DEPARTMENT OF ONCOLOGY- PATHOLOGY,
CANCER CENTRUM KAROLINSKA
Karolinska Institutet, Stockholm, Sweden

Redox-regulation of PTPs; mechanisms and impact on PDGFR signaling

Markus Dagnell

Karolinska Institutet

Stockholm 2013

Published by Karolinska Institutet. Printed by Larserics Digital Print AB.

ISBN 978-91-7549-190-5

Till mina föräldrar

ABSTRACT

Protein tyrosine phosphatases (PTPs) are reversibly oxidized upon activation of platelet-derived growth factor receptor beta (PDGFβR). Dys-regulation of the PDGFβR signaling pathway is associated with several diseases, including cancers and cardiovascular disease, and is thus a known driver of disease progression. Ligand dependent PDGFβR phosphorylation stimulates cell proliferation and migration. The aim of this thesis was to elucidate redox-regulatory mechanisms of protein tyrosine phosphatases impacting on PDGFβR signaling.

In Paper I, we analysed effects of mitochondria-derived ROS on PTP oxidation in models of hypoxia and hypoxia/re-oxygenation (H/R) *in vitro* and *in vivo*. We found an increase in PTP oxidation of multiple PTPs, including SHP-2, PTP1B and DEP-1, after exposure of NIH3T3 fibroblasts to H/R. An increase in total PTP oxidation and SHP-2 was also seen in rat cardiomyoblasts after H/R. Furthermore, H/R induced a delay of PDGFR dephosphorylation and also an antioxidant sensitive activation of downstream effectors ERK1/2. In addition, H/R enhanced PDGF-dependent cytoskeletal re-arrangements, which could be abolished by antioxidant treatment. Finally, we found an increase in total PTP oxidation and SHP2 oxidation in tissue extracts from an *ex-vivo* model of rat heart ischemia-reperfusion.

In paper II, we studied expression and activity of PDGFβR pathway components in human pulmonary artery smooth muscle cells (hPASMC) subjected to hypoxia. We show that hypoxia-induced HIF-1α in hPASMC, both *in vivo* and *in vitro*, negatively regulate expression of PDGFβR associated PTPs, including PTP1B, DEP-1, TC-PTP and SHP2. The negatively regulation of these PDGFβR-associated PTPs occurred together with an enhanced PDGF receptor activation and an increase in both proliferation and migration of hPASMC.

In paper III, we found that p66Shc dependent mitochondrial derived ROS contribute to inactivation of the PDGFβR associated PTPs PTP1B and SHP-2 upon ligand stimulation. In addition, deletion of p66Shc reduced downstream intracellular signaling after PDGF-BB stimulation. Furthermore, p66Shc KO cells displayed a decrease in migratory response to PDGF-BB treatment.

In the final study paper IV, we studied the reactivation of oxidized PTPs and its impact on PDGFβR signaling. We showed that cells lacking expression of thioredoxin reductase 1 (TrxR1) displayed an increase in oxidation of PTP1B but not of SHP-2. Furthermore, *in vivo* oxidized PTP1B was re-activated by addition of Trx system components to cell lysates, whereas SHP-2 was not re-activated. Oxidized recombinant PTP1B was also re-activated by treatment with Trx system components while SHP-2 remained largely unaffected. Intriguingly, the Trx related protein TRP14 also reactivated PTP1B but not SHP-2. Furthermore, PDGFβR phosphorylation and signaling was enhanced in Txnrd1$^{-/-}$ fibroblasts leading to an enhanced proliferative response after PDGF-BB stimulation.

LIST OF PUBLICATIONS

I. **Hypoxia followed by re-oxygenation induces oxidation of tyrosine phosphatases.**
Sandin A*, **Dagnell M*,** Gonon A, Pernow J, Stangl V, Aspenström P, Kappert K, Östman A.
Cell Signal. 2011 May;23(5):820-6
***** Both authors contributed equally

II. **Hypoxia enhances platelet-derived growth factor signaling in the pulmonary vasculature by down-regulation of protein tyrosine phosphatases**
ten Freyhaus H, **Dagnell M**, Leuchs M, Vantler M, Berghausen EM, Caglayan E, Weissmann N, Dahal BK, Schermuly RT, Ostman A, Kappert K, Rosenkranz S.
Am J Respir Crit Care Med. 2011 Apr 15;183(8):1092-102.

III. **Mitochondrial reactive oxygen species produced by p66Shc regulate PDGF signaling through protein tyrosine phosphatase oxidation**
Jeroen Frijhoff, **Markus Dagnell**, Elena Beltrami, Marco Giorgio, Arne Östman
Submitted

IV. **Thioredoxin-mediated selective activation of oxidized PTP1B modulates PDGFβ-receptor tyrosine kinase signaling**
Markus Dagnell, Jeroen Frijhoff, Irina Pader, Martin Augsten, Benoit Boivin, Pankaj K. Mandal, Nicholas K. Tonks, Carina Hellberg, Marcus Conrad, Elias S.J. Arnér, Arne Östman
Submitted

Additional papers not included in the thesis:

Targeting density-enhanced phosphatase-1 (DEP-1) with antisense oligonucleotides improves the metabolic phenotype in high-fat diet-fed mice
Janine Krüger, Manuela Trappiel, **Markus Dagnell**, Philipp Stawowy, Heike Meyborg, Christian Böhm, Sanjay Bhanot, Arne Östman, Ulrich Kintscher and Kai Kappert
Submitted

Cell transformation by FLT3 ITD in acute myeloid leukemia involves oxidative inactivation of the tumor suppressor protein-tyrosine phosphatase DEP-1/ PTPRJ.
Godfrey R, Arora D, Bauer R, Stopp S, Müller JP, Heinrich T, Böhmer SA, **Dagnell M**, Schnetzke U, Scholl S, Östman A, Böhmer FD.
Blood. 2012 May 10;119(19):4499-511

12/15-lipoxygenase-derived lipid peroxides control receptor tyrosine kinase signaling through oxidation of protein tyrosine phosphatases.
Conrad M, Sandin A, Förster H, Seiler A, Frijhoff J, **Dagnell M**, Bornkamm GW, Rådmark O, Hooft van Huijsduijnen R, Aspenström P, Böhmer F, Ostman A.
Proc Natl Acad Sci U S A. 2010 Sep 7;107(36):15774-9

FBXW7/hCDC4 is a general tumor suppressor in human cancer.
Akhoondi S, Sun D, von der Lehr N, Apostolidou S, Klotz K, Maljukova A, Cepeda D, Fiegl H, Dafou D, Marth C, Mueller-Holzner E, Corcoran M, **Dagnell M**, Nejad SZ, Nayer BN, Zali MR, Hansson J, Egyhazi S, Petersson F, Sangfelt P, Nordgren H, Grander D, Reed SI, Widschwendter M, Sangfelt O, Spruck C.
Cancer Res. 2007 Oct 1;67(19):9006-12

Oxidation sensitivity of the catalytic cysteine of the protein-tyrosine phosphatases SHP-1 and SHP-2.
Weibrecht I, Böhmer SA, **Dagnell M**, Kappert K, Ostman A, Böhmer FD.
Free Radic Biol Med. 2007 Jul 1;43(1):100-10

Interferon alpha-induced apoptosis in tumor cells is mediated through the phosphoinositide 3-kinase/mammalian target of rapamycin signaling pathway.
Thyrell L, Hjortsberg L, Arulampalam V, Panaretakis T, Uhles S, **Dagnell M**, Zhivotovsky B, Leibiger I, Grandér D, Pokrovskaja K.
J Biol Chem. 2004 Jun 4;279(23):24152-62

LIST OF ABBREVIATIONS

AML	Acute myeloid leukemia
ATP	Adenine triphosphate
Arg	Arginine
Asp	Asparagine
Bcr-Abl	Break point cluster region - Abelson
BCR	B-cell receptor
CML	Chronic myelogenous leukemia
Cys	Cysteine
CRC	Colo-rectal cancer
DEP-1	Density enhanced phosphatase-1
EC	Endothelial cell
EGFR	Epidermal growth factor
ER	Endoplamatic reticulum
ERK	Extracellular regulated kinase
FAD	Flavin adenine dinucleotide
FLT3	FMS-like tyrosine kinase 3
GBM	Glioblastom multiforme
GIST	Gastrointestinal stromal tumor
G6PD	Glucose-6-phosphate dehydrogenase
GPCR	G-protein coupled receptor
GPX	Glutathione peroxidase
GRX	Glutaredoxin
GSH/GSSG	Glutathione, reduced/oxidized form
HER-2	Human epidermal growth factor receptor-2
HIF-1α	Hypoxia-inducible factor-1α
PH	Pulmonary hypertension
hPSMCs	Human pulmonary smooth muscle cells
H/R	Hypoxia/reperfusion
IAA	Iodoacetic acid
IR	Insulin receptor
I/R	Ischemia/reperfusion
LOH	Loss of heterozygosity

MAPK	Mitogen activated protein kinase
MS	Mass spectroscopy
NAC	N-acetyl cysteine
NADPH	Nicotinamide adenine dinucleotide phosphate
NO	Nitirc oxide
NOX	NADPH oxidase
PDGF	Platelet-derived growth factor
PDGFβR	Platelet-derived growth factor β receptor
PH	Pulmonary hypertension
PI3K	Phosphatidyl-inositol-3-kinase
Prx	Peroxiredoxin
PTEN	Phosphatase and tensin homologe
PTP	Protein tyrosine phosphatase
PTK	Protein tyrosine kinase
ROS	Reactive oxygen species
RPTP	Receptor PTP
RTK	Receptor tyrosine kinase
SH2	Src homology 2
SHP-1/2	SH2-containing phosphatase 1/2
SOD	Superoxide dismutase
T-ALL	T-cell acute lymphoblastic leukemia
TC-PTP	T-cell PTP
TCR	T-cell receptor
TK	Tyrosine kinase
TNF-α	Tumor necrosis factor-α
Trx	Thioredoxin
TrxR1	Thioredoxin reductase
UV	Ultra violet
VEGFR	Vascular endothelial growth factor receptor
VSMCs	Vascular smooth muscle cells
XO	Xantine oxidase

TABLE OF CONTENTS

1. Introduction

Cellular processes such as proliferation, differentiation and migration are regulated through finely tuned mechanisms, which involve reversible phosphorylation of proteins. Net levels of tyrosine phosphorylation are determined by the balanced action of protein tyrosine kinases (PTKs) and PTPs. Ligand-induced receptor tyrosine kinase (RTK) activation is a well-described mechanism of regulation of tyrosine phosphorylation. However, increasing evidence suggests PTP activity as an important determinant of net tyrosine phosphorylation. Diseases like cancer and atherosclerosis are characterized by dys-regulated tyrosine kinase signaling. Therefore, detailed understanding of the regulatory network controlling tyrosine phosphorylation is of obvious medical interest.

2. Tumor suppressors and oncogenes affect cell signalling

2.1.1. PTKs

PTKs activate proteins through phosphorylation by catalyzing the transfer of the gamma-phosphoryl groups from adenosine triphosphate (ATP) to tyrosine hydroxyls. There are 90 known genes in the human genome coding for PTKs of which 58 encode transmembrane RTK while 32 encode cytosolic non-receptor PTKs. PTKs are involved in regulating intracellular signal-transduction pathways mediating development, growth, differentiation, adhesion, motility and programmed cell death (1, 2).

2.1.2. Phosphotyrosine signaling and cancer

PTKs play a significant role in many diseases such as cancer and diabetes. Cellular oncogenes contribute to cell transformation and cancer progression when mutated or overexpressed. Of the oncogene group PTKs comprise a large fraction. Tumor suppressor genes on the other hand have, in general, repressive effects on cell growth and tumorigenesis.

Oncogenic activation by tyrosine kinases in malignant cells occurs through three major mechanisms. One mechanism, chromosomal translocation, creates novel oncogenic fusion genes between e.g. tyrosine kinases and some other genes elsewhere in the genome. For example the fusion oncoprotein BCR-ABL is generated by fusion of the ABL1 PTK gene on chromosome 9 with the break point cluster region gene (BCR) on chromosome 22. The resulting protein is characterized by high constitutive kinase

activity and is a characteristic aberration associated with chronic myelogenous leukemia (3).

Amplification is another mechanism whereby RTKs can be oncogenically activated. Transcription of multiple copies of a particular gene results in overproduction of the encoded protein. One such example is the HER2/ErbB2 receptor which is amplified in 30% of all breast cancers (4).

The third mechanism for oncogenic activation involves point mutations or small deletions resulting in increased kinase activity. For example the cellular proto-oncogene c-KIT and PDGFRα are commonly mutated in gastrointestinal stromal tumors, and the epidermal growth factor (EGF) receptors are mutated in a subset of non-small cell lung cancer (5, 6).

Most solid tumors depend on the formation of supportive tumor stroma composed of tumor vessels and fibroblasts (7). The vascular endothelial growth factor receptor-2 (VEGFR2) which is expressed in endothelial cells is the most important tyrosine kinase involved in tumor angiogenesis (8). This receptor stimulates cell division, migration and differentiation of endothelial cells. Tumor vessels mature with the aid of pericytes which are mesenchymal cells lining the abluminal surface of capillaries, arterioles, and post-capillary venules (9). Pericytes are highly dependent on stimulation from PDGFRβ receptors (10). Fibroblasts are also important for tumor growth and are particularly dependent on stimulation of PDGF receptors (11). Besides stimulating the growth and migration of these cells, PDGF receptor stimulation of fibroblasts also affect their ability to induce an elevated interstitial fluid pressure, which is a characteristic of many solid tumors (12).

2.1.3. Drugs against PTK signaling pathways

Several therapeutic drugs against RTK have been developed and are now in use. Imitanib is a small molecule inhibitor targeting the fusion protein BCR-ABL in chronic myelogenous leukemia (CML) and activated c-KIT in gastrointestinal stromal tumor (GIST) (13). Trastuzumab, a monoclonal antibody, and erlotinib, a small molecule inhibitor, are two examples of drugs that target HER/ErbB2 receptor and EGFR, respectively (14). Although many patients respond to these treatments the majority will develop resistance to the drugs.

2.1.4. Phosphatases as modulators of PTK signaling.

PTPs are a protein family that have the capacity to dephosphorylate phosphotyrosines in proteins. Their catalytic activity is extremely high compared to tyrosine kinases. Several studies where PTP expression or activity has been manipulated reveal the impact that PTPs have on tyrosine kinase signaling.

Exposure of cells to the PTP inhibitor sodium orthovanadate, activate RTKs in a ligand-independent fashion (15). Also, inhibition of ligand activated RTKs with a kinase specific inhibitor rapidly leads to dephosphorylation of the receptor (16).

PTP1B knockout mice display an increased sensitivity to insulin as shown by glucose uptake studies, suggesting modulation of the insulin receptor signaling (17). In fact, overexpression of PTP1B abrogates insulin receptor (IR) signaling whereas osmotic loading of neutralizing antibodies against PTP1B enhances signaling (18, 19). Naturally occurring inactivating mutations of the gene coding for Src-homology 2 (SH2) PTP SHP-1 (murine *motheaten*, *me*, gene) has revealed functional interactions between SHP-1 and CSF-1 receptor signaling (20). Another study identified increased receptor activation of IR, EGFR and fibroblast – growth – factor receptor (FGFR) after antisense mediated down-regulation of RPTP-LAR (21, 22).

PTPs can also modulate RTK signaling in a positive manner. The SH2 domain PTP SHP-2 has been shown to be a positive regulator of PDGFR signaling and other RTKs (23). Also PTP1B has been shown to act as a positive mediator of the ErbB2 tyrosine kinase (24, 25).

In summary, the regulation of PTPs plays a significant role in determining net levels of tyrosine phosphorylation and downstream signaling.

3. PTPs

3.1.1. The PTP family

107 PTPs are found in the human genome (26). They can be divided into four different families based on the amino acid sequence in their catalytic domain. Three of these families, classical PTPs, dual-specificity PTPs and low-molecular-weight PTPs, have an active site based on a cysteine residue. The active site of the fourth family contains an asparagine residue.

The classical PTPs constitute a sub-group of 38 tyrosine-specific PTPs which will be the focus in this thesis. They are hereafter referred to as PTPs and can be divided into receptor-like PTPs (RPTPs) and cytosolic PTPs. The RPTPs span the cellular membrane and the extracellular domains are characterized by large structural variability. In most RPTPs the intracellular part consists of two tandem PTP domains, where the catalytic domain resides in the first domain. The extracellular part and the second domain are thought to exert regulatory functions. The cytosolic PTPs consist of one catalytic domain and by sequences outside the catalytic domain which regulate activity and location (27).

3.1.2. Catalytic mechanism

The catalytic motif of PTPs consists of a highly conserved amino acid sequence $HC(X_5)R$ that contains the catalytically essential cysteine (Cys) and Arginine (Arg) residues (28). The low pKa is a characteristic of the active site cysteine is suggested to be caused by an imidazole group of the neighbouring histidine in combination with a network of hydrogen bonds with the backbone of the so called P-loop (29, 30).

The positively charged pocket within the P-loop interacts with phosphotyrosine (pTyr) substrates that induce a conformational change from an open inactive cleft to a closed conformation containing bound substrate and a water molecule. The two step catalytic mechanism involves the formation of a cysteinyl-phosphate intermediate stabilized by the opposing Arg residue (31). A neighbouring Aspartic acid (Asp) residue function as an acid catalyst by protonating the leaving tyrosine residue of the substrate (32). In the final step of the mechanism, the same Asp residue mediates the release of inorganic phosphate through catalysis of a water molecule attack on the cysteinyl-phosphate intermediate (33, 34) **(Figure 1)**.

