DEPARTMENT OF ONCOLOGY-PATHOLOGY
CANCER CENTER KAROLINSKA

Karolinska Institutet, Stockholm, Sweden

Protein Tyrosine Phosphatases

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Taking a 'redoxionist' look at PDGFR signaling

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Karolinska Institutet

Stockholm 2014

Published by Karolinska Institutet. Printed by Åtta.45 Tryckeri AB.

ISBN 978-91-7549-521-7

"When I grow up, I don't want to BECOME anything. I want to DO something."
Seline Frijhoff

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-
Taking a 'redoxionist' look at PDGFR signaling

THESIS FOR DOCTORAL DEGREE (Ph.D.)

by

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ABSTRACT

Protein Tyrosine Phosphatases (PTPs) are oxidized and inactivated by reactive oxygen species (ROS) upon stimulation of a wide range of cell surface receptors, including the platelet-derived growth factor (PDGF) receptor β (PDGFβR). Signaling via this receptor tyrosine kinase stimulates cell proliferation and migration, and it is associated with cancer and cardiovascular diseases. **The general aim of this thesis was to achieve a better understanding of the redox regulation of PTPs in PDGFβR signaling.**

Paper I. We analyzed the involvement of mitochondrial ROS in PTP oxidation and PDGFβR signaling. We found that depletion of p66Shc, an enzyme that produces hydrogen peroxide from mitochondria, decreased PDGF-induced PDGFβR phosphorylation, oxidation of PTP1B, SHP2 and DEP1, and downstream activation of Akt, Erk, PLCγ-1 and FAK. Consistently we find that cells that lack p66Shc have a decreased migratory response to PDGF. Downregulation of p66Shc in breast cancer cells also led to a decrease in EGF-induced EGFR phosphorylation and downstream signaling. Finally, we could show that downregulation of the mitochondrial hydrogen peroxide scavenger Peroxiredoxin 3 increased PDGFβR phosphorylation. We therefore conclude that p66Shc and mitochondrial ROS contribute to PTP oxidation and growth factor signaling.

Paper II. Restenosis is a disease in which injury-induced proliferation and migration of vascular smooth muscle cells (VSMCs) leads to vessel wall thickening, which is partially dependent on PDGF signaling. We sought to investigate whether the *in vitro* findings from paper I have *in vivo* relevance for restenosis. We show that PDGF activates p66Shc also in VSMCs, and coincides with downstream signaling in a time-dependent manner. Downregulation of p66Shc decreased PDGF-induced PDGFβR phosphorylation in VSMCs, as well as PLCγ-1 phosphorylation and chemotaxis. An *in vivo* mouse model of restenosis showed that p66Shc knockout (KO) mice displayed decreased restenotic incidence and injury. We conclude that p66Shc contributes to PDGF signaling in VSMCs and restenosis in mice.

Paper III. In this paper we studied the potential role of Thioredoxin Reductase 1 (TrxR1) on the reactivation of oxidized PTPs and PDGFβR signaling. Cells that lack Thioredoxin Reductase 1 (*txnrd1*$^{-/-}$), and therefore have an impaired function of the thioredoxin (Trx) system, showed an increased oxidation of PTP1B, whereas the oxidation state of SHP2 was unchanged. Accordingly, *in vitro* studies showed that the Trx system, with either Trx1 or Trx-related protein 14 (TRP14), was capable of reducing oxidized PTP1B, but not SHP2. *Txnrd1*$^{-/-}$ cells also showed increased PDGF-induced PDGFβR phosphorylation at the PTP1B-targeted tyrosine-579/581 residue and increased proliferation. Moreover, deletion of PTP1B inhibited the increase in phosphorylation at tyrosine-579/581 after chemical inhibition of the Trx system compared to PTP1B-reconstituted cells. We conclude that the Trx system impacts on PDGFβR signaling through reactivation of oxidized PTP1B.

The findings of these studies have uncovered novel regulatory aspects of PTP oxidation in PDGFβR signaling that might have an impact on PDGFβR-regulated diseases.

LIST OF PUBLICATIONS

I. **The mitochondrial reactive oxygen species regulator p66Shc regulates PDGF-induced signaling and migration through protein tyrosine phosphatase oxidation**

Jeroen Frijhoff, Markus Dagnell, Martin Augsten, Elena Beltrami, Marco Giorgio, Arne Östman.
Free Radic Biol Med, 2014; 68:268-77.

II. **Deletion of p66shc attenuates PDGF signaling in vascular smooth muscle cells and restenosis injury in mice**

Jeroen Frijhoff, Olli Leppänen, Janna Paulsson, Marco Giorgio, Arne Östman.
Manuscript.

III. **Selective activation of oxidized PTP1B by the thioredoxin system modulates PDGF-β receptor tyrosine kinase signaling**

Markus Dagnell, **Jeroen Frijhoff**, Irina Pader, Martin Augsten, Benoit Boivin, Jianqiang Xu, Pankaj. K. Mandal, Nicholas K. Tonks, Carina Hellberg, Marcus Conrad, Elias S.J. Arnér, Arne Östman.
Proc Natl Acad Sci U S A, 2013: 110(33):13398-403.

Articles that are not included in this thesis:

I. **Differential upregulation of MAP kinase phosphatases MKP3/DUSP6 and DUSP5 by Ets2 and c-Jun converge in the control of the growth arrest versus proliferation response in MCF-7 breast cancer cells to phorbol ester**

Caroline E. Nunes-Xavier, Céline Tarréga, Rocío Cejudo-Martín, **Jeroen Frijhoff**, Åsa Sandin, Arne Östman, Rafael Pulido.
J Biol Chem, 2010: 285(34):26417-30.

II. **12/15-lipoxygenase-derived lipid peroxides control receptor tyrosine kinase signaling through oxidation of protein tyrosine phosphatases**

Marcus Conrad, Åsa Sandin, Heidi Förster, Alexander Seiler, **Jeroen Frijhoff**, Markus Dagnell, Georg W. Bornkamm, Olof Rådmark, Rob Hooft van Huijsduijnen, Pontus Aspenström, Frank Böhmer, Arne Östman.
Proc Natl Acad Sci U S A, 2010: 107(36)15774-9.

III. **Regulation of protein tyrosine phosphatases by reversible oxidation**

Arne Östman, **Jeroen Frijhoff**, Åsa Sandin, Frank D. Böhmer.
J Biochem, 2011: 150(4):345-56.

IV. **Regulation of Protein Tyrosine Phosphatase Oxidation in Cell Adhesion and Migration**

Jeroen Frijhoff, Markus Dagnell, Rinesh Godfrey, Arne Östman
Antioxid Redox Signal, 2013, Epub ahead of print

CONTENTS

LIST OF ABBREVIATIONS

ALS	Amyotrophic lateral sclerosis
AML	Acute myeloid leukemia
CAM	Chorioallantoic membrane
Cdc	Cell division cycle
CH	Collagen homology
CLL	Chronic lymphocytic leukemia
CM	Conditioned medium
COX	Cyclooxygenase
CR	Caloric restriction
DSP	Dual-specificity phosphatase
DUOX	Dual oxidase
EGF	Epidermal growth factor
EGFR	EGF receptor
ETC	Electron transport chain
FAK	Focal adhesion kinase
FN	Fibronectin
FOXO	Forkhead Box O
Gpx	Glutathione peroxidase
GR	Glutathione reductase
Grx	Glutaredoxin
GSH	Glutathione
GSSG	Oxidized glutathione
HUVEC	Human umbilical vein endothelial cell
LMW-PTP	Low-molecular weight PTP
LOX	Lipoxygenase
MEF	Mouse embryonic fibroblast
MKP	Map kinase phosphatase
NADPH	Nicotinamide adenine dinucleotide phosphate
NOX	NADPH oxidase
PDGF	Platelet-derived growth factor
PDGFR	PDGF receptor
PI3K	Phosphoinositide-3 kinase
Prx	Peroxiredoxin
Pin1	Prolyl isomerase 1
PKCβ	Protein Kinase Cβ
PTB	Phosphotyrosine binding
PTK	Protein tyrosine kinase
PTP	Protein tyrosine phosphatase
ROS	Reactive oxygen species
RPTP	Receptor-like PTP
RTK	Receptor tyrosine kinase
S1P	Sphingosine-1-phosphate
SH2	Src homology 2
SOD	Superoxide dismutase

T-ALL	T-cell acute lymphoblastic leukemia
Trx	Thioredoxin
TRP14	Thioredoxin-related protein of 14 kDa
TrxR	Trx Reductase
VEGF	Vascular endothelial growth factor
VEGFR	VEGF receptor
VSMC	Vascular smooth muscle cell
XO	Xanthine oxidase

1 INTRODUCTION

1.1 TYROSINE PHOSPHORYLATION

Receptor tyrosine kinases (RTKs) are an important class of enzymes that regulate vital developmental and physiological processes. Extracellular ligand binding to RTKs induces intracellular auto-phosphorylation on tyrosine residues, which then serve as binding sites for signaling mediators to regulate cellular processes such as gene transcription, cell cycle control, migration and differentiation. The phosphorylating reaction by tyrosine kinases is counterbalanced by the dephosphorylating activity of protein tyrosine phosphatases (PTPs). The activity of PTPs is regulated in many ways, one of which is inhibitory reversible oxidation of their active-site cysteine by reactive oxygen species (ROS). Cellular redox signaling that affects PTP activity therefore has the potential to also impact on RTK signaling (Fig. 1). Better knowledge of this integral part of PTP modulation will therefore also increase our understanding of RTK-mediated signal transduction, and potentially also of RTK-driven diseases.

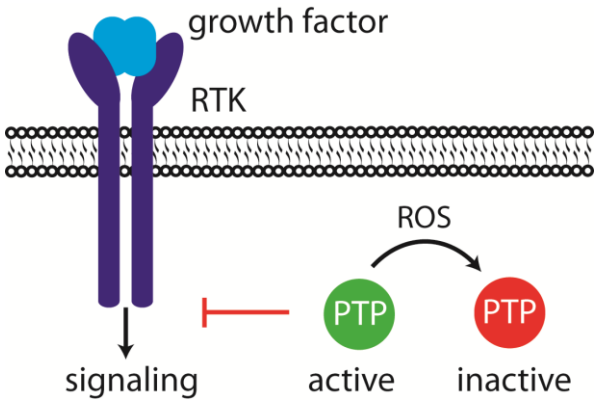

Fig. 1: Modulation of PTP oxidation regulates growth factor signaling.

