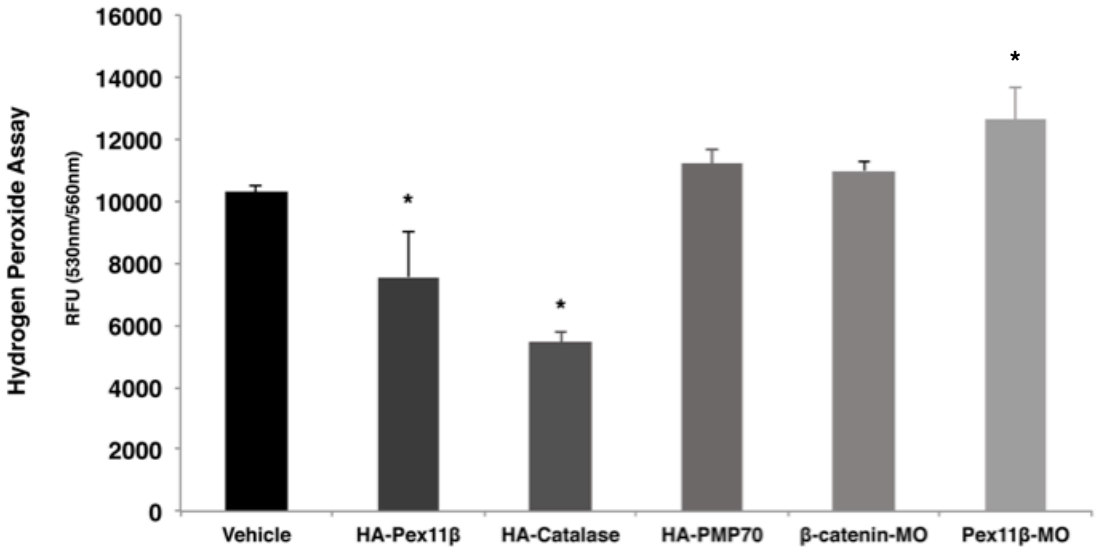

J
embryonic defects (%)
100
80
60
40
20
0
Partial
Complete
n=66
n=101
n=92
uninjected
Pex11β-MO
β-catenin-MO


K
viable stage 35
100
80
60
40
20
0
uninjected
Pex11β-MO
β-catenin-MO

4.3.2 Overexpression and inhibition of Pex11β decreases or increases H_2O_2 levels, respectively in X. laevis A6 cells

To determine changes in intracellular H_2O_2, levels were detected specifically using an Amplex Red Hydrogen Peroxide/Peroxidase Assay. Overexpression of HA-Pex11β resulted in a 1.25-fold decrease in H_2O_2 levels as show by decreases in relative fluorescence units (RFU) to control EV (vehicle) (Figure 4.2). Overexpression of HA-Catalase resulted in a 2-fold decrease in H_2O_2 levels as shown by decreases in RFU to control (Figure 4.2). Pex11β-MO resulted in a 1.25-fold increased H_2O_2 levels. HA-PMP70 and β-catenin-MO, used as positive controls, released no changes H_2O_2 levels as shown by no changes in RFU versus EV (Figure 4.2). Samples were run in triplicate to determine statistical significances as shown by a single asterisk.

Figure 4.2. H_2O_2 levels change in response to Pex11β expression in in *X. laevis* A6 cells. The levels of H_2O_2 were determined by measuring changes in resorufin generation in the Amplex Red Hydrogen Peroxide/Peroxidase Assay Kit. Overexpression of Pex11β and catalase resulted in a 1.25-fold and 2-fold decrease in H_2O_2 levels compared to empty vector (EV), respectively. Knockdown of Pex11β resulted in a 1.25-fold increased H_2O_2 levels compared to EV. Resorufin fluorescence was measured with excitation at 530-560 nm and emission at 590 nm. Data were analyzed by ANOVA, and means were compared by using Student's *t* test, $P<0.05$. Statistical significance indicated by single asterisks, compared to vehicle.


Hydrogen Peroxide Assay
RFU (530nm/560nm)
16000
14000
12000
10000
8000
6000
4000
2000
0
*
*
*
Vehicle
HA-Pex11β
HA-Catalase
HA-PMP70
β-catenin-MO
Pex11β-MO

4.3.3 Inhibition of Pex11β increases global ROS levels in X. laevis A6 cells

An oxidant signal in response to insulin was demonstrated in 3T3-L1 adipocytes loaded with CM-H_2DCF-DA, a redox indicator dye that is trapped intracellularly after cleavage by cellular esterases. When oxidized *in situ*, DCFDA generates a signal that is visualized by fluorescence microscopy. Following empty vector transfection, a strong oxidant signal was detected by DCFDA fluorescence as shown in Figure 4.3. We next determined whether changes in the levels of Pex11β could affect intracellular ROS levels. While overexpression of Pex11β studies revealed slight decreases in the relative fluorescence (Figure 4.3B), following electroporation of our Pex11β-MO we detected a strong fluorescence increase compared to our empty vector and overexpression results (Figure 4.3C versus A and B). Results were statistically compared in Figure 4.3D, which highlight a >2.5-fold increase in fluorescence following knockdown of Pex11β.

4.3.4 Inhibition of Pex11β increases mitochondrial ROS levels in X. laevis A6 cells

i next hypothesized that Pex11β expression could have an effect on mitochondrial ROS levels. In order to visualize mitochondrial ROS levels in A6 cells, we used a cell-permeable low toxicity fluorescent dye, MitoTracker Red CMX-ROS (Invitrogen), which stains mitochondria specifically and responds to changes in mitochondrial membrane potential. Following empty vector transfection, a weak MitoTracker Red signal was detected as shown in Figure 4.4A. i next determined whether changes in the level of Pex11β could affect mitochondrial ROS. While overexpression studies revealed slight decreases in the relative fluorescence (Figure 4.4B), consistent with our DCFDA data, following electroporation of our Pex11β-MO I detected a strong increase in

mitochondrial ROS compared to that from the empty vector and EV and overexpression experiments (Figure 4.4C versus A and B). Results were statistically compared in Figure 4.4D, which is highlighted by a >2.5-fold increase following knockdown of Pex11β.

Figure 4.3. Live cell DCFDA stain reveals increases in global ROS following inhibition of Pex11β. Endogenous ROS levels were detected in control, empty vector (EV) (A), with little changes following overexpression of Pex11β (B). Inhibition of Pex11β revealed increased levels of fluorescence compared to overexpression and EV (A and B versus C). Quantification of the mean fluorescence intensity generated by the oxidation of DCFDA was expressed as a percentage of fluorescence from ten randomly selected cells. A significant increase in fluorescence intensity was found following knockdown of Pex11β as noted by double asterisks (D). $P<0.05$. n=25 randomly selected cells.

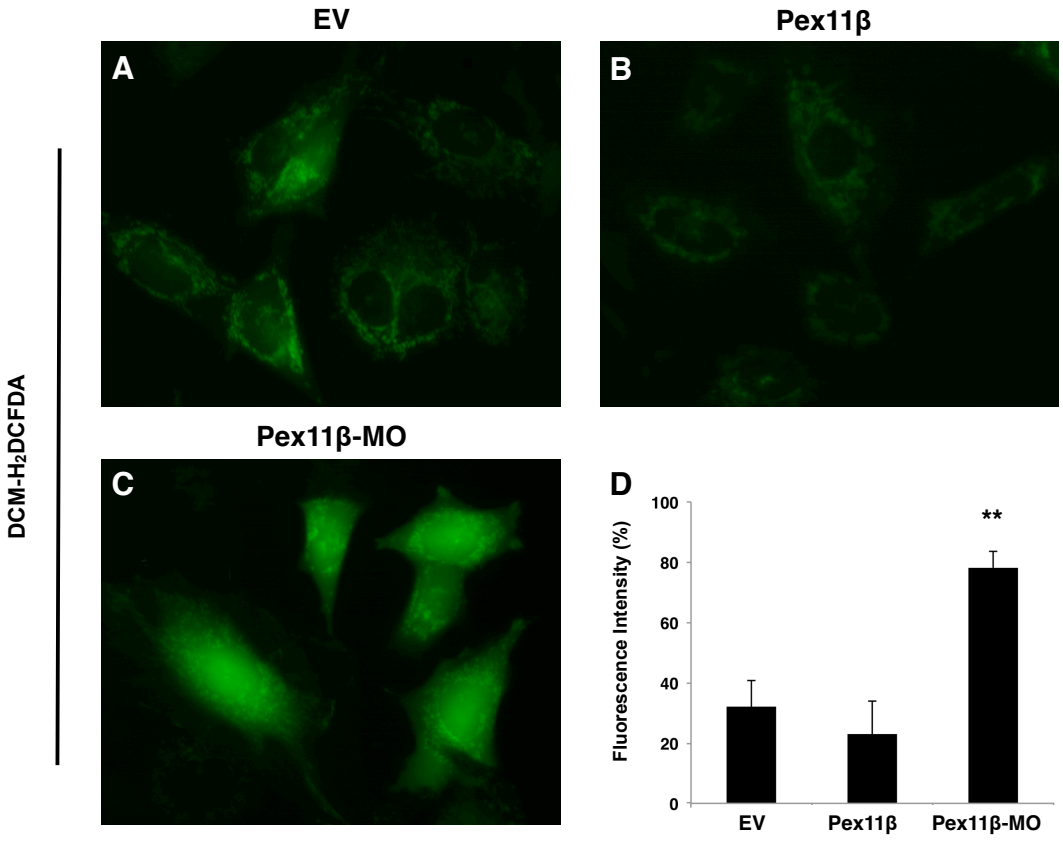
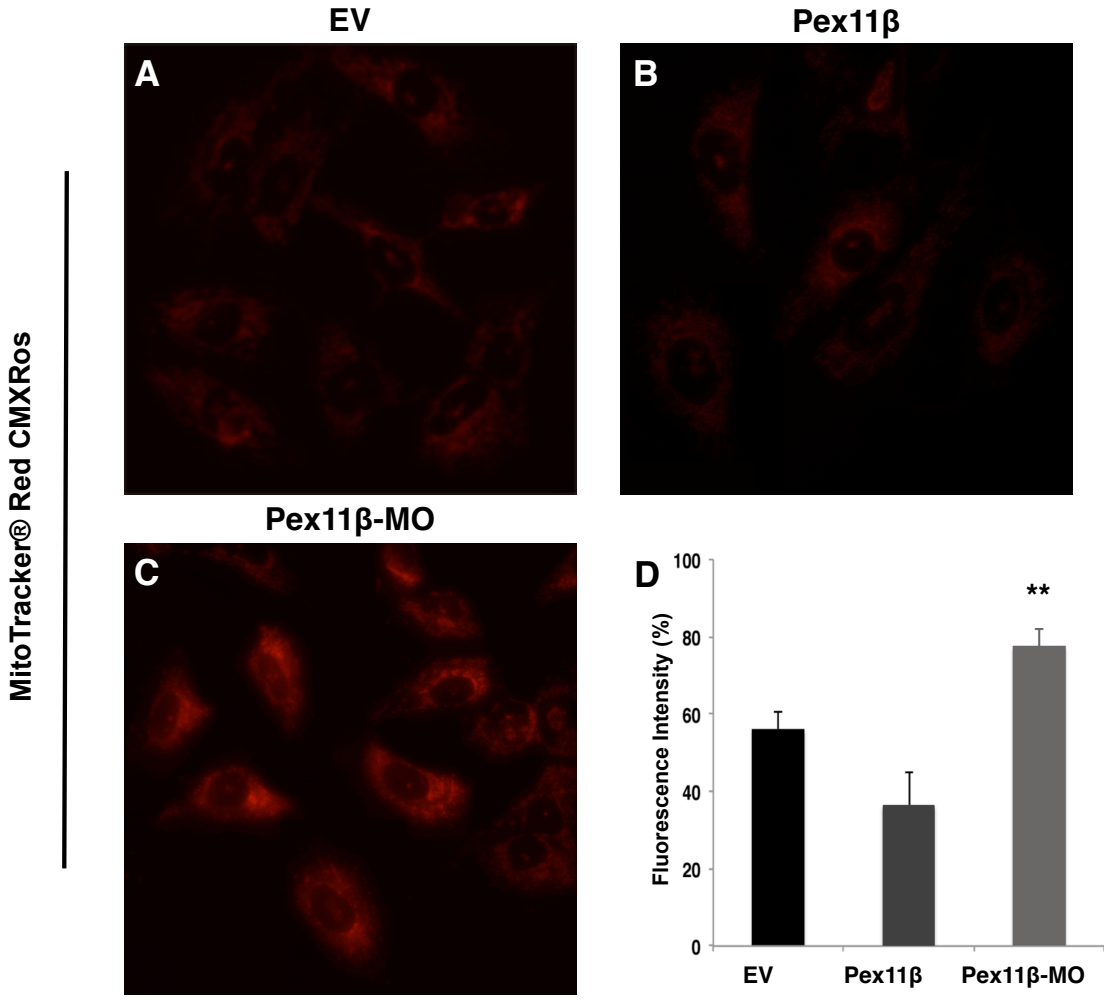