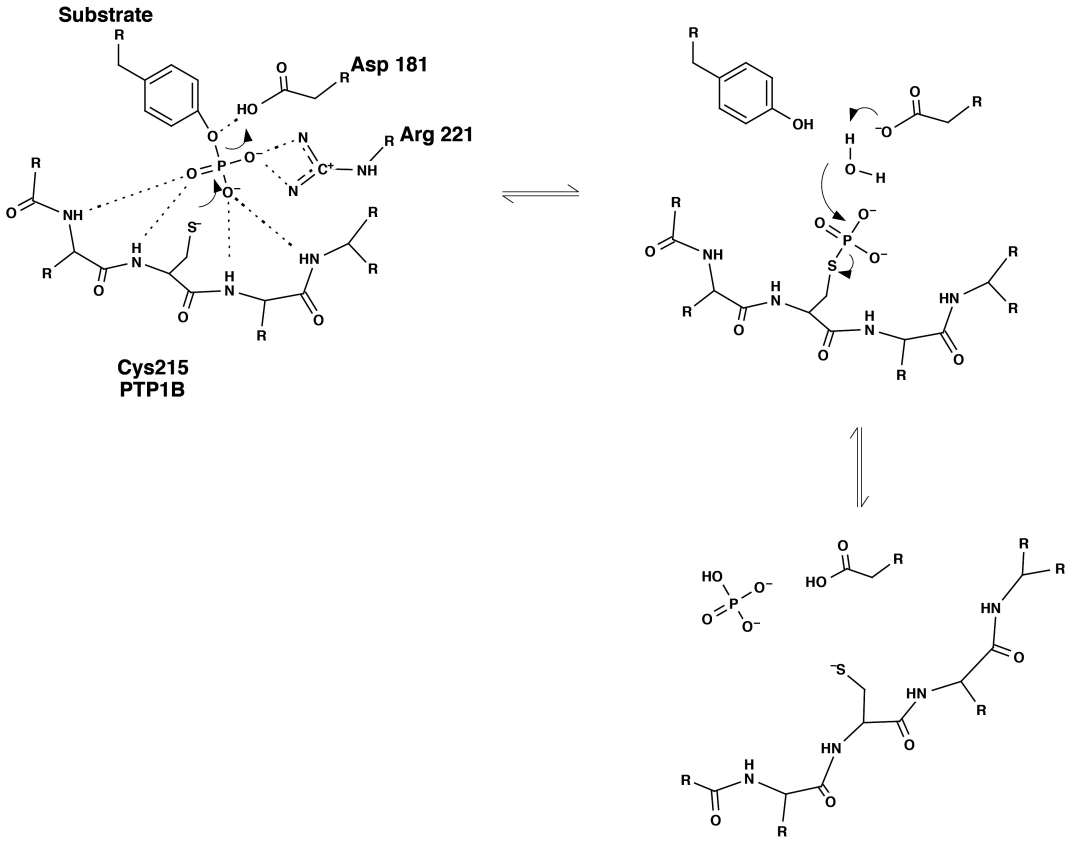

Figure 1. Catalytic mechanism of PTPs.

3.1.3. Substrate specificity

Although sharing a common catalytic domain, PTPs are highly selective towards protein substrates in different cellular contexts. The catalytic domain contains residues that determine substrate specificity. PTPs have been shown to have intrinsic differences with regard to catalytic efficiency and also display distinct specificity profiles (35).

The crystal structure of PTP1B in complex with a peptide derived from a phosphorylation site of the EGFR have revealed some mechanisms explaining PTP specificity and substrate recognition (33). The pTyr in the peptide is the main contributor to substrate recognition. Specifically, nonpolar side chains in close proximity to the active site cleft form hydrophobic interactions with the phenyl group of the pTyr of the peptide. Specificity is also achieved through flanking amino acids surrounding the catalytic site such as interactions between basic residues of the PTP and substrate peptide side chains (33). Also, another study of the PTP1B crystal structure highlights its ability to recognise several structurally different substrates due to conformation flexibility of Arg47. Depending on NH_2-terminal amino acids of the

substrate, Arg47 of PTP1B can adopt two different conformations (36). In addition, the presence of tandem pTyr residues on the substrate has been shown to increase affinity (37). Thus, the catalytic active site milieu determines the PTP affinity for its substrate (38).

Comparison between a set of PTPs toward their optimal substrate revealed differential intrinsic catalytic efficiency. PTP1B, SHP-1 and SHP-2 all shared similar k_{cat}/Km values in contrast to RPTPα, which had a 2-3 order of magnitude lower efficiency. In addition, RPTPα had the broadest substrate specificity among the four PTPs. PTP1B also showed a broad specificity but still more restrictive than RPTPα. SHP-1 and SHP-2 showed a narrower specificity with a restriction towards positively charged residue and instead a preference for acidic residues surrounding the phosphorylated tyrosine (35).

In vivo studies have also been performed analysing the site-selective preferences of different phosphatases for different sites in multiply phosphorylated substrates. For example, TC-PTP depletion results in hyper-phosphorylation of tyrosine residues Y1021 of the PDGFβ receptor. The increased Y1021 phosphorylation was associated with an increased migratory phenotype (39). In contrast depletion of PTP1B mainly increased phosphorylation at the Y579 site and corresponded to an increased proliferative response (39). Thus, although closely related in structure PTP1B and TC-PTP display differential site-specific preferences. These properties suggest that PTPs can modulate RTK signaling and determine the cellular response to growth factor stimulation.

Other studies have revealed a preference of DEP-1 for the PDGFβR pY1021 but not pY857 (40, 41). Intriguingly, phospho-peptides of Y1021 and Y857 displayed similar k_{cat} values. In contrast the pY1021 peptide showed a higher affinity (K_m value) compared to the pY857 peptide. Furthermore, substitution of key amino acids in both peptides could increase or decrease the affinity to DEP-1.

In addition to the catalytic domain, PTPs contain domains that bind to specific motifs in proteins or to sub-cellular structures. The cytosolic PTPs all contain one single catalytic domain with additional regulatory domains mediating PTP substrate interactions and subcellular localization (42). SHP-1 and SHP-2 both have a SH2 domain that directs

the phosphatase to its specific location (43). Several RTKs are dephosphorylated by SHP-1 that is recruited to the receptors by SH2 domains (23). The SH2 domain of SHP-2 and SHP-1 also regulate the active site by blocking the active site when not bond to its pY-partner (43, 44).

Substrate specificity can also be directed by sub-cellular localization, as exemplified by the C-terminal localization signal of PTP-1B mediating targeting to the endoplasmatic reticulum (ER) (45).

3.2. Regulation of PTPs

Several mechanisms regulate PTP-activity, and PTPs are subject to multiple types of modifications, leading to either impaired or increased PTP activity.

Differential expression of PTPs is one obvious mechanism for regulation of PTP activities in the cell. Up-regulation of DEP-1 is achieved in cells reaching high densities (46). Promotor methylation leading to downregulation of PTPs has been shown in cancer cells (47).

Subcellular localization is also important for regulation of PTP activity. PTPs accumulate at the plasma membrane and form complexes with RTKs and thereby regulate those (48). The SH2-domain containing PTPs (SHP-1 and SHP-2) can also accumulate at the plasma membrane through SH2 domain/phosphotyrosine interactions (23, 49). Studies have also identified a targeting signal in the C-terminus of SHP-1, with high affinity for acidic phospholipids (50). This targeting signal is important for localization of SHP-1 to lipid rafts in T lymphocytes where SHP-1 regulate T-cell receptor signaling (51).

Alternative splicing is a mechanism that might change the domain structure of PTPs leading to functionally different splice variants. The receptor like PTPs are frequently undergoing alternative splicing resulting in structural variants of extracellular domains (52, 53). Alternative splicing can also change regulatory domains in PTPs affecting protein/protein interactions. For example alternative splicing alters the PDZ domain in PTP-BAS and thereby affects its binding affinity to its substrate APC (54).

Regulated proteolysis can also control PTP activity. PTP-PEST undergoes limited proteolysis in a manner related to apoptotic signaling (55). Furthermore, ultra violet (UV) irradiation-induced PTP oxidation has been shown to result in calpain-mediated degradation of oxidized PTPs (56). Furthermore, in vitro studies of oxidized PTP1B revealed a calpain-dependent inactivating cleavage (57).

Concerning receptor-like PTPs, dimerization of the catalytic domains has been proposed to function as an inhibitory mechanism. Two dimers of RPTPα, consisting of a helix-loop-helix wedge like structure, block each other's catalytic cleft leading to inhibition (58). Also ligand binding to extracellular domains of RPTPs can either activate or inactivate the catalytic domain. Pleiotrophins inactivate the catalytic activity of RPTP β/ζ and thereby increase phosphorylation of several substrates such as β-adductin, β-catenin, p190Rho-GAP and ALK (59-62). Ligand activation and inactivation of PTP-LAR has been shown to be critical during synaptic development in *Drosophila*. The two heparan-sulfate proteoglycans syndecan and dallylike modulate the activity of PTP-LAR and thereby also the phosphorylation state of its substrate Ena (63, 64). In another study, the extracellular-matrix preparation "matrigel" was shown to increase the activity of DEP-1 by interacting with extracellular domains of the PTP (65).

Covalent post-translational modifications of PTP can also regulate their activity. Phosphorylation of specific serine, threonine or tyrosine residues, affecting affinity, has been shown for CD45, PTP1B and PTP-PEST (66-69). Another example is the phosphorylation of two serine residues on the juxtamembrane domain of RPTPα that leads to an increased activity (70). These phosphorylation sites are located in the wedge-like helix-loop-helix structure that is essential for dimer formation. Both SHP-1 and SHP-2 are also phosphorylated on serine residues upon protein kinase C activation. SHP-2 activity was shown to be unaffected, whereas SHP-1 activity was decreased, after these phosphorylations (71, 72).

Recently, reversible oxidation has emerged as an additional regulatory mechanism (see detailed description in chapter 4 below).

3.3. PTPs and cancer

3.3.1. PTPs as tumor suppressors

PTPs are potential tumor suppressors due to their antagonistic effect on oncogenic RTK signaling. Experimental support for this notion was provided through early studies showing that overexpression of PTPs *in vitro* reverses tyrosine kinase-dependent transformation (73). Furthermore, DEP-1 has been described to inhibit the RAS pathway by dephosphorylating ERK1/2 kinases and to induce G1 arrest through stabilization of cyclin-dependent-kinase-inhibitor p27 (74). Overexpression of the inactive DEP1-K1017N mutant impairs the interaction between DEP-1 and ERK, leading to increased tyrosine kinase signaling (75). Furthermore, re-constitution of DEP-1 expression in cultured breast cancer cell lines was shown to reduce growth (76), and over-expression of DEP-1 in cultured breast cancer, pancreatic thyroid and colon cancer cells reduced cell growth (74, 77, 78). These initial findings in cancer cell lines have also been substantiated with analysis of tumors with regard to inactivating aberrations in PTP genes.

Inactivation through point mutations has been shown in a large study, by Wang et al, involving sequencing of PTPs in colorectal cancer (CRC) where mutations were found in both cytosolic and receptor-like PTPs (79). Examples of mutated PTPs are PTP-BAS, PTPD2 and PTPγ. These mutations commonly altered the amino acid sequence arguing for a functional relevance. A subset of the mutations were functionally tested and shown to have a loss of biological function (79). Furthermore, Korff et al have analyzed another set of CRC samples and found many frame-shift mutations in PTP genes. The highest number of mutations occurred in HDPTP (26%) and PTPBAS (22%) whereas the most frequently mutated PTPs were PTPD1 (17%), PTPRS (12%) and STEP (6%) (80). In this study the authors selected CRCs with microsatellite instability, in contrast to the Wang study where the CRCs where unselected. Interestingly the two studies reveal different subsets of affected PTP genes.

Loss of heterozygosity is also a mechanism that has been reported to affect PTPs. Allelic loss has been found for PTPRJ, encoding PTP DEP-1, in carcinoma of colon (49%), lung (50%) and breast (78%) (81). PTPN2 (TC-PTP), was found to be deleted in t-cell lymphoblastic leukemia (T-ALL). Furthermore, knock down of PTPN2 in T-ALL cells increased proliferation and sensitivity to cytokines (82).

Epigenetic changes, such as methylation of the promoter region of SHP-1, have been shown to occur frequently in lymphoma and leukemia (83). The gene coding for PTPRD is inactivated by epigenetic hypermethylation, genetic deletion and point mutation in over 50% of glioblastoma multiforme (GBM) tumors. (84).

3.3.2. PTPs as oncogenes

SHP-2 is a PTP with oncogenic properties. It activates mitogenic and pro- migratory signals from different RTKs (23). Examples of receptors dependent on SHP-2 activity are the EGFR and FGFR3 (85). Also transformation by ErbB2 and BCR-ABL seem to depend on SHP-2 activity (47). Dominant autosomal mutations in the PTPN11 (gene encoding SHP-2) is the cause of Noonan Syndrome in 50% of cases (86). Noonan Syndrome patients develop facial abnormalities and heart failure and they also have an increased risk of developing several different myelomonocytic leukemias. Also somatic mutations in SHP-2 have been shown to be involved in various types of leukemias (87).

Some studies in mouse breast cancer models have also indicated that PTP1B is an important positive regulator of ErbB2 signaling and that inhibition of its function attenuates mammary tumorigenesis and malignancy. Inhibiton of PTP1B in NDL2 transgenic mice either by cross-breeding with Ptpn1 deficient mice or by treatment with a specific PTP1B inhibitor results in significant mammary tumor latency and resistance to lung metastasis (24, 25). Furthermore, overexpression of PTP1B in the mammary glands leads to spontaneous breast cancer development (73).

4. Redox regulation of PTP activity

4.1.1. ROS and antioxidants

4.1.1.1. ROS introduction

Reactive oxygen species (ROS) are formed by the incomplete reduction of oxygen (reduction can be defined as a gain of electrons by an atom) forming reactive molecules such as superoxide anion ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2) and the hydroxyl radical ($^{\bullet}OH$). Free radicals can be described as reactive molecules or molecular fragments with one or more unpaired electrons. The hydroxyl radical reacts with any biological molecule due to its high reactivity whereas $O_2^{\bullet-}$ and H_2O_2 each have preferred molecular targets such as iron-sulphur clusters and cysteine/methionine residues,

respectively. H_2O_2 can be converted to $^{\bullet}OH$ by a metal-catalysed fenton-reaction (Fe(II) + H_2O_2 → Fe(III) + $^{\bullet}OH$ + OH^-) (88).

ROS have for a long time been considered as a harmful by-product of metabolism. The elevation of ROS levels can lead to damage of cellular lipids, proteins and DNA with a disturbed function as a consequence. Many human diseases such as cardiovascular disease, cancer and ischemia/reperfusion involve oxidative stress (89, 90). Net levels of ROS in cells are a consequence both of ROS production and ROS scavenging.

4.1.1.2. Sources of ROS:

ROS can be produced by various sources such as the mitochondria, NADPH oxidase (NOX), xanthine oxidase (XO), cyclooxygenases (COX) and lipoxygenases (LOX).

The bulk amount of ROS is generated by the electron transport chain within the mitochondria that produces $O_2^{\bullet-}$ through non-enzymatically redox-reactive compounds such as semiquinone. Complex I (NADPH/ubiquinone oxidoreductase) and complex III (ubiquinol/cytochrome c oxidoreductase) are the major site for $O_2^{\bullet-}$ production (91). The produced $O_2^{\bullet-}$ will rapidly be dismutated into H_2O_2 by MnSOD (92). Nowadays eight sites of $O_2^{\bullet-}$ generation are known in mitochondria (93).

The NOX enzyme was discovered in phagocytes and found responsible for the generation of local bursts of free radicals in so-called “respiratory burst”. By consuming high amounts of oxygen, the NOX enzymes generate $O_2^{\bullet-}$ through one electron-reduction. The phagocyte production of free radicals is part of the host defence system against invading microbes. NOX enzymes have also been found as ROS producers in non-phagocytic cells. Specifically, activation of membrane bound RTKs generates a NADPH dependent localized burst of ROS.

NOX enzymes 1-4 all share the flavoprotein domain as well as the NOX flavocytochrome domain, common for all NOX enzymes. NOX5 is the single NOX enzyme containing an amino-terminal calmodulin-like domain that contains four calcium-binding EF hand structures. Another $O_2^{\bullet-}$ -forming group of enzymes are the dual oxidases (DUOX), that also generate $O_2^{\bullet-}$ through reduction of molecular oxygen (94). The DUOX enzymes contain, in addition to the NADPH oxidase domain, also a

domain homologous to heme-containing peroxidases. One example of this is the myeloperoxidase present in neutrofils (95).

Xanthine oxidase is another oxygen-free radical producing enzyme widely distributed within various tissues. It catalyses hydroxylation of purines where reduction of molecular oxygen generates both $O_2^{\bullet-}$ and H_2O_2. Transition metals such as Fe^{2+} and Cu^{+} can break down H_2O_2 to the highly reactive $^{\bullet}OH$ trough the Fenton reaction (96). ROS are also produced during metabolism of arachidonic acid metabolism by COX and LOX (97).

4.1.2. Antioxidants introduction

ROS levels are kept low by several different free-radical scavengers. There are two main categories, enzymatic and non-enzymatic antioxidant systems.

Both glutathione and thioredoxin (Trx) belong to the thiol- redox buffer in the cell. Glutathione, a non-enzymatic antioxidant, is present at mM concentration in contrast to thioredoxin which is present at 1000- fold lower concentrations. Oxidized glutathione (GSSG) and (Trx) are reduced by glutathione reductase (GR) and thioredoxin reductase (TrxR), respectively, utilizing NADPH as cofactor. These two main antioxidant nodes reduce target proteins such as peroxiredoxins (Prx) and glutathione peroxidase (Gpx) family members.

Superoxide dismutase (SOD), catalase and glutathione peroxidase (GPx) all belong to the enzymatic antioxidants and are also the most efficient in quenching the free radicals. SOD catalyses reduction of $O_2^{\bullet-}$ through the dismutation reaction $2O_2^{\bullet-} + 2H^{+} \rightarrow H_2O_2 + O_2$ and exists in three different isoforms with location in the mitochondria, cytosol and extracellularly (96). Catalase is located in the peroxisomes and converts H_2O_2 to oxygen and water with great efficiency (96). Five out of a total of eight glutathione peroxidases are selenium-dependent antioxidant enzymes with a major task to protect against low levels of oxidative stress. GPxs catalyse the reduction of H_2O_2 to water and lipid hydroperoxides (98).

4.1.2.1. Thioredoxin system

The Trx system in mammalian cells consists of Trx, TrxR and NADPH (99). One isoform is located in the cytosol (Trx1/TrxR1) and the other in mitochondria (Trx2/TrxR2). A third testis-specific TrxR3, also named Thioredoxin glutathione reductase TGR, is expressed in germ cells (100, 101). Cytosolic Trx1 and mitochondrial Trx2 are highly conserved small proteins with a size of 10-12 kDa, that are present either in a reduced dithiol form or as an oxidized disulphide form. Reduced Trx functions as a potent electron donor for disulfide substrates that when reduced generate oxidized Trx. Oxidized Trx can in turn be reduced back utilizing NADPH and the flavin adenine dinucleotide (FAD)-containing enzyme TrxR (99). These three components comprise the Trx system and are responsible for maintaining a reduced redox balance in the cell. Interestingly, knock-down of individual components of the Trx system results in embryonic lethality (102-104).

4.1.2.2. Glutathione system

Reduced glutathione (GSH) is a highly abundant low molecular weight protein, present in all living cells with the major function to protect cells from oxidative stress, together with the GPx family of proteins, (105). The glutathione dependent enzyme glutaredoxin (Grx) reduces disulfide substrates or glutathionylated proteins. Both GPx and Grx utilize GSH in the ROS detoxification process and increase the pool of oxidized glutathione (GSSG) as a result.

Glutathione reductase (GR) re-reduces GSSG using NADPH as an electron donor (106). The availability of NADPH is maintained by the glucose-6-phosphate dehydrogenase (G6PD) enzyme in the pentose phosphate pathway (107).

Reactive cysteines in proteins can in their deprotonated thiolate form react with GSSG and form glutathionylated adducts.