1.2 TYROSINE PHOSPHORYLATION AS A TARGET IN DISEASE

The dysregulation of RTK signaling is associated with many diseases, including cancer and restenosis.

Cancer is a complex disease, with interactions between cancer cells and stromal host cells, and tyrosine kinase signaling is relevant to both compartments. In cancer cells, tyrosine kinases are often subject to genetic alterations such as translocation, point mutation and amplification, to achieve high or constitutive kinase activity. An example is the chromosomal translocation in leukemia between chromosome 8 and 22, creating the Philadelphia chromosome with the Bcr-Abl fusion gene [1]. Another leukemia-associated translocation is between chromosome 5 and 12, creating the Tel-PDGFRβ fusion protein [2]. Examples of point mutations in tyrosine kinases are those in C-kit and PDGFRα in gastrointestinal stromal tumors [3], as well EGFR mutations in lung cancer [4]. Some notable examples of tyrosine kinase

amplification are Her2/Neu in breast cancer [5], c-Met in gastric cancers [6] and EGFR and PDGFRα in glioblastoma [7]. The stromal compartment of cancer consists of many different cell types, such as endothelial cells, fibroblasts, pericytes, immune cells and also extracellular matrix. Much of the communication between cancer cells and these stromal cells occurs via paracrine signaling, in which one cell produces the ligand for the tyrosine kinase receptor of the other cell. Cancer cells can secrete for example vascular endothelial growth factor (VEGF), which binds to the VEGF receptor (VEGFR) on endothelial cells and promote the vascularization of the tumor to supply it with oxygen and energy [8]. Similarly cancer cells can secrete PDGF that binds the PDGF receptor and attract fibroblasts that support tumor growth [9].

Much effort has been put in finding compounds and antibodies that will inhibit (receptor) tyrosine kinases, considering their apparent supportive role in cancer. Some have been very successful, such as the treatment of Her2-positive breast cancer with the inhibitory antibody Trastuzumab. Studies have shown that Her2 positive breast cancer patients have a decreased risk of relapse when they receive adjuvant Trastuzumab treatment [10, 11]. Another example is imatinib/gleevec that is used in the treatment of certain leukemias, as it inhibits the tyrosine kinase activity of the aforementioned Bcr-Abl [12].

Atherosclerosis is a disease in which plaque builds up in arteries, which over time hardens and narrows the arterial lumen. This condition can be treated by angioplasty, alongside changes in lifestyle and diet. However, a significant number of patients suffer from restenosis, the renarrowing of the artery through migration and proliferation of vascular smooth muscle cells (VSMCs) [13-15]. A lot of focus has been put into studying PDGFR signaling, which is a potent driver for the VSMCs to migrate and proliferate. Many preclinical studies have shown efficacy of inhibitors of PDGFR kinase activity in inhibiting restenosis, while the first clinical studies have shown inconclusive results [16-20].

2 PROTEIN TYROSINE PHOSPHATASES AND GROWTH FACTOR SIGNALING

2.1 THE PROTEIN TYROSINE PHOSPHATASE FAMILY

The protein tyrosine phosphatase (PTP) family consists of 107 members that are characterized by their active site signature motif HCX_5R [21]. It can be divided into four different classes based on the amino acid sequence of their catalytic domains, which translates into structural differences and substrate specificities. The class I PTPs contains the "classical" tyrosine-specific phosphatases, which are the focus of this thesis. The dual-specificity phosphatases (DSPs) are also part of this class, and have a wide range of substrates, namely phosphotyrosine, phosphoserine, phosphothreonine and also phosphoinositides that are subject to dephosphorylation by the myotubularins and PTEN phosphatases. The class II contains only the low-molecular weight PTP (LMW-PTP) that targets phosphotyrosine, and the class III consists of the three cell division cycle (Cdc) phosphatases. In contrast to class I-III that are cysteine-based phosphatases, class IV is made up by the aspartic acid-based Eya phosphatases [21].

The 38 classical PTPs can be subdivided into receptor-like PTPs (RPTPs) and non-receptor-like PTPs. Each contains at least one catalytically active PTP domain, while most RPTPs contain a second membrane-distal PTP domain that is catalytically inactive. Most PTPs contain additional structural features such as SH2, FERM and immunoglobulin-like domains (Fig. 2)[22]. These additional domains serve to either facilitate binding of the PTP to its substrate or localize it to a particular part of the cell [23].

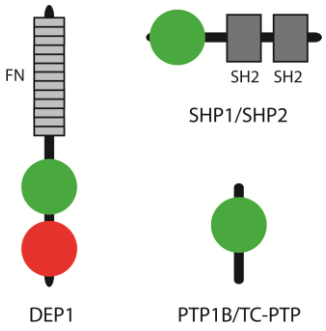

Fig. 2: Examples of PTPs with different domain structures. FN: Fibronectin domains. SH2: Src homology 2 domain

2.2 THE PTP CATALYTIC MECHANISM

The specificity of PTPs towards phosphotyrosine is determined by the deep active site pocket, for which phosphoserine and phosphothreonine are too small to reach down into [24]. The structure of the catalytic site of PTPs is organized in a way that it lowers the pKa of the catalytic cysteine, allowing it to exist as a thiolate anion [25, 26]. The interaction of the phosphotyrosine substrate through hydrogen and ionic bonds with the active site induces a conformational change from an open conformation to one where the WPD loop closes over the active site. The dephosphorylation reaction starts by the nucleophilic attack of the thiolate anion on the tyrosine-phosphate and yields a phosphoryl-cysteine intermediate [27] (Fig. 3). The aspartic acid residue serves as an acid catalyst and protonates the hydroxyl group of the leaving substrate [28]. In the second step of catalysis, the same aspartic acid now functions as a base to catalyze the attack of a glutamine-coordinated water

molecule on the phosphoryl-cysteine intermediate. This leads to release of phosphate and regeneration of the active site [24, 29].

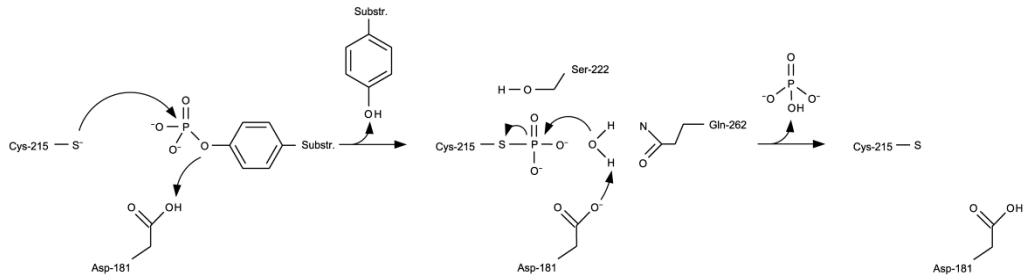

Figure 3: The catalytic reaction mechanism of PTPs.

2.3 PTP SUBSTRATE SPECIFICITY

The specificity of PTPs towards substrates is regulated at multiple levels. Firstly, structural domains and target sequences outside the PTP domain regulate both the substrate-PTP interactions and intracellular localization. The PTPs SHP1 and SHP2 initially bind a phosphotyrosine of their substrates with one or both SH2 domains [30, 31]. The N-terminal SH2 domain is normally folded over the active site, but the interaction with the phosphotyrosine leads to a structural change that exposes the active site to allow for phosphotyrosine dephosphorylation [30, 32]. This mechanism serves to activate the PTP only when it is bound to its substrate. Examples of substrate restriction by subcellular localization are demonstrated by PTP1B, which has a C-terminal sequence that targets this phosphatase to the ER [33], and SHP1 that contains a nuclear localization signal [34].

Sequences within the PTP domain have also been found to determine substrate selectivity. In general, comparative studies determined that PTPs in general prefer acidic residues in the phosphotyrosine substrate, although with little preference of their position relative to the phosphotyrosine [35, 36]. However, some PTPs (PTP-PEST, SHP1 and SHP2) showed a high selectivity for acidic compared to basic phosphotyrosine peptides, whereas RPTPα and RPTPδ showed little selectivity [36]. PTP1B showed high reactivity towards multiply phosphorylated peptides [35]. Very different catalytic efficiencies were found when comparing the activity of individual PTPs towards their optimal substrate, which could differ over 10^5 fold [36]. Overall the identified profile was in very good agreement with known *in vivo* substrates of the PTPs, and the preference for different acidic substrates is likely due to different electrostatic interactions between positively charged residues close to the PTP catalytic site and acidic residues in the substrate.

Specificity of PTPs to *in vivo* substrates has been demonstrated for several RTKs. These receptors contain multiple tyrosine-phosphorylated sites. Knockout of the phosphatase TC-PTP showed a preferential increase of phosphorylation at tyrosine-1021 of the PDGFβR, whereas PTP1B knockout preferentially increased phosphorylation at tyrosine-579 [37]. Similarly, DEP1 dephosphorylates tyrosine-1175 in VEGFR2, but not tyrosine-1214, whereas it is the opposite for TC-PTP [38, 39].

2.4 REGULATION OF PTPS

PTP activity is regulated by multiple mechanisms, which can either be stimulatory or inhibitory. PTP expression is regulated by e.g. cell density for DEP1 [40], promotor methylation for SHP1 [41] and capillary formation for VE-PTP [39]. Some of the receptor-like PTPs have been reported to be regulated by ligand binding. For example, binding of the ligand Galectin-1 to CD45 inhibits its activity [42, 43], whereas binding of DEP1 to extracellular matrix increases its activity [44]. Dimerization is another inhibitory mechanism that has been described for RPTPα [45, 46] and CD45 [47].

PTPs are also regulated by multiple posttranslational modifications. Serine and tyrosine phosphorylation has been described for multiple PTPs with different effects on their catalytic activity. Tyrosine phosphorylation of SHP1 and SHP2 has been suggested to increase their activity [48-50]. Serine phosphorylation of SHP2 does not influence its activity [51], whereas SHP1 serine phosphorylation increases its activity [52]. Sumoylation has been reported for PTP1B and inhibits its activity. This type of regulation was shown to regulate both insulin signaling and mitosis [53, 54]. Other PTP posttranslational modifications include proteolysis, ubiquitination, glycosylation and finally oxidation [55], which will be discussed in detail in chapter 5.

