Redox regulation of protein phosphatase-1 and ER stress regulation of connective tissue growth factor in cardiomyocytes

Doctoral Thesis

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Submitted by

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Dedicated to the Family

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Affidavit

Here I declare that my doctoral thesis entitled:

"Redox regulation of protein phosphtase-1 and ER stress regulation in connective tissue growth factor in cardiomyocytes"

has been written independently with no other sources and aids than quoted.

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Abbreviations

α	alpha
Á	Ångström
Asp	aspartic acid
Asn	asparagine
Arg/R	arginine
ATF6	activating transcription factor 6
ATF4	activating transcription factor 4
APS	Ammonium persulfate
AMC	7-Amino-4-methylcoumarin
ABC	ammonium bicarbonate
β	beta
BiP	binding protein
BrdU	5'-bromo-2'-deoxyuridine
BFA	Brefeldin A
BPS	bottom precoll solution
CBFHH	Calcium- and bicarbonate- free Hanks with HEPES
cMyBP-C	cardiac myosin binding protein-C
CVD	cardiovascular disease
CTGF	connective tissue growth factor
ºC	degree Celsius
cDNA	complementary DNA
CO_2	carbon dioxide
CHOP	C/EBP homologous protein
cAMP	cyclic adenosine monophosphate
Ca^{2+}	calcium
Cys	cysteine
CT	cysteine knot
CR	cysteine-rich
CM	cardiomyocyte medium
DAPI	4',6-diamidino-2-phenylindole
DTT	Dithiothreitol
DMSO	dimethylsulfoxide
SS	disulfide
DNA	deoxyribonucleic acid
DiFMUP	6,8-difluoro-4-methylumbelliferyl phosphate
ER	endoplasmic reticulum
EBM-2	endothelial basal medium-2
e.g.	exempli gratia (for example)
EGM-2	endothelial growth medium-2
ECM	extracellular matrix
EDTA	ethylendiamine-tetraacetic acid

ECC	excitation-contraction coupling
ESI	electrospray ionization
eIF2α	eukaryotic translation initiation factor 2α
ESI	Electrospray ionization
et al.	et altera
FBS	fetal bovine serum
F-actin	filamentous actin
FITC	fluorescein thioisocyanate
g	gram
gDNA	genomic DNA
His	histidine
HCl	hydrochloric acid
hEGF	human epidermal growth factor
hFGF	human fibroblast growth factor
h	hour
H_2O_2	hydrogen peroxide
$^{\bullet}OH$	hydroxyl radical
HUVEC	human umbilical vein endothelial cells
HRP	Horseradish peroxidase
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HPLC	high-performance liquid chromatography
I-1	inhibitor-1
I/R	ischemia/reperfusion
IRE-1	inositol-requiring enzyme-1
IGFBP	insulin-like growth factor binding protein
IF	immunofluorescence
IAA	iodoacetamide
i.d	internal diameter
l	liter
LTCC	L-type Ca^{2+} channels
LC	liquid chromatography
Lys/K	lysine
kDa	kilo dalton
MALDI	matrix-assisted laser desorption/ionization
MS	mass spectrometry
m/z	mass/charge
μ	micro
min	minute
M	molarmolar
mRNA	messenger RNA

mM	millimolar
μ	micro
μl	microliter
ms	milliseconds
n	nano
NOX	NADPH oxidases
NOS	nitric oxide synthases
NCM	non-cardiomyocyte medium
NRCM	neonatal rat cardiac cardiomyocytes
OM	opti-MEM
O_2	oxygen
PLB	phospholamban
PKA	protein kinase A
pH	negative logarithm of the H^+-ions
PP	Protein phosphatase
PP-1	protein phosphatase type-1
PP-2	protein phosphatase type-2
PTP	protein tyrosine phosphatase
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PERK	protein kinase R-like ER kinase
PTM	post-translational modifications
PFA	paraformaldehyde
PDGF	platelet-derived growth factor
%	percent
P/S	penicillin/streptomycin
PBS	phosphate-buffered saline
ppm	parts per million
Q	quadrupole
ROS	reactive oxygen species
qRT-PCR	quantitative reverse transcription polymerase chain reaction
RNA	ribonucleic acid
RNase	ribonuclease
RT	room temperature
RyR2	ryanodine receptors
R110	bisamide rhodamine 110 peptide
rpm	rounds per minute
rPP-1	recombinant protein phosphatase type-1
SR	sarcoplasmic reticulum
SERCA2a	sarcoplasmic reticulum Ca-ATPase
Ser	serine
S.D.	standard deviation

sec	second
siRNA	small interfering RNA
$O_2^{\bullet-}$	superoxide
SOD	superoxide dismutases
SDS	sodium dodecyl sulfate
SAD-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
TEMED	tetramethylethylenediamine
TGN	thapsigargin
Thr	threonine
ToF	time of flight
TnI	troponin I
TSP1	thrombospondin motif
TGF-β	transforming growth factor-β
TPS	top precoll solution
UPR	unfolded protein response
vWF-C	von-Willebrand factor type C
v/v	volume by volume
W.B	western blot
H_2O	water
XO	xanthine oxidase
XBP-1	X-box binding protein 1 mRNA

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Abstract

Heart failure is one of the most common causes for morbidity and hospitalization in the western civilization. The prognosis is still poor and new therapies are needed. For decades, variations in phosphorylation and redox status of cardiac proteins have been characterized in different heart diseases to identify new drug targets. Both abnormal phosphorylation-levels of cardiac key proteins and elevated reactive oxygen species (ROS) production were found to contribute to contractile dysfunction and fibrosis in failing hearts.