EV
Pex11β
A
B
DCM-H2DCFDA
Pex11β-MO
C
D
Fluorescence Intensity (%)
100
80
60
40
20
0
**
EV
Pex11β
Pex11β-MO

Figure 4.4. Live cell MitoTracker Red stain reveals increases mitochondrial ROS following inhibition of Pex11β. Mitochondrial ROS production was observed from control, empty vector (EV), and following overexpression and inhibition of Pex11β in A6 cells. Endogenous mitochondrial ROS levels were detected in control EV (A), with little change following overexpression of Pex11β (B). Inhibition of Pex11β revealed increased levels of fluorescence compared to overexpression and EV (A and B versus C). Quantification of the mean fluorescence intensity generated by the oxidation of MitoTracker Red was expressed as a percentage of fluorescence from ten randomly selected cells. A significant increase in fluorescence intensity was found following knockdown of Pex11β as indicated by double asterisks (D). $P<0.05$. n=25 randomly selected cells.


EV
Pex11β
A
B
MitoTracker® Red CMXRos
Pex11β-MO
C
D
100
80
60
40
20
0
Fluorescence Intensity (%)
**
EV
Pex11β
Pex11β-MO

4.3.5 Inhibition of Pex11β increases Wnt/PCP signaling in X. laevis A6 cells

Since we found that inhibition of Pex11β results in bent/double axis phenotype during *X. laevis* development (Figure 4.1) and ROS levels are directly related to the number of peroxisomes (Figure 4.2, 4.3 and 4.4), then one would predict the possibility of aberrant cell signaling due to loss of Pex11β. As it was previously elucidated that Nrx regulates the Wnt/PCP pathway in *X. laevis* (Funato, Michiue et al. 2008), we sought to determine if inhibition of Pex11β, resulting in decreased peroxisome numbers and increases ROS levels, resulted in the activation of the redox-sensitive Wnt/PCP signaling pathway.

We first examined changes in JNK/AP-1 cell signaling using pAP-1-Luciferase that serves as a reporter for changes in the Wnt/PCP cell singling pathway. Our results indicate a >4-fold increase in the amount of luciferase detected following inhibition of Pex11β versus both overexpression and control EV (Figure 4.5). As the current known downstream effectors of the PCP pathway are Rho-like GTPases and c-Jun terminal kinase (JNK) kinase, we investigated whether knockdown of Pex11β increases the levels of JNK and the active forms phospho-JNK. Our results indicate increases in levels of phospho-JNK following inhibition of Pex11β, suggesting a relationship between increased ROS and Wnt/PCP cell signaling (Figure 4.6 A and B).

To elucidate whether Nrx negatively regulated Wnt/PCP cell signaling we performed a coimmunoprecipitation to determine the interaction of Dvl and Nrx, by immunoprecipitating (IP) with anti-Dvl antibodies, followed by immunoblotting (IB) using for Nrx. Our results reveal that knockdown of Pex11β, decreased the levels of Nrx detected compared to both untransfected and GFP transfected cells (Figure 4.7). These

results suggest that a redox-sensitive mechanism exists for Wnt/PCP cell signaling in *X. laevis* A6 cells.

Figure 4.5. Knockdown of Pex11β increases AP-1 associated cell signaling in *X. laevis* A6 cells as evident by a reporter gene expression assay. *X. laevis* A6 cells were transfected pRL-TK to normalize luciferase levels and with pAP-1-Luciferase reporter constructs to monitor the activity of AP-1 regulated signal transduction in response to inhibition and overexpression of Pex11β. Inhibition of Pex11β resulted in a >4-fold increase in the levels of luciferase compared to that of overexpression of Pex11β and empty vector (EV), as quantified using Student T-test between treatments.

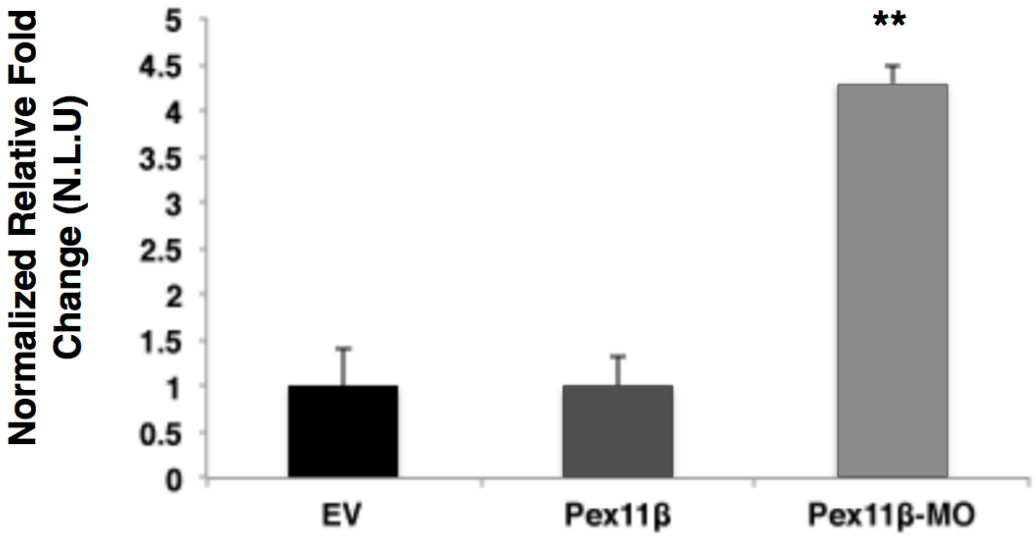

Normalized Relative Fold Change (N.L.U)
5
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
**
EV
Pex11β
Pex11β-MO

Figure 4.6. Knockdown of Pex11β increases phosphorylated-active-JNK protein levels. Immunoblot analyses of active (phosphorylated) and inactive forms of JNK were performed to determine changes relative protein levels. Inhibition of Pex11β resulted in a >2-fold increase in the relative protein levels of phosphorylated Jnk compared to Pex11β overexpression and electroporation of EV (**A** and **B**).