4.1.2.3. GPx

GPxs are a family of enzymes that catalyse the reduction of H_2O_2, organic hydroperoxides and lipid hydroperoxide (98). There are five main mammalian selenium dependent GPx enzymes expressed in various tissues and with different subcellular localization (98, 108). GPx1 is expressed in red blood cells, liver, lung and

kidney and localize in the cytosol, nucleus and mitochondria within the cell. GPx2 is found in the gastrointestinal tract and localizes in the cytosol and nucleus. GPx3 is located in the cytosol and expressed in several different organs such as lung and kidney. Phospholipid hydroperoxide glutathione peroxidase (GPx4) is present in most tissues and localizes and binds to the membranes of the mitochondria, cytosol and nucleus (98). GPx5 is present in epididymis and lacks the active site selenocysteine and instead utilizes a cysteine. The recently found GPx6 is located in the epithelial of the olfactory system in humans (109). GPx1-3 utilizes GSH for regeneration in contrast to GPx4 that can be regenerated by thioredoxin (105).

The substrate specificity of GPx4 is much broader compared to the other members of the GPx family. In addition to hydroperoxides and lipid hydroperoxide, a common substrate for all GPx, GPx4 also reduces phospholipid-associated hydroperoxides in biological membranes to alcohols (110). GPx4 is emerging to be one of the most important GPx as it controls non-apoptotic cell death (111). Its deletion leads to embryonic lethality (112).

4.1.2.4. Peroxiredoxins

The protein family of six peroxiredoxins (Prx) is an abundantly expressed group of enzymes that catalyses the reduction of H_2O_2, organic hydroperoxides and peroxynitrite (113). Oxidized Prx1, 2, 3 and 5 are reduced by the Trx system and Prx4 can be reduced by both Trx and GSH as an electron donor. Prx6 only uses GSH for its reduction.

The conserved peroxidatic cysteine exists in a thiolate anion, which makes it highly susceptible towards H_2O_2-induced oxidation to a sulfenic acid form (114). All six Prx enzymes share this first step of cysteine oxidation. The second part of the catalytic mechanism, where a sulfenic acid cysteine is reduced back to a thiol, involves a resolving cysteine attack on the sulfenic acid cysteine and formation of a disulfide bond subsequently reduced by Trx. This second part of the mechanism divides the different isoforms into three subclasses. The cytosolic 2-Cys Prx, which comprise Prx1-4 are homodimers whereas atypical 2-Cys Prx5 is monomeric and 1-Cys Prx are homodimeric that do not contain a resolving cysteine but instead use GSH as a reductant (115-117).

4.1.2.5. Catalase

In addition to Prx and GPx, catalase is another main enzyme responsible for removal of H_2O_2 in the cell (118). It is located in the peroxisomes in almost all aerobic cells and the reaction mechanism involves complete reduction of H_2O_2 into H_2O. The active site Fe^{3+} ion mediates the two-electron transfer with high efficiency due to lack of need for cellular reductants such as GSH or NADPH (118).

4.1.2.6. Superoxide dismutase

CuZnSOD (SOD1) exists in all eukaryotes and prokaryotes. The localization of SOD1 is mainly in the cytosol but it is also present in nucleus and in the inner membrane space of mitochondria. Manganese containing SOD (SOD2) is localized in the matrix of mitochondria. The SOD enzyme function is to accelerate the dismutation of $O_2^{\bullet-}$ into H_2O_2 and O_2 (119). Superoxide can react with nitric oxide ($NO^{\cdot}$) to form peroxynitrite, and also react with different transition metals to take part in the Fenton reaction and form hydroxyl radicals. Therefore fast clearance by SOD is of great importance for protection against formation of reactive compounds (119).

4.2. Effects of ROS on proteins and signaling

Recently the production of ROS has emerged as an important signaling mechanism involved in both extracellular and intracellular signal transduction pathways. Redox-regulation involves oxidation of reactive cysteines in proteins. There are over 200 000 cysteine residues encoded in proteins in the human genome and around 20-40 000 of these are suggested to be sensitive to oxidation (120). Previous work has suggested a correlation between number of cysteines in proteins and organism complexity.

Proteins that contain redox-sensitive thiols can react with free radicals, such as $O_2^{\bullet-}$, or nonradical oxidants H_2O_2. Reversible oxidation of thiols can regulate biological functions by several different mechanisms.

Macro-molecular interactions may be altered by oxidation. For example actin filaments have been shown to be glutathionylated during oxidative stress leading to impaired microfilament organization (121). Oxidation of cysteines within proteins may also induce conformational changes mediating allosteric regulation. One example is the S-nitrosylation of an specific cysteine residue in p21RAS which induces guanine

nucleotide exchange and downstream signaling (122). Also, reversible s-glutathionylation of caspase-3 mediated by Grx, modulates Tumor Necrosis Factor-α (TNFα) induced apoptosis (123). Recently, the EGFR has been shown to be oxidized at an active site cysteine resulting in a enhanced RTK activity (124). In addition, the transcription factor nuclear factor erythoid 2-related factor 2 (Nrf2) is targeted for ubiquitin-mediated degradation by kelch-like ECH-associated protein 1 (Keap-1) under non-stressed conditions. This involves elevated ROS levels which oxidize specific cysteines in Keap-1 that promote dissociation of the Nrf2-Keap-1 complex. Nrf2 can upon release translocate to the nucleus and activated ARE response elements (125, 126).

Finally redox-regulation of the active site cysteine in PTPs has emerged as a regulatory mechanism and will be discussed in the following section (127).

4.3. Oxidation of PTPs

The active site cysteine of almost all classical PTPs exists as a thiolate (RS^-) form due to its low pKa value (4-6). Its nucleophilic property makes the cysteine more susceptible to oxidation in comparison to most cysteines with display pKa values of 8-9 (29, 128).

Oxidants involved in PTP oxidation: Several different types of oxidants have been analysed *in vitro* with regard to their ability to oxidize PTPs. Due to the relative stable properties of H_2O_2 it has for long been considered to be the most likely candidate to oxidize thiols in proteins. Several studies have investigated H_2O_2 effects on PTPs and showed oxidative inactivation. Examples of these PTPs are PTP1B (129-131), SHP-1 (132, 133), SHP-2 (132, 133), PTP-LAR (130), RPTPμ (130), RPTPα (134), VHR (129), PTEN (135), MKP3 (136), LMW-PTP (137) and Cdc25 (138).

Nitric oxide ($NO^{\cdot}$) is another oxidant involved in PTP oxidation. S-nitrosylation has been shown for several PTPs such as PTP1B, SHP-1, SHP-2 and PTEN (131, 139-142). Peroxynitrite, formed by $O_2^{\bullet-}$ and $NO^{\cdot}$, has been shown to irreversibly oxidize PTP1B, LAR and CD45 (143).

Other oxidants which have been described to oxidize PTPs, directly or by induction in cell systems, include superoxide (144), GSSG (144-147), hydrogen sulfide (131), lipid

peroxides (148, 149), hydroperoxides (150), peroxymonophosphate (151), hypothiocyanous acid (152), arcenic trioxide (153), pyrroloquinoline (154), polyaromatic quinones (155, 156) and peroxymonocarbonate (157).

Oxidation variants of active site cysteine of PTPs: Several different oxidative forms of the active site cysteine of PTPs have been reported depending on the oxidant involved and structural features of the particular PTP.

Upon exposure to ROS the active site cysteine forms an initial sulfenic acid (-SOH). Exposure to higher levels of ROS leads to irreversibly oxidized sulfinic ($-SO_2$) or sulfonic ($-SO_3$) forms (158).

Two separate studies identified a sulphenyl-amide form of PTP1B that functions as a protective intermediate against irreversible forms (129, 159, 160). The mechanism involves conversion of the sulphenic acid intermediate into a sulphenyl-amide where the sulphur atom of the catalytic cysteine is covalently linked to the main chain nitrogen of an adjacent residue. In addition to PTP1B the sulphenylamide form has also been shown for the D2 domain of RPTPα (161).

Other oxidative forms described for PTPs are intramolecular disulfides. Upon oxidation PTEN forms a disulfide between cysteine 71 and 124 (135). Furthermore, analysis of SHP-1 and SHP-2 treated with oxidants showed formation of a disulfide involving a nearby "backdoor cysteine" with an adjacent amino acid (135). PTP Lyp has also been described to form intramolecular disulfides (162). Oxidized versions of non-classical PTPs, including LMW-PTP and MKP3, containing disulfides have also been described (138, 163-166) (136).

Inter-molecular disulfides involving the catalytic site cysteine have been described for RPTPα D2 domain (167). Interestingly, the dimer formation was still maintained after reduction of the disulfide indicating other stabilizing interactions. In the same study, disulfide-mediated dimerization was shown for PTP-LAR although the specific cysteines involved were not determined (167). Furthermore reductant-sensitive dimerization was also found to inactivate PTPRO although the exact mechanism was not determined (168). Regarding cytosolic PTPs, SHP-2 has been described to be

regulated by inter-molecular dimerization though the specific mechanism is yet to be determined (169).

Nitrosylation of PTPs has been described both *in vitro* and in cell-based systems. This has been seen after treatment with S-nitrosothiols or NO^{-} releasing compounds (139, 170). Analysis of PTP1B using quantitative MS demonstrated that the active site cysteine was the cysteine most susceptible to S-nitrosylation. Interestingly, the S-NO form of the active site cysteine seems to protect from further irreversible oxidation (171). Also SHP-1 and SHP-2 were shown to be S-nitrosylated by oxidative stress as indicated by regained PTP activity after reduction of with ascorbate (139).

PTPs can also be modified through glutathionylation of the active site cysteine (172). Glutathionylation have been shown *in vitro* for a panel of PTPs, such as PTP1B, SHP-1, PTPL1 and PTEN (146, 147).

Sulfhydration has been described in a recent publication. Hydrogen sulfide was found to oxidize PTP1B through sulfhydration with same efficiency as H_2O_2 and $NO^{\cdot}$. Production of hydrogen sulfide (H_2S) was induced by endoplasmatic reticulum (ER) stress resulting in PTP1B oxidation (131).

Reductants involved in PTP oxidation: Growth factor-induced oxidative inactivation of PTPs has been shown to be a reversible process. Re-activation of oxidized PTPs is dependent on the two major cellular reducing systems, the Trx system and GSH system (173-175). Until now most studies have been done *in vitro*, exposing different PTPs to cellular reductants. Several PTPs (PTP1B (131, 176), PTEN (147), SHP-1 (132), SHP-2 (132), Cdc25A (177) and Cdc25B (178)) have been shown to be affected by Trx mediated reactivation. With regard to the glutathione system, GSH mediated reduction has been shown for PTP1B (131, 176) , PTEN (147), SHP-1 (132) and SHP-2 (132) . In addition, Grx has also been shown to reduce PTP1B (176) and LMW-PTP (137). A more detailed description of potential differential sensitivity of different PTPs to the two major reducing systems is provided below (see 4.3.2).

4.3.1. Methods detecting oxidation of PTPs

Different methods have been developed to monitor the redox state of PTPs. The majority of methods rely on blocking the reduced pool of PTPs and restoration of oxidized PTPs followed by subsequent analysis. In more detail, after stimulation of cells, PTPs are oxidized into a sulfenic acid form or a sulfenylamide intermediate. This will create one pool of catalytically inactive oxidized PTPs and another pool of reduced and catalytically active PTPs. By use of alkylating agents, such as iodoacetic acid (IAA), it is possible to block the reduced fraction of PTPs. Different technical approaches enable analysis of an increase in the oxidized fraction of PTPs as described below (See also **Figure 2**). To minimize experimentally induced oxidation all procedures are performed under anaerobic condition. Also all solutions are degassed prior to use to eliminate the majority of soluble oxygen in the solutions.

4.3.1.1. Modified phosphatase in-gel assay

Originally the in-gel assay was developed to monitor PTP expression by denaturing proteins from total cell lysate on a SDS/PAGE gel containing radioactive PTP substrates (179). In the modified in-gel assay, developed by *Meng et al.* (127), reduced PTPs are irreversibly alkylated for selection of oxidized PTPs. PTPs of total cell lysate, or immunoprecipitated PTPs, are subsequently recovered by refolding on a SDS-polyacrylamide gel under reducing conditions. The gel is contains a radioactively labeled substrate (p^{32}-labeled poly (GluTyr)) allowing reaction with re-activated PTPs. The modified in-gel assay works well with cytosolic PTPs but many receptor-like PTPs fail to fully renature. Furthermore, identification of the phosphatase of interest can be done by immuno-depletion experiments or by size estimates (127).

4.3.1.2. Antibody-based OxPTP assay

The OxPTP assay utilizes an antibody developed to recognize the conserved PTP signature motif (VHCSAG) and the hyperoxidized sulfonic acid form (SO_3) of the active site cysteine. In this method stimulated cells are lysed under presence of an alkylating agent such as IAA to block the reduced pool of PTPs. PTPs are immobilized using immuno-precipitation followed by hyper-oxidation to stable sulfonic acid forms by pervanadate. Visualization of oxidized PTPs is performed using immunoblotting with the designated OxPTP antibody. With good performing immunoprecipitating antibodies this assay is able to recognize endogenous oxidized PTPs (180).

4.3.1.3. PTP activity assay

In this assay PTP activity is determined using a radioactive labeled phosphotyrosine containing peptide as a substrate (AEEEIpYGEFEAKKK). The activity of total cell lysates or immunoprecipitated PTPs is measured with or without reducing agent (DTT). The oxidized fraction is deduced from the two different activity assays (181).

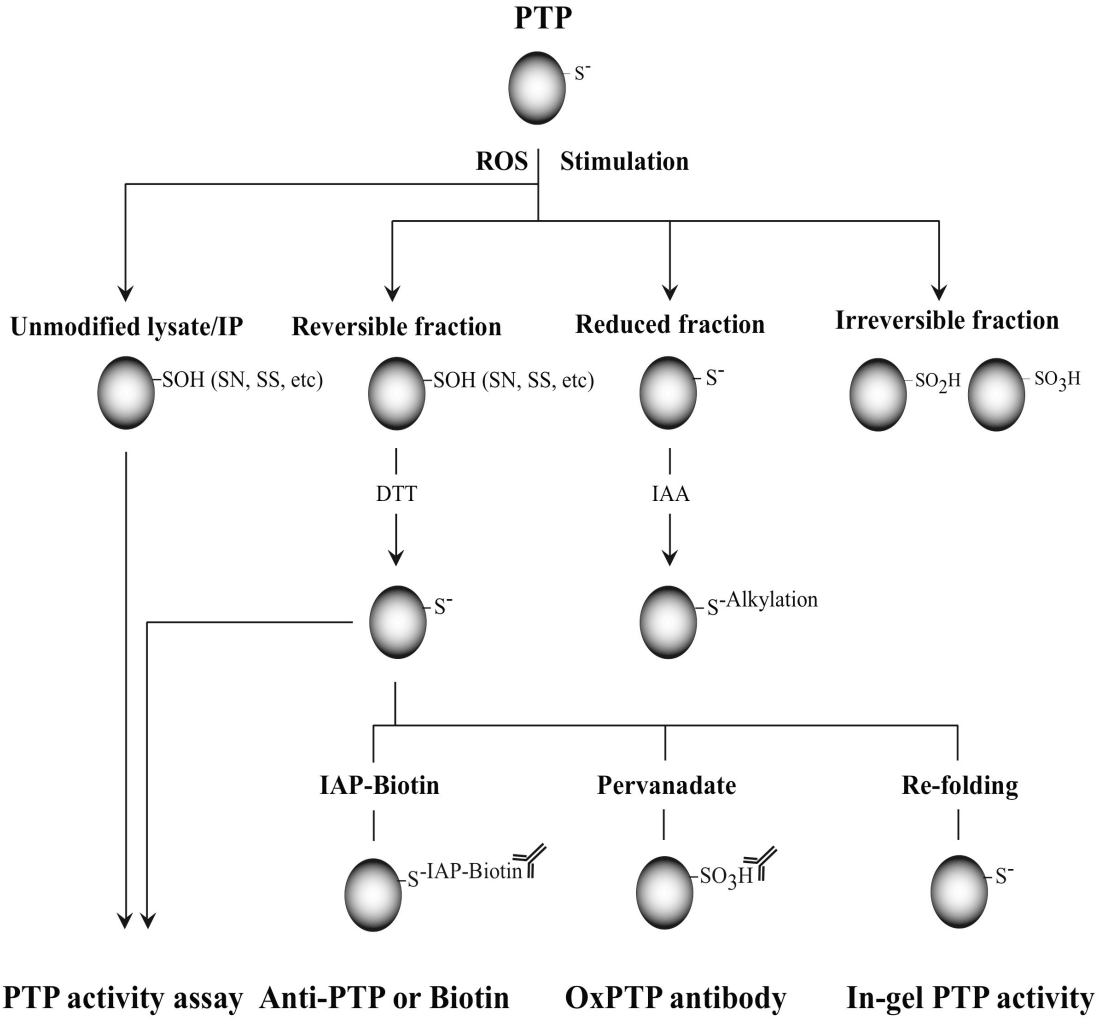

Figure 2. Schematic overview of methods for detection of reversible oxidized PTPs.

4.3.1.4. Cysteine-labeling assay

The modified cysteine-labeling assay involves detection of oxidized PTPs using a biotin-tagged alkylating agent IAP-biotin (182). Cells are lysed under alkylating conditions using IAA that block the reduced pool of PTPs. Next step involves a buffer exchange for removal of IAA. Subsequently, oxidized PTPs are reduced by dithiothreitiol (DTT) followed by labeling with IAP-biotin that alkylates previously oxidized PTPs. Labeled PTPs can be purified using streptavidin-Sepharose beads and

visualized using immuno-blot analysis. The assay is performed under mildly acidic conditions (pH 5.5) to limit labeling of cystein containing-proteins. At low pH conditions most cysteines in proteins will be in their protonated thiol (SH) state as opposed to the catalytic cysteine of PTPs that have a low pKa and therefore remain as a thiolate (S^-).

4.3.2. Specificity of PTP oxidation

Inactivation of PTPs by oxidation is an intrinsic component of signaling following activation of many types of cell surface receptors. It is therefore crucial that PTP oxidation is spatially and temporally regulated. Several distinct mechanisms have been shown to contribute to specificity of PTP oxidation:

- An intrinsic differential sensitivity between different PTPs, and PTP domains in RPTPs, with regard to susceptibility to oxidation and to the activity of reducing systems.

- Temporally and spatially controlled productions of ROS, and modulated activity of ROS scavenging enzymes, which lead to inactivation of a restricted key fraction of PTPs.

Differential sensitivity to oxidation of different PTPs and PTP domains: Several studies have compared PTPs and PTP domains with regard to sensitivity to oxidation. Some of these studies have compared the PTP domains of receptor-like PTPs, which commonly have two tandem domains where the catalytic activity resides in the first domain (D1) (183, 184). The second domain (D2) has been proposed to have regulatory functions (185).