In this context type-1 phosphatase (PP-1) was demonstrated to be a principal contributor to Ser/Thr PP activity (~45%) and has been implicated particularly in the regulation of basal cardiac contractility and in the responses to β-adrenergic stimulation (El-Armouche et al, 2009; Yin et al, 2009). Up until now redox sensitivity of cardiac PP-1 has not been addressed, despite well-known perturbations in PP-1 regulation in failing hearts. Therefore, one goal of this project was to identify the underlying mechanisms of PP-1 oxidation and to test whether oxidized PP-1 contributes to the pathophysiology of abnormal protein phosphorylation and myocardial dysfunction in failing myocardium. Immunoblotting revealed that the phosphorylation status of classical PP-1 downstream target proteins, such as phospholamban (PLB) and cardiac myosin binding protein-C (cMyBP-C) were differentially affected by H_2O_2, indicating a complex layer of regulation of both redox sensitive kinases and phosphatases. Consistently, the phosphorylation status of protein phosphatase inhibitor-1 (I-1), a crosstalk protein between protein kinase A and PP-1 signaling, showed a bell-shaped phosphorylation response with a maximal peak at 100 µM., For the first time we demonstrated with mass spectrometry that PP-1 shows various post-translational modifications on the incubation with H_2O_2 as one of the majorly available intracellular reactive oxygen species. In summary, for PP-1 a mechanism is purposed which states that PP-1's cysteine residues in the presence of H_2O_2, first form sulfenic acid with a fast response to protect higher oxidations by glutathione that enables a self-protective mechanism by forming transient inter-disulfide bridges. Intra-protein disulfide bridges with Cys^{127} to form a dimer formation of PP-1 at 70 kDa might also play a role for the activity of the protein. In contrast, in the absence of glutathione, direct formation of sulfonic acid would make the protein irreversible inactive. The discovery of reversibility of PP-1 in the presence of the reducing agent (TCEP) after inactivation upon H_2O_2 treatment clearly shows that disulfide bridges are playing a crucial role in maintaining the activity of PP-1.

In addition, to the changed redox status of cytosolic proteins like PP-1 in diseased cardiomyocytes, an impairment of the redox balance in organelles was described. With this respect the occurring endoplasmic reticulum (ER) stress is of high interest as it could influence transmembrane and secreted proteins. Therefore, the redox- and ER stress-dependent regulation of the secreted connective tissue growth factor (CTGF) was investigated. CTGF is a cysteine-rich protein highly expressed during embryonic development and in fibrotic diseases, including cardiac fibrosis (Winter et al., 2008; Lok et al., 2015). Due to its high content in cysteines and intramolecular disulfide bonds, we hypothesize that ER stress modulates the oxidation status of CTGF, which in turn affects its activity and structure in cardiomyocytes. Moreover, it was unknown whether ER stress can be modulated by the expression of cysteine-rich proteins like CTGF. We first analyzed CTGF expression in human diseased heart samples and were able to show an up-regulation in

ischemic cardiomyopathy (ICM), which is associated with increased ER stress and changes in redox signaling. To further link the regulation of CTGF to these processes, isolated neonatal rat cardiomyocytes (NRCMs) were treated with pharmacological ER (DTT, thapsigargin) and oxidative (H_2O_2) stress inducers. DTT altered the molecular weight of CTGF in non-reducing immunoblots, suggesting conformational changes in the protein structure. In contrast, thapsigargin increased intracellular CTGF content, reaching the maximum after 6 hours of exposure to NRCMs. H_2O_2 had only a modest effect increasing intracellular CTGF within minutes. To further analyze the crosstalk of ER stress and CTGF regulation, CTGF expression was reduced with a specific siRNA in NRCMs, which led to a decrease in the expression of ER stress markers like PDI, BIP and IRE1-α. This data argues for an interconnection of CTGF and ER stress, as ER stress modulates CTGF and *vice versa,* CTGF expression modulates proteins of the ER stress cascade.

In summary, this thesis gives mechanistic insight in the redox-dependent regulation of PP1 and CTGF, which represent not only the cytosolic and secretory compartments of cardiomyocytes, respectively, but also the two mayor pathomechanisms contractile dysfunction and fibrosis in heart disease.

Key words: cysteine, disulfide bridges, heart failure, redox, cardiomyocytes, PP-1, CTGF