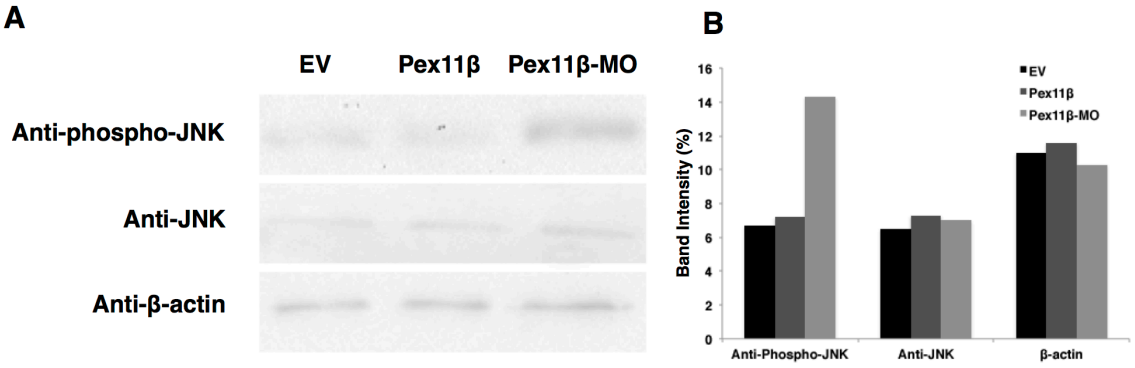
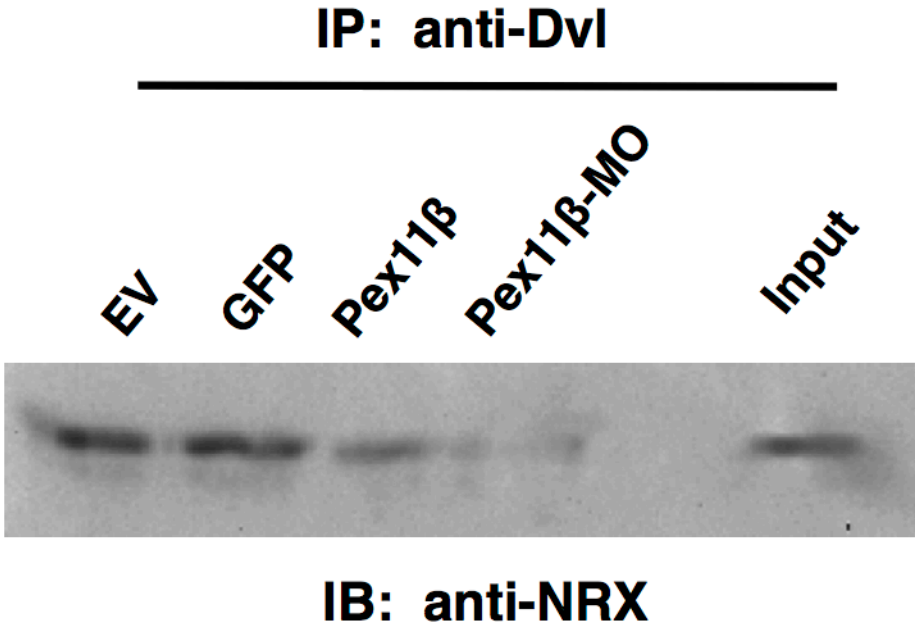

A
EV
Pex11β
Pex11β-MO
Anti-phospho-JNK
Anti-JNK
Anti-β-actin
B
Band Intensity (%)
16
14
12
10
8
6
4
2
0
EV
Pex11β
Pex11β-MO
Anti-Phospho-JNK
Anti-JNK
β-actin

Figure 4.7. Immunoprecipitation of Dvl revealed that knockdown of Pex11β decreases Dvl-Nrx association in *X. laevis* A6 cells. Immunoprecipitation (IP) with anti-Dvl followed by immunoblotting (IB) using anti-Nrx following overexpression and knockdown of Pex11β reveals knockdown of Pex11β decreases Nrx levels versus untransfected and GFP transfected cells. Empty vector (EV) and GFP transfected cells were used as positive control. Total cell lysates were loaded under input lane.


IP: anti-Dvl
EV
GFP
Pex11β
Pex11β-MO
Input
IB: anti-NRX

4.4. Discussion

We have previously revealed the pivotal role of Pex11β in regulating peroxisome number and catalase levels (Fox, Walsh et al. 2011). Overexpression of Pex11β increased the number of peroxisomes and increased levels of catalase, whereas the knockdown decreased the number of peroxisomes, while catalase levels remained unchanged. As catalase is related to ROS elimination, these results lead us to investigate whether a relationship exists between peroxisome number, ROS levels and ROS induced changes in cell signaling in *X. laevis* A6 cells.

4.4.1. Peroxisome numbers contribute to regulation of redox balance

High peroxisomal consumption of O_2 has supported the notion that these ubiquitous organelles play a key role in both the production and scavenging of ROS, particularly H_2O_2 (Schrader and Fahimi 2006). Therefore, we first elucidated whether changes in peroxisome numbers affect cellular levels of H_2O_2 in A6 cells. We carried out a fluorogenic assay to quantify the relative levels of H_2O_2 following overexpression or knockdown of Pex11β. We detected a significant decrease in H_2O_2 following overexpression of Pex11β, and significant increase in H_2O_2 following knockdown of Pex11β, indicating changes in peroxisome number directly affect intracellular levels of H_2O_2. A previous study in liver cells revealed that 20-60% of total H_2O_2 generated inside of peroxisomes diffuses to the surrounding media (Boveris, Oshino et al. 1972). In addition, it was demonstrated that H_2O_2 could rapidly cross the peroxisomal membrane, likely through the newly identified porin-like channel (Rokka, Antonenkov et al. 2009). These studies, along with our results, suggest that peroxisomes not only contribute to

levels of cytosolic H_2O_2, however, their numbers may also greatly affect the amount of H_2O_2 present in surrounding tissue and media.

In addition to H_2O_2, peroxisomes contain enzymatic sources of membrane-permeant superoxide and nitric oxide (Fransen, Nordgren et al. 2011). Therefore, peroxisomes are likely a cellular source of various types of ROS. Consequently, we tested whether peroxisome number has an effect on global ROS levels by measuring ROS levels within cells. Our results demonstrated that knockdown of Pex11β significantly increased cellular ROS levels.

To further investigate these peroxisome induced changes in ROS, we next examined the relationship between Pex11β and mitochondrial ROS. Similar to peroxisomes, mitochondria have the ability to adapt in number in response to metabolic cues, and there is evidence suggesting these two organelles may cooperate via cross talk (Schrader and Yoon 2007). In addition, it has become clear that the relationship between peroxisomes and mitochondria is redox-sensitive (Fransen, Nordgren et al. 2011). Recently, it was found that cells lacking catalase or functional peroxisomes resulted in mitochondrial redox imbalance (Ivashchenko, Van Veldhoven et al. 2011). We investigated whether changes to peroxisome number could result in redox changes that are communicated to the mitochondria. Consistent with our previous findings on global ROS levels, we demonstrated that knockdown of Pex11β decreased peroxisome number, and significantly increased the levels of mitochondrial ROS. In summary, these findings suggest that peroxisome-derived oxidative stress may trigger signaling/communication events that ultimately result in increased levels of H_2O_2, as well as changes in the global levels of ROS that directly induce mitochondrial stress. This altered redox state can have

varying cellular consequences, and can particularly effect ROS sensitive cell signaling pathways.

4.4.2. Peroxisome generated ROS mediates cell signaling

It is well known that ROS can modulate the activity of redox sensitive proteins. The intracellular localization and activity of numerous proteins may be, directly or indirectly, controlled by the oxidation of thiol groups on redox-sensitive cysteine residues. Peroxisomes have intricate protective mechanisms to counteract oxidative stress and maintain redox balance. An imbalance in ROS levels may damage biomolecules and perturb cellular thiol levels, resulting in deregulation of cellular signaling pathways. Thus, the levels of peroxisome generated ROS must be tightly regulated, as certain levels of ROS may act as regulators of intracellular signaling (Masters 1996).

Of the many signaling molecules that are ROS sensitive, we investigated the Wnt signaling pathways, as Wnt is an important mediator of normal development. The redox sensitivity of Wnt/β-catenin and Wnt/PCP cell signaling were first elucidated during *X. laevis* embryogenesis (Funato, Michiue et al. 2006; Funato, Michiue et al. 2008; Funato and Miki 2010). Nrx is a redox-sensitive protein that can negatively regulate both Wnt pathways through inhibition of Dvl. When oxidized, Nrx detaches from Dvl, thereby activating each pathway in the absence of their respective ligands. We show that knockdown of Pex11β during *X. laevis* embryogenesis resulted in a bent-axis phenotype that is typically observed in embryos with abnormal PCP activation (Sokol 1996; Wallingford and Harland 2001). Additionally, a similar phenotype is also observed following redox-sensitive activation of Wnt/PCP cell signaling (Funato, Michiue et al.

2008).

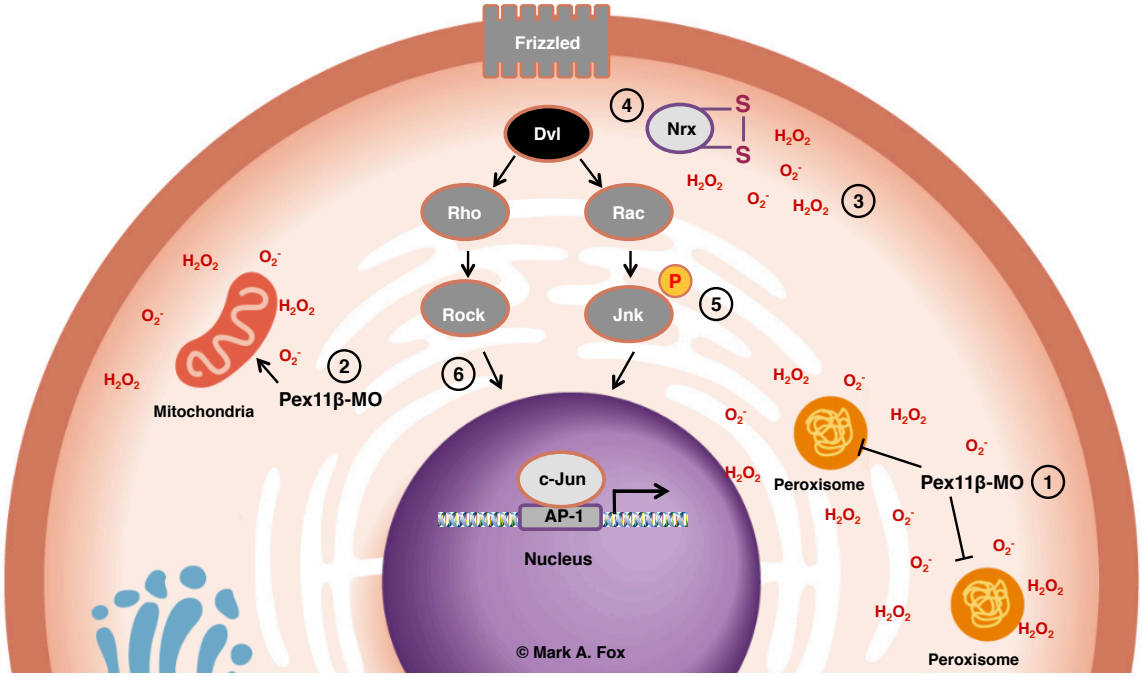
Using mouse intestinal cells, it was shown that Wnt treatment induces the production of ROS through NADPH oxidase (Nox1), thereby increasing levels of cytosolic H_2O_2, oxidizing Nrx and activating Wnt/β-catenin signaling (Kajla, Mondol et al. 2012). On the basis of this data and our current results, we hypothesized that knockdown of Pex11β would result in redox-sensitive activation of Wnt/PCP cell signaling in *X. laevis* A6 cells. Our research verifies the recently published *X. laevis* embryonic work mentioned above (Kajla, Mondol et al. 2012). We examined the activation of Wnt/PCP cell signaling by pAP-1-luciferase reporter gene expression assays, and following inhibition of Pex11β demonstrated a significant increase in Wnt/PCP cell signaling. Additionally, we saw increases in the amount of active (phosphorylated) Jnk, a downstream target of the Wnt/PCP pathway. To corroborate this, we also found through immunoprecipitation analyses that knockdown of Pex11β reduced the interaction between Dvl and Nrx. Therefore, consistent with previous *X. laevis* embryo studies on redox-sensitive Wnt signaling, we highlight the importance of peroxisome produced ROS as secondary messengers in *X. laevis* cells (Figure 4.8).