Intriguingly, *in vitro and in vivo* studies of RPTPα have revealed differential sensitivity of the two domains towards oxidation. Utilizing the OxPTP antibody method, described above, higher sensitivity to oxidation was observed of the membrane distal domain D2 when compared to D1 upon UV-irradiation *in vivo*, and *in vitro* treatment with H_2O_2 (185, 186). In addition, it is well recognized that catalytic activity is pH dependent (134). Susceptibility to oxidation has also been found to be highly dependent on pH (130). Moreover, *in vitro* analyses using an activity-based assay, and H_2O_2 as

oxidant, found PTEN and Sac-1 to be highly sensitive, PTPL1/FAP-1 to be moderately sensitive and the myotubularin phosphatase to be almost resistant to inactivation (147).

Domains outside of the PTP domain have also been shown to affect sensitivity to oxidation. Studies utilizing the OxPTP antibody method investigated differential sensitivity towards oxidation between SHP-1 and SHP-2. The two structurally homologous PTPs containing tandem SH2 domains were not readily oxidized compared to other PTPs. Interestingly, the SH2 domain seemed to protect from oxidative inactivation as determined from analyses comparing the full length proteins with catalytic domains only. This effect was particularly prominent for SHP-1 (133).

Intrinsic differences in sensitivity to reductants: Some initial work has also been done to characterize potential differences in sensitivity of PTPs to different reductants.

Early work investigated the reducing effects of the Trx system, the Grx system, DTT and GSH alone on recombinant PTP1B (176). The Trx system, used at a concentration of Trx (3.8μM), TR (0.2 μM), NADPH (200 μM), was almost as efficient in reducing recombinant PTP1B as 4mM DTT. On the contrary, the Grx system showed less capacity to reduce PTP1B, and treatment of GSH alone had least effect (176). Another study compared reactivation of recombinant PTP1B inactivated by H_2O_2, H_2S or NO. Strikingly, the Trx system was 190-fold faster in reactivating PTP1B inactivated with H_2S, compared to DTT, while oxidized or nitrosylated PTP1B showed a similar rate of reactivation. GSH showed much lesser effect on reactivation, as compared to Trx and DTT, irrespectively of the oxidant used (131).

SHP-2 and SHP-1 has also been characterized with regard to reactivation by various reducing systems. Using a set-up where recombinant protein was inactivated until only 5% remained, both SHP-1 and SHP-2 was able to be re-activated to 80% with DTT and GSH. The Trx system had no ability to reactivate either SHP-1 or SHP-2. Interestingly, in variants lacking the SH2 domains, ΔSHP-2 was sensitive to Trx system but not ΔSHP-1 (132).

Localized production of oxidants at the plasma membrane enable inactivation of PTPs: Several types of stimuli, such as growth factor receptor stimulation, activation of GPCR and UV-irradiation, results in a local production of ROS at the plasma membrane. These free radicals are short-lived entities, and a localized concentrated accumulation of ROS is thus occurring allowing a spatially restricted oxidative inactivation of specific PTPs.

Induction of migration in endothelial cells has been shown to induce a localized accumulation of ROS that leads to inactivation of PTP-PEST. In this system, the scaffold protein Hic-5 and the adaptor protein TRAF4 bind to the NADPH oxidase subunit p47phox that mediates ROS production responsible for oxidation and inactivation of PTP-PEST (187).

Early work by Tonks and co-workers showed PDGF induced oxidation of SHP-2 in Rat-1 cells. Intriguingly, only the recruited fraction of SHP-2 was inactivated by oxidation suggesting a localized ROS accumulation in close proximity to the receptor (127). Furthermore, PDGF-stimulation of cells has been shown to induce inactivation of membranous associated PTPs, but not cytosolic PTPs (188).

Localized inactivation of a ROS scavenger upon growth factor activation: Recent work has identified a localized redox-dependent inactivation of the ROS scavenger Prx1. Stimulation of various receptors such as PDGF, EGFR, B-cell receptor (BCR) and T-cell receptor (TCR) in different cells types induced a time-dependent inhibitory phosphorylation of Prx1 at Tyr194 (189). NOX1 deficient cells reduced the Prx1 specific phosphorylation suggesting a ROS dependent regulation. Only Prx1 present at the membrane was found to be inactivated where Src family kinases contributed to phosphorylation. In cells with downregulated c-Src expression a partial decrease in PDGF-induced Prx1 phosphorylation was seen. Furthermore, Prx1 was found to be phosphorylated at Tyr194 during wound healing of a cutaneous injury in mice. Finally, Prx2 was shown to display a different and distinct mode of regulation including hyperoxidation in cells exposed to global stress (189).

4.3.3. PTP oxidation in cell signaling

It is now well established that stimulation of a wide variety of cell surface receptors lead to a ROS mediated increase in PTP oxidation (127, 176, 190) **(Figure 3)**. A number of different cell surface receptor-controlled mechanisms for modulation of ROS have been identified.

Most analysis has addressed the RTK-mediated activation of NOX enzymes responsible for ROS production in close proximity to the RTKs. ROS production induced by NOX enzymes has been connected to PDGF, EGF and insulin-receptor (IR) signaling (191, 192). However, other sources such as mitochondrial produced ROS have been shown to impact on PTP oxidation. Mitochondrial-derived ROS have been shown to inactive PTPs during ischemia/reperfusion (181). In addition, p66Shc dependent mitochondrial-derived ROS have been shown to oxidize PTEN upon insulin signaling (193). A connection between growth factor-induced p66Shc dependent ROS production and PTP oxidation have also been suggested from analyses of PDGF signaling in p66Shc deficient fibroblasts which revealed decreased PTP oxidation in the knock-out cells (*Paper III*). ROS production through mitochondria and 5-lipoxygenase (5-LOX) enzyme, leading to inactivation of SHP-2, has been shown following engagement of integrin receptors (194). Furthermore, studies have implicated arachidonic acid release by phospholipase A as a source of oxidants leading to PTP oxidation (195, 196). As outlined above, activation of cell-surface receptors can also modulate ROS production through inhibitory effects on ROS scavengers, such as Prx1 (189).

The following paragraphs give some examples of studies which have demonstrated that PTP oxidation is an intrinsic component of signaling leading to cell proliferation and migration. Most of these studies have been performed using cell based model systems. It should be noted however, that some studies have also showed cell signaling-relevant PTP oxidation *in vivo (181)*.

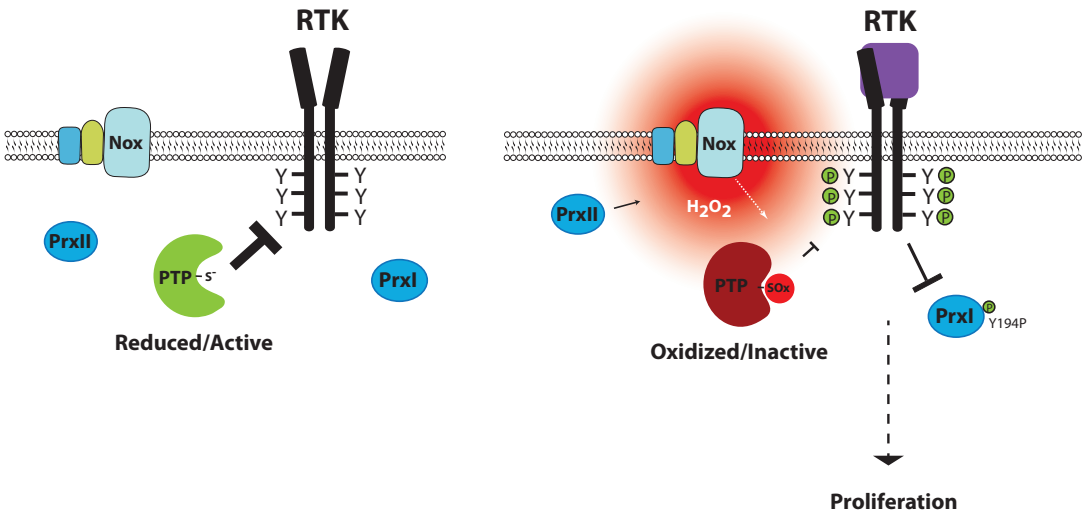

Figure 3. Schematic overview of receptor tyrosine kinase (RTK) mediated ROS production and subsequent PTP oxidation.

Reversible PTP oxidation and its impact on cell proliferation: The first and most important studies linking PTP oxidation to mitogenic growth factor signaling analyzed PDGF signaling in vascular smooth muscle cells (VSMCs) and EGF responses in A431 cells.

The study by Sundaresan *at al.* demonstrated that treatment of vascular smooth muscle cells (VSMCs) with PDGF ligand transiently increased the intracellular concentrations of H_2O_2. The cellular effects, such as tyrosine phosphorylation, proliferation and chemotaxis could be abrogated by treatment with antioxidants N-acetylcysteine (NAC) or catalase (197). Furthermore, exogenous H_2O_2 treatment of human fibroblasts (NIH3T3) cells increased activation of the mitogenic ERK signaling pathway, which also could be reverted by antioxidant treatment (198).

Rhee and co-workers showed the first evidence of PTP oxidation. Stimulation of human epidermoid carcinoma cells (A431) with EGF ligand was shown to reversibly inactivate PTP1B as shown by radiolabelled alkylation of the reduced fraction (176).

A contribution of PTP oxidation to the signaling leading to transactivation of EGFR by GPCRs was demonstrated in analyses of cardiomyocytes (199). Upon U-II treatment, activating the AT^1R receptor, oxidation of SHP-2 but not PTP1B was demonstrated. Importantly, transactivation of the EGF receptor could be abrogated by NAC treatment.

It was thus suggested that U-II mediated ROS induction is responsible for ligand independent EGFR activation (199)

Since the original study by Sundaresan et al, additional studies have confirmed the role of PTP oxidation in mitogenic PDGF signaling. PDGF induces reversible oxidation of SHP-2 upon activation of PDGF receptors in Rat-1 cells (127). In these cells, recruitment of GAD and the p85 subunit of the PI3 kinase, to the receptor seemed dependent on oxidative inactivation of SHP-2. Induction of PDGFR also led to increased activation of downstream effectors ERK MAP kinases. Furthermore, inactivation was critical for a ROS-induced proliferative response. In another study, antioxidant treatment decreased PDGF-dependent proliferation of VSMCs in an *in vivo* restenosis model. The antioxidants NAC or tempol decreased PDGF induced proliferation of VSMCs in rat carotid arteries (200).

Interestingly, different studies have revealed a regulatory effect of ROS scavengers on PDGFR signaling. Prx2 was shown to modulate PDGFR activation and the specific activity of membrane associated PTPs. Overexpression of the ROS scavenging enzyme suppressed both phosphorylation of the receptor and PLC-γ in human aortic VSMCs. In addition, neointimal thickening in injured carotid arteries was decreased in Prx2 KO mice compared to WT (188). In another study, overexpression of Grx decreased PDGF dependent proliferation of myocardiac H9c2 cells by modulating activity of LMW-PTP (137). Additionally, PTEN oxidation has been analysed upon stimulation of cells with various growth factors. PDGF and EGF receptor activation induced PTEN oxidation which, increased PI3 Kinase activity and downstream effectors AKT. These pro-proliferative signals could be abrogated by overexpression of Prx2 or increased by overexpression of NOX1 (192).

Reversible PTP oxidation and its impact on cell migration and adhesion: Several studies have identified reversible oxidation of PTPs as a central component of cell signaling also in the context of adhesion and migration.

Early work identified PTP oxidation as a central component of integrin signaling and cell adhesion (201). Activation of integrin receptors in fibroblasts induced a Rac-1 dependent ROS where both NOX but mainly LOX enzymes were identified as sources. Inactivation of LMW-PTP and tyrosine phosphorylation of FAK was crucial for cell spreading and cell attachment. Reactivation of PTP activity by antioxidant treatment abrogated the cell phenotype.

A subsequent study from the same group revealed the importance for mitochondrial-induced ROS at an early stage of cell attachment (194). Later stages of the cell spreading process specifically depended on LOX-induced ROS production. Furthermore, SHP-2 oxidation increased FAK and SHPS-1 phosphorylation (194) **(Figure 4).** Studies have identified the 5-LOX metabolite LTB4 to be responsible for stimulation of NOX to produce ROS (97).

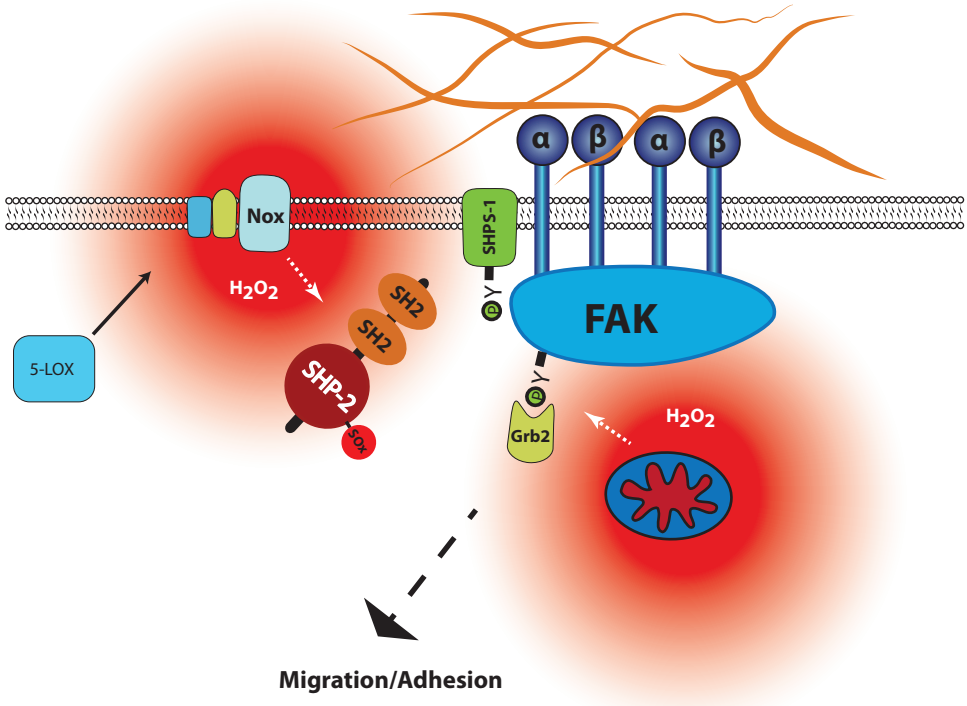

Figure 4. Overview of integrin-mediated ROS production.

PDGF is known to be a potent chemotactic factor targeting fibroblasts. Several studies have linked PTP oxidation and PDGF receptor signaling to “membrane-ruffling” which is a migration related phenotype. Lipid peroxides were identified as strong inducers of TC-PTP- and SHP-2- oxidation in GPx4 deficient cells exposed to PDGF ligand (148). “Membrane-ruffling” was significantly increased in GPx4 deficient cells induced by PDGF ligand. Another study also demonstrated a PDGF dependent induction of “membrane-ruffling” after subjecting fibroblasts to H/R. H/R induced oxidation of multiple PDGFR-associated PTPs including SHP-2, DEP-1and PTP1B (181).

In endothelial cells VEGF is a known regulator of migration and cell adhesion. In a recent study oxidation of VEGF-induced ROS were linked to LMW-PTP oxidation important for FAK phosphorylation and endothelial cell migration. Intriguingly, the LMW-PTP oxidative form was identified as a S-glutathione and peroxynitrite was suggested as main oxidant (145). Another study has shown that extracellular SOD-derived ROS increases phosphorylation of the VEGFR leading to an induced migration by oxidizing PTPs (PTP1B and DEP-1) (202).

Adhesion of T-cells is partially regulated by a cross-talk between T-cell receptors and integrins. SHP-2 oxidation after T-cell receptor activation is a crucial component for activation of this signaling pathway. The two SHP-2 substrates involved, ADAP and Vav1, are important for T-cell receptor-induced adhesion (203).

Growth factor receptor independent redox environment is regulated by both ROS producing enzymes and major antioxidant nodes such as Trx and GSH system. Several studies have identified ROS modulators such as Prx and GPx to impact on PTP oxidation (204). Inactivation of GPx4 induces an increase in cellular lipid peroxides oxidizing PTPs (148). Intriguingly, in vitro analysis revealed lipid peroxides more potent in inactivating PTPs as compared to H_2O_2.

4.3.4. PTP oxidation and disease

Several different pathological diseases such as reperfusion injury, inflammation and cancer have been associated with deregulated levels of free radicals and antioxidant defences. For example, proliferating cancer cells have been shown to display an increased production of free radicals (205). Other reports have described a compensatory up-regulation of antioxidant enzymes through the Nrf2 system (206). ROS-sensitive signaling pathways have been described to be elevated in many different types of cancers such as mitogen-activated (MAP) kinase/ERK cascade, PI3K/AKT and NF-κB. Other studies have linked NOX1-mediated ROS production to a transforming phenotype (207). Furthermore, NOX1 has been shown be crucial for Ras dependent transformation (208).

Several studies thus implicate imbalances between ROS production and antioxidant defences in pathological settings. Some studies have addressed to what extent these imbalances impact on PTP oxidation. Previous work has shown oxidative inactivation of the tumor suppressor PTEN upon mitogenic stimulation (192). Mitochondria-derived ROS in cancer cells increase Akt activation and also leads to oxidative inactivation of the PTEN phosphatase (209). Higher levels of ROS and oxidative inactivation of PTEN has been documented in T-ALL. Treatment with reductants increased T-ALL cell death, suggesting an apoptosis-protective role of the constitutively high ROS levels (210).

The carcinoma cell lines A431 and HepG2 also showed a constitutively oxidative inactivation of PTP1B. Interestingly, inhibition of ROS levels in HepG2 cells decreased anchorage-independent growth indicating an involvement of ROS and PTP oxidation in maintaining the transformed phenotype (158).

As outlined above the receptor-like PTP DEP-1 has been suggested to have tumor suppressor properties. DEP-1 has also been characterized as a negative regulator of WT FLT3 autophosphorylation and signaling (211). An important study established that oncogenic signaling through the ITD-FLT3 onco-protein requires oxidative inaction of DEP-1 (212). This study showed that AML-related mutant version of FLT3 ITD-expressing cells produces higher levels of ROS than WT FLT3. In these cells, DEP-1

was found to be partially inactivated by oxidation. Inhibition of ROS production reactivated DEP-1and attenuated transformation both *in vivo* and *in vitro* (212).

Finally, PTP oxidation has also been implicated in monocytes of metabolic disorder such as obesity and diabetes. The chronic inflammation state of these disorders involves release of chemoattractant MCP-1 that primes monocytes and recruits macrophages into vascular lesions where transformation of these cells into a hyper-migratory and pro-inflammatory phenotype takes place. The priming of monocytes involves induction of ROS production by activation of the NOX4 enzyme. The increased ROS levels were shown to inactivate MKP-1 and thereby promote monocyte migration and adhesion. These findings indicate a connection between redox regulation of MKP-1 and a monocyte-dependent induction of chronic inflammation (213).