1 Introduction

1.1 Oxidative stress

During normal cellular aerobic function reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2), hydroxyl radical ($^\bullet OH$) and superoxide ($O_2^{\bullet-}$) are produced in cells which react at both cellular and tissue levels (Griendling and FitzGerald, 2003). Intracellular ROS are formed from a single electron reduction of oxygen (O_2), which leads to the formation of radical $O_2^{\bullet-}$. In the presence of superoxide dismutase (SOD) enzymes, two molecules of $O_2^{\bullet-}$ can be converted to one molecule of H_2O_2 and one molecule of water (H_2O). In addition, by accepting an electron from free Fe^{2+} ions (Fenton reaction), H_2O_2 is converted to $^\bullet OH$. With the potential presence of glutathione peroxidases, peroxiredoxins or catalase, H_2O_2 can be reduced to water. H_2O_2 can also potentiate the modification of redox-sensitive Cys residues to alter cellular signaling (Sullivan and Chandel, 2014; Figure 1). Further details about Cys redox modifications are discussed in Section 1.2.
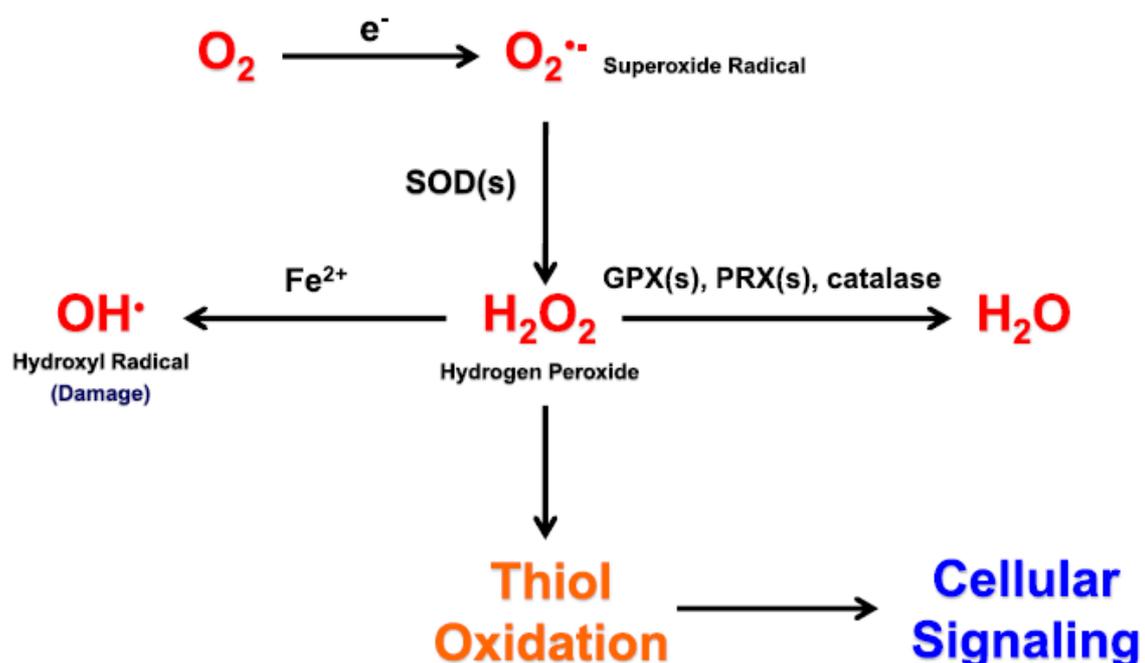
Figure 1 | Chemical reactions involved in generating ROS. Signaling pathway showing the formation of ROS species within the cell (Sullivan and Chandel, 2014).

Classically, oxidants have been considered as harmful elements mediating pathology. Oxidants are counterbalanced by antioxidants to maintain homeostatic levels of ROS. The most well-known antioxidant molecules are GSH, ubiquinol, thioredoxin, lipoic acid, beta carotene, retinol (vitamin A), ascorbic acid (vitamin C) and alpha-tocopherol (vitamin E)

(Charles and Eaton, 2008). Whenever oxidants and antioxidants are imbalanced, leads to an increase in ROS levels, a condition which is called oxidative stress (Sies, 1997).

On the other hand, redox signaling involves O_2 or O_2-derived ROS to alter the cardiac function at the post-translational level. From a clinical perspective, oxidative stress has been associated with cardiac disease development (e.g., heart failure) with antioxidants in clinical trials showing little or no impact on rescuing the heart from diseases (Johnston et al., 2015).

1.1.1 Sources of ROS and their role in the heart

The primary sources of ROS in cardiac metabolism are known to be mitochondria, endoplasmic reticulum (ER), nicotinamide adenine dinucleotide phosphate (NADPH) oxidases (NOX), nitric oxide synthases (NOS), cytochrome P450 oxidases and xanthine oxidase (XO) (Sag et al., 2014; Figure 2). Electron leak from complexes I and III are the most well characterized sources of mitochondrial ROS production and have a significant impact on both disease pathogenesis and redox signaling transduction in the cardiovascular system (Chen and Zweier, 2014). However, recent studies suggest excessive mitochondrial ROS production in apoptosis, which leads to an abrupt remodeling of the heart (van Empel, Vanessa P M et al., 2005; Matsushima et al., 2006). Within the human end-stage of heart failure and in various heart failure models such as myocardial infraction and pressure overload–induced myocardial hypertrophy, XO has been found to be highly expressed (Berry and Hare, 2004; Maytin et al., 2004; Stull et al., 2004). In contrast, human clinical studies suggested that XO inhibitor treatment is not related to increased or decreased risk of cardiovascular diseases (Seoyoung C. Kim et al., 2015).

All seven isoforms of NADPH oxidase enzymes contain a core subunit NOX1-5 and DUOX1-2. NOX2 and NOX4 are expressed mainly in endothelial cells, cardiomyocytes, and fibroblasts. Studies of NOX2 knockout mice suggest that during pressure overload, ROS produced by NOX2 can affect the development of interstitial fibrosis and cardiac contractile dysfunction, but it is not shown to be important for the development of cardiac hypertrophy (Grieve et al., 2006). In eukaryotic cells, ER provides a typical oxidation environment for protein folding and disulfide bridge formation. Over time, as unfolded proteins are accumulated in the ER, leading to ER stress in the cell, an increase in ROS species can be observed due to a decrease in antioxidant levels (Rahal et al., 2014). More information related to ER-associated ROS will be discussed in Section 1.6.