4.2.3. Concluding remarks

Taken together, these results suggest that peroxisomes are key elements in maintaining oxidative balance. Perturbation to the number of peroxisomes can independently affect intracellular ROS levels, damaging various cellular constituents; thus, their numbers must be tightly regulated. Additionally, our studies have extended the current understanding of the redox-sensitive mechanism of Wnt/PCP cell signaling.

It will be interesting to further investigate the mechanisms through which changes in peroxisome number may alter cell signaling.

Figure 4.8. Schematic model of redox-sensitive Wnt/PCP activation in *X. laevis* A6 cells following Pex11β inhibition. 1) Knockdown of Pex11β, decreasing peroxisome number, results in significant increases in H_2O_2, global ROS and 2) mitochondrial ROS levels. 3) Increases in ROS resulted in the oxidation of Nrx, 4) liberating Nrx from Dvl thereby activating Wnt/PCP cell signaling 5) resulting in the phosphorylation and subsequent activation of Jnk, 6) leading to changes in cell polarity and cytoskeletal rearrangements.


Frizzled
Dvl
Nrx
S
S
4
Rho
Rac
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Rock
Jnk
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6
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Pex11β-MO
Mitochondria
Peroxisome
Pex11β-MO
1
c-Jun
AP-1
Nucleus
© Mark A. Fox
Peroxisome
H_2O_2
O_2^-

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CHAPTER 5

Conclusions and General Discussion

5.1. Summary and Conclusions

By examining peroxisomes during *X. laevis* embryogenesis and in *X. laevis* A6 cells, I have shown for the first time that changes to peroxisome numbers directly effect the levels of ROS, which subsequently result in altercations to redox-sensitive Wnt/PCP cell signaling. As the physiological roles of peroxisomes are well characterized, however, the functionality of these organelles in terms of biogenesis and division, is less established. In addition to understanding how peroxisomes contribute to normal cellular functioning, the importance of also understanding how peroxisome division is regulated is highlighted by a group of fatal human diseases termed peroxisome biogenesis disorders (PBD). Patients with PBDs have either a lack of, or very small numbers of functional peroxisomes, which results in a neurological abnormalities (Weller, Gould et al. 2003). Studies characterizing the mechanisms underlying peroxisome division, suggest that various peroxins are involved in governing organelle numbers, and that the cell signals regulating overall numbers may be linked to metabolism (Yan, Rayapuram et al. 2005). One model for division suggests that Pex11β interacts with a translipid bilayer on the luminal side of the peroxisomal membrane, inducing peroxisome elongation (Platta and Erdmann 2007). Once the organelle membrane has elongated, which signals membrane division, various peroxins and dynamin-related proteins facilitate membrane constriction and fission. Pex11β and other peroxins have been implicated in membrane elongation, although few have been demonstrated to induce peroxisome division via overexpression analyses, thereby increasing peroxisome numbers in the absence of extracellular stimuli (Li and Gould 2002). To my knowledge, there has been no mechanistic link between Pex11β, peroxisome number and cellular ROS levels. Over the past decades it was

revealed that peroxisomes participate not only in the generation of ROS, which can negatively affect cell fate and result in malignant degeneration, but also in cell rescue from the damaging effects of such radicals (Schrader and Fahimi 2006). High levels of ROS exert a toxic effect on biomolecules such as DNA, proteins, and lipids, leading to the accumulation of oxidative damage in diverse cellular locations. The accumulation of ROS may contribute to the deregulation of redox-sensitive metabolic and signalling pathways, and to various pathological conditions including cancers, diabetes and neurodegeneration. Due to their oxidative metabolism, peroxisomes are considered a source of oxidative stress. However, peroxisomes can also respond to ROS that have been generated in other intracellular or extracellular locations, indicating that they likely also play a role in protecting the cell against oxidative damage. I altered the amount of ROS in A6 cells through overexpression and knockdown of Pex11β, which resulted in an increase or decrease in the number of peroxisomes, respectively. Along with the notion that peroxisomes adapt in number due to changes in metabolic load, my results shed light on the possibility that perhaps peroxisome numbers are a reflection of the cellular oxidation state. This illustrates the importance of these organelles in regulating oxidative stress, suggesting that peroxisomes may behave as signaling compartments through redox-orchestrated cell-signaling events.

During *X. laevis* development, Wnt/PCP cell signaling regulates the convergent extension movements that underlie normal gastrulation. Disruption of Wnt/PCP signaling during *X. laevis* development causes convergent extension errors, resulting in defects in gastrulation and a bent-axis phenotype in later developmental stages (Wallingford and Harland 2001). Following knockdown of Pex11β during

embryogenesis, I observed a bent/double-axis phenotype in developing embryos, suggesting that changes in peroxisome number may lead to altercations in Wnt/PCP cell signaling. Indeed, using *X. laevis* A6 cells, I have confirmed that there is a link between peroxisome number and Wnt/PCP signaling. I have previously demonstrated that altered levels of Pex11β can affect the numbers of peroxisomes and cellular ROS levels, and I also investigated whether a redox-sensitive mechanism for Wnt/PCP cell signaling exists in A6 cells. My results indicate that altered ROS levels, which occur as a result of changes to peroxisome number induced by knockdown of Pex11β, resulted in increased Wnt/PCP cell signaling. This study sheds light on the possibility that peroxisomes, along with their well-established physiological functions, are intricate organelles involved in the maintenance of intracellular oxidative balance.

5.2. Contributions to the Current Knowledge of Peroxisome Division

The current understanding is that peroxisome divisions require several steps, namely i) the induction of proliferation, ii) elongation of the organelle, iii) constriction and membrane fission and iv) maturation of the newly formed organelle by matrix and membrane protein import. Of the many proteins implicated in this process, the first identified to be crucial for peroxisome division was Pex11, classified in *Saccharomyces cerevisiae* (Erdmann, Veenhuis et al. 1989). Moreover, Pex11 is the most abundant component of the peroxisomal membrane, and is an integral membrane protein containing two transmembrane spans, with both termini facing the cytosol (Abe, Okumoto et al. 1998; Lorenz, Maier et al. 1998). The importance of Pex11 in peroxisome division is emphasized by its imperative role in initiating elongation of the

organelle membrane, a required step in the divisionary process (Huber, Koch et al. 2011).

In *S. cerevisiae,* deletion of the Pex11 gene led to the occurrence of fewer and enlarged peroxisomes, whereas overexpression of Pex11 resulted in increased numbers of these organelles (Erdmann and Blobel 1995). Homologues of *S. cerevisiae*-Pex11 are known in most eukaryotic organisms and these usually contain more than one Pex11 protein (Marshall, Krimkevich et al. 1995). Depending on the species, up to three members of the Pex11 family were identified in yeasts, plants typically contain five, whereas vertebrates harbor three, namely Pex11α, Pex11β and Pex11γ. While the functions of Pex11 proteins have been well characterized in mammals, no current data exits on the role of this family of peroxisomal proteins in other vertebrates.

Previous studies have suggested that there is a metabolic regulation of peroxisome division. Chang et al. (1999) found that defects in peroxisomal fatty acid β-oxidation enzymes reduced peroxisome abundance in mammalian cells. These results suggested that the recruitment of Pex11 to the peroxisomal membrane, and the induction of membrane elongation, was in fact linked to metabolism. More recently, Pex11β was found to drive peroxisome division in the absence of peroxisome metabolism, and that the loss of mammalian Pex11β causes a reduction in peroxisome abundance in the absence of peroxisomal metabolic substrates (Li and Gould 2002). As a result, it was proposed that Pex11β has a direct role in peroxisome division, and that loss of Pex11β inhibits peroxisome metabolism indirectly, perhaps due to altered membrane structure or dynamics.

No current data is available on the role of Pex11 proteins in vertebrate development, and it is unknown whether peroxisomes exist in fertilized eggs or in early

stage vertebrate embryos. It is for these reasons that I have attempted to elucidate the molecular mechanisms of Pex11β during *X. laevis* embryogenesis and in A6 cells. This study has revealed for the first time when functional peroxisomes are first formed during embryogenesis, and has demonstrated that overexpression or knockdown of Pex11β during this fragile time can induce an early- or late-onset to peroxisome biogenesis, respectively. These results confirm that Pex11β not only partakes in membrane elongation to induce the divisionary process, it also suggests that Pex11β has a functional role in *de novo* biogenesis. If the onset of Pex11β expression during embryogenesis serves as a molecular switch to induce peroxisome biogenesis, other peroxisomal components should modulate their expression patterns accordingly. To corroborate this, I have also demonstrated that alterations to the levels of Pex11β, both *in vitro* and *in vivo*, directly affect the levels of expression of peroxisomal genes and proteins that encode peroxisomal cytosolic chaperones, membrane proteins, ATPases, membrane recruitment factors and antioxidant enzymes found within the organelle itself. Additionally, I found that the expression pattern for PPAR-γ was either decreased or increased following overexpression or inhibition of Pex11β, respectively, both *in vitro* and *in vivo*. Collectively, these studies suggest for the first time that Pex11β can modulate peroxisome number and may be involved in the *de novo* biogenesis pathway.