5. Aim of studies

- To investigate if hypoxia followed by reoxygenation is associated with an increase in oxidation of PTPs both in cell culture and tissue model.
- To investigate if long term hypoxia (24h) affects PTP expression and protein level.
- To investigate if mitochondrial produced ROS modulate growth factor signaling by oxidation of PTPs.
- To evaluate potential differential antioxidant effects of the thioredoxin system on PTP1B and SHP-2.

6. Results

6.1. Paper I

Hypoxia followed by re-oxygenation induces oxidation of tyrosine phosphatases

Previous work has shown that hypoxia followed by re-oxygenation/reperfusion (H/R) is associated with an increase in levels of ROS molecules such as H_2O_2, $O_2^{\cdot-}$ and $^{\cdot}OH$ (90, 214, 215). H/R studies on cardiomyoblasts have revealed mitochondria as the major site for H_2O_2 and $O_2^{\cdot-}$ production (216). Furthermore, it is well established that PTPs are negatively regulated by reversible oxidation upon several different stimuli. Still the impact of elevated ROS levels on PTPs after H/R has yet not been explored.

Here we investigate whether H/R-induced mitochondrial ROS results in increased PTP oxidation.

We show that NIH3T3 cells subjected to H/R increase oxidation of two classical tyrosine phosphatases, the cytosolic SHP-2 and receptor-like DEP-1. Oxidation was detected using the OxPTP assay that utilizes a monoclonal antibody recognizing the sulfonic acid forms of the active site cysteine. In addition, PTP activity assays on total cell lysate and immuno-precipitated PTP1B showed a decrease in the fraction of reduced total PTPs, as well as PTP1B. To substantiate these findings we performed activity assays in cardiomyoblasts. Cardiomyoblasts subjected to H/R show an increase in total PTP oxidation as well as in the oxidation of immuno-precipitaed SHP-2. Since PTPs regulate phosphorylation state of tyrosine kinase signaling, we analyzed the effects of H/R on the downstream targets Erk1/2. NIH3T3 cells treated with H/R indeed showed an increase in Erk1/2 phosphorylation. Pretreatment of cells with the antioxidant NAC abrogated Erk1/2 activation. This indicates a link between H/R mediated Erk1/2 activation and ROS-induced inactivation of PTPs.

Several PTPs have been shown to modulate PDGFR signaling by dephosphorylating tyrosine residues located outside the kinase domain. When these specific tyrosine sites on the PDGFβR are activated, SH2 domain containing signal transduction molecules can dock and propagate downstream signaling. Previous studies have shown inactivation of PTPs upon growth factor signaling. We therefore investigated if cells subjected to H/R displayed an enhanced response following PDGF stimulation. Indeed,

PDGF-induced formation of membrane ruffles was increased in NIH3T3 cells subjected to H/R. The enhanced signaling upon H/R could be abolished by pretreatment of cells with the antioxidant NAC supporting the hypothesis of oxidative inactivation of PTPs. To further consolidate these findings we specifically analysed the activity of PDGFβR associated PTPs. H/R-induced NIH3T3 cells were stimulated with PDGF in the presence of a PDGFR tyrosine kinase inhibitor AG1296. The activity of receptor associated PTPs was determined through measurement of the decrease in PDGFβR phosphorylation. Cells exposed to hypoxia displayed a significant decrease in PDGFβR dephosphorylation compared to control-treated cells.

We conclude from these cell-based studies that hypoxia-induced ROS production results in increased PTP oxidation and increased PDGFβR growth factor signaling.

Finally, we tested if the H/R induced PTP oxidation found in cell culture also could be reproduced in an *ex-vivo* tissue model; the Langendorf model of perfused heart. Ischemia followed by reperfusion (I/R) of *ex-vivo* hearts has previously been shown to induce transient ROS production (90). PTP activity measurements were performed on total cell lysates from control hearts, and hearts treated with I/R. A significant increase in PTP oxidation was shown in tissue extracts of hearts subjected to I/R. In addition immuno-precipitated SHP-2 from I/R subjected heart extracts displayed a significant increase in oxidation.

Thus, these experiments demonstrate for the first time that *ex vivo* ischemia followed by reperfusion is associated with an increased oxidation of classical PTPs.

6.2. Paper II

Hypoxia enhances platelet-derived growth factor signaling in the pulmonary vasculature by down-regulation of protein tyrosine phosphatases

The pulmonary arterial wall is composed of an outer adventitial fibroblast layer, intervening vascular smooth muscle cells (VSMCs), and an inner endothelial cell wall. Pulmonary hypertension (PH) is a disease of the pulmonary arteries that is characterized by increased vascular resistance and sustained elevation of pulmonary arterial pressure leading to right heart failure. PH is associated with structural damage in the vascular architecture, including increased proliferation and migration of VSMC and adventitial fibroblasts as well as injury of endothelial cells (ECs) (217-219). The combination of these vascular insults results in hypoxia. PDGFβR signaling plays an important role in vascular development and remodelling (220, 221).

In this paper we investigate if hypoxia modulates PDGFβR signaling in pulmonary vasculature. Treatment of cultured human pulmonary smooth muscle cells (hPSMCs) with PDGF induced a dose-dependent increase in proliferation and migration of hPSMCs under normoxic and hypoxic condition. Cells subjected to 24h hypoxia showed a significant increase in both proliferation and migration as compared to normoxic cells. Previous work has identified PI3K and PLCγ as critical mediators of both proliferation and migration of VSMCs (222). Therefore we investigated if treatment of hPSMCs with the inhibitors of PI3K (Ly294002) and PLC-gamma (U73122) would block hypoxia induced proliferation and migration. Indeed, both inhibitors attenuated proliferation and migration in both normoxic and hypoxic cells. Expression levels of the PDGFβR displayed no significant difference between normoxic and hypoxic condition. In addition, total phosphorylation levels of the receptor were not affected by hypoxia treatment. Intriguingly, when cells were treated with PDGF, a marked increase in total receptor phosphorylation was seen in cells subjected to hypoxia as compared to normoxia. To further elucidate the specific mechanism involved, the specific phosphotyrosine sites of PI3K and PLCγ were analyzed. Indeed, the PDGFβR binding sites of PI3K and PLCγ (Y751 and Y1021) demonstrated a marked increase in phosphorylation under hypoxic condition and treatment with PDGF. In addition, hypoxia was also associated with an increased PDGF-induced activation of downstream effectors AKT and ERK1/2.

The observed increase in hypoxia-induced PDGFβR phosphorylation could be due to the downregulation of PTP expression and activity. To test this, we performed qRTPCR analysis on hypoxia treated hPSMCs. Analysis of PTPs antagonizing the PDGFβR showed a significant decrease in mRNA levels of PTP1B, DEP-1 and TC-PTP, but not SHP-2. In addition, the total PTP activity and the activity of PTP1B, SHP-2 and DEP-1 were significantly decreased upon treatment with hypoxia. Thus, the increase in tyrosine phosphorylation of the PDGFβR was accompanied by a decrease in antagonizing PTP activity.

To further understand the molecular mechanism, the expression level of HIF-1α was determined. In culture, hPSMCs subjected to hypoxia showed a time-dependent increase of hypoxia-inducible factor 1- α (HIF-1α) expression peaking between 4 and 6 hours. Most interestingly, silencing of HIF-1α by RNA interference resulted in loss of hypoxia-induced PDGFβR phosphorylation. Importantly the effects were decreased to the levels of normoxic cells. In line with these observations, the hypoxia-induced down-regulation of PTP1B, SHP-2, TC-PTP and DEP-1 was blocked. Strikingly, down-regulation of HIF-1α with siRNA resulted in a marked up-regulation of DEP-1 expression under hypoxic treatment. In an independent set of experiments, a pharmacological inhibitor of HIF-1α (2ME2) was applied to hypoxia-induced hPSMCs. Indeed, the enhanced PDGF-dependent proliferation and migration was reversed by treatment of 2ME2.

To determine whether hypoxia regulates PTP expression in *vivo*, a previously established mouse model of PH was used. C57B1/6J mice were subjected to chronic hypoxia (10% O_2) for 3 weeks, which increased their ventricular systolic pressure and right ventricular hypertrophy. Mice treated with chronic hypoxia displayed an increase in muscularization of pulmonary arterioles indicating ventricular hypertrophy. Consistent with the in *vitro* findings, hypoxia-induced vascular pulmonary re-modelling induced an increased PDGFβR phosphorylation in the vascular wall. Additionally, expression levels of PTP1B, TC-PTP and DEP-1 were significantly decreased.

Our results suggest that hypoxia-induced PH results in the down-regulation of PTPs and concomitant increase in PDGFβR phosphorylation levels.

6.3. Paper III

Mithochondial Reactive Oxygen Species produced by p66Shc regulated PDGF signaling through Protein Tyrosine Phosphatase Oxidation

Growth factor receptor signaling trigger NOX enzyme dependent ROS production at the plasma membrane (191). p66Shc is a redox enzyme that generates mitochondrial ROS utilizing electrons from the respiratory transport chain for reduction of O_2 to H_2O_2 (223). Mitochondrial-derived ROS production upon p66Shc activation has been shown to be critical for stress-induced apoptosis. Previous studies have described growth factor induced, oxidative inactivation of PTPs where NOX enzymes at the plasma membrane are identified as the source of ROS production. It is possible that growth factor induced signaling also induces mitochondrial ROS production, but this remains to be shown.

The aim of this study is to investigate if growth factor induced signaling is modulated by oxidative inactivation of PTPs by mitochondrial ROS dependent on p66Shc. For these studies p66Shc knockout MEFs (p66Shc KO) were used.

Activation of the p66Shc enzyme is mediated by phosphorylation at serine-36 (S36P) and subsequent translocation of p66Shc to the mitochondria where ROS production is induced. To investigate possible involvement of p66Shc in PDGFβR signaling, NIH3T3 cells were stimulated with PDGF-BB for analysis of S36P phosphorylation. Activation of PDGFβR with ligand induced activation of p66Shc by phosphorylation of S36. After stimulation with ligand, total PDGFβR phosphorylation was reduced in p66Shc KO cells as compared to WT. PDGF-BB stimulation of WT cells induced site-specific phosphorylation of the PDGFβR on tyrosine pY771 and pY1021. In contrast, a smaller increase in phosphorylation was seen upon ligand stimulation of p66Shc KO cells indicating that p66Shc-derived ROS is required for PDGFβR phosphorylation. To further investigate if these effects were dependent on p66Shc induced ROS production, analysis was performed in presence of the antioxidant NAC. Results revealed a clear decrease in phosphorylation of the PDGFβR in WT cells but not in p66Shc KO cells. NAC treatment had no effects on the phosphorylation status of individual phosphotyrosine sites in p66Shc KO cells. From these results, we concluded that PDGFβR phosphorylation is dependent upon p66Shc activation and mitochondrial ROS production.

Ligand stimulation of the PDGFβR leads to phosphorylation and activation of downstream effectors AKT, ERK1/2 and PLCγ-1. Interestingly, p66Shc KO cells showed reduced phosphorylation of these three downstream effectors after ligand stimulation strengthening the role of p66Shc ROS production in growth factor signaling. Thus, these experiments identify a connection between the reduced phosphorylation of the PDGFβR in p66Shc KO cells and decreased activation of known downstream targets, AKT, ERK1/2 and PLCγ-1.

Previous studies have shown inactivation of PTPs by reversible oxidation upon growth factor stimulation. Plasma membrane-localized NOX enzymes have been hypothesized to be the main source of ROS production. Little is known if mitochondrial ROS contributes to growth factor induced inactivation of PTPs. To elucidate the possible contribution of mitochondrial-derived ROS on PTP oxidation, PTP oxidation was directly assessed in WT and p66Shc KO MEFs using different assays. Stimulation with increasing concentrations of PDGF increased oxidation of a panel of soluble PTPs. Specific PTP oxidation after PDGF stimulation was seen for PTP1B and SHP-2. However, PDGF induced PTP oxidation was less prominent in p66Shc KO cells indicating a role of p66Shc induced mitochondrial ROS on PTP oxidation. These results led us to conclude that mitochondrially-derived ROS contribute to oxidative inactivation of PTPs during PDGFβR signaling.

Ligand stimulation of the PDGFRβ is linked to an increase in proliferation and migration where activation PLCγ-1 is known to induce migration. Since PDGFβR pY1021 phosphorylation, and PLCγ-1 activation, is reduced in p66Shc KO cells we performed analysis of ligand-induced migration. Indeed, KO cells showed no migratory response after PDGF stimulation. In contrast, WT cells responded with a 2-fold increase. Effects seen in WT cells were abrogated with antioxidant treatment whereas KO cells showed no response to anti-oxidant treatment.
In summary, this study identifies a previously unrecognised role for p66Shc-derived mitochondral ROS in regulating PDGF signaling through oxidation of PTPs.

6.4. Paper IV

Thioredoxin-mediated selective activation of oxidized PTP1B modulates PDGFβ-receptor tyrosine kinase signaling

There are two main NADPH dependent antioxidant systems, Trx and GSH, responsible for re-activating oxidized proteins in cells. The detailed processes of re-activating oxidized PTPs are still largely unknown. Here we investigate the role of the Trx system on reactivating two different PTPs and its possible role on PDGF signaling.

We analyzed the oxidation status of PTP1B and SHP-2 in wild-type (WT) or *Txnrd1*$^{-/-}$ mouse embryonic fibroblasts (MEFs). Activity measurements of immunoprecipitated PTP1B showed a decrease in activity in *Txnrd1*$^{-/-}$ MEFs compared to WT. In agreement with reduced PTP1B activity, direct measurement of oxidation using cysteinyl-labeling assay revealed an increase in oxidation of PTP1B in *Txnrd1*$^{-/-}$ compared to WT MEFs. On the other hand, no significant difference was seen in SHP-2 oxidation between *Txnrd1*$^{-/-}$ MEFs and WT. The glutathione reduction system is upregulated in *Txnrd1*$^{-/-}$ MEFs to compensate for redox imbalance (224). In agreement with these findings no significant difference in ROS levels was found between *Txnrd1*$^{-/-}$ and control cell lines using CellROX redox assay.

To further explore above described findings NIH3T3 cells were treated with H_2O_2 and subsequent lysis. Exogenous Trx system components (Trx1, TrxR1 and NADPH) were added to cell lysates where after PTP activity was assayed. As a control, the strong reducing agent DTT was added to cell lysates to completely restore PTP activity. Results revealed a strong potency of the Trx system to reduce oxidized PTP1B but not SHP-2.

As an additional way to measure the effects of Trx on PTP redox state, we analysed the effects of Trx system components on oxidized PTP1B and SHP-2 in vitro. Activity measurements of PTP1B in combination with Trx, TrxR1 and NADPH showed a dephosphorylation equal to that seen with DTT. In addition, Trx1 re-activated PTP1B in a dose-dependent manner. A combination of NADPH and TrxR1 had a modest ability to reactivate PTP1B. The Trx system had no effects on the redox state of recombinant SHP-2.

Two major conclusions were derived from these experiments. First, we found that PTP1B oxidation is reversible by the Trx system. Secondly, these experiments reveal that different PTPs are differentially sensitive to Trx system components.

PDGFβR signaling is regulated by several different phosphatases including PTP1B and SHP-2. Analysis of total PDGFβR phosphorylation and PTP1B specific tyrosine (pY579-581) upon PDGF-BB stimulation showed an increase in phosphorylation in *Txnrd1*$^{-/-}$ MEFs compared to WT. The enhanced receptor activation in *Txnrd1*$^{-/-}$ MEFs correlated with an increase in ligand dependent proliferation.

We further explored if the enhanced phosphorylation of the PTP1B specific tyrosine (pY579-581) was due to an increase in PTP1B oxidation. Comparison of PTP1B deficient MEFs (*Ptpn1*$^{-/-}$) and PTP1B-reconstituted *Ptpn1*$^{-/-}$ cells revealed an increase in pY579-581 tyrosine phosphorylation of the PDGFβR in MEFs lacking PTP1B as previously described (39). Treatment with the TrxR1 inhibitor auranofin increased the phosphorylation of the PTP1B site in PTP1B reconstituted cells but to a lesser extent in *Ptpn1*$^{-/-}$ MEFs. Thus, these data support the hypothesis of a TrxR1 dependent redox regulation of PTP1B and PDGFβR signaling.

The above-described in vitro data identifies Trx1 as a potent reductant of oxidized PTP1B in combination with TrxR1 and NADPH. It is possible though that other substrates of TrxR1 can contribute to the effects seen in *Txnrd1*$^{-/-}$ MEFs. Recently, a thioredoxin-related protein TRP14 was identified and characterized (225). We performed in vitro experiments with oxidized PTP1B treated with a combination of TRP14 and TrxR1 and NADPH. Most interestingly, TRP14 reduced oxidized PTP1B in a dose-dependent manner. This experiment identifies TRP14 as an additional reductant of possible in vivo relevance for oxidized PTP1B.

7. Discussion

This discussion will be limited to some speculation on the potential roles of regulated and specific PTP oxidation in tumor biology. The discussion is integrating the fact that PTPs have been implied both as tumor suppressors and as oncogenes. It is also considering the notion, emphasized in this thesis, that individual PTPs display distinct and specific profiles with regard to their sensitivity to various oxidants and reducing agents.

The first part of the discussion is based on the assumption that oxidative inhibition of PTPs with tumor suppressor characteristics contributes to the tumor-supportive effects of hypoxia, whereas the second part will discuss the possibility that tumor cell-dependency of the Nrf2-anti-oxidant-system involves maintenance of oncogenic PTPs in a reduced and active state.

Hypoxia and hypoxia/re-oxygenation have been associated with an increase in ROS production (90). Tumor cells are exposed to intermittent hypoxia and largely exist in a microenvironment with irregular blood flow creating a hypoxic/re-oxygenated condition which act as a stimulus for development of a more aggressive phenotype (226). Furthermore, treatment of tumors with anti-vascular endothelial growth factor therapy has been associated with development of a pro-invasive/metastatic phenotype of the remaining tumor cells (227). Finally, pericyte depletion has been shown to enhance metastasis through mechanisms involving increased hypoxia (228). In all these above settings the responses to hypoxia are believed to involve transcription regulated by HIF-1α.

In this thesis we present a series of findings which demonstrate that hypoxia negatively regulates PTP activity in a manner that facilitates receptor tyrosine kinase signaling. Firstly, hypoxia/reperfusion was shown to increase PTP oxidation (paper 1); secondly, hypoxia was shown to reduce transcription of multiple PTPs (paper 2); and thirdly, mitochondria-derived ROS, which is believed to be increased in hypoxia and hypoxia/reperfusion, was shown to also increase PTP oxidation (paper 3). In all these cases the increased PTP oxidation, and decreased PTP transcription, was shown to be associated with an enhanced response to growth factor signaling.

Collectively these findings suggest that the hypoxic microenvironment of tumors should be associated with a reduction in activity and expression of PTPs that would normally act as antagonists of growth factor signaling. It would therefore be highly interesting to profile PTP oxidation and expression in hypoxic tumor tissue to experimentally validate this notion. Positive findings from such studies would also suggest re-activation of oxidized PTP, with tumor-suppressor activity, as a novel candidate approach for anti-cancer therapy.