For the physiological functioning and signaling of the cardiac cells, high amounts of endogenous H_2O_2 lead to various diseases, whereas lower levels are essential. Within

diseased tissues H_2O_2 concentrations of up to 100 μM have been measured (Burgoyne et al., 2007; Hartzell, 2007). However, 1-15 μM of H_2O_2 appears to be the peak level in normal physiological contexts. During scientific experimentation, intracellular concentrations of 1-15% of externally applied H_2O_2 have been reported. External incubation with $10-10^3$ μM H_2O_2 could largely mimic the discharge of H_2O_2 endogenously by growth factors and therefore such concentrations of exogenous H_2O_2 are physiologically relevant (Schroder and Eaton, 2008).
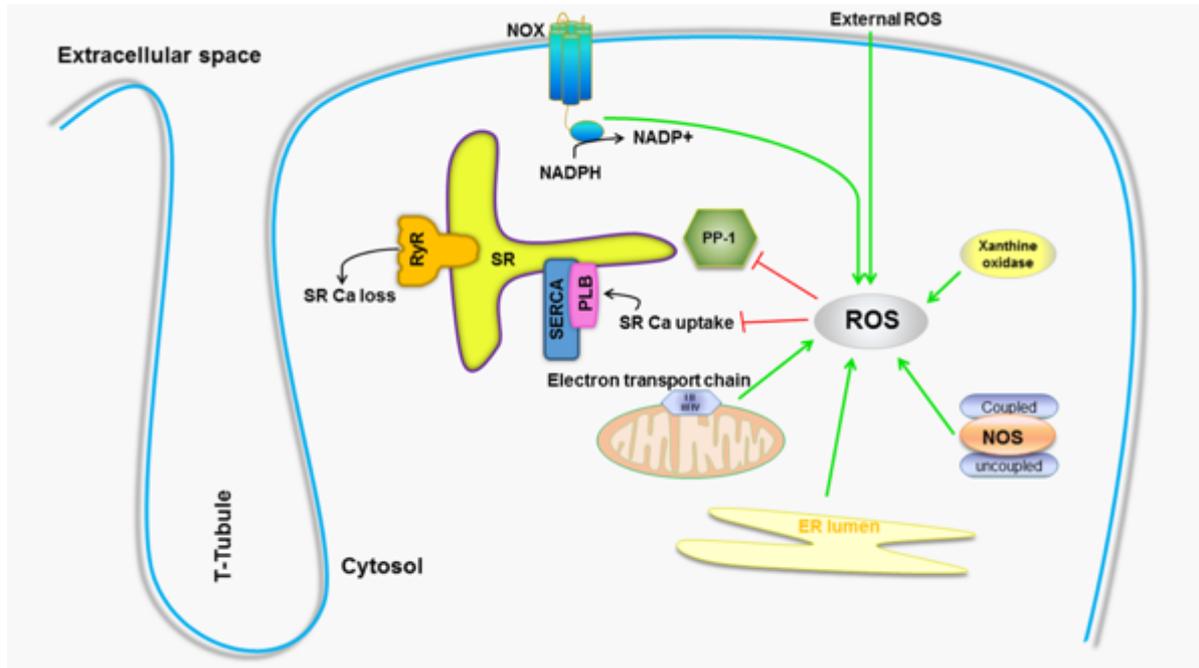
Figure 2 | Sources of ROS in cardiomyocytes. Under both baseline and pathophysiological conditions, ROS is produced by various sources in cardiac tissue. PP-1 is inhibited by ROS, playing an important role in calcium and ROS signaling within the cell (adapted from Erickson et al., 2011).

Previous clinical experiments using antioxidants have been largely disappointing as despite promising pre-clinical data in animal models, anti-oxidants failed to show any protective effects in large scale clinical trials (Steinhubl, 2008). These unanticipated results have been largely attributed to the unspecific nature of antioxidants. In such cases, exogenous application of anti-oxidants would also disrupt the physiological processes that oxidants contribute to. This therefore permits further research on the pathological and physiological roles of oxidants in order to develop specific treatments to target these mechanisms.

1.2 Redox-nano switches – Cysteine residues

Cysteine (Cys) is a molecule that consists of sulfur, carbon, nitrogen and hydrogen. In general, due to the presence of the thiol group, Cys side chains are known to be a highly potent nucleophile under physiological conditions (Figure 3). Within the thiol group, the

average pKa of Cys is 8.2 (Tajc et al., 2004). Thiol group reactivity is linked with its pKa value (Shaked et al., 1980).
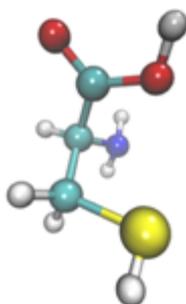
Figure 3 | Cys structure. Cartoon representation of the Cys molecule showing sulfur (yellow), carbon (cyan), oxygen (red), nitrogen (blue) and hydrogen (gray-white).