5.3. Contributions to the Current Knowledge of Peroxisomes and Oxidative Stress

The current knowledge on the relationship between peroxisomes and ROS is centred on the high peroxisomal consumption of O_2, the subsequent large production of ROS byproducts, and the discovery of several ROS metabolizing oxidative enzymes

found within these organelles. These facts alone support the notion that these ubiquitous organelles play a key role in both the production and scavenging of ROS in the cell, in particular H_2O_2 (Fransen, Nordgren et al. 2011). The main metabolic processes contributing to the generation of H_2O_2 in peroxisomes are the β-oxidation of fatty acids and the disproportionation of superoxide radicals. It is obvious that due to their oxidative metabolism, peroxisomes are considered a source of oxidative stress. However, peroxisomes can also respond to oxidative stress and ROS, which have been generated in other intra- or extracellular locations, most likely to protect the cell against oxidative damage.

An interesting feature of peroxisomes is their ability to proliferate and multiply, or be degraded in response to nutritional and environmental stimuli (Fransen, Nordgren et al. 2011). In mammalian cells for example, the number and size of peroxisomes as well as the expression of peroxisomal β-oxidation enzymes are increased substantially when activators of PPARs are applied (Fahimi, Reinicke et al. 1982). Such conditions are considered to generate peroxisome-induced oxidative stress, which may overwhelm the antioxidant capacity and lead to disease. These studies suggest that if peroxisome number can adapt to certain cellular conditions, then changes to their numbers could mediate changes to the cellular oxidative environment; a concept I examined in my research.

When elucidating the molecular mechanisms of Pex11β, I performed various fluorogenic experiments to determine whether changes in peroxisome numbers affected ROS levels in A6 cells. I have shown that Pex11β inhibition, resulted in decreased peroxisome numbers and increased levels of H_2O_2, as well as increased levels of global

and mitochondrial ROS. Through my overexpression and inhibition analyses of Pex11β, I have demonstrated that a dynamic relationship exists between the levels of Pex11β and catalase expression. Interestingly, overexpression of Pex11β significantly increased both the relative gene and protein levels of catalase. Immunohistochemical analysis for catalase revealed increases in punctate-like structures, which are indicative of peroxisomes, consistent with previous overexpression analyses for Pex11β in human fibroblast cells (Li, Baumgart et al. 2002). However, inhibition of Pex11β resulted in no significant increase or decrease in catalase expression or the appearance of localized structures in A6 cells.

Along with catalase levels, overexpression of Pex11β significantly increased the levels of Pex5, a cytosolic receptor for PTS1-like proteins such as catalase (Freitas, Francisco et al. 2011). Inhibition of Pex11β revealed no changes in Pex5 levels, however, it did result in significant decreases in Pex1 – an ATPase involved in the energy dependent steps of matrix protein import. Thus, induction of the divisionary process through Pex11β overexpression, increases the number of pre-peroxisomal vesicles that mature through matrix and membrane protein import. Perhaps knockdown of Pex11β results in membrane defects, creating nonfunctional "giant" peroxisomes, a common phenotype in yeast that occurs when peroxisomes are unable to segregate (Erdmann and Blobel 1995). Therefore, in the absence of Pex11β peroxisome division may begin, however, without proper elongation machinery, matrix protein import is halted, and peroxisomes become nonfunctional. This could explain why I observed decreased levels of Pex1 following inhibition of Pex11β, while Pex5 levels remained unchanged. Consequently, I have determined that the decreased numbers of functional peroxisomes,

following Pex11β inhibition, resulted in significant increases in ROS levels.

5.4. Contributions to the Current Knowledge on Redox-sensitive Wnt signaling

Our current understanding of redox-regulated Wnt signaling is based on the results of several experiments conducted using *X. laevis* embryos. Both the canonical Wnt/β-catenin and noncanonical Wnt/PCP pathways have been reported as redox-sensitive through the negative regulation of Nrx, a thioredoxin-related protein. Upon oxidation, Nrx is liberated from Dvl, resulting in the activation of Wnt signaling in the absence of the Wnt ligand. The redox mechanisms of Wnt signaling was extensively studied through the canonical signaling pathway (Funato and Miki 2010), and it has been suggested that the same mechanisms apply to noncanonical Wnt/PCP signaling (Funato, Michiue et al. 2008). In *X. laevis* embryos, Funato et al., (2008) found that knockdown of Nrx yields a bent-axis phenotype that is typically observed in embryos with abnormal Wnt/PCP cell signaling (Funato, Michiue et al. 2008). Additionally, Nrx overexpression can inhibit Dvl-induced phosphorylation of c-Jun terminal kinase (Jnk), a downstream component of the noncanonical Wnt/PCP cell-signaling pathway (Funato, Michiue et al. 2008). Conclusively, these data suggest that redox-regulated Wnt signaling functions through the canonical and noncanonical Wnt/PCP pathways.

More recently, a study investigating ROS, Wnt and Nrx, revealed that superoxide-generating NADPH oxidase1 (Nox1) is involved in the redox sensitive mechanisms of Wnt signaling (Kajla, Mondol et al. 2012). It was demonstrated in mouse epithelial cells that treatment with a Wnt ligand, activating canonical Wnt signaling, consequently increases ROS levels through active Nox1. Increases in ROS resulted in the oxidation

and inactivation of Nrx, thereby releasing Nrx from Dvl and activating Wnt/β-catenin cell signaling.

Since knockdown of Pex11β resulted in a bent/double-axis in *X. laevis* embryos, similar to the phenotype observed following abnormal activation of Wnt/PCP cell signaling, I investigated whether increases in ROS via knockdown of Pex11β, could independently activate Wnt/PCP signaling. My data suggests that increases in ROS, by Pex11β knockdown, can induce Wnt/PCP cell signaling through redox-sensitive liberation of Nrx. In addition to this, I have also shown that this activation induces the phosphorylation of Jnk, increasing the activity of this pathway. Collectively, I have revealed both the importance of peroxisome numbers to the cellular oxidation state and shed light on the redox-sensitive mechanisms of Wnt/PCP cell signaling in *X. laevis* A6 cells.

5.5. Limitations of Research and Suggestions for Future Studies

5.5.1. Pex11β induces peroxisomal gene expression and alters peroxisome number during early Xenopus laevis development.

Although I have shown for the first time a *in vivo* the link between Pex11β and peroxisome division in vertebrates, the major limitation of this study is the lack of evidence supporting the idea that Pex11β overexpression is inducing the formation of fully functional mature peroxisomes. As mentioned previously, co-overexpression studies in *Xenopus* embryos and A6 cells with Pex11β and GFP-SKL, resulted in increases in GFP-containing bodies suggestive of functional peroxisomes. In order for PTS1-type cargoes (GFP-SKL) to be imported into the peroxisomal matrix, all

components of the importomer must be present. These data presented in this study suggest they are present. However, identifying the functionalities of these organelles, for example in terms of their ability to chain shorten VLCFA, and synthesize etherphospholipids such as plasmalogen and cholesterol, would shed insight as to whether or not these GFP-SLK containing bodies are functional mature peroxisomes. In future experiments, measuring levels of these metabolic substrates would increase the veracity of this study. Additionally, I also believe using a Pex11β-GFP fusion protein would confirm the ability of Pex11β to induce peroxisomal elongation, and demonstrate the formation of tubular peroxisomal membrane compartments. Together, these additional experiments would lead to a more complete understanding of the mechanism of Pex11 β induced peroxisome division that was proposed earlier.

5.5.2. Morpholino-induced knockdown of Xenopus laevis Pex11-Beta reveals its pivotal role in peroxisome biogenesis during embryonic development

As these experiments recapitulate my previous studies, using knockdown, I first suggest examining the levels of metabolic byproducts of peroxisomes. This data will shed light on the role of Pex11β, in its ability to reduce the number of functional mature organelles. Another possible improvement to my *in vivo* studies relates to whether or not knockdown of Pex11β interrupts the *de novo* formation of peroxisomes. The specific experiment discussed in this study examined the localization patterns of GFP-SKL following knockdown of Pex11β in early embryos. Interestingly, the fluorescence staining of GFP-SKL was much different from the typical punctate-like structures representative of peroxisomes. Rather, I observed irregularly shaped GFP-like structures

after knockdown of Pex11β. These data, in corroboration with the overexpression of Pex11β in embryos, suggest that Pex11β may interfere with *de novo* biogenesis in embryos. The staining pattern observed is suggestive of lager structures possibly representing "blebs" of ER, which peroxisomes utilize for *de novo* biogenesis. To examine this further, I would propose confirming the role of Pex11β expression on the formation of functional peroxisomes and their relationship to the ER. In these experiments I would use peroxisomal markers (GFP-SKL) in combination with ER-specific fluorescent stains to determine the association between these two organelles, and examine how fluorescence patterns change based on Pex11β expression. Although there would be many experiments needed to confirm these findings, especially *in vivo*, I have still revealed a novel mechanism for Pex11β during embryonic development.

5.5.3. Peroxisome numbers directly affect levels of ROS and the redox-sensitive mechanism of Wnt signaling

The results of this study give insight as to how the numbers of peroxisomes affect intracellular ROS levels, and suggest that changes in ROS resulting from modulations in peroxisome number may induce the redox-sensitive activation of Wnt/PCP cell signaling pathway. As a result, I have established that peroxisomes are important regulators of oxidative stress. To further investigate how ROS levels fluctuate based on Pex11β expression, in future experiments I would examine the relative gene expression of antioxidants housed in peroxisomes, mitochondria and the cytosol. In combination with this study, I would investigate the protein expression of antioxidants, such as peroxiredoxin, via 2D-PAGE, to visualize changes in oxidation state. Once again, these

results would give insight on changes in ROS levels resulting from Pex11β expression.

The most intriguing part of my study indicates that changes in the levels of ROS via knockdown of Pex11β, result in irregular Wnt/PCP cell signaling. In addition to the experiments I have already performed, in order to confirm the redox-sensitivity association of Nrx and Dvl, I would recapitulate my coimmunoprecipitation studies using both oxidizing and reducing agents in A6 cells. This would verify that, along with changes in peroxisome number, exogenous oxidants would have a similar effect on the interaction between these two proteins.