2.5 PTPS AS GROWTH FACTOR SIGNALING MODIFIERS

The classical view on PTPs has been as endogenous negative regulators of protein tyrosine kinase signaling. This has been demonstrated for a wide number of cytosolic and receptor tyrosine kinases. The PDGFR has been shown to be negatively regulated my many PTPs, such as PTP1B [37, 56], SHP2 [57], TC-PTP [37], DEP1 [58] and LMW-PTP [59]. Similarly the VEGFR2 is targeted by DEP1 [60], TC-PTP [38], PTP1B [61] and also VE-PTP [39]. Other PTP-regulated RTKs include c-MET [62, 63], EGFR [64, 65] and Flt3 [66, 67]. In addition cytoplasmic tyrosine kinases, adaptor proteins and transcription factors are also subjected to PTP-mediated dephosphorylation, such as focal adhesion kinase (FAK) [68], p130Cas [69] and STAT proteins [70]. These are just a few examples of many reported in the literature.

The interplay between PTPs and RTKs is however much more complex and some PTPs have also been described to promote tyrosine kinase signaling. SHP2 for example has been demonstrated to have a positive role on tyrosine kinase signaling, by coupling the PDGFR to Grb2 binding and Ras activation [71]. RPTPα and RPTPε were demonstrated to dephosphorylate an inhibitory phosphotyrosine on Src, thereby promoting RTK signaling through Src activation [72-75]. PTP1B as well was demonstrated to exert a positive role on Her2/Neu signaling in breast cancer by removing the inhibitory activity of p62Dok towards Ras [76, 77]. However, in insulin signaling PTP1B clearly has a negative role by dephosphorylating both the insulin receptor and molecules downstream of the insulin receptor [78, 79].
It is clear already from these few examples that PTPs do not exist to simply turn off tyrosine kinase signaling. Their activity is highly coordinated to modulate tyrosine kinase signaling, both positively and negatively, in a highly context-dependent manner.

3 P66SHC

3.1 MOLECULAR SIGNALING OF P66SHC

The Shc locus was first identified to support EGFR signaling and transformation [80]. The two smaller gene products of 46 and 52 kDa were identified, p46Shc and p52Shc, and shown to be responsible for the oncogenic properties of this locus [80]. The proteins contain a collagen homology (CH) domain a Src Homology 2 (SH2) domain and phosphotyrosine-binding (PTB) domain. Five years later a larger 66 kDa Shc gene product was reported, which was shown to originate from a different promotor and include an additional N-terminal CH2 domain, and termed p66Shc [81]. Additional experiments showed that p66Shc had a very different function from p46/52Shc, namely to negatively regulate EGFR signaling, presumably due to competitive binding to EGFR and/or Grb2 [81]. The first indications of an altogether different function of the p66Shc protein that involved the CH2 domain were revealed by a whole-body knockout mouse specific for the p66Shc isoform [82]. These mice were reported to be long-lived, and to better cope with treatments that induce oxidative stress, as were the embryonic fibroblasts isolated from these knockout embryos. Stimuli such as UV irradiation and staurosporin treatment induce the phosphorylation of p66Shc at serine-36 within the CH2 domain. Additional studies went on to show that these oxidative stresses activate protein kinase Cβ (PKCβ) to phosphorylate p66Shc at serine-36, followed by its interaction with prolyl isomerase 1 (Pin1), dephosphorylation at serine-36 and TIM/TOM-mediated import into mitochondria [83-85]. It is here, in the mitochondrial intermembrane space, that p66Shc exerts its unique function, which is to act as a copper-dependent oxidoreductase. It reduces oxygen to hydrogen peroxide, using cytochrome c as electron donor [83].

Several studies indicate that p66Shc can promote intracellular ROS through two additional pathways (Fig. 4). The first is the notion that p66Shc can promote the activity of Rac1, a component of NADPH oxidase (NOX) complexes. VEGF was shown to promote association of non-phosphorylated p66Shc with Rac1, which correlated with Rac1 activity [86]. p66Shc has also been reported to form a complex with Grb2 and α-1-synotrophin. This leads to displacement of Sos1 from Grb2 to allow complex formation with Ets8 and Rac1 to promote the GDP-to-GTP exchange that is necessary for Rac1 activation [87]. Macrophages from long-lived p66Shc mice also display decreased superoxide production, due to decreased phosphorylation and activation of indivual NOX-complex components [88]. In neuronal cells however, p66Shc was shown to induce the inactivation of Rac1 [89]. It appears therefore that p66Shc might have different effects on Rac1 activity depending on the cell type and signaling event. Another pathway through which p66Shc can elevate intracellular ROS levels is through modulation of Forkhead Box O (FOXO) activity. FOXO proteins are transcription factors that are activated upon oxidative stress and respond by transcribing antioxidant genes such as catalase and superoxide dismutase [90]. p66Shc can promote Akt activation, leading to inhibitory phosphorylation of FOXO by Akt [91, 92], thereby decreasing transcription of antioxidant genes.

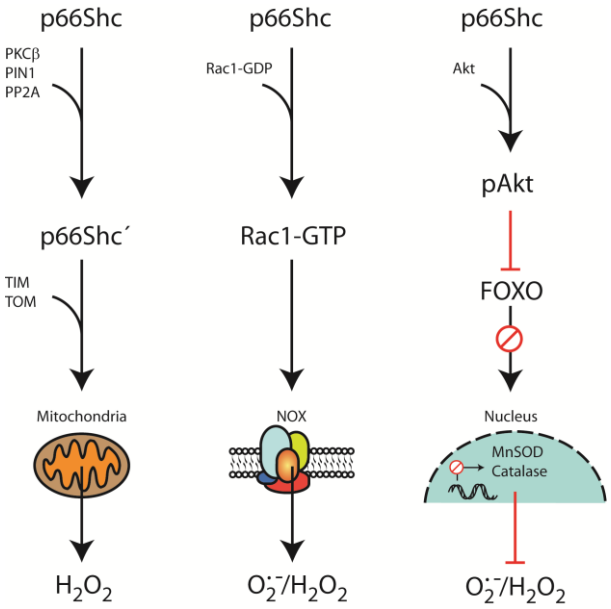

Figure 4: The three described pathways for p66Shc-mediated ROS production

A few studies have contested the ROS-producing activity of p66Shc, and suggest it rather acts as a redox sensor. One study showed that the p66Shc CH2 domain exists as a dimer that can tetramerize through formation of disulfide bonds between cysteine-59 residues, which could be reduced by either thioredoxin or glutathione. The CH2 tetrameric form had the capacity to induce rupture of isolated mitochondria, while surprisingly producing less ROS *in vitro* than the dimeric CH2 form [93]. A second study found peroxiredoxin I (PrxI) as an interaction partner of the p66Shc CH2 domain [94]. As will be discussed in chapter 4, peroxiredoxins are enzymes whose activity is regulated through oligomerization. In a dimeric state they degrade hydrogen peroxide, while in their decameric state they function as chaperones. The p66Shc CH2 domain was found to induce disassembly of the PrxI decameric form to dimers. The authors speculated that the interaction of p66Shc with PrxI serves to counteract p66Shc-induced ROS formation in the cytosol, which is disrupted by phosphorylation of p66Shc upon oxidative stress. It remains to be seen whether these *in vitro* findings also applies to cells with endogenous full-length p66Shc. Indeed, the shorter p66Shc CH2 domain was unable to produce ROS using cytochrome c as an electron donor [93], which is in contrast to results that have been published for full length p66Shc [83] and may indicate differential behavior of the isolated p66Shc CH2 domain.

3.2 P66SHC IN CELLULAR SIGNALING

p66Shc has been described to act as a downstream effector of p53-dependent stress-induced apoptosis, through its production of mitochondrial hydrogen peroxide that leads to mitochondrial permeability transition pore opening and leaking of cytochrome c to the cytosol [85, 95]. In addition p66Shc has been implicated in promoting anoikis, which is cell death induced by loss of cellular adhesion to extracellular matrix [96]. Many of the *in vivo* effects of p66Shc are attributed to its role in promoting apoptosis, which are further discussed in chapter 3.3.

In addition, p66Shc has been described to act on other signaling pathways than apoptosis. In adipocytes p66Shc positively regulates insulin signaling [91]. Stimulation of adipocytes with insulin activates p66Shc to generate mitochondrial ROS, which leads to Akt activation, nuclear exit and inactivation of FOXO1 and regulation of selected insulin target genes, all of which are decreased in p66Shc-depleted adipocytes. These effects were attributed to an observed decrease of PTEN

oxidation in p66Shc-depleted adipocytes after insulin stimulation. These knockout adipocytes also displayed increased fatty acid oxidation, increased mitochondrial uncoupling and decreased triglyceride accumulation. p66Shc has also been described to promote VEGF signaling and angiogenesis in a cell-autonomous and paracrine manner. Conditioned medium (CM) from T-cells that are exposed to hypoxia stimulate angiogenesis in a chick chorioallantoic membrane assay (CAM) through upregulation of HIF1α and VEGF secretion. CM from T-cells overexpressing p66Shc showed increased VEGF secretion and an increased capacity of promoting CAM angiogenesis, which was not seen with an inactive mutant of p66Shc or when using spleen cells from p66Shc knockout mice [97]. In a separate study VEGF was shown to stimulate p66Shc activation in endothelial cells, and downregulation of p66Shc in human umbilical vein endothelial cells (HUVECs) decreased VEGF-induced phosphorylation of VEGFR2 and p38, but not Erk1/2. In addition downregulation of p66Shc decreased endothelial cell proliferation, migration and capillary network formation on matrigel [86]. A recent study identified p66Shc as an inhibitor of glucose metabolism [98]. Cells with p66Shc depletion showed an increase in anabolic metabolism, which depended on mammalian target of rapamycin (mTOR). These effects were however specific for insulin, as EGF stimulation failed to show these p66Shc-dependent effects on mTOR [98]. Additional pathways/receptors that have been described to be regulated by p66Shc are Angiotensin-II signaling [99], the chemokine receptor CXCR4 [100], androgen receptor signaling [101, 102] and endothelin-1 signaling [103-105].