Independent from the studies discussed above there is emerging evidence that overexpression of oncogenes such as Kras, Braf and Myc, known to increase ROS production, also induce an antioxidant response program through activation of the transcription factor Nrf2 (206). Interestingly, depletion of Nrf2 was shown to revert the transformed phenotype, and to prevent tumor growth in a ras-dependent mouse model of pancreatic cancer (206).

Based on our findings in paper 4, which demonstrate the differential sensitivity of two different PTPs (PTP1B and SHP-2) to the reducing activity of the Trx system, it is legitimate to speculate that the particular redox phenotype of oncogene-transformed cells, characterized both by an enhanced ROS production, and an up-regulation of a certain set of anti-oxidants, will generate a special profile of PTP oxidation which is overall favourable for tumor growth. This profile is predicted, in general terms, to be characterized by an oxidative inactivation of tumor suppressor PTPs and a reduced active state of the oncogenic PTPs. This concept is possible to experimentally test by performing global analyses of PTP oxidation in e.g. ras-transformed cells with or without Nrf2-depletion.

The discussion above suggests full characterization of redox regulation of PTP oxidation would enable new treatment strategies where targeted reactivation or oxidation of a distinct set of PTPs would abrogate tumor development. A promising exemple of this approach has been provided though the generation of a conformation-specific antibody that stabilizes PTP1B in its oxidized state (229). Interestingly, this antibody was shown to block PTP1B reactivation in a manner that also enhanced insulin signaling.

8. Acknowledgements

My supervisor Arne Östman:
Thank you for your patience and endless support. You have given me the opportunity to work independently. At the same time, your door has always been open, and you have been there to support, discuss and help me progress, at any time! I appreciate your stability and warm heart. Nu...”Framåt och vidare!”

To all past and present members of the lab:
Thank you all for a great time together!
Thank you for your patience with me and my absent mindedness!
Jeroen, Martin, Janna, Carina, Elin, Magnus, Monica, Artur, Giovanni, Hanna, Ceran, Sara and Sara, Kai, Patrick, Daniel, Christina, Maja, Marcus, Patrik, Claudia, Francesca, Alessandra, Sabine, Ismael, Helene, Daniel, Chern, Jai, Cristina, Janine and Akira.

Special thanks to my PTP oxidation colleagues, for all hard work together: Åsa (for struggles against the oxygen but also many fun times together), Kai (thanks for all support, help and inspiration) and Jeroen (you´ve really helped me tremendously during this tuff period, with manuscript, figures, reading my thesis, backing me up with experiments and so on. Things wouldn’t have gone so smoothly without you!).

Present and past colleagues at CCK:
Sören, Mehran, Jacob and Arindam; Thanks you guys for great discussions and laughs!
Ann-Gitt, thank you for all support during the years and for good talks.
Lotte, you took great care of me when I first came to CCK and I could always turn to you for help during the years.
Marcela; Thank you for great conversations about art and photography.
Micke, Emma and Martin for great talks about everything and nothing and for keeping my spirit up.

3:rd Floor: Olle, Alena, Natalie, Nimesh, Diana, Martin, Lotte, Linn, Lena, Katja, Eva, Masako, Iryna, Caroline, Per, Dan, Stefan, Jacob, Karin, Mira, Tanya, Anders, Niina, Anna, Bhavesh, Arindam, Nathalie, Karen, Staffan, Simona, Zheng, Irmeli, Sebastian, Madhi, Lars, Sophia, Claire, Aris, Pedram, Veronica, Karin, Suzanne, Carolina, Sattar, Alireza, Marianne, Rainer, Marja, Miguel, Jens, Edel, Ulrika, Johanna, Naveen, Xianli, Bertrand, My, Lina, Mimmi, Ran, Johan, Jonas.

5th floor: Ulrika, Rona, Tamador, Sanna-Maria, Mikael, Inga, Christer, Xiaobing and Monica.

4th floor: Marianne, Salah, Anna, Sofia, Hanif, Fredrik, Klas, Elisabeth, Nina, Sylvia, Dudi, Cinzia, Emarndeena, Susanne.

1th floor: Therese, Andreas, Eva, Anders, Erik, Veronika, Tina, Marteen, Helena, Isabel.

0 floor: Linda, Walid, Emma, Maria, Maria, Giuseppe, Padraig, Angelo, Slavica, Paola, Stig, Barry, Bertha.

Z house: Maria, Dali.

The administration:
To all at the administration and especially: Maria, Monica, Maria, Anne, Annelie och Liselotte.

Others at CCK:
Eva-Lena, Marie, Elle, Elisabeth, Evi,

Collaborators:
Frank and Annette Böhmer, Nick Tonks and Ben Boivin; Thank you for all help and great hospitality during my visits.
Elias Arnér and his group; Thank you for support, advice and great work.
Henrik ten Freyhaus and Stephan Rosenkranz.
Marcus Conrad, thanks for all help during the years.

Vänner:
Pelle, Camilla, Daniel, Maria, Catarina Å, Magnus Å, Catharina U, Hanna, Magnus L, Staffan, Greta, Helena, Magnus C, Linda, Palmar, Sandra, Martin, Thomas, Ben, Katherine, Daniel L, Arindam, Per G, Moses och Gustavo Garcia.

Familj:
Marianna och Georg Bukovinszky. Kedves Bukó! Neked köszönhetem, hogy megszerettem a kémiát és biológiát. Ha Te nem segitettél volna, ma nem jutottam volna el idáig. Mindenkor hálás köszönettel gondolok Rád. (Tack Bukó för all hjälp med kemin!)

Bengt-Arne och Karin, tack för stöd och många trevliga pratstunder.
Gunnar och Margareta, tack för allt stöd och uppmuntran under åren. Ser fram emot sommaren och vin på trallen!
Min käre bror Andreas, jag är glad att jag har dig.

Min kära mor, tack för ditt enorma stöd. Du pendlar varje vecka från Göteborg för att finnas tillhands för dina barnbarn. Din hjälp är för oss som familj är ovärderlig.

Charlotta, Matilda och Elias, älskar er innerligt!

9. Populärvetenskaplig sammanfattning

Vår kropp är uppbyggd av olika typer av celler med unika egenskaper. Exempel på celltyper är muskel-, immun-, lever- och hjärnceller. Kommunikation celler emellan är en viktig strategi för att bibehålla organfunktion samt reglera cellers tillväxt och livstid. Celler kommunicerar med varandra via utsöndring av olika signalsubstanser, till exempel tillväxtfaktorer. PDGF är en tillväxtfaktor som när den binder till sin specifika PDGF receptor på cellens yta, ger signal till cellkärnan vilket kan leda till att cellen delar sig eller förflyttar sig. PDGF receptorn tillhör en proteinfamilj som heter tyrosinkinaser (TKs). TKs är enzymer som genom kemisk modifiering (fosforylering) av andra proteiner inuti cellen fortplantar signalen från cellytan vidare in mot cellkärnan. En annan proteinfamilj, tyrosinfosfataser (PTPs) gör det omvända och tar bort fosforgrupper på proteiner. Tyrosinkinaser och tyrosinfosfataser avgörsålunda tillsammans om en signal utifrån fortplantas inuti cellen eller inte, och avgör därmed om man får en effekt av t.ex. en tillväxtfaktor.

I denna avhandling har jag på närmare håll studerat hur aktiviten av PTPs kan regleras inuti celler. Mer specifikt har jag studerat samspelet mellan PTPs, fria radikaler, antioxidanter och tillväxtfaktorsignalering. Man vet sedan tidigare att PTPs kan modifieras (oxideras) av fria radikaler vilket hämmar aktiviteten av PTPs. Fria radikaler bildas bl.a. i syrefattiga (hypoxiska) miljöer i kroppen, t.ex. i hjärtmuskeln i samband med hjärtinfarkt samt i tumörer. Fria radikaler neutraliseras av s.k. antioxidanter.

I arbete 1 har vi simulerat hjärtinfarkt i en djurmodell. För att simulera hjärtinfarkt skapades tillfällig syrebrist (hypoxi) i hjärtmuskeln. Vi kunde visa att hypoxin resulterade i ökad oxidering av tyrosinefosfataser (PTPs) och ökad tillväxtfaktorsignalering via PDGF-receptorn.

I arbete 2 var syftet att studera PTP aktivitet och tillväxtfaktorsignalering vid långvarig hypoxi i samband med pulmonell hypertension (förhöjt blodtryck i lungkretsloppet). Vid denna kroniska sjukdom ses syrebrist och ökad celldelning i lungans kärl. Vi kunde visa att långvarig syrebrist i celler från lungartärer resulterade i minskad mängd och lägre aktivitet av PTPs Vidare visade vi att minskad PTP aktivitet resulterade i ökad tillväxtfaktorsignalering via PDGF-receptorn samt ökad celldelning och cellmigration.

I arbete 3 har vi närmare studerat mekanismer som reglerar frisättning av fria radikaler inuti celler i samband med tillväxtfaktor (PDGF) signalering. Vi har kunnat visa att tillväxtfaktor stimulering leder till frisättning av fria radikaler från mitokondrien. Dessutom visade vi att denna mitokondriella frisättning var beroende av proteinet p66Shc.

I arbete 4 var syftet att studera hur kroppens egna system för att ta hand om fria radikaler, s.k. antioxidantsystem, kan reglera specifika PTPs och därmed påverka tillväxtfaktor (PDGF) signalering. Resultaten visade att antioxidantsystemet thioredoxin, selektivt motverkar oxidering av vissa PTPs (PTP1B) och därigenom har möjlighet att reglera tillväxtfaktorsignalering via PDGF receptorn.

Sammantaget har resultaten i denna avhandling ökat kunskapen kring hur aktiviteten av intracellulära PTPs regleras av fria radikaler och antioxidanter. Detta kan i sin tur påverka kommunikationen mellan celler, och specifikt tillväxtfaktorsignalering via PDGF-receptorn. Dessa mekanismer kan i ett större perspektiv ha betydelse för olika sjukdomstillstånd som hjärtinfarkt och cancer där syrebrist är en viktig komponent.

10. References

1. Hunter T, Lindberg RA, Middlemas DS, Tracy S, & van der Geer P (1992) Receptor protein tyrosine kinases and phosphatases. *Cold Spring Harb Symp Quant Biol* 57:25-41.
2. Manning G, Whyte DB, Martinez R, Hunter T, & Sudarsanam S (2002) The protein kinase complement of the human genome. *Science* 298(5600):1912-1934.
3. Wong S & Witte ON (2004) The BCR-ABL story: bench to bedside and back. *Annu Rev Immunol* 22:247-306.
4. Slamon D & Pegram M (2001) Rationale for trastuzumab (Herceptin) in adjuvant breast cancer trials. *Semin Oncol* 28(1 Suppl 3):13-19.
5. Heinrich MC*, et al.* (2006) Molecular Correlates of Imatinib Resistance in Gastrointestinal Stromal Tumors. *J Clin Oncol*.
6. Sequist LV, Bell DW, Lynch TJ, & Haber DA (2007) Molecular predictors of response to epidermal growth factor receptor antagonists in non-small-cell lung cancer. *J Clin Oncol* 25(5):587-595.
7. Pietras K & Ostman A (2010) Hallmarks of cancer: interactions with the tumor stroma. *Exp Cell Res* 316(8):1324-1331.
8. Carmeliet P (2005) Angiogenesis in life, disease and medicine. *Nature* 438(7070):932-936.
9. Armulik A, Genove G, & Betsholtz C (2011) Pericytes: developmental, physiological, and pathological perspectives, problems, and promises. *Dev Cell* 21(2):193-215.
10. Armulik A, Abramsson A, & Betsholtz C (2005) Endothelial/pericyte interactions. *Circ Res* 97(6):512-523.
11. Kalluri R & Zeisberg M (2006) Fibroblasts in cancer. *Nat Rev Cancer* 6(5):392-401.
12. Ostman A (2004) PDGF receptors-mediators of autocrine tumor growth and regulators of tumor vasculature and stroma. *Cytokine Growth Factor Rev* 15(4):275-286.
13. Druker BJ*, et al.* (2001) Efficacy and safety of a specific inhibitor of the BCR-ABL tyrosine kinase in chronic myeloid leukemia. *N Engl J Med* 344(14):1031-1037.
14. Baselga J (2006) Targeting tyrosine kinases in cancer: the second wave. *Science* 312(5777):1175-1178.
15. Jallal B, Schlessinger J, & Ullrich A (1992) Tyrosine phosphatase inhibition permits analysis of signal transduction complexes in p185HER2/neu-overexpressing human tumor cells. *J Biol Chem* 267(7):4357-4363.
16. Sorby M & Ostman A (1996) Protein-tyrosine phosphatase-mediated decrease of epidermal growth factor and platelet-derived growth factor receptor tyrosine phosphorylation in high cell density cultures. *J Biol Chem* 271(18):10963-10966.
17. Elchebly M*, et al.* (1999) Increased insulin sensitivity and obesity resistance in mice lacking the protein tyrosine phosphatase-1B gene. *Science* 283(5407):1544-1548.
18. Kenner KA, Anyanwu E, Olefsky JM, & Kusari J (1996) Protein-tyrosine phosphatase 1B is a negative regulator of insulin- and insulin-like growth factor-I-stimulated signaling. *J Biol Chem* 271(33):19810-19816.
19. Ahmad F, Li PM, Meyerovitch J, & Goldstein BJ (1995) Osmotic loading of neutralizing antibodies demonstrates a role for protein-tyrosine phosphatase 1B

in negative regulation of the insulin action pathway. *J Biol Chem* 270(35):20503-20508.
20. Paulson RF, Vesely S, Siminovitch KA, & Bernstein A (1996) Signalling by the W/Kit receptor tyrosine kinase is negatively regulated in vivo by the protein tyrosine phosphatase Shp1. *Nat Genet* 13(3):309-315.
21. Kulas DT, Goldstein BJ, & Mooney RA (1996) The transmembrane protein-tyrosine phosphatase LAR modulates signaling by multiple receptor tyrosine kinases. *J Biol Chem* 271(2):748-754.
22. Kulas DT, Zhang WR, Goldstein BJ, Furlanetto RW, & Mooney RA (1995) Insulin receptor signaling is augmented by antisense inhibition of the protein tyrosine phosphatase LAR. *J Biol Chem* 270(6):2435-2438.
23. Neel BG, Gu H, & Pao L (2003) The 'Shp'ing news: SH2 domain-containing tyrosine phosphatases in cell signaling. *Trends Biochem Sci* 28(6):284-293.
24. Bentires-Alj M & Neel BG (2007) Protein-tyrosine phosphatase 1B is required for HER2/Neu-induced breast cancer. *Cancer Res* 67(6):2420-2424.
25. Julien SG*, et al.* (2007) Protein tyrosine phosphatase 1B deficiency or inhibition delays ErbB2-induced mammary tumorigenesis and protects from lung metastasis. *Nat Genet* 39(3):338-346.
26. Alonso A*, et al.* (2004) Protein tyrosine phosphatases in the human genome. *Cell* 117(6):699-711.
27. Salmeen A & Barford D (2005) Functions and mechanisms of redox regulation of cysteine-based phosphatases. *Antioxid Redox Signal* 7(5-6):560-577.
28. Andersen JN*, et al.* (2001) Structural and evolutionary relationships among protein tyrosine phosphatase domains. *Mol Cell Biol* 21(21):7117-7136.
29. Zhang ZY & Dixon JE (1993) Active site labeling of the Yersinia protein tyrosine phosphatase: the determination of the pKa of the active site cysteine and the function of the conserved histidine 402. *Biochemistry* 32(36):9340-9345.
30. Stuckey JA*, et al.* (1994) Crystal structure of Yersinia protein tyrosine phosphatase at 2.5 A and the complex with tungstate. *Nature* 370(6490):571-575.
31. Pannifer AD, Flint AJ, Tonks NK, & Barford D (1998) Visualization of the cysteinyl-phosphate intermediate of a protein-tyrosine phosphatase by x-ray crystallography. *J Biol Chem* 273(17):10454-10462.
32. Lohse DL, Denu JM, Santoro N, & Dixon JE (1997) Roles of aspartic acid-181 and serine-222 in intermediate formation and hydrolysis of the mammalian protein-tyrosine-phosphatase PTP1. *Biochemistry* 36(15):4568-4575.
33. Jia Z, Barford D, Flint AJ, & Tonks NK (1995) Structural basis for phosphotyrosine peptide recognition by protein tyrosine phosphatase 1B. *Science* 268(5218):1754-1758.
34. Denu JM, Lohse DL, Vijayalakshmi J, Saper MA, & Dixon JE (1996) Visualization of intermediate and transition-state structures in protein-tyrosine phosphatase catalysis. *Proc Natl Acad Sci U S A* 93(6):2493-2498.
35. Ren L*, et al.* (2011) Substrate specificity of protein tyrosine phosphatases 1B, RPTPalpha, SHP-1, and SHP-2. *Biochemistry* 50(12):2339-2356.
36. Sarmiento M*, et al.* (2000) Structural basis of plasticity in protein tyrosine phosphatase 1B substrate recognition. *Biochemistry* 39(28):8171-8179.
37. Salmeen A, Andersen JN, Myers MP, Tonks NK, & Barford D (2000) Molecular basis for the dephosphorylation of the activation segment of the insulin receptor by protein tyrosine phosphatase 1B. *Mol Cell* 6(6):1401-1412.
38. Barr AJ*, et al.* (2009) Large-scale structural analysis of the classical human protein tyrosine phosphatome. *Cell* 136(2):352-363.