Although this study may serve as the basis for many different areas of future research, recapitulating these experiments during *X. laevis* development will highlight an important avenue of investigation in our lab. I would propose looking at the interaction of Nrx and Dvl by first overexpression and inhibition of Pex11β, and then through microinjection of oxidizing and reducing agents in *Xenopus* embryos. Next, I would determine if I could rescue the bent/double-axis phenotype caused by knockdown of Pex11β, by co-microinjection Pex11β-MO and Nrx into early developing embryos. Again, these studies would act as a series of pilot experiments that will be continued in our lab as they offer great potential for increasing our understanding of the dynamic relationship between Pex11β, peroxisome number, ROS levels and the mechanisms behind redox-sensitive Wnt/PCP signaling during *X. leaves* embryogenesis.

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Appendix A: Additional Data

Figure A.1. Embryonic inhibition of Pex11β increased PPARγ levels during early *X. laevis* embryogenesis. The respective mRNA levels represent measures of mid-log phase RT-PCR product band intensities, relative to levels of EF1α. RT-PCR analysis of RNA isolated from control stage 10 embryos and Pex11β-MO injected embryos revealed significant changes in the expression of PPARγ, but not PPARα, and PPARδ. PPARα levels were decreased by treatment. $P<0.05$, n=3. Values presented are the means ± SE.

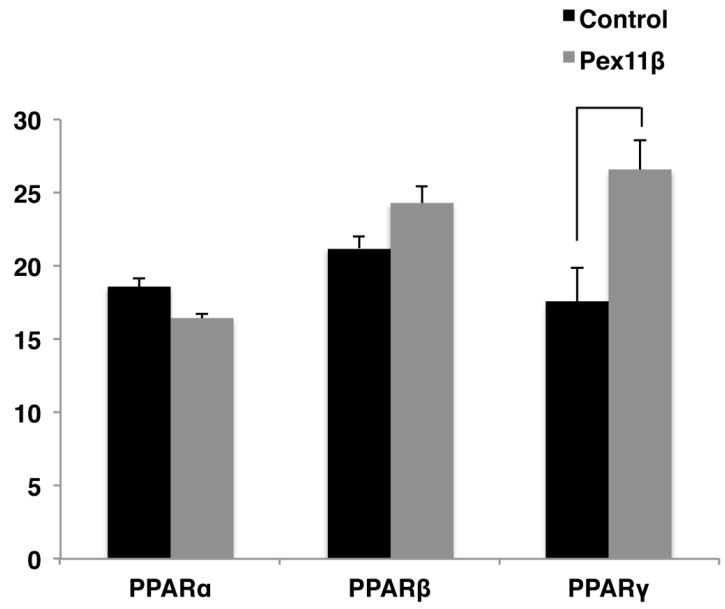

Control
Pex11β
30
25
20
15
10
5
0
PPARα
PPARβ
PPARγ

Figure A.2. Overexpression of Pex11β decreases peroxidase activity in *X. laevis* A6 cells. Peroxidase activity were determined by measuring changes in resorufin generation in the Amplex Red Hydrogen Peroxide/Peroxidase Assay Kit. Overexpression of Pex11β and catalase resulted in a 1.25-fold and 2-fold decrease peroxidase activity compared to EV, respectively. Resorufin fluorescence was measured with excitation at 530-560 nm and emission at 590 nm. Data were analyzed by ANOVA, and means were compared by using Student's *t* test.

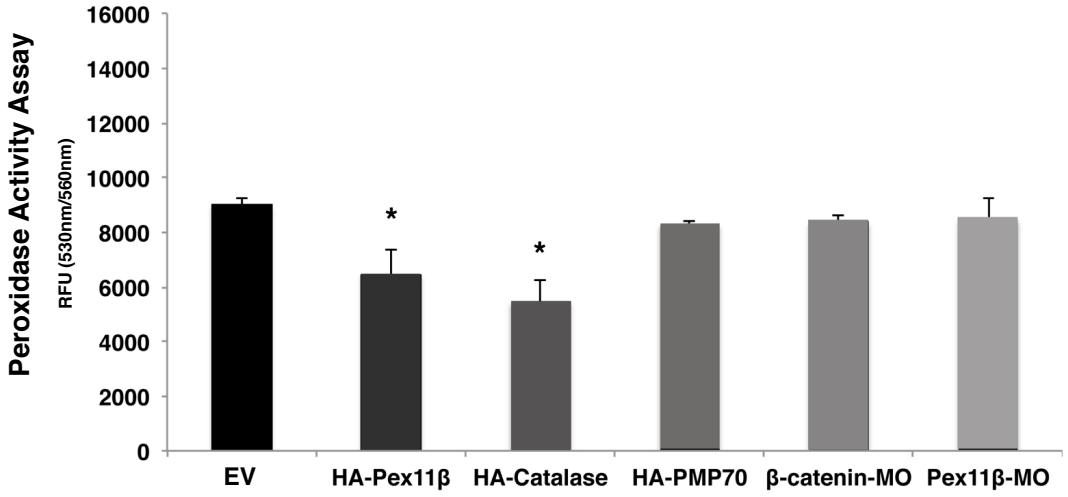

Peroxidase Activity Assay
RFU (530nm/560nm)
16000
14000
12000
10000
8000
6000
4000
2000
0
*
*
EV
HA-Pex11β
HA-Catalase
HA-PMP70
β-catenin-MO
Pex11β-MO

Figure A.3. Comicroinjection of Pex11β and Pex11β-MO rescues PMP70 and Catalase protein levels during *X. laevis* embryogenesis. Western blot analysis revealed comicroinjection Pex11β and Pex11β-MO rescues protein levels during embryogenesis. Catalase protein levels reveal slight increases following Pex11β, however now changes were found after injection of GFP, Pex11β-MO, while comicroinjection of Pex11β and Pex11β-MO reveal levels similar to GFP (**A**). PMP protein levels reveal increases following Pex11β, decreases following Pex11β-MO, while comicroinjection of Pex11β and Pex11β-MO reveal levels similar to GFP (**A**). Protein loading in each lane was confirmed and standardized via a β-actin antibody (**A**). Western blot signals were digitized and data were quantified and analyzed (**B**), comicroinjection indications that protein levels are rescued to their endogenous levels.