3.3 P66SHC IN DISEASE

The first study using mice with a genetic deletion of the p66Shc isoform reported a substantial increase in the longevity of these mice [82]. However, when p66Shc knockout mice are maintained in a natural environment, the p66Shc deletion has a negative selective advantage [106]. The same group together with collaborators recently reported the findings from a much larger cohort of laboratory mice with the p66shc deletion in the 129Sv, C57Bl/6J and mixed 129Sv-C57Bl/6 background, that were fed either a 5% or 40% caloric restriction (CR) diet [107]. The results did not show any difference between the genotypes in life span, prevalence of neoplasms or other measures of end-of-life pathology, although the 70th percentile survival was increased in C57Bl/6J p66Shc KO mice that were fed a 40% CR diet. The authors concluded from these data that p66Shc is not a longevity protein, while suggesting the need for additional studies to assess how p66Shc may influence the onset and severity of age-related diseases. Indeed, a substantial amount of literature has reported on the beneficial effect of the deletion of p66Shc in mouse models of human disease. Deletion of p66Shc protected mice from early atherogenic lesions when fed a high-fat diet, being characterized by decreased lesion size, decreased vascular apoptosis and decreased vascular oxidized LDL [108]. Knockout mice are also protected from diabetes-induced loss of cardiac-progenitor cells and heart failure [109], ischemia-reperfusion damage of the hindlimb [110] and heart [111], high-fat diet-induced obesity [91] and hyperglycemia-induced endothelial dysfunction and oxidative stress [112]. Increased p66Shc expression was also detected in peripheral blood monocytes of patients with acute coronary syndrome compared to stable coronary artery disease [113]. Deletion of p66Shc also shows

protective effects in a mouse model of amyotrophic lateral sclerosis (ALS), in which mice carry a mutant form of superoxide dismutase (SOD1). Transgenic mutant SOD1 mice with the p66Shc deletion showed delayed onset of disease, improved motor performance and increased survival compared to wildtype transgenic mice [89]. p66Shc deletion has however also been reported to have detrimental consequences. p66Shc acts as a negative regulator of lymphocyte development, and aged p66Shc knockout mice develop a lupus-like autoimmune disease that is characterized by autoimmune glomerulonephritis and alopecia [114].

The role of p66Shc in cancer is still incompletely understood. Reports show both loss of p66Shc expression in Her2/Neu overexpressing breast cancer cells [115, 116], overexpression of p66Shc in highly metastatic breast cancer cell lines and patient samples [117] and in relapsing tamoxifen-treated breast cancer patients [118]. Studies on prostate cancer have reported no correlation between p66Shc expression and prostate cancer [119] and a positive correlation between p66Shc protein level and prostate cancer cell proliferation [120, 121] and experimental tumor onset and growth [101]. Recent studies identified that deletion of p66Shc does not decrease the incidence of spontaneous tumors [122], whereas UVB or TPA-induced papillomas were slightly fewer in numbers in p66Shc knockout mice. Furthermore, p66Shc deletion in a $p53^{-/-}$ background delayed tumor initation and increased survival [122]. On the other hand, p66Shc expression has been reported to be impaired in chronic lymphocytic leukemia (CLL) B cells, which favored the expression of anti-apoptotic Bcl-2 family members [123] and downregulation of sphingosine-1-phosphate 1 (S1P1) to prevent exit of B cells from the bone marrow [124].

4 CELLULAR REDOX SYSTEMS

Organisms have adapted to life with oxygen by evolving systems that cope with oxygen-derived reactive species, which can damage proteins, lipids and DNA. It is now clear though that nature has not simply evolved these redox systems to combat oxidative stress, but that cells make use of these systems to fine-tune intracellular and organism signaling.

4.1 ANTIOXIDANT SYSTEMS

4.1.1 Superoxide Dismutase

Mammals express three different forms of superoxide dismutase (SOD). SOD1 is a copper and zinc-dependent dismutase (also called Cu-Zn SOD) that is present in the cytosol. SOD2 is expressed in mitochondria and is manganese-dependent. SOD3 also has copper and zinc in its active site and is secreted and therefore also called extracellular SOD. All SODs catalyze the dismutation of superoxide to oxygen and hydrogen peroxide, using the metal ions in their catalytic site as catalysts [125]. The categorization of SOD as an antioxidant enzyme is ambiguous, as the product of its reaction is also an oxidant, hydrogen peroxide. Nevertheless, superoxide is one of the main oxidants produced in cells, and SODs are essential to counterbalance superoxide production. The important physiological role of SODs is shown by the severe pathologies that the various SOD knockout mice develop. These range from hepatocellular carcinoma and reduced lifespan of $SOD1^{-/-}$ mice [126], and death shortly after birth of $SOD2^{-/-}$ mice [127]. In addition mutation of SOD1 has been linked to the cause of amyotrophic lateral sclerosis [128, 129], while overexpression of SOD1 has been linked to neurological phenotypes of Down syndrome [130].

4.1.2 Catalase

Catalase is an enzyme found in virtually all organisms that are exposed to oxygen. It is a heme-dependent enzyme that is located in peroxisomes [131]. It catalyzes the breakdown of hydrogen peroxide into water and oxygen using a prosthetic heme group [132]. Mice that lack catalase do not show any overt phenotypes, although it might have a more tissue-specific role in antioxidant defense [133]. Overexpression of catalase in mitochondria, but not in peroxisomes or nuclei, increases lifespan in mice by several months [134].

4.1.3 Glutathione Peroxidases

Glutathione peroxidases (Gpx) are part of a family of 8 enzymes, for which Gpx6 is specific for humans [135]. The different Gpx members differ structurally, such as oligomerization states, but most notably Gpx1-4 and human Gpx6 contain a selenocysteine in their active site, while Gpx5, 7 and 8 have a cysteine residue. In addition a single Gpx enzyme can be expressed as isoforms with different intracellular localization [135]. Gpx enzymes have a wide variety of substrates, ranging from hydrogen peroxide to lipid peroxides. Notably, the lipid peroxide scavenger Gpx4 was shown to regulate PTP oxidation and PDGFR signaling [136].

Several knockout mice are available, but only full knockout of Gpx4 is embryonically lethal [137-139]. Other Gpx knockouts show various phenotypes, such as infertility [140], increased sensitivity to airway inflammation [141] and increased insulin sensitivity [142].

4.1.4 Peroxiredoxins

Peroxiredoxins (Prxs) come in 6 variants that either have specific intracellular localization and/or tissue-specific expression [143]. Peroxiredoxin I and II are cytosolic enzymes, Peroxiredoxin III is expressed in mitochondria, Peroxiredoxin IV in the endoplasmic reticulum and Peroxiredoxin V and VI have a more distributed expression pattern [144]. Peroxiredoxins have a dual function depending on their oligomerization state. As decamers they promote protein folding by acting as chaperones [145]. When these decamers are disassembled Prxs catalyze the conversion of hydrogen peroxide into water and oxygen, which for mammalian peroxiredoxins proceeds via a two-step mechanism [146]. The catalytic cysteine reacts with hydrogen peroxide to form a sulfenic acid. This sulfenic acid then forms a disulfide with a resolving cysteine, either from the same monomer or from a dimeric partner, depending on the type of peroxiredoxin. The monomer or dimer disulfides are then reduced to their active state, typically via the thioredoxin system [147]. They are considered one if not the most important hydrogen peroxide consuming enzymes, due to their high reactivity with hydrogen peroxide combined with their high expression levels. Knockout mice for PrxI develop normally, but have a reduced lifespan owing to the development of severe hemolytic anemia and malignant cancers [148]. PrxII protects erythrocytes from oxidative stress, and erothrocytes from PrxII knockout mice have a shortened lifespan and these mice develop hemolytic anemia [149]. PrxIII and PrxVI knockout mice show no obvious developmental defects [150, 151], although they are more susceptible to oxidative stress [150, 152-154]. In addition, peroxiredoxins have been shown to regulate mammalian circadian rhythms [155].

4.1.5 Thioredoxin system

The thioredoxin system consists of thioredoxin (Trx), thioredoxin reductase (TrxR) and NADPH. Thioredoxin comes in a cytosolic (Trx1) and mitochondrial form (Trx2), and in addition thioredoxin-like proteins (TRP) have been described that act as disulfide reductases [156, 157]. Two different genes encode for the complementary cytosolic (TrxR1) and mitochondrial (TrxR2) Trx reductase proteins. The predominant function of the Trx system is to reduce disulfides, although other types of oxidized substrates have been described. The catalytic cysteine of Trx (or TRP) performs a nucleophilic attack on the substrate disulfide, thereby forming a Trx-substrate disulfide,

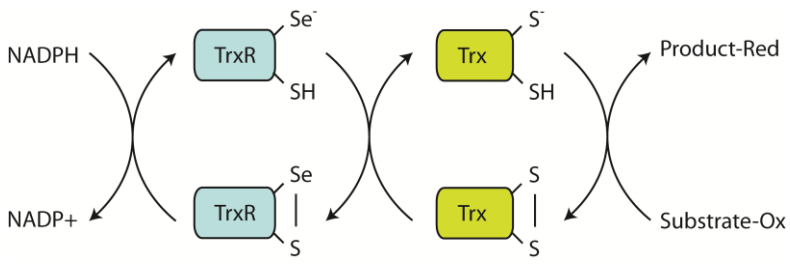

Figure 6: The thioredoxin system reaction mechanism

which is subsequently hydrolyzed and transferred to Trx to form an intramolecular disulfide bond. This intramolecular disulfide bond is then reduced by the Trx reductase, leading to TrxR oxidation. TrxR is in turn regenerated by NADPH (Fig. 6). The Trx system is indispensable for organisms, as genetic depletion of Trx1, Trx2, TrxR1 or TrxR2 leads to embryonic lethality in mice [158-162]. The dysregulation of the Trx system has been implicated in many diseases, including cancer, cardiovascular diseases and neurodegenerative diseases [157].