39. Persson C*, et al.* (2004) Site-selective regulation of platelet-derived growth factor beta receptor tyrosine phosphorylation by T-cell protein tyrosine phosphatase. *Mol Cell Biol* 24(5):2190-2201.
40. Kovalenko M*, et al.* (2000) Site-selective dephosphorylation of the platelet-derived growth factor beta-receptor by the receptor-like protein-tyrosine phosphatase DEP-1. *J Biol Chem* 275(21):16219-16226.
41. Persson C, Engstrom U, Mowbray SL, & Ostman A (2002) Primary sequence determinants responsible for site-selective dephosphorylation of the PDGF beta-receptor by the receptor-like protein tyrosine phosphatase DEP-1. *FEBS Lett* 517(1-3):27-31.
42. Tonks NK & Neel BG (2001) Combinatorial control of the specificity of protein tyrosine phosphatases. *Curr Opin Cell Biol* 13(2):182-195.
43. Barford D & Neel BG (1998) Revealing mechanisms for SH2 domain mediated regulation of the protein tyrosine phosphatase SHP-2. *Structure* 6(3):249-254.
44. Hof P, Pluskey S, Dhe-Paganon S, Eck MJ, & Shoelson SE (1998) Crystal structure of the tyrosine phosphatase SHP-2. *Cell* 92(4):441-450.
45. Frangioni JV, Beahm PH, Shifrin V, Jost CA, & Neel BG (1992) The nontransmembrane tyrosine phosphatase PTP-1B localizes to the endoplasmic reticulum via its 35 amino acid C-terminal sequence. *Cell* 68(3):545-560.
46. Ostman A, Yang Q, & Tonks NK (1994) Expression of DEP-1, a receptor-like protein-tyrosine-phosphatase, is enhanced with increasing cell density. *Proc Natl Acad Sci U S A* 91(21):9680-9684.
47. Ostman A, Hellberg C, & Bohmer FD (2006) Protein-tyrosine phosphatases and cancer. *Nat Rev Cancer* 6(4):307-320.
48. Ostman A & Bohmer FD (2001) Regulation of receptor tyrosine kinase signaling by protein tyrosine phosphatases. *Trends Cell Biol* 11(6):258-266.
49. Pao LI, Badour K, Siminovitch KA, & Neel BG (2007) Nonreceptor protein-tyrosine phosphatases in immune cell signaling. *Annu Rev Immunol* 25:473-523.
50. Frank C, Keilhack H, Opitz F, Zschornig O, & Bohmer FD (1999) Binding of phosphatidic acid to the protein-tyrosine phosphatase SHP-1 as a basis for activity modulation. *Biochemistry* 38(37):11993-12002.
51. Sankarshanan M, Ma Z, Iype T, & Lorenz U (2007) Identification of a novel lipid raft-targeting motif in Src homology 2-containing phosphatase 1. *J Immunol* 179(1):483-490.
52. Ogata M, Sawada M, Kosugi A, & Hamaoka T (1994) Developmentally regulated expression of a murine receptor-type protein tyrosine phosphatase in the thymus. *J Immunol* 153(10):4478-4487.
53. O'Grady P, Krueger NX, Streuli M, & Saito H (1994) Genomic organization of the human LAR protein tyrosine phosphatase gene and alternative splicing in the extracellular fibronectin type-III domains. *J Biol Chem* 269(40):25193-25199.
54. Kachel N*, et al.* (2003) Structure determination and ligand interactions of the PDZ2b domain of PTP-Bas (hPTP1E): splicing-induced modulation of ligand specificity. *J Mol Biol* 334(1):143-155.
55. Halle M*, et al.* (2007) Caspase-3 regulates catalytic activity and scaffolding functions of the protein tyrosine phosphatase PEST, a novel modulator of the apoptotic response. *Mol Cell Biol* 27(3):1172-1190.
56. Gulati P, Markova B, Gottlicher M, Bohmer FD, & Herrlich PA (2004) UVA inactivates protein tyrosine phosphatases by calpain-mediated degradation. *EMBO Rep* 5(8):812-817.

57. Trumpler A, Schlott B, Herrlich P, Greer PA, & Bohmer FD (2009) Calpain-mediated degradation of reversibly oxidized protein-tyrosine phosphatase 1B. *FEBS J* 276(19):5622-5633.
58. Majeti R, Bilwes AM, Noel JP, Hunter T, & Weiss A (1998) Dimerization-induced inhibition of receptor protein tyrosine phosphatase function through an inhibitory wedge. *Science* 279(5347):88-91.
59. Meng K*, et al.* (2000) Pleiotrophin signals increased tyrosine phosphorylation of beta beta-catenin through inactivation of the intrinsic catalytic activity of the receptor-type protein tyrosine phosphatase beta/zeta. *Proc Natl Acad Sci U S A* 97(6):2603-2608.
60. Pariser H, Perez-Pinera P, Ezquerra L, Herradon G, & Deuel TF (2005) Pleiotrophin stimulates tyrosine phosphorylation of beta-adducin through inactivation of the transmembrane receptor protein tyrosine phosphatase beta/zeta. *Biochem Biophys Res Commun* 335(1):232-239.
61. Tamura H, Fukada M, Fujikawa A, & Noda M (2006) Protein tyrosine phosphatase receptor type Z is involved in hippocampus-dependent memory formation through dephosphorylation at Y1105 on p190 RhoGAP. *Neurosci Lett* 399(1-2):33-38.
62. Perez-Pinera P, Zhang W, Chang Y, Vega JA, & Deuel TF (2007) Anaplastic lymphoma kinase is activated through the pleiotrophin/receptor protein-tyrosine phosphatase beta/zeta signaling pathway: an alternative mechanism of receptor tyrosine kinase activation. *J Biol Chem* 282(39):28683-28690.
63. Fox AN & Zinn K (2005) The heparan sulfate proteoglycan syndecan is an in vivo ligand for the Drosophila LAR receptor tyrosine phosphatase. *Curr Biol* 15(19):1701-1711.
64. Johnson KG*, et al.* (2006) The HSPGs Syndecan and Dallylike bind the receptor phosphatase LAR and exert distinct effects on synaptic development. *Neuron* 49(4):517-531.
65. Sorby M, Sandstrom J, & Ostman A (2001) An extracellular ligand increases the specific activity of the receptor-like protein tyrosine phosphatase DEP-1. *Oncogene* 20(37):5219-5224.
66. Flint AJ, Gebbink MF, Franza BR, Jr., Hill DE, & Tonks NK (1993) Multi-site phosphorylation of the protein tyrosine phosphatase, PTP1B: identification of cell cycle regulated and phorbol ester stimulated sites of phosphorylation. *EMBO J* 12(5):1937-1946.
67. Garton AJ & Tonks NK (1999) Regulation of fibroblast motility by the protein tyrosine phosphatase PTP-PEST. *J Biol Chem* 274(6):3811-3818.
68. Ostergaard HL & Trowbridge IS (1991) Negative regulation of CD45 protein tyrosine phosphatase activity by ionomycin in T cells. *Science* 253(5026):1423-1425.
69. Yamada A*, et al.* (1990) Effect of activation of protein kinase C on CD45 isoform expression and CD45 protein tyrosine phosphatase activity in T cells. *Eur J Immunol* 20(8):1655-1660.
70. den Hertog J, Sap J, Pals CE, Schlessinger J, & Kruijer W (1995) Stimulation of receptor protein-tyrosine phosphatase alpha activity and phosphorylation by phorbol ester. *Cell Growth Differ* 6(3):303-307.
71. Strack V*, et al.* (2002) The Protein-tyrosine-phosphatase SHP2 is phosphorylated on serine residues 576 and 591 by protein kinase C isoforms alpha, beta 1, beta 2, and eta. *Biochemistry* 41(2):603-608.
72. Liu Y, Kruhlak MJ, Hao JJ, & Shaw S (2007) Rapid T cell receptor-mediated SHP-1 S591 phosphorylation regulates SHP-1 cellular localization and phosphatase activity. *J Leukoc Biol* 82(3):742-751.

73. Brown-Shimer S, Johnson KA, Hill DE, & Bruskin AM (1992) Effect of protein tyrosine phosphatase 1B expression on transformation by the human neu oncogene. *Cancer Res* 52(2):478-482.
74. Trapasso F*, et al.* (2000) Rat protein tyrosine phosphatase eta suppresses the neoplastic phenotype of retrovirally transformed thyroid cells through the stabilization of p27(Kip1). *Mol Cell Biol* 20(24):9236-9246.
75. Sacco F*, et al.* (2009) Tumor suppressor density-enhanced phosphatase-1 (DEP-1) inhibits the RAS pathway by direct dephosphorylation of ERK1/2 kinases. *J Biol Chem* 284(33):22048-22058.
76. Keane MM, Lowrey GA, Ettenberg SA, Dayton MA, & Lipkowitz S (1996) The protein tyrosine phosphatase DEP-1 is induced during differentiation and inhibits growth of breast cancer cells. *Cancer Res* 56(18):4236-4243.
77. Iuliano R*, et al.* (2003) An adenovirus carrying the rat protein tyrosine phosphatase eta suppresses the growth of human thyroid carcinoma cell lines in vitro and in vivo. *Cancer Res* 63(4):882-886.
78. Trapasso F*, et al.* (2004) Restoration of receptor-type protein tyrosine phosphatase eta function inhibits human pancreatic carcinoma cell growth in vitro and in vivo. *Carcinogenesis* 25(11):2107-2114.
79. Wang Z*, et al.* (2004) Mutational analysis of the tyrosine phosphatome in colorectal cancers. *Science* 304(5674):1164-1166.
80. Korff S*, et al.* (2008) Frameshift mutations in coding repeats of protein tyrosine phosphatase genes in colorectal tumors with microsatellite instability. *BMC Cancer* 8:329.
81. Ruivenkamp CA*, et al.* (2002) Ptprj is a candidate for the mouse colon-cancer susceptibility locus Scc1 and is frequently deleted in human cancers. *Nat Genet* 31(3):295-300.
82. Kleppe M*, et al.* (2010) Deletion of the protein tyrosine phosphatase gene PTPN2 in T-cell acute lymphoblastic leukemia. *Nat Genet* 42(6):530-535.
83. Khoury JD, Rassidakis GZ, Medeiros LJ, Amin HM, & Lai R (2004) Methylation of SHP1 gene and loss of SHP1 protein expression are frequent in systemic anaplastic large cell lymphoma. *Blood* 104(5):1580-1581.
84. Veeriah S*, et al.* (2009) The tyrosine phosphatase PTPRD is a tumor suppressor that is frequently inactivated and mutated in glioblastoma and other human cancers. *Proc Natl Acad Sci U S A* 106(23):9435-9440.
85. Zhan Y & O'Rourke DM (2004) SHP-2-dependent mitogen-activated protein kinase activation regulates EGFRvIII but not wild-type epidermal growth factor receptor phosphorylation and glioblastoma cell survival. *Cancer Res* 64(22):8292-8298.
86. Tartaglia M*, et al.* (2001) Mutations in PTPN11, encoding the protein tyrosine phosphatase SHP-2, cause Noonan syndrome. *Nat Genet* 29(4):465-468.
87. Tartaglia M*, et al.* (2003) Somatic mutations in PTPN11 in juvenile myelomonocytic leukemia, myelodysplastic syndromes and acute myeloid leukemia. *Nat Genet* 34(2):148-150.
88. D'Autreaux B & Toledano MB (2007) ROS as signalling molecules: mechanisms that generate specificity in ROS homeostasis. *Nat Rev Mol Cell Biol* 8(10):813-824.
89. Blume-Jensen P & Hunter T (2001) Oncogenic kinase signalling. *Nature* 411(6835):355-365.
90. Zhu X, Zuo L, Cardounel AJ, Zweier JL, & He G (2007) Characterization of in vivo tissue redox status, oxygenation, and formation of reactive oxygen species in postischemic myocardium. *Antioxid Redox Signal* 9(4):447-455.

91. Fleury C, Mignotte B, & Vayssiere JL (2002) Mitochondrial reactive oxygen species in cell death signaling. *Biochimie* 84(2-3):131-141.
92. Turrens JF (2003) Mitochondrial formation of reactive oxygen species. *J Physiol* 552(Pt 2):335-344.
93. Brand MD (2010) The sites and topology of mitochondrial superoxide production. *Exp Gerontol* 45(7-8):466-472.
94. Suh YA*, et al.* (1999) Cell transformation by the superoxide-generating oxidase Mox1. *Nature* 401(6748):79-82.
95. Lambeth JD, Kawahara T, & Diebold B (2007) Regulation of Nox and Duox enzymatic activity and expression. *Free Radic Biol Med* 43(3):319-331.
96. Valko M*, et al.* (2007) Free radicals and antioxidants in normal physiological functions and human disease. *Int J Biochem Cell Biol* 39(1):44-84.
97. Kim C, Kim JY, & Kim JH (2008) Cytosolic phospholipase A(2), lipoxygenase metabolites, and reactive oxygen species. *BMB Rep* 41(8):555-559.
98. Margis R, Dunand C, Teixeira FK, & Margis-Pinheiro M (2008) Glutathione peroxidase family - an evolutionary overview. *FEBS J* 275(15):3959-3970.
99. Luthman M & Holmgren A (1982) Rat liver thioredoxin and thioredoxin reductase: purification and characterization. *Biochemistry* 21(26):6628-6633.
100. Nordberg J & Arner ES (2001) Reactive oxygen species, antioxidants, and the mammalian thioredoxin system. *Free Radic Biol Med* 31(11):1287-1312.
101. Sun QA*, et al.* (2005) Reaction mechanism and regulation of mammalian thioredoxin/glutathione reductase. *Biochemistry* 44(44):14528-14537.
102. Nonn L, Williams RR, Erickson RP, & Powis G (2003) The absence of mitochondrial thioredoxin 2 causes massive apoptosis, exencephaly, and early embryonic lethality in homozygous mice. *Mol Cell Biol* 23(3):916-922.
103. Matsui M*, et al.* (1996) Early embryonic lethality caused by targeted disruption of the mouse thioredoxin gene. *Dev Biol* 178(1):179-185.
104. Jakupoglu C*, et al.* (2005) Cytoplasmic thioredoxin reductase is essential for embryogenesis but dispensable for cardiac development. *Mol Cell Biol* 25(5):1980-1988.
105. Barbazette J (2008) Redox Biochemistry. *Redox Biochemistry*, ed Ruma Banerjee DFB MB, Dickman, Vadim N Gladyshev, Stephen W. Ragsdale (John Wiley & Sons, Inc, New jersey).
106. Thieme R, Pai EF, Schirmer RH, & Schulz GE (1981) Three-dimensional structure of glutathione reductase at 2 A resolution. *J Mol Biol* 152(4):763-782.
107. Cappellini MD & Fiorelli G (2008) Glucose-6-phosphate dehydrogenase deficiency. *Lancet* 371(9606):64-74.
108. Brigelius-Flohe R & Kipp A (2009) Glutathione peroxidases in different stages of carcinogenesis. *Biochim Biophys Acta* 1790(11):1555-1568.
109. Kryukov GV*, et al.* (2003) Characterization of mammalian selenoproteomes. *Science* 300(5624):1439-1443.
110. Conrad M, Schneider M, Seiler A, & Bornkamm GW (2007) Physiological role of phospholipid hydroperoxide glutathione peroxidase in mammals. *Biol Chem* 388(10):1019-1025.
111. Seiler A*, et al.* (2008) Glutathione peroxidase 4 senses and translates oxidative stress into 12/15-lipoxygenase dependent- and AIF-mediated cell death. *Cell Metab* 8(3):237-248.
112. Yant LJ*, et al.* (2003) The selenoprotein GPX4 is essential for mouse development and protects from radiation and oxidative damage insults. *Free Radic Biol Med* 34(4):496-502.

113. Rhee SG & Woo HA (2011) Multiple functions of peroxiredoxins: peroxidases, sensors and regulators of the intracellular messenger H(2)O(2), and protein chaperones. *Antioxid Redox Signal* 15(3):781-794.
114. Noguera-Mazon V, Krimm I, Walker O, & Lancelin JM (2006) Protein-protein interactions within peroxiredoxin systems. *Photosynth Res* 89(2-3):277-290.
115. Hofmann B, Hecht HJ, & Flohe L (2002) Peroxiredoxins. *Biol Chem* 383(3-4):347-364.
116. Rhee SG, Kang SW, Netto LE, Seo MS, & Stadtman ER (1999) A family of novel peroxidases, peroxiredoxins. *Biofactors* 10(2-3):207-209.
117. Wood ZA, Schroder E, Robin Harris J, & Poole LB (2003) Structure, mechanism and regulation of peroxiredoxins. *Trends Biochem Sci* 28(1):32-40.
118. Fritz R*, et al.* (2007) Compartment-dependent management of H(2)O(2) by peroxisomes. *Free Radic Biol Med* 42(7):1119-1129.
119. Fridovich I (1995) Superoxide radical and superoxide dismutases. *Annu Rev Biochem* 64:97-112.
120. Jones DP (2008) Radical-free biology of oxidative stress. *Am J Physiol Cell Physiol* 295(4):C849-868.
121. Pastore A*, et al.* (2003) Actin glutathionylation increases in fibroblasts of patients with Friedreich's ataxia: a potential role in the pathogenesis of the disease. *J Biol Chem* 278(43):42588-42595.
122. Lander HM*, et al.* (1996) Redox regulation of cell signalling. *Nature* 381(6581):380-381.
123. Sykes MC, Mowbray AL, & Jo H (2007) Reversible glutathiolation of caspase-3 by glutaredoxin as a novel redox signaling mechanism in tumor necrosis factor-alpha-induced cell death. *Circ Res* 100(2):152-154.
124. Paulsen CE*, et al.* (2012) Peroxide-dependent sulfenylation of the EGFR catalytic site enhances kinase activity. *Nat Chem Biol* 8(1):57-64.
125. Brigelius-Flohe R & Flohe L (2011) Basic principles and emerging concepts in the redox control of transcription factors. *Antioxid Redox Signal* 15(8):2335-2381.
126. Kensler TW, Wakabayashi N, & Biswal S (2007) Cell survival responses to environmental stresses via the Keap1-Nrf2-ARE pathway. *Annu Rev Pharmacol Toxicol* 47:89-116.
127. Meng TC, Fukada T, & Tonks NK (2002) Reversible oxidation and inactivation of protein tyrosine phosphatases in vivo. *Mol Cell* 9(2):387-399.
128. Peters GH, Frimurer TM, & Olsen OH (1998) Electrostatic evaluation of the signature motif (H/V)CX5R(S/T) in protein-tyrosine phosphatases. *Biochemistry* 37(16):5383-5393.
129. Denu JM & Tanner KG (1998) Specific and reversible inactivation of protein tyrosine phosphatases by hydrogen peroxide: evidence for a sulfenic acid intermediate and implications for redox regulation. *Biochemistry* 37(16):5633-5642.
130. Groen A*, et al.* (2005) Differential oxidation of protein-tyrosine phosphatases. *J Biol Chem* 280(11):10298-10304.
131. Krishnan N, Fu C, Pappin DJ, & Tonks NK (2011) H2S-Induced sulfhydration of the phosphatase PTP1B and its role in the endoplasmic reticulum stress response. *Sci Signal* 4(203):ra86.
132. Chen CY, Willard D, & Rudolph J (2009) Redox regulation of SH2-domain-containing protein tyrosine phosphatases by two backdoor cysteines. *Biochemistry* 48(6):1399-1409.