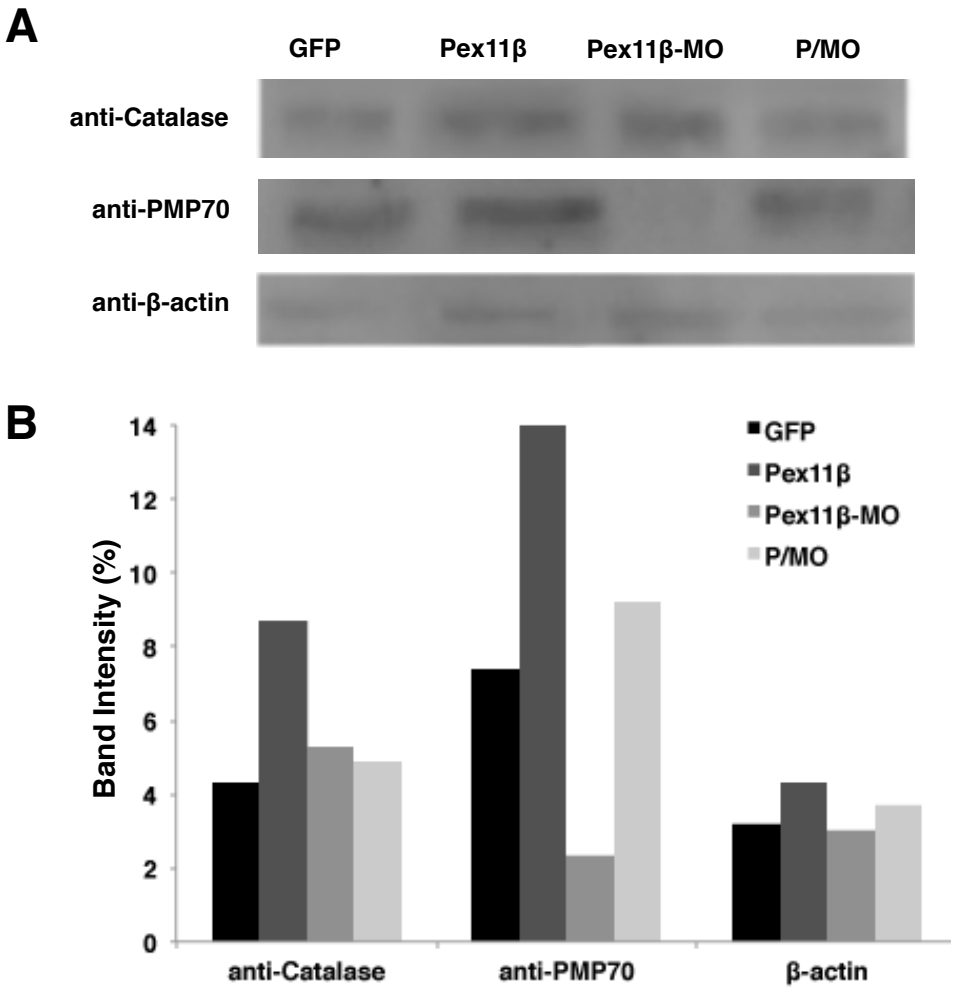
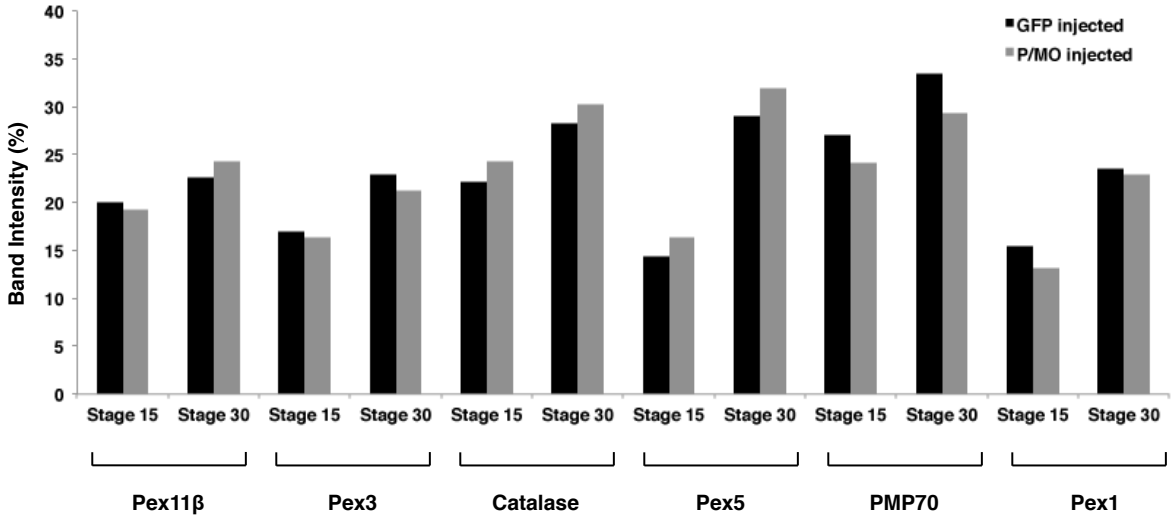
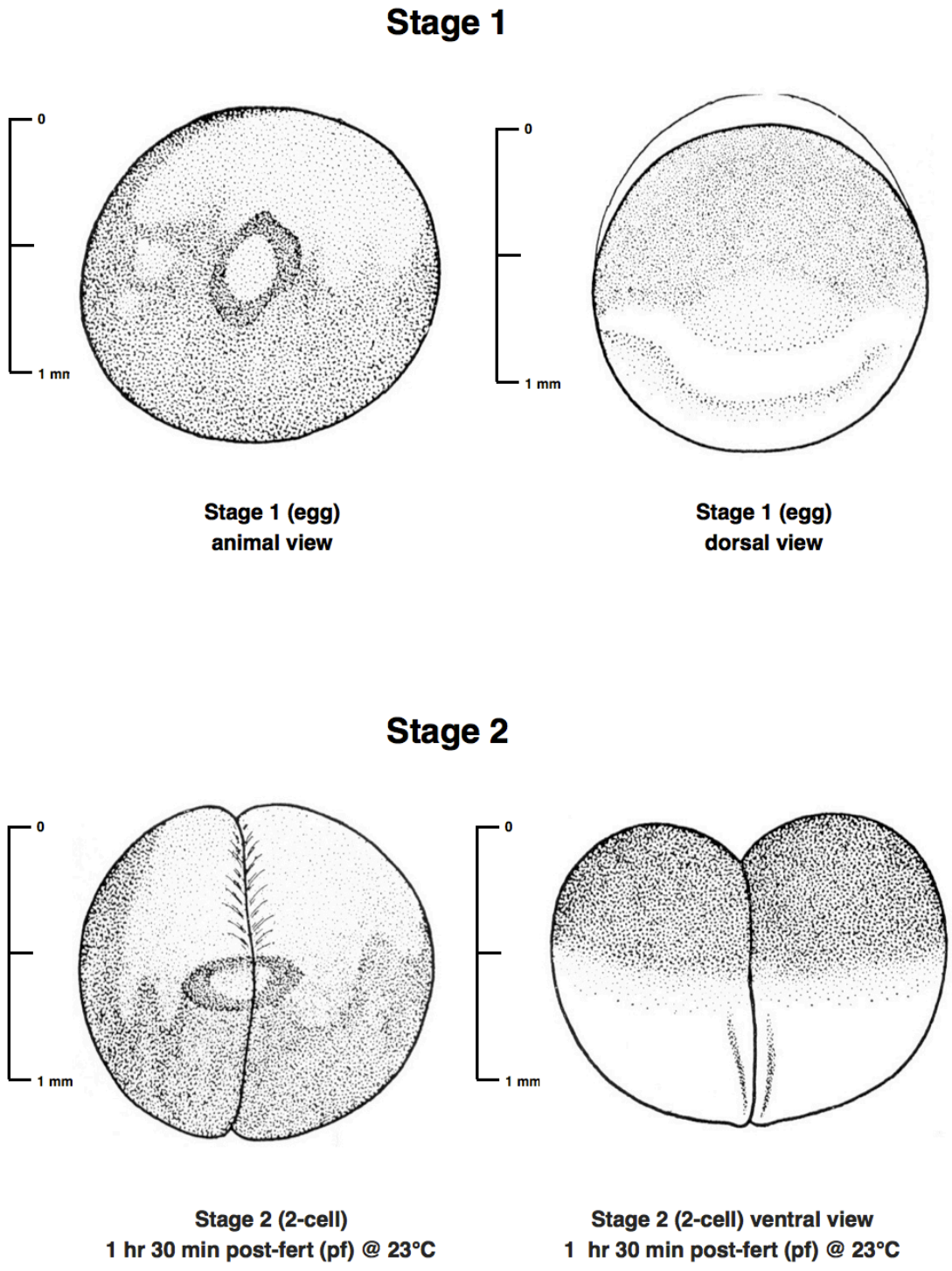

A
GFP
Pex11β
Pex11β-MO
P/MO
anti-Catalase
anti-PMP70
anti-β-actin
B
Band Intensity (%)
14
12
10
8
6
4
2
0
GFP
Pex11β
Pex11β-MO
P/MO
anti-Catalase
anti-PMP70
β-actin

Figure A.4. Comicroinjection of Pex11β and Pex11β-MO rescues peroxisome related gene levels during *X. laevis* embryogenesis. RT-PCR analysis during normal embryogenesis revealed that the levels of all genes exampled increased as development progressed from stage 15 to 30, both following injection of GFP and comicroinjection of Pex11β and Pex11β-MO. Following comicroinjection of Pex11β and Pex11β-MO, band intensities of RT-PCR products were unchanged compared to that of GFP control injected embryos. The respective bar heights represent measures of mid-log phase RT-PCR products band intensities relative to the levels of EF1α.

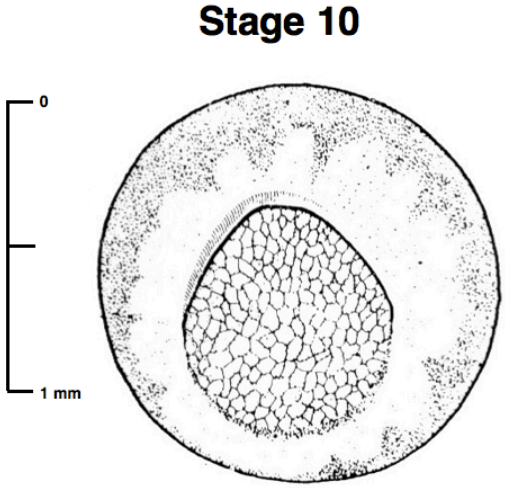

GFP injected
P/MO injected
Band Intensity (%)
40
35
30
25
20
15
10
5
0
Stage 15
Stage 30
Pex11β
Pex3
Catalase
Pex5
PMP70
Pex1

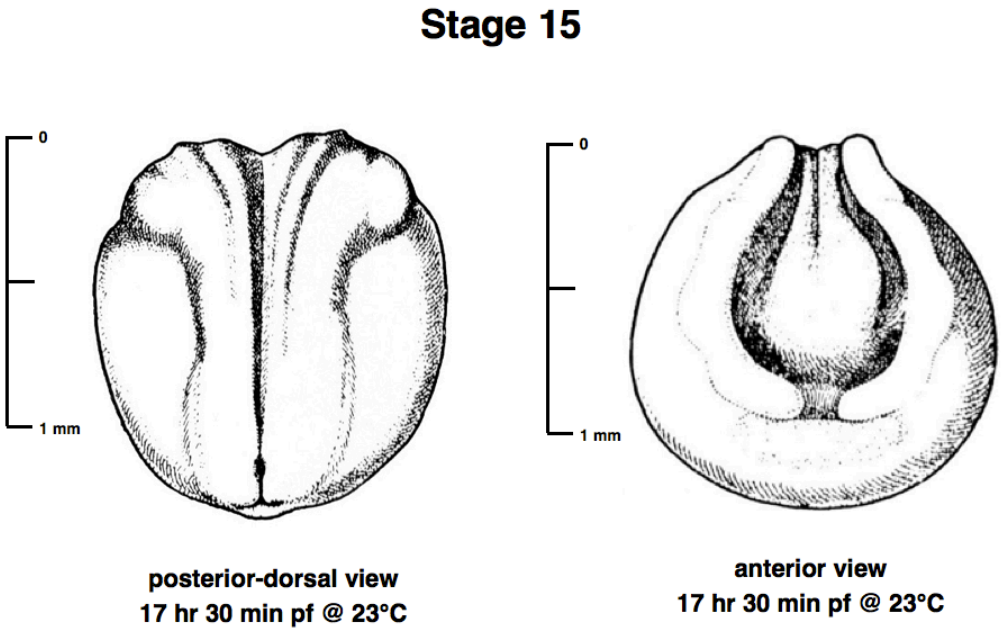
Appendix B:
***Xenopus laevis* developmental stages 1 and 2**



Adapted and modified from Nieuwkoop and Faber 1956.