4.1.6 Glutathione system

The glutathione system consists of glutathione (GSH), glutathione reductase (GR), glutaredoxin (Grx) and NADPH. Glutaredoxin is encoded by two different genes; Grx1 is mainly cytosolic and Grx2 is located in mitochondria and the nucleus. The glutathione system has several functions. GSH acts together with Gpx to remove hydrogen peroxide. One of the products of this reaction is oxidized glutathione (GSSG), which is reduced back to GSH by glutathione reductase at the expense of NADPH [163, 164]. Glutaredoxins can reduce protein disulfides via a dithiol mechanism, similarly to thioredoxins, or mixed disulfides between proteins and glutathione via a monothiol mechanisms. In each reaction Grx will form a glutathione-mixed disulfide, which is reduced by GSH. The resulting GSSG is again regenerated by GR to GSH. Two of the major functions of the glutathione system is to reduce ribonucleotide reductase, which provides the cell with ribonucleotides, as well as to reduce enzymes that are involved in cellular metabolism, such as phosphofructokinase [164, 165]. A rare genetic disorder exists that causes decreased activity of GR, leading to decreased lifespan of erythrocytes, shortened respiratory burst in leukocytes, cataract and early onset of deafness [166].

4.2 PRO-OXIDANT SYSTEMS

4.2.1 Xanthine Oxidase

Xanthine oxidase (XO) is an enzyme that can oxidize hypoxanthine to xanthine, and further oxidize xanthine to uric acid. In this process it reduces oxgen to superoxide or hydrogen peroxide. The protein binds molybdenum, flavin and iron that are required for enzymatic activity [167]. Xanthine oxidase is normally found in serum and lungs. Xanthinuria is a rare genetic disorder, in which lack of XO leads to increased levels of xanthine in the blood, which can lead to renal failure [168, 169].

4.2.2 Cyclooxygenases

There are currently three Cyclooxygenase (COX) isoforms known, COX1-3. COX3 is a splice variant of COX1 and has a limited expression profile [170]. COX1 is ubiquitously expressed, whereas COX2 expression is mainly induced upon inflammatory stimuli [171]. COX enzymes are involved in prostaglandin synthesis by converting arachidonic acid into prostaglandin H_2, through addition of oxygen and reduction of the resulting peroxide prostaglandin G_2 [172]. COX enzymes are inhibited by aspirin, which acetylates a serine residue at their active site. COX inhibitors, both general and isoform-specific, have been used to decrease inflammation, although treatments are associated with cardiovascular side-effects.

4.2.3 Lipoxygenases

Lipoxygenase (LOX) enzymes oxidize arachidonic acid to produce peroxidized lipids [173]. Examples are 5-LOX, 12-LOX and 15-LOX, where the number stands for which carbon it modifies in arachidonic acid. These peroxidized lipids are precursors for leukotrienes, which can act as chemoattractants for neutrophils in inflamed tissues [174]. They have therefore been implicated in inflammation-associated diseases, such as cancer and rheumatoid arthritis.

4.2.4 NADPH Oxidases

NADPH oxidases (NOX) are well described enzymes that consist of NOX1-5 and dual oxidase (DUOX) DUOX1-2. They vary in structure and activation mechanism. Whereas NOX1-2 are activated by assembly of cofactors, NOX4 activity is mainly regulated by expression level, and NOX5 is activated after calcium binding. NOX enzymes produce either superoxide or hydrogen peroxide [175, 176]. They were first described for their role in combating pathogens, by producing an oxidative burst in macrophages. However, the role of NOX enzymes extends to regulation of growth factor signaling, and changes in NOX expression or activity have been implicated in a number of diseases such as cancer, diabetes and atherosclerosis [177, 178].

4.2.5 Mitochondria

Mitochondria produce energy in the form of ATP, through a series of electron transfer reactions from Complex I-IV. Superoxide is generated when electrons from the electron transport chain (ETC) leak onto oxygen. However, the high concentration and reactivity of SOD in mitochondria leads to the rapid turnover of superoxide to hydrogen peroxide, making this the predominant species in mitochondria. The two major sites of superoxide production in mitochondria are complex I and complex III [179]. Dysregulation of mitochondrial ROS production has been observed in many diseases, such as cancer [180] and diabetes [181].

5 PTP OXIDATION

5.1 REDOX SIGNAL TRANSDUCTION

The mammalian genome encodes over 200000 cysteine residues, of which an estimated 10-20% is involved in biological redox reactions [182]. Peroxiredoxins are highly expressed proteins and highly reactive with hydrogen peroxide, often several orders of magnitude higher than most other proteins. A still unanswered question within the redox field is how ROS can reach all these different targets in an environment that is dominated by high expression and reactivity of peroxiredoxins. Different hypotheses have been put forward to explain these findings (Fig. 7).

The floodgate hypothesis argues that peroxiredoxins need to be hyperoxidized in order for ROS to reach other targets [183]. Although this scenario is conceivable for high levels of ROS, no hyperoxidized peroxiredoxins have been detected during growth factor stimulation of cells [184, 185].
A second hypothesis also describes peroxiredoxin inactivation, but through phosphorylation. This has been shown for peroxiredoxin I (PrxI), which upon growth factor stimulation is transiently tyrosine phosphorylated by Src and inactivated in confined membrane-associated areas [185]. This local inactivation would presumably lead to a local accumulation of ROS, subsequent inactivation of PTPs and efficient tyrosine kinase signal transduction. In the same context peroxiredoxin II (PrxII) was not inhibited by tyrosine phosphorylation, indicating specificity in phosphorylation-mediated inactivation of peroxiredoxins.
Lastly, a popular hypothesis is that peroxiredoxins react with ROS first, and then transfer their oxidation to target proteins via disulfide interactions that are mediated through additional protein-protein interactions. The notion of disulfide-mediated transoxidation has not only been seen for peroxiredoxins. The bacterial glutathione peroxidase 3 (Gpx3) was shown to transfer its hydrogen peroxide-induced oxidation to the transcription factor Yap1, which then transcribes antioxidant genes [186]. FOXO4 has also been shown to become odizided and form specific disulfide-interaction partners with each of its four cysteine residues [187, 188]. Recently similar reactions have been seen for PrxI [189]. PrxI was shown to become oxidized and form a disulfide intermediate with Ask1, thereby releasing an oxidized Ask1, which allowed for phosphorylation of p38. Knockdown of PrxI, but also overexpression of PrxII inhibited Ask1 oxidation, indicating that peroxiredoxins either show substrate specificity or differences in redox transfer capability.

When applied to RTK signaling and PTPs, peroxiredoxin downregulation would potentially lead to decreased PTP oxidation and decreased tyrosine kinase signaling. This has been observed for T cell receptor signaling, although PTP oxidation status was not investigated [190]. Similarly downregulation of PrxII decreases VEGFR2 signaling, although the molecular mechanism was described to be due to inhibitory disulfide formation of the VEGFR2 dimer [191]. Other studies have shown the opposite, where downregulation of PrxI [185], PrxII [184, 192] or PrxIII [193] leads to a decrease of tyrosine phosphorylation and/or signaling. Indeed, interaction of PrxI with PTEN [194] and MKP1 and MKP5 [195] has been reported, but in both cases served a role to protect the phosphatases from oxidation, rather than to transduce

the oxidative signal. It appears therefore that there might not be a unifying mechanism for peroxiredoxin-mediated redox signal transduction to PTPs.

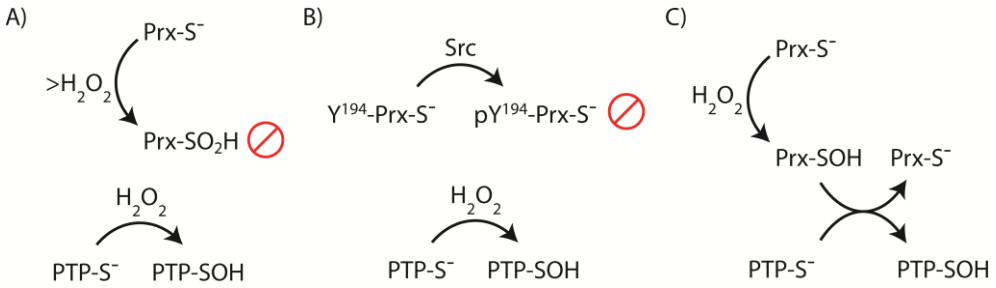

Figure 7: Three mechanism for PTP oxidation in a Prx-dominated environment. A) Floodgate hypothesis. B) Phosphorylation-mediated inactivation of Prx. C) Prx-mediated redox transfer.

5.2 PTP OXIDATION IN CELL SIGNALING

Already since the first isolation of PTPs it was observed that their activity depended on at least one highly reactive cysteine residue [196, 197]. Seven years later it was shown that PDGF-induced tyrosine phosphorylation and signaling requires the production of hydrogen peroxide [198], and PTP oxidation could therefore potentially serve as a physiological regulation (Fig. 8). Since then, many PTPs have been shown to become oxidized upon stimulation of cell-surface receptors. These range from integrins, to immune cell receptors, receptor tyrosine kinases and many more [57, 91, 142, 192, 199-220]. The dominating view is that ligand binding to RTKs stimulates activation of phosphoinositide-3-kinase (PI3K) and subsequently Rac translocation to and activation of NOX enzyme complexes [37, 57, 200, 221-223]. These NOX enzymes produce ROS that inactivate PTPs to then allow for signal transduction. Also arachidonic acid release, through the action of cytosolic phospholipase A, can impact on PTP oxidation, either through acting on NOX enzymes or after conversion to peroxidized lipids to directly act on PTPs [136, 224-228]. In addition mitochondria have also been shown to play a role in PTP oxidation [91, 202, 213].

ROS are unstable molecules that have a limited diffusion range, and PTP oxidation in cell signaling is therefore hypothesized to be highly localized. Some of the evidence stems from the observed localized activation of NOX enzymes [199, 229] and translocation of mitochondria to membrane receptors [230, 231]. In addition localized inactivation of ROS scavengers has been suggests as an additional type of regulation. This was shown for PrxI, which is inhibited by tyrosine phosphorylated in membrane fractions, which would allow a local increase of hydrogen peroxide due to decreased scavenging [185]. Direct evidence for localized PTP oxidation was first demonstrated in PDGF signaling, in which only the PDGFR-associated fraction of SHP2 was oxidized [57]. Subsequent studies have also shown inactivation of only the membrane-associated PTPs upon PDGF stimulation [184] and oxidation of membrane-associated DEP1 and PTP1B upon VEGF stimulation [208]. There have been no reports on direct *in situ* detection of PTP oxidation in cells. However, a recently described intrabody that specifically detects oxidized PTP1B might

represent a methodology that would allow the first *in situ* visualization of an oxidized PTP [232].