133. Weibrecht I*, et al.* (2007) Oxidation sensitivity of the catalytic cysteine of the protein-tyrosine phosphatases SHP-1 and SHP-2. *Free Radic Biol Med* 43(1):100-110.
134. Persson C*, et al.* (2004) Preferential oxidation of the second phosphatase domain of receptor-like PTP-alpha revealed by an antibody against oxidized protein tyrosine phosphatases. *Proc Natl Acad Sci U S A* 101(7):1886-1891.
135. Lee SR*, et al.* (2002) Reversible inactivation of the tumor suppressor PTEN by H2O2. *J Biol Chem* 277(23):20336-20342.
136. Seth D & Rudolph J (2006) Redox regulation of MAP kinase phosphatase 3. *Biochemistry* 45(28):8476-8487.
137. Kanda M*, et al.* (2006) Glutaredoxin modulates platelet-derived growth factor-dependent cell signaling by regulating the redox status of low molecular weight protein-tyrosine phosphatase. *J Biol Chem* 281(39):28518-28528.
138. Savitsky PA & Finkel T (2002) Redox regulation of Cdc25C. *J Biol Chem* 277(23):20535-20540.
139. Barrett DM*, et al.* (2005) Inhibition of protein-tyrosine phosphatases by mild oxidative stresses is dependent on S-nitrosylation. *J Biol Chem* 280(15):14453-14461.
140. Li S, Wang H, Xian M, & Whorton AR (2012) Identification of protein nitrosothiols using phosphine-mediated selective reduction. *Nitric Oxide* 26(1):20-26.
141. Shi ZQ*, et al.* (2013) S-nitrosylated SHP-2 contributes to NMDA receptor-mediated excitotoxicity in acute ischemic stroke. *Proc Natl Acad Sci U S A* 110(8):3137-3142.
142. Yu CX, Li S, & Whorton AR (2005) Redox regulation of PTEN by S-nitrosothiols. *Mol Pharmacol* 68(3):847-854.
143. Takakura K, Beckman JS, MacMillan-Crow LA, & Crow JP (1999) Rapid and irreversible inactivation of protein tyrosine phosphatases PTP1B, CD45, and LAR by peroxynitrite. *Arch Biochem Biophys* 369(2):197-207.
144. Barrett WC*, et al.* (1999) Roles of superoxide radical anion in signal transduction mediated by reversible regulation of protein-tyrosine phosphatase 1B. *J Biol Chem* 274(49):34543-34546.
145. Abdelsaid MA & El-Remessy AB (2012) S-glutathionylation of LMW-PTP regulates VEGF-mediated FAK activation and endothelial cell migration. *J Cell Sci* 125(Pt 20):4751-4760.
146. Barrett WC*, et al.* (1999) Regulation of PTP1B via glutathionylation of the active site cysteine 215. *Biochemistry* 38(20):6699-6705.
147. Ross SH*, et al.* (2007) Differential redox regulation within the PTP superfamily. *Cell Signal* 19(7):1521-1530.
148. Conrad M*, et al.* (2010) 12/15-lipoxygenase-derived lipid peroxides control receptor tyrosine kinase signaling through oxidation of protein tyrosine phosphatases. *Proc Natl Acad Sci U S A* 107(36):15774-15779.
149. Covey TM, Edes K, & Fitzpatrick FA (2007) Akt activation by arachidonic acid metabolism occurs via oxidation and inactivation of PTEN tumor suppressor. *Oncogene* 26(39):5784-5792.
150. Bhattacharya S, Labutti JN, Seiner DR, & Gates KS (2008) Oxidative inactivation of protein tyrosine phosphatase 1B by organic hydroperoxides. *Bioorg Med Chem Lett* 18(22):5856-5859.
151. LaButti JN, Chowdhury G, Reilly TJ, & Gates KS (2007) Redox regulation of protein tyrosine phosphatase 1B by peroxymonophosphate (=O3POOH). *J Am Chem Soc* 129(17):5320-5321.

152. Lane AE, Tan JT, Hawkins CL, Heather AK, & Davies MJ (2010) The myeloperoxidase-derived oxidant HOSCN inhibits protein tyrosine phosphatases and modulates cell signalling via the mitogen-activated protein kinase (MAPK) pathway in macrophages. *Biochem J* 430(1):161-169.
153. Wan X, *et al.* (2011) Oxidative inactivation of the lipid phosphatase phosphatase and tensin homolog on chromosome ten (PTEN) as a novel mechanism of acquired long QT syndrome. *J Biol Chem* 286(4):2843-2852.
154. Kimura K, Takada M, Ishii T, Tsuji-Naito K, & Akagawa M (2012) Pyrroloquinoline quinone stimulates epithelial cell proliferation by activating epidermal growth factor receptor through redox cycling. *Free Radic Biol Med* 53(6):1239-1251.
155. Samet JM & Tal TL (2010) Toxicological disruption of signaling homeostasis: tyrosine phosphatases as targets. *Annu Rev Pharmacol Toxicol* 50:215-235.
156. Wang Q, *et al.* (2004) Catalytic inactivation of protein tyrosine phosphatase CD45 and protein tyrosine phosphatase 1B by polyaromatic quinones. *Biochemistry* 43(14):4294-4303.
157. Zhou H, *et al.* (2011) The biological buffer bicarbonate/CO2 potentiates H2O2-mediated inactivation of protein tyrosine phosphatases. *J Am Chem Soc* 133(40):15803-15805.
158. Lou YW, *et al.* (2008) Redox regulation of the protein tyrosine phosphatase PTP1B in cancer cells. *FEBS J* 275(1):69-88.
159. van Montfort RL, Congreve M, Tisi D, Carr R, & Jhoti H (2003) Oxidation state of the active-site cysteine in protein tyrosine phosphatase 1B. *Nature* 423(6941):773-777.
160. Salmeen A, *et al.* (2003) Redox regulation of protein tyrosine phosphatase 1B involves a sulphenyl-amide intermediate. *Nature* 423(6941):769-773.
161. Yang J, *et al.* (2007) Reversible oxidation of the membrane distal domain of receptor PTPalpha is mediated by a cyclic sulfenamide. *Biochemistry* 46(3):709-719.
162. Tsai SJ, *et al.* (2009) Crystal structure of the human lymphoid tyrosine phosphatase catalytic domain: insights into redox regulation. *Biochemistry* 48(22):4838-4845.
163. Caselli A, *et al.* (1998) The inactivation mechanism of low molecular weight phosphotyrosine-protein phosphatase by H2O2. *J Biol Chem* 273(49):32554-32560.
164. Caselli A, *et al.* (1994) Nitric oxide causes inactivation of the low molecular weight phosphotyrosine protein phosphatase. *J Biol Chem* 269(40):24878-24882.
165. Fauman EB, *et al.* (1998) Crystal structure of the catalytic domain of the human cell cycle control phosphatase, Cdc25A. *Cell* 93(4):617-625.
166. Reynolds RA, *et al.* (1999) Crystal structure of the catalytic subunit of Cdc25B required for G2/M phase transition of the cell cycle. *J Mol Biol* 293(3):559-568.
167. van der Wijk T, Overvoorde J, & den Hertog J (2004) H2O2-induced intermolecular disulfide bond formation between receptor protein-tyrosine phosphatases. *J Biol Chem* 279(43):44355-44361.
168. Hower AE, Beltran PJ, & Bixby JL (2009) Dimerization of tyrosine phosphatase PTPRO decreases its activity and ability to inactivate TrkC. *J Neurochem* 110(5):1635-1647.
169. Nardozza AP, *et al.* (2012) Reactive oxygen species and epidermal growth factor are antagonistic cues controlling SHP-2 dimerization. *Mol Cell Biol* 32(10):1998-2009.

170. Lim S & Clement MV (2007) Phosphorylation of the survival kinase Akt by superoxide is dependent on an ascorbate-reversible oxidation of PTEN. *Free Radic Biol Med* 42(8):1178-1192.
171. Chen YY*, et al.* (2008) Cysteine S-nitrosylation protects protein-tyrosine phosphatase 1B against oxidation-induced permanent inactivation. *J Biol Chem* 283(50):35265-35272.
172. Xiong Y, Uys JD, Tew KD, & Townsend DM (2011) S-glutathionylation: from molecular mechanisms to health outcomes. *Antioxid Redox Signal* 15(1):233-270.
173. Arner ES & Holmgren A (2000) Physiological functions of thioredoxin and thioredoxin reductase. *Eur J Biochem* 267(20):6102-6109.
174. Lillig CH, Berndt C, & Holmgren A (2008) Glutaredoxin systems. *Biochim Biophys Acta* 1780(11):1304-1317.
175. Lillig CH & Holmgren A (2007) Thioredoxin and related molecules--from biology to health and disease. *Antioxid Redox Signal* 9(1):25-47.
176. Lee SR, Kwon KS, Kim SR, & Rhee SG (1998) Reversible inactivation of protein-tyrosine phosphatase 1B in A431 cells stimulated with epidermal growth factor. *J Biol Chem* 273(25):15366-15372.
177. Beillerot A, Battaglia E, Aline B, & Bagrel D (2012) Protection of CDC25 phosphatases against oxidative stress in breast cancer cells: evaluation of the implication of the thioredoxin system. *Free Radic Res* 46(5):674-689.
178. Sohn J & Rudolph J (2003) Catalytic and chemical competence of regulation of cdc25 phosphatase by oxidation/reduction. *Biochemistry* 42(34):10060-10070.
179. Burridge K & Nelson A (1995) An in-gel assay for protein tyrosine phosphatase activity: detection of widespread distribution in cells and tissues. *Anal Biochem* 232(1):56-64.
180. Persson C, Kappert K, Engstrom U, Ostman A, & Sjoblom T (2005) An antibody-based method for monitoring in vivo oxidation of protein tyrosine phosphatases. *Methods* 35(1):37-43.
181. Sandin A*, et al.* (2011) Hypoxia followed by re-oxygenation induces oxidation of tyrosine phosphatases. *Cell Signal* 23(5):820-826.
182. Boivin B, Zhang S, Arbiser JL, Zhang ZY, & Tonks NK (2008) A modified cysteinyl-labeling assay reveals reversible oxidation of protein tyrosine phosphatases in angiomyolipoma cells. *Proc Natl Acad Sci U S A* 105(29):9959-9964.
183. Wang Y & Pallen CJ (1991) The receptor-like protein tyrosine phosphatase HPTP alpha has two active catalytic domains with distinct substrate specificities. *EMBO J* 10(11):3231-3237.
184. Streuli M, Krueger NX, Thai T, Tang M, & Saito H (1990) Distinct functional roles of the two intracellular phosphatase like domains of the receptor-linked protein tyrosine phosphatases LCA and LAR. *EMBO J* 9(8):2399-2407.
185. Blanchetot C, Tertoolen LG, & den Hertog J (2002) Regulation of receptor protein-tyrosine phosphatase alpha by oxidative stress. *EMBO J* 21(4):493-503.
186. van der Wijk T, Blanchetot C, Overvoorde J, & den Hertog J (2003) Redox-regulated rotational coupling of receptor protein-tyrosine phosphatase alpha dimers. *J Biol Chem* 278(16):13968-13974.
187. Wu RF*, et al.* (2005) Subcellular targeting of oxidants during endothelial cell migration. *J Cell Biol* 171(5):893-904.
188. Choi MH*, et al.* (2005) Regulation of PDGF signalling and vascular remodelling by peroxiredoxin II. *Nature* 435(7040):347-353.

189. Woo HA, *et al.* (2010) Inactivation of peroxiredoxin I by phosphorylation allows localized H(2)O(2) accumulation for cell signaling. *Cell* 140(4):517-528.
190. Chiarugi P, *et al.* (2001) Two vicinal cysteines confer a peculiar redox regulation to low molecular weight protein tyrosine phosphatase in response to platelet-derived growth factor receptor stimulation. *J Biol Chem* 276(36):33478-33487.
191. Chen K, Kirber MT, Xiao H, Yang Y, & Keaney JF, Jr. (2008) Regulation of ROS signal transduction by NADPH oxidase 4 localization. *J Cell Biol* 181(7):1129-1139.
192. Kwon J, *et al.* (2004) Reversible oxidation and inactivation of the tumor suppressor PTEN in cells stimulated with peptide growth factors. *Proc Natl Acad Sci U S A* 101(47):16419-16424.
193. Berniakovich I, *et al.* (2008) p66Shc-generated oxidative signal promotes fat accumulation. *J Biol Chem* 283(49):34283-34293.
194. Taddei ML, *et al.* (2007) Integrin-mediated cell adhesion and spreading engage different sources of reactive oxygen species. *Antioxid Redox Signal* 9(4):469-481.
195. Cho KJ, Seo JM, & Kim JH (2011) Bioactive lipoxygenase metabolites stimulation of NADPH oxidases and reactive oxygen species. *Mol Cells* 32(1):1-5.
196. de Carvalho DD, *et al.* (2008) Nox1 downstream of 12-lipoxygenase controls cell proliferation but not cell spreading of colon cancer cells. *Int J Cancer* 122(8):1757-1764.
197. Sundaresan M, Yu ZX, Ferrans VJ, Irani K, & Finkel T (1995) Requirement for generation of H2O2 for platelet-derived growth factor signal transduction. *Science* 270(5234):296-299.
198. Guyton KZ, Liu Y, Gorospe M, Xu Q, & Holbrook NJ (1996) Activation of mitogen-activated protein kinase by H2O2. Role in cell survival following oxidant injury. *J Biol Chem* 271(8):4138-4142.
199. Liu JC, Chen CH, Chen JJ, & Cheng TH (2009) Urotensin II induces rat cardiomyocyte hypertrophy via the transient oxidization of Src homology 2-containing tyrosine phosphatase and transactivation of epidermal growth factor receptor. *Mol Pharmacol* 76(6):1186-1195.
200. Kappert K, *et al.* (2006) Antioxidants relieve phosphatase inhibition and reduce PDGF signaling in cultured VSMCs and in restenosis. *Arterioscler Thromb Vasc Biol* 26(12):2644-2651.
201. Chiarugi P, *et al.* (2003) Reactive oxygen species as essential mediators of cell adhesion: the oxidative inhibition of a FAK tyrosine phosphatase is required for cell adhesion. *J Cell Biol* 161(5):933-944.
202. Oshikawa J, *et al.* (2010) Extracellular SOD-derived H2O2 promotes VEGF signaling in caveolae/lipid rafts and post-ischemic angiogenesis in mice. *PLoS One* 5(4):e10189.
203. Kwon J, *et al.* (2005) Receptor-stimulated oxidation of SHP-2 promotes T-cell adhesion through SLP-76-ADAP. *EMBO J* 24(13):2331-2341.
204. Loh K, *et al.* (2009) Reactive oxygen species enhance insulin sensitivity. *Cell Metab* 10(4):260-272.
205. Szatrowski TP & Nathan CF (1991) Production of large amounts of hydrogen peroxide by human tumor cells. *Cancer Res* 51(3):794-798.
206. DeNicola GM, *et al.* (2011) Oncogene-induced Nrf2 transcription promotes ROS detoxification and tumorigenesis. *Nature* 475(7354):106-109.

207. Arnold RS, *et al.* (2001) Hydrogen peroxide mediates the cell growth and transformation caused by the mitogenic oxidase Nox1. *Proc Natl Acad Sci U S A* 98(10):5550-5555.
208. Mitsushita J, Lambeth JD, & Kamata T (2004) The superoxide-generating oxidase Nox1 is functionally required for Ras oncogene transformation. *Cancer Res* 64(10):3580-3585.
209. Pelicano H, *et al.* (2006) Mitochondrial respiration defects in cancer cells cause activation of Akt survival pathway through a redox-mediated mechanism. *J Cell Biol* 175(6):913-923.
210. Silva A, *et al.* (2008) PTEN posttranslational inactivation and hyperactivation of the PI3K/Akt pathway sustain primary T cell leukemia viability. *J Clin Invest* 118(11):3762-3774.
211. Arora D, *et al.* (2011) Protein-tyrosine phosphatase DEP-1 controls receptor tyrosine kinase FLT3 signaling. *J Biol Chem* 286(13):10918-10929.
212. Godfrey R, *et al.* (2012) Cell transformation by FLT3 ITD in acute myeloid leukemia involves oxidative inactivation of the tumor suppressor protein-tyrosine phosphatase DEP-1/ PTPRJ. *Blood* 119(19):4499-4511.
213. Kim HS, Ullevig SL, Zamora D, Lee CF, & Asmis R (2012) Redox regulation of MAPK phosphatase 1 controls monocyte migration and macrophage recruitment. *Proc Natl Acad Sci U S A* 109(41):E2803-2812.
214. Zweier JL, Flaherty JT, & Weisfeldt ML (1987) Direct measurement of free radical generation following reperfusion of ischemic myocardium. *Proc Natl Acad Sci U S A* 84(5):1404-1407.
215. Garlick PB, Davies MJ, Hearse DJ, & Slater TF (1987) Direct detection of free radicals in the reperfused rat heart using electron spin resonance spectroscopy. *Circ Res* 61(5):757-760.
216. Ambrosio G, *et al.* (1993) Evidence that mitochondrial respiration is a source of potentially toxic oxygen free radicals in intact rabbit hearts subjected to ischemia and reflow. *J Biol Chem* 268(25):18532-18541.
217. Perros F, *et al.* (2008) Platelet-derived growth factor expression and function in idiopathic pulmonary arterial hypertension. *Am J Respir Crit Care Med* 178(1):81-88.
218. Schermuly RT, *et al.* (2005) Reversal of experimental pulmonary hypertension by PDGF inhibition. *J Clin Invest* 115(10):2811-2821.
219. Das M, Burns N, Wilson SJ, Zawada WM, & Stenmark KR (2008) Hypoxia exposure induces the emergence of fibroblasts lacking replication repressor signals of PKCzeta in the pulmonary artery adventitia. *Cardiovasc Res* 78(3):440-448.
220. Sano H, *et al.* (2001) Functional blockade of platelet-derived growth factor receptor-beta but not of receptor-alpha prevents vascular smooth muscle cell accumulation in fibrous cap lesions in apolipoprotein E-deficient mice. *Circulation* 103(24):2955-2960.
221. Hart CE, *et al.* (1999) PDGFbeta receptor blockade inhibits intimal hyperplasia in the baboon. *Circulation* 99(4):564-569.
222. Vantler M, Caglayan E, Zimmermann WH, Baumer AT, & Rosenkranz S (2005) Systematic evaluation of anti-apoptotic growth factor signaling in vascular smooth muscle cells. Only phosphatidylinositol 3'-kinase is important. *J Biol Chem* 280(14):14168-14176.
223. Giorgio M, Trinei M, Migliaccio E, & Pelicci PG (2007) Hydrogen peroxide: a metabolic by-product or a common mediator of ageing signals? *Nat Rev Mol Cell Biol* 8(9):722-728.

224. Mandal PK, *et al.* (2010) Loss of thioredoxin reductase 1 renders tumors highly susceptible to pharmacologic glutathione deprivation. *Cancer Res* 70(22):9505-9514.
225. Jeong W, Yoon HW, Lee SR, & Rhee SG (2004) Identification and characterization of TRP14, a thioredoxin-related protein of 14 kDa. New insights into the specificity of thioredoxin function. *J Biol Chem* 279(5):3142-3150.
226. Toffoli S & Michiels C (2008) Intermittent hypoxia is a key regulator of cancer cell and endothelial cell interplay in tumours. *FEBS J* 275(12):2991-3002.
227. Paez-Ribes M, *et al.* (2009) Antiangiogenic therapy elicits malignant progression of tumors to increased local invasion and distant metastasis. *Cancer Cell* 15(3):220-231.
228. Cooke VG, *et al.* (2012) Pericyte depletion results in hypoxia-associated epithelial-to-mesenchymal transition and metastasis mediated by met signaling pathway. *Cancer Cell* 21(1):66-81.
229. Haque A, Andersen JN, Salmeen A, Barford D, & Tonks NK (2011) Conformation-sensing antibodies stabilize the oxidized form of PTP1B and inhibit its phosphatase activity. *Cell* 147(1):185-198.