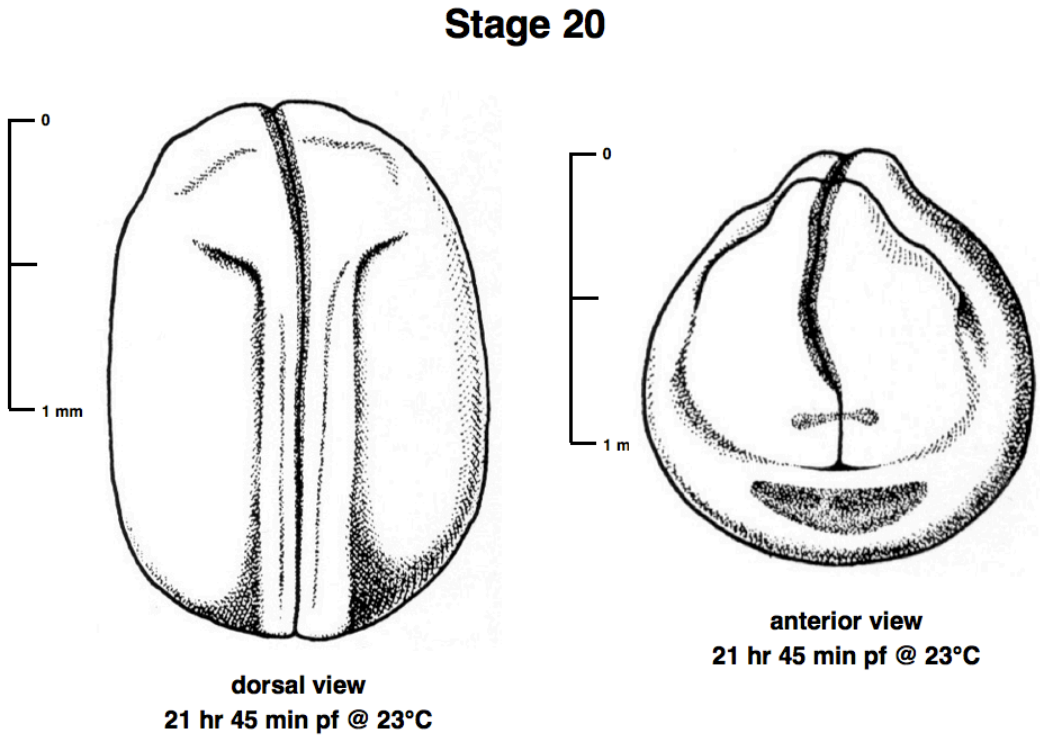
***Xenopus laevis* developmental stages 10 and 15.**





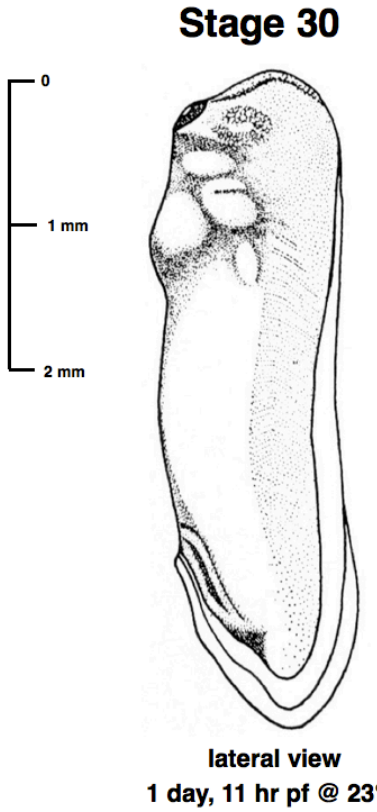
Adapted and modified from Nieuwkoop and Faber 1956.

***Xenopus laevis* developmental stages 20.**



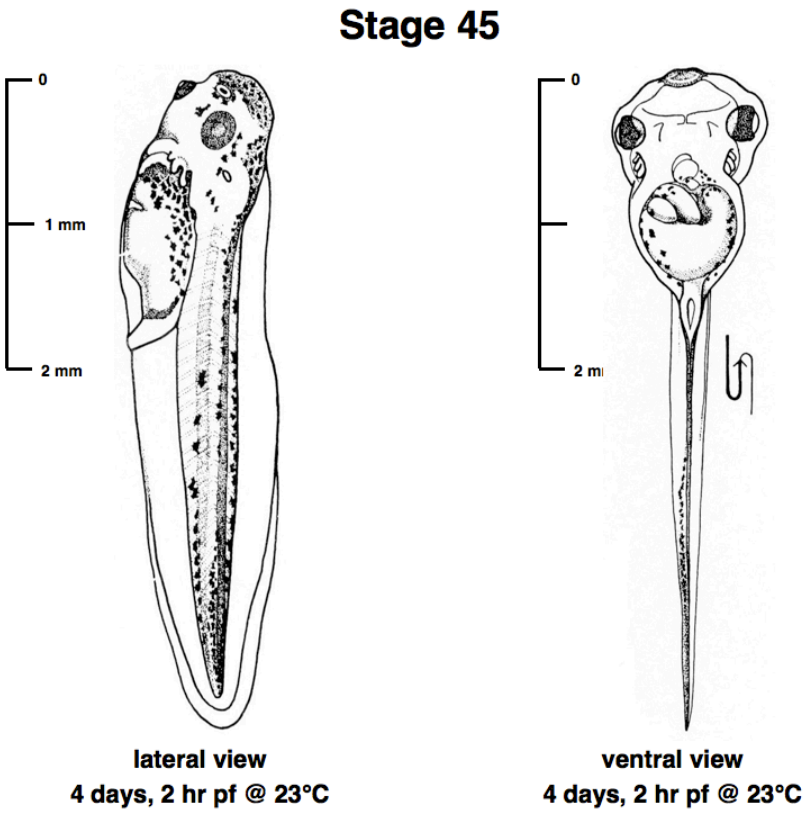
Adapted and modified from Nieuwkoop and Faber 1956.

***Xenopus laevis* developmental stage 30.**



Adapted and modified from Nieuwkoop and Faber 1956.

***Xenopus laevis* developmental stage 45.**



Adapted and modified from Nieuwkoop and Faber 1956.

Appendix C:

AUP Number: 2009-044 PI Name: Damjanovski, SashkoAUP Title: MMP Activation During Xenopus Development

The YEARLY RENEWAL to Animal Use Protocol (AUP) 2009-044 has been approved.

1. This AUP number must be indicated when ordering animals for this project.
2. Animals for other projects may not be ordered under this AUP number.
3. Purchases of animals other than through this system must be cleared through the ACVS office.Health certificates will be required.

REQUIREMENTS/COMMENTS Please ensure that individual(s) performing procedures on live animals, as described in this protocol, are familiar with the contents of this document.

The holder of this Animal Use Protocol is responsible to ensure that all associated safety components (biosafety, radiation safety, general laboratory safety) comply with institutional safety standards and have received all necessary approvals. Please consult directly with your institutional safety officers.

Submitted by: Thompson, Sharla H on behalf of the Animal Use Subcommittee

The University *of* Western Ontario
Animal Use Subcommittee / University Council on Animal Care
Health Sciences Centre, • London, Ontario • CANADA – N6A 5C1
PH: 519-661-2111 ext. 86768 • FL 519-661-2028
Email: auspam@uwo.ca • http://www.uwo.ca/animal/website/

Researcher: Dr. S. Damjanovski

Biosafety Approval Number: BIO-UWO-0141

Expiry Date: June 16, 2014

June 21, 2011

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,

Appendix D:

University of Western Ontario
Biology and Geology 2053
London, Ontario
N6A 5B7

Curriculum Vitae

Mark A. Fox, PhD

Education

2009 – 2012 University of Western Ontario
Doctor of Philosophy – Cell and Molecular Biology

2004 – 2009 University of Western Ontario
Honors Specialization in Biology

Publications

Fox, MA, Walsh LA, Nieuwesteeg M, Damjanovski S (2011). PEX11beta induces peroxisomal gene expression and alters peroxisome number during early Xenopus laevis development. *BMC Dev Biol*;**11**:24.

Fox, MA, Hwanger J, Cumming R, Kelly G, Damjanovski S. Peroxisome number regulates intracellular ROS levels and redox sensitive-Wnt signaling in *Xenopus laevis*. (In preparation for submission)

Fox, MA, Nieuwesteeg M, Damjanovski S (Submitted – Under Review). Morpholino-induced knockdown of *Xenopus laevis* Pex11β reveals its pivotal role in

peroxisome biogenesis during embryonic development. BMC Developmental Biology

Graves HA, **Fox MA**, Mai, S (In preparation for submission). Induced C-MYC activity generates telomere uncapping via formation of a TRF2-ORC1-MYC protein complex. *Genes to Cells*

Nieuwesteeg M, Walsh LA **Fox MA**, and Damjanovski S. (2011). Domain specific overexpression of TIMP-2 and TIMP-3 reveals MMP-independent functions of TIMPs during *X. laevis* development. *Biochem Cell Biol*.

Nieuwesteeg M, **Fox MA**, and Damjanovski S. Conseuqences of domain specific overexpression of TIMP-1 during Xenopus laevis development. (In preparation for submission).

Awards and Nominations

2008 – 2012 Western Graduate Research Scholarship, Department of Biology, University of Western Ontario

2011 Graduate Student Travel Award, Department of Biology, University of Western Ontario

2010 Society of Graduate and Postdoctoral Students – Nomination for Graduate Student Teaching Award, Department of Biology, University of Western Ontario

2011 Society of Graduate and Postdoctoral Students - Graduate Student Teaching Award, Department of Biology, University of Western Ontario

2004 – Western Scholarship of Distinction, University of Western Ontario

2003 – Ontario Scholar

Conferences

2010 – American Society for Cell Biology 50th Annual Meeting. Philadelphia, PA. **Mark A. Fox** and Sashko Damjanovski. PEX11beta induces peroxisomal gene expression and alters peroxisome number during early Xenopus laevis development.

Teaching

Graduate Teaching Assistant – Analysis and Interpretation of Biological Data 224A - 2008

Graduate Teaching Assistant – Introduction to Cell Biology 238A - 2009

Graduate Teaching Assistant – Developmental Biology 338A – 2009

Graduate Teaching Assistant – Introduction to Cell Biology 2382A - 2010

Graduate Teaching Assistant – Developmental Biology 3338A – 2010

Graduate Teaching Assistant – Introduction to Cell Biology 2382B – 2011

Graduate Teaching Assistant – Developmental Biology 3338A – 2011.

Graduate Teaching Assistant – Introduction to Cell Biology 2382B – 2012.

Certifications

Animal Care and Veterinary Services Animal Care

Laboratory and Environmental Waste Management

Biological Safety Cabinet Management

Transportation of Dangerous Goods

Biosafety Management

Level C CPR W.H.M.I.S

	Confocal Microscopy Certificate - Zeiss LSM 5 Duo
	Fluorescence Microscopy Certificate - Zeiss Axio Imager Z1 Microscope
Volunteer Experience	Hospice of London 2009 – 2012 Two and a half years of volunteering providing palliative care for 19-year-old Micah Pleasant, who was diagnosed with Hodgkin's lymphoma, Micah is 21 years old currently remains in a state of remission