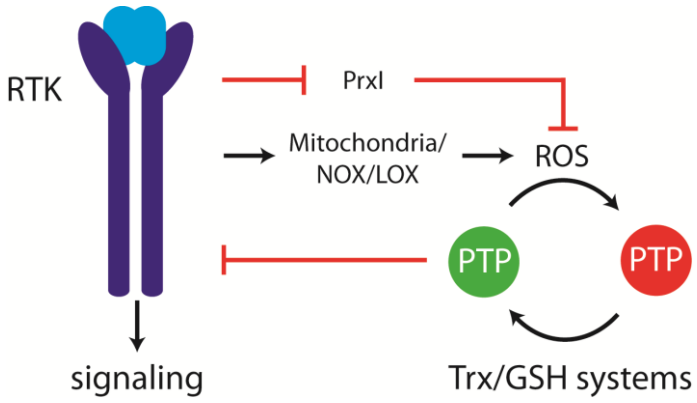

Figure 8: Redox systems that affect PTP oxidation can impact on growth factor signaling.

5.3 BIOCHEMISTRY AND SPECIFICITY OF PTP OXIDATION

PTPs can be oxidized by many different types of molecules, ranging from hydrogen peroxide to lipid peroxides and nitric oxide, and they show differential sensitivity towards these oxidants. PTP1B for example is more readily oxidized by superoxide than by hydrogen peroxide [233], while it is completely unaffected by t-butyl hydroperoxide or cumene hydroperoxide [234]. Various PTPs also show differential reactivity to the same oxidant. The closely related SHP1 and SHP2 show differential oxidation sensitivity towards hydrogen peroxide treatment, and the SH2 domain of SHP1 was found to confer a more protective effect to oxidation than in SHP2 [235]. In addition the two PTP domains of RPTPα were shown to have differential sensitivity towards oxidation, and interestingly the catalytically inactive D2 domain was shown to be more sensitive towards oxidation [236, 237]. Very few studies have been performed to find the molecular determinants that dictate oxidation sensitivity, but the relative position of the active-site arginine to the catalytic cysteine was suggested to play a role [238]. Individual PTPs also display different oxidation states. Hydrogen peroxide for example can oxidize PTPs to sulfenic acid (SOH), cyclic sulfenamide (SN) and inter- and intramolecular disulfide bridges (SS) (Fig. 9).

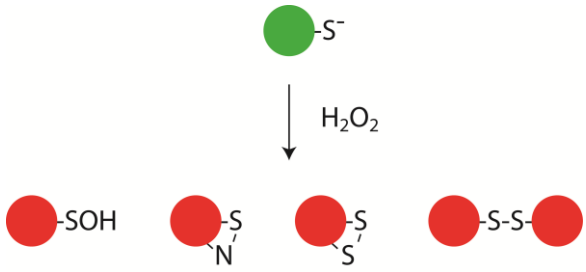

Figure 9: Reversible oxidation of PTPs by hydrogen peroxide can lead to different oxidation states, such as sulfenic acid, cyclic sulfenamide and intra- and intermolecular disulfides

The specificity of PTP reduction has been studied to a much lesser extent, although studies with recombinant PTPs and PTP domains also show specificity for this reaction. Oxidized PTP1B was shown to be efficiently reduced by the Trx system and DTT, whereas the glutathione system showed to be less capable of reducing PTP1B

[204]. SHP2 and SHP1 on the other hand were able to be reduced by glutathione, but not by the Trx system. Deletion of the SH2 domain sensitized SHP2 to reduction by the Trx system, whereas this was not the case for SHP1 [239]. Presumably the type of active-site cysteine modification will also impact on the activity of reducing systems towards PTPs. This was demonstrated for PTP1B when comparing the activity of the Trx system towards differently inactivated forms of PTP1B. It was determined that the Trx system was approximately 200-fold faster compared to DTT in reactivating sulfhydrated PTP1B, whereas oxidized or nitrosylated PTP1B showed a similar rate of reactivation by the Trx system. Glutathione on the other hand was much less effective in reactivating PTP1B, regardless of the cysteine modification [240].

5.4 PTP OXIDATION IN DISEASE

PTPs are involved in many diseases, both positively and negatively. Their negative role in tyrosine kinase signaling spurred interest in PTPs as potential tumor suppressors, which has been demonstrated for a number of PTPs [241, 242]. For example, large-scale studies have shown point mutations in both cytosolic and receptor-like PTPs [243, 244]. Loss of heterozygosity has been observed for DEP1 in multiple cancers [245]. Promotor methylation was shown for SHP1 in lymphoma and leukemia [246]. In addition, individual tumors may apply different events to inactivate one PTP. This was shown for RPTPδ in glioblastoma, which was inactivated by either promotor methylation, genetic loss or point mutation in over 50% of the tumors [247].

An increasing number of PTPs are also reported to have oncogenic properties in many different types of cancer [241, 242]. Activating mechanism include overexpression, point mutations and amplification. SHP2 was the first described oncogene, and mutational activation promotes leukemia by activating the Ras signaling pathway [248, 249]. As mentioned earlier, RPTPα is involved in activating Src, and overexpression of RPTPα is seen in colorectal cancer, head and neck cancer and gastric cancer, where it is also associated with metastasis [250-252]. In addition SHP1, which is inhibited in lymphomas and leukemias, is overexpressed in ovarian cancer, indicating that PTPs can act both as tumor suppressors and oncogenes depending on the context [253].

PTPs are also extensively studied in the field of obesity and diabetes. Multiple PTPs act on insulin and leptin signaling. Considerable interest came after studies that showed that PTP1B knockout mice remain insulin-sensitive when put on a high fat diet, and are furthermore resistant to obesity [254, 255]. A plethora of studies followed that explored different tissue-specific PTP knockout mice to understand their involvement in diabetes, obesity and insulin resistance [256, 257].

ROS have long been considered key factors in many different types of diseases, such as cardiovascular diseases, cancer, neurodegenerative diseases and more. The damaging effects of ROS have been well documented. At the same time ROS are increasingly recognized to impact on signaling pathways through redox regulation of proteins. This crosstalk with redox signaling adds another layer of complexity to

the control of cell signaling and behavior, and potentially also to cellular dysregulation in disease.

A significant number of studies suggest that cancer cells have a modulated intracellular redox balance. PTEN is commonly found mutated or lost in cancer. Additional studies indicate that inactivation of PTEN by oxidation is another way to promote activation of Akt and cancer cell survival. Mitochondrial respiration defects were attributed to the increased ROS and PTEN oxidation [258]. In primary T-cell acute lymphoblastic leukemia (T-ALL) PTEN was also found to be inactivated by oxidation, leading to hyperactivation of Akt. Restoring PTEN activation by antioxidant treatment promoted T-ALL cell death without affecting normal T cell precursors [259]. PTEN oxidation has also been demonstrated in prostate, pancreatic and breast cancer cells [194, 225, 260, 261]. The tyrosine kinase receptor Flt3 is frequently mutated (Flt3-ITD) in acute myeloid leukemia (AML). It was shown that the transforming ability of Flt3-ITD depended on the ROS-mediated oxidation of DEP1. Decrease of ROS levels through overexpression of antioxidant enzymes or inhibition of NOX enzymes restored DEP1 activity, while antioxidant enzymes showed less of an anti-transforming effect when DEP1 was downregulated [262]. The oncogene Bcr-Abl has also been described to promote ROS from both mitochondria and NOX enzymes to decrease PTP activity [263-265]. PTP1B has also been reported to be both reversibly and irreversible oxidized in A431 and HepG2 cancer cells, which have high levels of ROS. Inhibition of NOX enzymes decreased ROS levels, PTP oxidation, tyrosine phosphorylation and anchorage-independent growth, indicating a requirement of high ROS levels and PTP oxidation to support the transforming phenotype [266]. A recently described mass-spectrometric method to analyze family-wide PTP oxidation identified different PTP oxidation profiles for multiple cancer cell lines that might be functionally relevant [267].

PTP oxidation has also been implicated in a variety of cardiovascular diseases and diabetes. Antioxidants were shown to alleviate the restenosis phenotype in a rat model of restenosis. These changes were accompanied by an increase of PTP activity, indicating that the VSMC-driven restenosis depends on PTP oxidation [268]. Similarly, mice that lack the antioxidant enzyme PrxII show a PDGF-dependent increased neointima size, accompanied by increased PDGFR phosphorylation. This study confirmed the positive role of ROS in neointima formation [184]. SHP2 oxidation has been reported in hypoxia/reoxygenation in cells and ischemia/reperfusion in rat hearts [269], and during hypertrophic signaling and cardiac hypertrophy [205]. Impaired migration of monocytes from diabetic patients is accompanied with increased ROS and PTP1B oxidation, implying a role for oxidative inhibition of PTP1B in diabetes [270]. Metabolic stress conditions in monocytes, known risk factors for atherosclerosis, were shown to enhance NOX4-mediated MKP1 oxidation and subsequent degradation, leading to enhanced p38 and Erk signaling [271]. In a mouse model of high fat diet-induced obesity ROS served to enhance insulin sensitivity. Mice that lacked Gpx1 showed an enhanced insulin response and increased PTEN oxidation, arguing for a positive role of ROS and PTEN inhibition in the treatment of diabetes [142].

6 AIMS, RESULTS AND DISCUSSION

6.1 THESIS AIMS

PTP oxidation is increasingly recognized as an integral part of growth factor signaling. However, many questions still remain unanswered, such as the exact source(s) of ROS production, and the regulation of PTP reduction in cell signaling. The work in this thesis aimed to gain a better understanding of these. We focused on PDGFβR signaling as a model, which is commonly perturbed in different types of cancer and cardiovascular disease. In particular we explored the potential role of mitochondrial ROS, produced by p66Shc, in PDGFβR-dependent PTP oxidation and signaling *in vitro* and PDGF-dependent restenosis *in vivo*. The thioredoxin system was investigated as a potential reducing system for PTPs in PDGFβR signaling.