Thiol acidity could be increased by three to four-fold, if the thiol groups are in proximity to positively charged residues, i.e. lysine or arginine (Copley et al., 2004). Interactions with distinct residues and metal ions can also lead to stabilization of the thiolate form. Within the ER (a highly oxidizing compartment), Cys residues have a tendency to form disulfide bridges under physiological conditions. Whereas in the cytoplasm (a highly reducing environment), Cys residues are in the free thiol state.
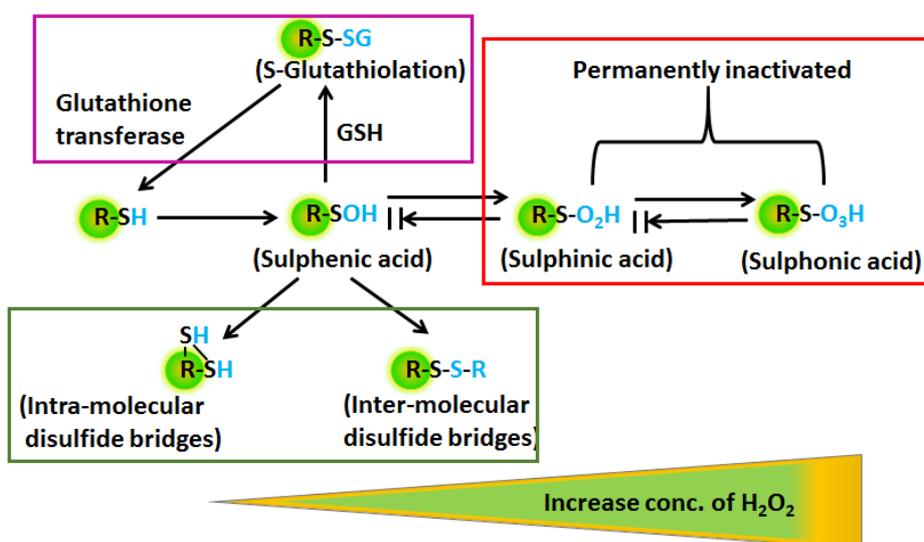
Figure 4 | Redox modification pathway of Cys by H_2O_2. The catalytic thiol groups, representing oxidative modification by H_2O_2: reversible modification (sulfenic acid, disulfide, and glutathionylation) and irreversible modification (sulfinic acid and sulfonic acid) (adapted from Meng C.T. et al., 2004).

When Cys is oxidized, kinases and phosphatases are activated and inactivated, respectively (Denu and Tanner, 1998; Brennan et al., 2006). Cys thiol groups are highly reactive and can trigger many biological pathways and act as a primary site for post translational modifications (PTM). Thiol modifications can be reversible, such as the formation of sulfenic acid (R-SOH), inter or intra-disulfide bonds and glutathionylation (R-S-SG) (Lim et al., 2001), or irreversible,

such as the formation of sulfinic acid (R-S-O$_2$H) and sulfonic acid (R-S-O$_3$H) (for review see Murray and van Eyk, 2012; Figure 4).

1.2.1 Reversible thiol modifications

1.2.1.1 Disulfide bridges

At the post-translational level, only Cys residues can undergo oxidation, which leads to the formation of disulfide bridges, which are particularly enhanced in secreted and membranous proteins. Disulfide bridges help to stabilize the protein's secondary structure through stronger covalent bonds (and not ionic or H-bridge formation) between two parts of the protein, or by linking various polypeptide chains. Normally proteins with only a single disulfide are available, but due to the presence of multiple Cys residues within one protein, formations of many disulfide bridges are also possible. Disulfide bridges formation within the cellular environment is also dependent on the oxidants present in the various cell compartments. Disulfide bridge formations are favorable within the lumen of ER due to a suitable pH (~7.2) for redox reactions of a thiol group (Kim et al., 1998). On the contrary, in the cytosol glutathione is the primary thiol-containing molecule, which prevents the formation of disulfide bridges. Within the cytosol of a resting cell, glutathione is present in either its reduced form (GSH) or oxidized state (GSSG) in the ratio 100:1; a ratio which has been demonstrated to reduce to 10:1 or even 1:1 in various models of oxidative stress (Chai et al., 1994).

Disulfide bridges can be of two types: intra, and inter - disulfide bridges. Some proteins such as thioredoxin, glutaredoxin, and protein disulfide isomerases contain CXXC motifs, which are a signature style of all proteins that form intra-disulfide bridges (Go et al., 2015). In addition, kinases are also well known to be redox regulated. It has been established that serine/threonine kinases PKG-1α (Burgoyne, JR et al., 2007) and ATM (Guo et al., 2010) activation mechanism is dependent on the formation of intermolecular disulfide bridges between homodimers.

1.2.1.2 S-glutathionylation

One of the most common PTMs inside the cells is S-glutathionylation. When a protein undergoes the oxidation process, first the most unstable form of thiol-oxidation sulfenation happens, which is then resolved by a Cys GSH. This reaction stops further oxidation of the protein to form an irreversible form of thiol oxidation (sulfinic and sulfonic acid). The resulting product of the reaction is S-glutathionylation (R-S-SG), which can be reversed to the original state of the protein (R-SH), in the presence of glutathione transferase (GST). GSSG ←→

GSH transfer to Cys residues is primarily dependent on the action of GST. This in return is mainly dependent on continued yield of GSH from the synthetic enzymes, such as glutathione synthetase and gamma-glutamylcysteine. This further helps in the removal of conjugates of GSH as a defined transporter. The principle role of GST is detoxification of xenobiotics by catalyzing the nucleophilic attack by GSH, which is possible with electrophilic carbon, nitrogen or sulfur atoms. This prevents any communication with crucial nucleic acids and cellular proteins (Ferre and Clote, 2005).