6.2 RESULTS AND DISCUSSION

6.2.1 Paper I

The mitochondrial reactive oxygen species regulator p66Shc regulates PDGF-induced signaling and migration through protein tyrosine phosphatase oxidation.

Previous studies that have analyzed PTP oxidation in growth factor signaling have mainly focused on NADPH Oxidases. Mitochondria are an additional source of ROS, and have been demonstrated to play a role in PTP oxidation in integrin signaling. The aim of Paper I was to explore the role of mitochondria in growth factor-induced PTP oxidation, focusing in particular on the role of p66Shc in promoting mitochondrial ROS in PDGF signaling.

The first step of activation of p66Shc is phosphorylation at serine-36, which ultimately leads to mitochondrial translocation. Stimulation of fibroblasts with PDGF resulted both in a time-dependent increase of serine-36 phosphorylation of p66Shc and accumulation of p66Shc in mitochondria. Fibroblasts with downregulation or knockout of p66Shc showed decreased PDGF-induced PDGFβR phosphorylation, both regarding total phosphotyrosine and individual phosphotyrosine levels. Overexpression of wildtype p66Shc, but not a non-phosphorylatable mutant, increased PDGFβR phosphorylation, indicating that PDGF-induced activation of p66Shc promotes PDGFβR activation. Downregulation of p66Shc in MDA-MB-231 breast cancer cells also decreased PDGF-induced PDGFβR phosphorylation and EGF-induced EGFR phosphorylation. These results indicate that p66Shc can regulate PDGFβR activation in multiple cell types and also multiple tyrosine kinase receptors.

Activation of p66Shc leads to production of mitochondrial ROS. We therefore pretreated wildtype and p66Shc knockout fibroblasts with antioxidants before PDGF stimulation, to assess whether the p66Shc-dependent effects on PDGFR phosphorylation occurs through ROS. Pretreatment with the antioxidant N-acetylcysteine (NAC) decreased PDGF-induced total and site-specific PDGFβR phosphorylation as expected. Antioxidants had little to no effect on PDGF-induced

PDGFβR phosphorylation in p66Shc knockout fibroblasts, which was already much lower compared to wildtype. Tyrosine phosphorylation of the PDGFβR leads to activation of distinct signaling pathways. Consistent with the decrease in PDGFβR phosphorylation, p66Shc knockout fibroblasts also showed decreased phosphorylation of the PDGFβR downstream proteins Akt, Erk1/2 and PLCγ-1, which was largely antioxidant insensitive, compared to wildtype fibroblasts.

The above-described results strongly suggest that p66Shc promotes PTP oxidation in PDGFβR signaling. We confirmed PDGF-induced oxidation of PTP1B and SHP2 in fibroblasts, as has been demonstrated in the literature [57, 272]. Deletion of p66Shc leads to a complete loss of PDGF-induced oxidation of PTP1B. Similarly, treatment with NAC or DPI, a general NOX inhibitor, completely inhibited PTP1B oxidation, suggesting crosstalk exists between NOX enzymes and p66Shc. SHP2 oxidation levels were also lower in p66Shc knockout cells, but the PDGF-induced fraction of oxidized SHP2 did not differ. This suggest that different sources of ROS potentially impact on different PTPs. p66Shc-dependent PTP oxidation was confirmed in MDA-MB-231 cells, that show lack of PDGF-induced oxidation of DEP1, PTP1B and SHP2 upon p66Shc downregulation.

PDGF is a potent migratory stimulus, and our results on PDGFβR tyrosine-1021 and PLCγ-1 phoshorylation suggested that p66Shc also regulates PDGF-induced migration. Indeed, whereas PDGF stimulated antioxidant-sensitive migration of wildtype fibroblasts, p66Shc knockout fibroblasts did not show a PDGF-dependent migration. Consistently p66shc knockout fibroblasts lack PDGF-induced phosphorylation of focal adhesion kinase (FAK), an important mediator of migration. These results so far confirm that p66Shc promotes ROS-dependent PTP oxidation in PDGF signaling.

We then sought to determine whether ROS from mitochondria in general promote PDGF signaling. Peroxiredoxin III (PrxIII) is one of the major mitochondrial antioxidant enzymes that scavenge mitochondrial hydrogen peroxide. Downregulation of PrxIII led to a clear increase in PDGF-induced PDGFβR phosphorylation, confirming that mitochondrial ROS promote PDGF signaling.

Altogether we have identified p66Shc as an important mediator of PTP oxidation in PDGF signaling, and suggest mitochondria as a not to be neglected source of ROS in growth factor signaling. Our finding that downregulation of p66Shc also decreases EGFR phosphorylation moreover indicates that this could be a general phenomenon for receptor tyrosine kinases. Further studies are needed to characterize the precise nature of crosstalk between p66Shc and NOX enzymes, which could e.g. control temporal or spatial regulation of PTP oxidation in growth factor signaling.

6.2.2 Paper II

Deletion of p66Shc attenuates PDGF signaling in vascular smooth muscle cells and restenosis injury in mice.

Restenosis after percutaneous coronary interventions represent a significant clinical problem. The pathological features include migration and proliferation of vascular smooth muscle cells (VSMCs), leading to neointima formation and narrowing of the vascular lumen. One of the key factors in this process is PDGF that acts as a potent migratory and proliferative cue for VSMCs. Preliminary clinical trials with PDGFR inhibiters have however shown inconclusive results, and a better understanding is needed of the role of PDGFR signaling in restenosis. The aim of this study was to analyze the contribution of p66Shc to PDGF-induced signaling in VSMCs and restenosis *in vivo.*

Our previous results have shown that p66Shc regulates PDGF signaling in fibroblasts and breast cancer cells. In this study we show that PDGF also activates p66Shc serine-36 phosphorylation in VSMCs. The kinetics of phosphorylation showed a peak between 5-10 minutes, which was similar to phosphorylation of PDGFβR, PLCγ-1, Erk1/2 and p38, while Akt phosphorylation steadily increased over time.

Consistent with our previous findings, downregulation of p66Shc decreased PDGF-induced PDGFβR phosphorylation in VSMCs. Phosphorylation on tyrosine-1021 is also decreased in p66Shc downregulated VSMCs, which is accompanied with a decrease in PLCγ-1 phosphorylation. PLCγ-1 promotes PDGF-induced migration, and the decrease of PLCγ-1 phosphorylation also leads to a decrease of PDGF-induced migration in p66Shc downregulated VSMCs. Together these data show that p66Shc regulates PDGF-induced signaling and migration in VSMCs.

Together these data show that p66Shc regulates PDGF-induced signaling and migration in VSMCs. We performed a mouse model of restenosis by catheter-mediated de-endothelialization of the carotid artery in wildtype and p66Shc knockout mice, to determine whether our findings also have *in vivo* relevance. Only 50% of the p66Shc knockout mice developed detectable neointima, whereas all wildtype mice developed neointima. The neointimal size was significantly decreased in p66Shc knockout mice, as were the amount of PCNA-positive intimal cells, medial area, intima/media ratio and intimal nuclei. The lumen size did not differ significantly between the two groups.

Taken together these data show that p66Shc promotes PDGF signaling and migration in VSMCs, and neointima formation in mice. It will be important to determine whether these signaling effects are mediated through oxidative inactivation of PTPs, as we have shown for fibroblasts and breast cancer cells. In addition, further characterization of downstream signaling and cell proliferation should be carried out to determine extent of regulation of p66Shc on PDGF signaling in VSMCs. Further tissue analyses can determine whether the observed phenotype also correlates with changes in PDGFβR phosphorylation *in vivo.*

6.2.3 Paper III

Selective activation of oxidized PTP1B by the thioredoxin system modulates PDGF-β receptor tyrosine kinase signaling.

The majority of studies that have analyzed PTP oxidation in cell signaling have focused on oxidation, rather than reduction of PTPs. *In vitro* studies on PTP reduction suggest specificity of cellular reducing systems towards individual PTPs, although the relevance to signal transduction is not clear. The aim of paper III was to analyze the relevance of PTP reduction to PDGF signaling.

The thioredoxin (Trx) system is one the major cellular reducing systems and has been shown to be able to reduce PTPs *in vitro*. We have made use of cells that lack Thioredoxin Reductase 1 (*Txnrd1*$^{-/-}$) to determine whether this system also regulates RTK signaling and PTP oxidation *in vivo*. Analyses of PTP activity and oxidation showed that *Txnrd1*$^{-/-}$ cells have increased oxidation of PTP1B, whereas SHP2 was unaffected. These changes occurred in the absence of changes in ROS levels, suggesting that these are due to loss of thioredoxin reducing activity. Also *in vivo* oxidized PTP1B, but not SHP2, could be efficiently reduced *in vitro* by the Trx system, further suggesting that the Trx system selectively acts on oxidized PTP1B, but not SHP2.

In vitro assays confirmed these findings, and showed that the Trx system is as efficient as DTT in reducing oxidized PTP1B, but not SHP2. The Trx system reduces oxidized PTP1B in a concentration-dependent manner, and all components of the Trx system are required for efficient reduction of oxidized PTP1B. Using a Trx substrate-trapping mutant we could furthermore confirm that Trx binds PTP1B *in vitro*.

These results suggested that the differential PTP oxidation seen in the *Txnrd1*$^{-/-}$ cells could also impact on PDGFR signaling. Indeed, stimulation with PDGF leads to an enhanced phosphorylation of tyrosine-579/581 of the PDGFβR, which is the main target of PTP1B. PDGF-induced phosphorylation of tyrosine-771, the main SHP2 target, did not differ between wildtype and *Txnrd1*$^{-/-}$ cells, which is consistent with the inability of the Trx system to reduce oxidized SHP2. Phosphorylation at tyrosine-579/581 is known to promote a proliferative response, and *Txnrd1*$^{-/-}$ cells accordingly show an increase in proliferation. These results suggested that PTP1B is the main target of the Trx system in PDGF signaling. We confirmed these findings by using *Ptpn1*$^{-/-}$ and PTP1B reconstituted (*hPtpn1*) cells, which are characterized by an increased phosphorylation of the PDGFβR, mainly on tyrosine-579/581. Treatment with auranofin, an inhibitor of the Trx system, increased tyrosine-579/581 phosphorylation in *hPtpn1* cells, but had no effect in *Ptpn1*$^{-/-}$ cells, showing that PTP1B is a main Trx target *in vivo*.

The *in vitro* data identified PTP1B as a substrate of Trx1. However, the Trx system can utilize other Trx-related proteins as effectors, which could also account for the observed phenotypes of the *Txnrd1*$^{-/-}$ cells. We therefore tested whether

Thioredoxin-related protein 14 (TRP14) is also capable of reducing oxidized PTP1B. Indeed, TRP14 dose-dependently reduced oxidized PTP1B *in vitro*, but not SHP2. This indicates that, while the Trx system shows specificity towards PTP1B versus SHP2, multiple Trx-molecules can contribute to the observed differences in *Txnrd1*$^{-/-}$ cells and future studies are needed to delineate their individual contribution.

Altogether the data presented here shows for the first time PTP substrate specificity of the Trx system in cellular signaling. Future studies are needed to understand the selectivity of the Trx system towards PTP1B, whether these are spatial restrictions or depend on the specific oxidative modification of the PTP in question. It will be interesting to see also whether other reducing systems, such as the glutathione system, show a similar substrate preference and regulation of RTK signaling. The Trx system is often dysregulated in diseases such as cancer. It will therefore also be highly interesting to determine the contribution of PTP oxidation and RTK signaling to these Trx system-dependent disease phenotypes.

7 GENERAL OUTLOOK

It is now well established that PTP oxidation is an integral part of growth factor signaling. The exact regulation however of growth factor-induced PTP oxidation and reduction is still under investigation.

Many oxidants have been shown to induce PTP oxidation in vitro [273], while the common assumption is that hydrogen peroxide is the most relevant oxidant *in vivo* [274]. Future studies are therefore needed to assess the relative contribution of different molecules to PTP oxidation. In this context peroxiredoxins have also been suggested to act as intermediates in PTP oxidation [275], although convincing experimental data for such a universal mechanism is as of yet lacking. Multiple scenarios of oxidants and intermediates are conceivable that might each have its place in a specific signaling context. Continued studies on the reduction of oxidized PTPs in RTK signaling will also likely reveal spatial, temporal and substrate specificities.

Of the 38 classical PTPs, only about 15% have been shown to be regulated by oxidation in a signaling context. While some of this can be attributed to specificity in the oxidation of PTPs, much is likely due to the lack of good antibodies that most PTP oxidation methods rely on [276]. A recently described mass spectrometric-based method, qOx-PTPome, allows the quantification of the oxidation state of all classical PTP domains, and has shown differential patterns of PTP oxidation in different cancer cells [267]. Future qOx-PTPome studies with increased sensitivity should allow the analysis of oxidation of all classical PTPs in growth factor signaling.

The conclusions from this thesis support a model where activation of RTKs induces signaling by oxidizing PTPs, and that PTP oxidation could therefore be an underlying mechanism of RTK-dependent diseases. Reactivation of oxidized PTPs in turn could serve to inhibit this pathological signaling by RTKs. This rationale is supported by a recent study that showed reactivation of oxidized DEP1 by antioxidant treatment to be beneficial in Flt3-ITD dependent experimental tumorigenesis [262].

The use of antioxidants to reactivate PTPs seems straight-forward and easy, as they are readily available in the form of vitamins. However, clinical trials with e.g. diabetic patients that received supplementary antioxidants have generally been unsuccessful, and in some cases even showed increased cancer incidence [277-279]. These studies suffered however from the lack of criteria to be able to select patients that could benefit from such treatments, presumably due to technological limitations of measuring redox status *in vivo* and an incomplete understanding of redox (patho)physiology.

An alternative approach is to inhibit the pro-oxidant system that is responsible for the production of ROS that oxidizes the PTP in question. Such inhibitors are available for e.g. NADPH oxidases, and are under investigation in preclinical and clinical settings [178]. Some of the more unspecific NOX inhibitors have shown the anticipated effects on PTP oxidation in cell culture systems. It remains to be seen

however whether the effect on PTP oxidation is also relevant in pre-clinical and clinical settings.

A third conceivable alternative for PTP reactivation are small molecule compounds that directly reactivate specific PTPs. Although these have not been reported for PTPs in the literature, examples exist for other proteins such as p53 where a thiol-targeting compound modulates its activity. This compound, APR-246, reactivates mutant p53 by covalently binding cysteine thiols [280, 281]. This example shows that it is feasible to design redox-active compounds that have the ability to reactivate enzymes. Future studies are needed to analyze whether oxidized PTPs can be specifically reduced by such redox-active molecules.

In the context of diabetes ROS might actually serve to enhance insulin sensitivity [142], and tilting the balance towards an oxidized PTP would be beneficial in this setting. Recently an intrabody was described that specifically detects and stabilizes oxidized PTP1B, and thereby also sustained insulin signaling in cells [232]. This early proof-of-concept study should stimulate to further efforts to create drugs acting through stabilization of oxidized PTPs, which might contribute to the development of clinically useful PTP inhibitors [282, 283].

8 ACKNOWLEDGEMENTS

First of all I'd like to thank my main supervisor **Arne Östman**. There's such a multitude of different projects in the group, and yet you manage to keep track of them and to get to the heart of things. I find that truly amazing. You allow people to find their own balance between their personal life and work in the lab, and present them with the opportunities to grow professionally through research visits, courses, conferences and so much more. I really respect you for that and it is a great driving force for everyone to do well in the lab and something for everyone to strive for.

All the past and present Östman group members. Åsa, Janna, Carina, Martin, Markus, Elin, Linda, Christina, Monica, Hanna, Ceren, Patrik, Cristina, Sara C, Sara M, Sarah, Laura M, Laura L, Maja, Alessandro, Alessandra, Francesca, Helene, Chern, Jai, Janine, Akira, Artur, Magnus, Giovanni, Daniel, Sarah.
A special thanks to **Åsa** and **Markus**, for sharing the pain, sweat, tears and victories in our small world of PTP oxidation. Markus, it's been great to work together on our projects and see them lift off. Thanks for assuring us a heavy-duty pump and anaerobic box! You're the only one at CCK that enjoys a lengthy talk about the best to way stir and degass a lysis buffer, and I thoroughly enjoyed it too ☺!

Past and present people at floor 3 at CCK. **Miguel, Xianli and Bertrand**, it's been great to be part of your success story. **Masako** and your crazy diets! I hope we'll see each other again in the future. **Nathalie**, the PhD student representative driving force. It was great to restart the CCK pubs with you and see them turn into a big success. Other people from the 3rd floor: Mahdi, Sara, Arindam (the Bengal Tiger, keep going!), Zheng, Lars, Aravindh, Per, Matheus, Sören, Sebastian, Sophia, Aris, Pedram, Claire, Suzanne, Ali, Rainer, Marianne, Jens, Johanna, Poppi, My, Lina, Mimmi, Ran, Lotte, Edel, Caroline, Dan, Iryna, Veronica, Suzanne, Irmeli, Staffan, Mathilde, Katja, Inez, Lina, Karthik, Lisa, Amir, Bavesh, Mikke, Tanya, Tao, Per, Nick, Tiago, Linda, Hans, and many more.

Other past and present people at CCK; Walid, Barry, Giuseppe, Paola, Xiaobing, Bertha, Klas, Cinzia, Sofia, Anna-Maria, Maarten, Hogir, Hanif, Helena, Erik, Jelena, Inga, Emma, Jeremy, Maria, Dudi, Sylvia, Ahmed, Slavica, Angelo, Maria, Salah, Diana, Ulrika, Rona, Sylvia, Dali, Pinar, Shahab, Ninib, Moritz, Toota, Jian, Mikael, Kaveh, Emarndeena, Christina, Serhiy, Michael, Nimesh, Pádraig, Lidi, Elisabeth, Marianne, and many, many more.

A big thank you also to the people that make the department run smoothly: the administration staff for their much-wanted help with all the 'reseräkningar' and the copious amounts of EU Marie Curie paperwork, the CCK shop ladies and Eva-Lena.

The very first CCK Gentlemen's club members, **Walid**, **Dali** and the biggest gentlemen of all **Mahdi**. It's been so secretive that even we didn't know exactly what it was all about.

Our collaborators. **Frank Böhmer**, also my cosupervisor, it's great to see you every time you visit Stockholm again. **Ben Boivin,** it was real good seeing you at the Phosphatase meetings and in our lab. All the best with your group in Montreal! **Ben Neel** and **Rob Karisch** in Toronto, for hosting me in your lab and helping me with the PTP oxidation mass spectrometry. **Janne Lehtiö** and **Davide Tamburro** for starting this technique at SciLife (I'm sure we'll get there!). **TC Meng**, although we don't collaborate on any papers, it's always great to meet you, talk and get suggestions at the PTP meetings.

All the other **Marie-Curie FP6 PTP-NET members**, Frank, Rafa, Ari, Andy, Jeroen, Sheila, Wiljan, Rob, Lydia, Yvonne, Stefan, Viki, Caroline, Thomas, Deepika, Vincent, Barbara, Irene, Monique, Leo, Vasu, Deepankar and Fanny. It's been great and lots of fun to get together all over Europe, from the first beer in the pub/launderette in Jena to the last cava in the bar in Rehovot! Hope to see all of you again in the future!

And the people that mean the most to me, **Lianne, Seline and Matthias**. Seline, who 'wants to work with cells like daddy' later on in life. So lively, talkative, opinioned, curious, independent and smart! You're such an amazing little person. You bring smiles on people's faces with your enthusiasm, and you get excited when we 'feed the cells' and look at them under the microscope. And Matthias, already 1 year old by now, such a happy, curious and determined boy! It makes me happy when you make your wobble-run to me with your short legs when I get home. It will be a joy to watch you grow up and discover the person behind that cute "I-know-I'm-not-allowed-to-do-this-but-I-will-do-it-anyhow-smile".
And Lianne, my lovely wife. Life in Sweden hasn't always been easy, but then again our life usually seems to follow the rule 'why easy when it can be difficult'. It's hard to put into words how lucky I feel to be with you, other than to say I love you very much! The world is much more beautiful with you in it ☺.

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