1.2.1.3 S-sulfenylation

When proteins are exposed to oxidants, sulfenic acid (R-SOH) is formed which can alter protein structure and hence protein activity. As this modification is highly unstable, the identification of this state remains challenging (Lo Conte and Carroll, 2013). It has been found that mainly protein tyrosine phosphatase (PTPs) are inactivated by H_2O_2. Within the SH2 domain-containing PTPs (SHP-1 and SHP-2), two Cys residues form a stable disulfide bridge. This modification leads to an increase in catalytic pK_a value and hence a decrease in the activity of PTP protein (Chen et al., 2009).

1.2.2 Irreversible thiol modifications

1.2.2.1 Sulfinic and sulfonic acid

Two modifications of the thiol groups are considered to be permanently inactivated and irreversible: sulfinic (R-S-O_2H) and sulfonic (R-S-O_3H) acid. Recently, 181 R-S-O_2H/R-S-O_3H sites were recognized from rat myocardial tissue incubated with a physiological estimation of H_2O_2 (<100 µM) or from ischemia/reperfusion (I/R) injury using the Langendorff perfusion. This study showed that I/R not only substantially increases both modified peptides from proteins involved in energy utilization and contractility, but also those engaged in oxidative damage and repair (Paulech et al., 2015).

1.3 Mass spectrometry

Mass spectrometry (MS) is an analytical technique mainly used to measure the molecular mass of a sample. It can also be used for more complex protein samples and structure analysis. The MS techniques is known to be very versatile due to the following attributes: (i) high sensitivity, (ii) detection of every molecule independent from its chemical nature, (iii) enabling of minor mass changes, e.g. alteration of one amino acid for another, and (iv) detection of PTMs with their exact modification site. The most common instruments in

biopharmaceutical MS are based on the matrix-assisted laser desorption/ionization (MALDI) and electrospray ionization (ESI) principle because they are available to large biomolecules. In principle, the liquid or gaseous sample first needs to be transferred from atmospheric pressure to the high vacuum regions and also from a non-charged molecule into the charged ion state. This is done by one of several ion sources available at front of the mass spectrometry unit. Then the particles pass through the mass analyzer, where they are separated according to their m/z ratio. At the end, they hit the detector plate that consumes the ions. Detection is enabled by the multiplication of a molecule in a secondary ion cascade, and this package of ions is recorded both the detector and by a PC framework. The PC shows the signals graphically as a mass spectrum: a two-dimensional plot of intensity versus m/z.
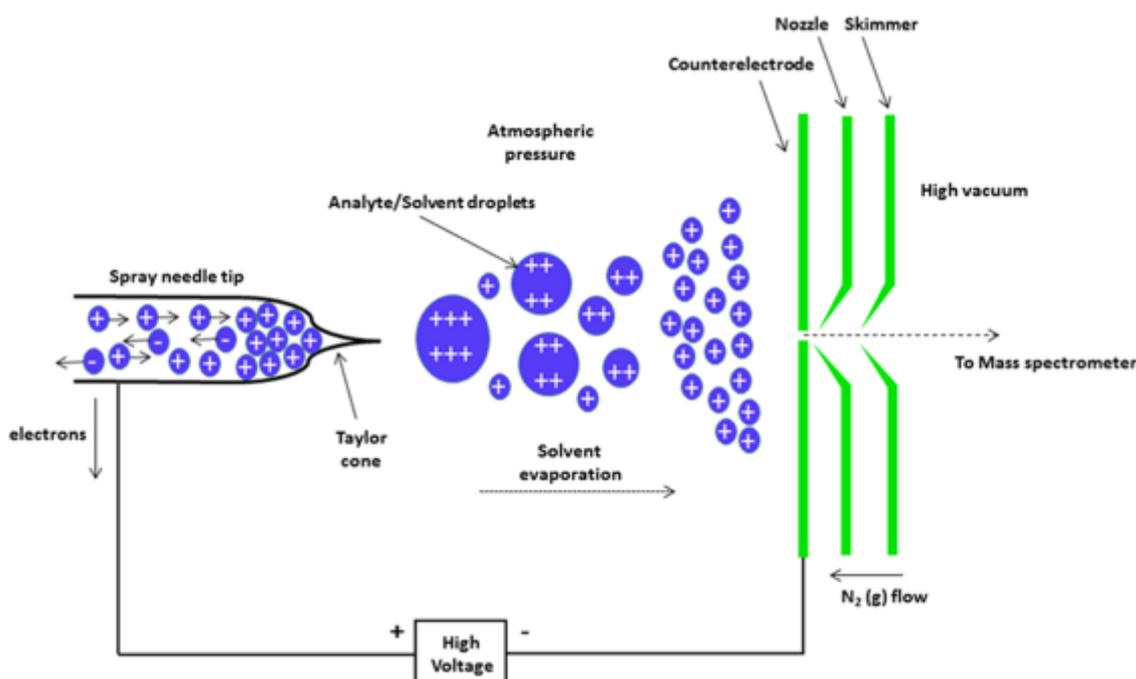
Figure 5 | An overview of electrospray ionization. ESI source produced a continuous stream of the sample solution, which is passed through quartz silica capillary, producing ions into multiple charge states that are trapped by MS.

Currently, most MS instrumentation is coupled with ESI, which has been established as one of the most important methods for small-scale chromatography coupling. A sample of interest is first brought into the tip of the conductive capillary, i.e. the ionization source of the MS. Then an electric field is established between the capillary and the mass spectrometer, which leads to the initial procuration of positive or negative charges. In ESI the molecules are present in solution and are then transformed into the gaseous state using high voltage. This process generates charged analyte/solvent droplets at the tip. Furthermore, atmospheric pressure reduces the size of the charged droplets and the solvent evaporates. Consequently,

droplets continue to contracting until the surface strain can no longer support the charge and the droplets are separated, and then analyzed by MS (for review see Yates et al., 2009; Figure 5).

1.3.1 General strategies for peptide and disulfide bridge identification

As discussed in Section 1.2, disulfide bridges formation is most common with secretory proteins, which could become one of the main targets in biopharmaceutical industry. Certainly, the PTMs in general, assume an essential role in the structural stabilization of the protein, and in the near future, it will be necessary to study the advancement of novel protein biopharmaceutical interventions (Sandra et al., 2014). Currently, detection of SS bridges or glutathionylation in proteins is difficult in MS analysis due to the high tendency for false–positive results. In MS-based disulfide mapping, the general rule is to produce and dissect fragments with a single disulfide bridge associating two peptides via proteolytic enzymatic digestion of the non-reduced protein. Further, it can then be recognized either by mass alone or by MS/MS sequencing. The predominantly used enzyme in proteomics is Trypsin, but in SS analysis it might be beneficial to digest at low pH, in such case Pepsin would be an ideal enzyme (Liu et al., 2014). Such cases result in the integration of an absence of enzymatic cleavage sites amongst Cys residues, and smaller disulfide bridges centers with firmly dispersed or even adjacent Cys residues (Goyder et al., 2013; Reinwarth et al., 2014).
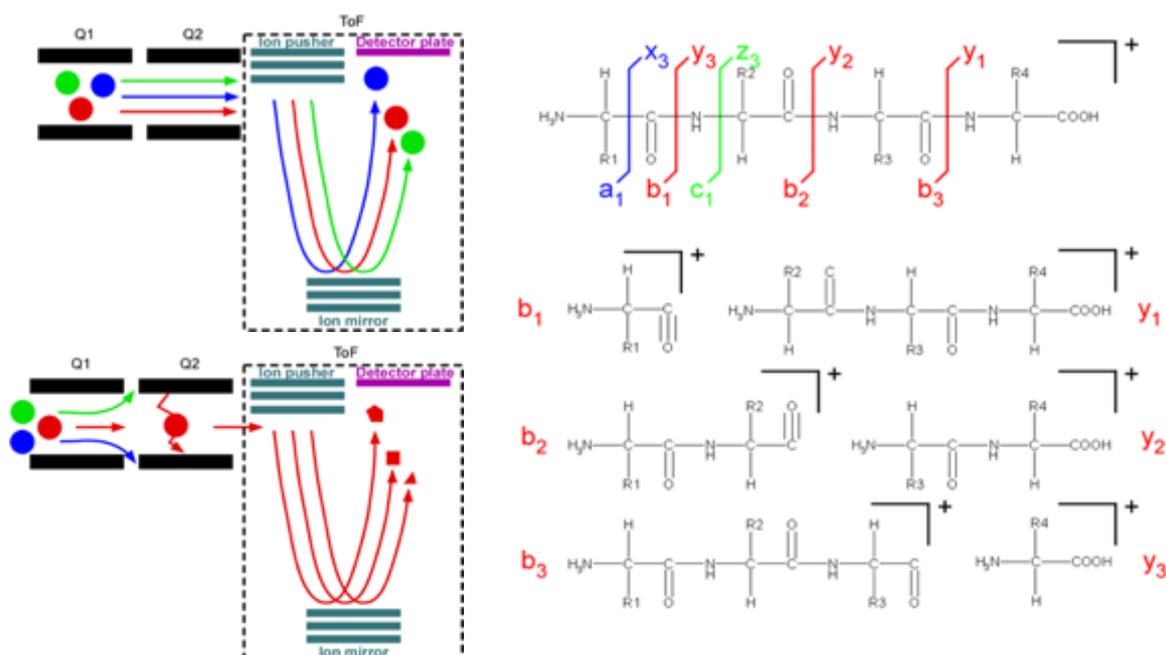
Figure 6 | An overview of the generation of MS and MS/MS spectra used for the identification of peptides. On the top left, the MS analysis of three different ions is shown. They bypass Q1 and Q2 and are separated in the ToF according to m/z. On the lower left, selection of one ion in Q1 and fragmentation in Q2 with subsequent detection in the ToF is shown. On the right hand, the

fragmentation pattern with the most typical ions of a short model peptide is represented (according to Biemann, 1988).

To understand the general strategy behind the unequivocal identification of a linear peptide, an example of a QToF instrument is explained (Figure 6). A QT of instruments consists of the following parts: (i) a selection Quadrupole (Q1), (ii) a fragmentation Quadrupole (Q2), and (iii) a Time of Flight (ToF) high-resolution mass analyzer. The latter can be interchanged with an Orbitrap which turns the instrument into the state of the art QExactive. When analyzing the intact mass of the peptides, the mixture transitions from Q1 to Q2, and is then separated according to its m/z in the ToF analyzer.

When the sequence or structural information of a peptide is required, the peptide is first selected in Q1, then fragmented in Q2 (by applying collision energy which is achieved by a lower vacuum value when inflating the cell with He or N_2 molecules), and the resulting fragments are separated according to their m/z in the ToF analyzer. Combined information on the intact mass and the sequence readout can be submitted to a database search for unequivocal identification of the peptide. Fragmentation of the peptide depends on the method and the gas used. Generally, the peptide breaks along the backbone exactly at the peptide bond, resulting in y-type (C-terminal) and b-type (N-terminal) ions. If using Trypsin as a proteolytic enzyme, the placement of a positive charge at the C-terminal Arginine (Arg/R) or Lysine (Lys/K) residue is visible, and another mobile proton is available for b-type ions.
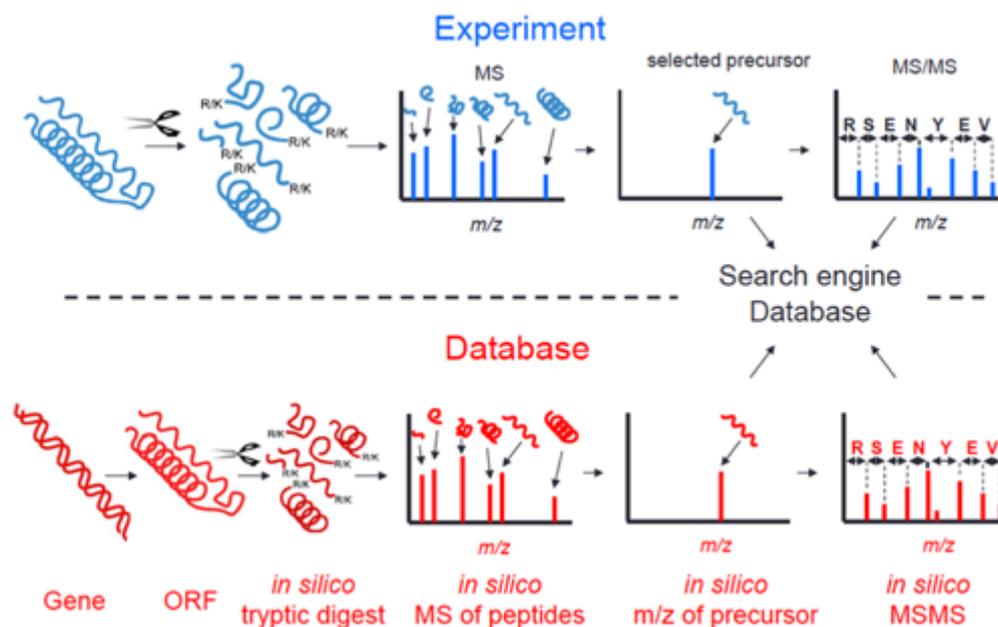
Figure 7 | A general strategy explaining the identification of peptide using MS/MS data. In silico generated MS/MS data from a complex database is compared with spectra obtained from the LC-MS/MS experiment. The blue side represents the real experiment, while the red side is an *in-silico* experiment performed by the search engine.

Identification of a peptide in a proteomics experiment is assisted with databases as shown in Figure 7. The intact protein is digested with a site-specific endoproteinase like Trypsin, leaving Arg/R or Lys/K residues at the C-terminus. These peptides are detected in the MS regime, one precursor ion (one peptide) is selected for fragmentation, and the sequence can be read out according to Figure 7.

Starting with a gene, the open reading frame (ORF) is in-silico transcribed, but modern proteome databases begin immediately with a curated database that contains only existing proteins. The proteins are in-silico digested and then an automated database search requires a spectrum comparison, which can be performed by two approaches: (1) One approach starts with comparing the intact mass of a peptide with all suitable intact masses from the *in-silico* digested database. Further spectrum searches are performed on a subset of corresponding peptide spectra. (2) The other approach starts with the determination of peptides sequences and searches them against a much smaller subset of possible precursor masses going along with it, which will greatly improve the confidence of peptide identification, particularly when PTMs are involved.

1.4 The cardiac β-adrenergic signaling pathway

Cardiovascular diseases (CVDs) are the primary cause of mortality worldwide, accounting for 17.3 million deaths per year, and mortalities are expected to increase to more than 23.6 million deaths per year by 2030. Even though the death rate from CVDs has fallen to 39% between 2001 and 2011, the concern and risk are high (Mozaffarian et al., 2015). Cardiac homeostasis is maintained by various post-translational modifications (PTMs), including phosphorylation, glycosylation, acetylation, hydroxylation, proteolytic cleavage as well as oxidative modifications. These altered PTMs can lead to heart failure, including contractile dysfunction and arrhythmias (Herren et al., 2013; Hoshino et al., 2014; Prysyazhna and Eaton, 2015).

During physical activity or stress the sympathetic nervous system initiates a 'fight or flight' response. Within ventricular cardiomyocytes, norepinephrine and epinephrine act upon the β-adrenergic signaling system - activating adenylyl cyclase (AC) via stimulatory G proteins (Gs). This leads to an increase in cyclic adenosine monophosphate (cAMP), and hence activates protein kinase A (PKA) (Reuter H., 1983). Activated PKA further phosphorylates various pivotal proteins, such as ryanodine receptors (RyR2), phospholamban (PLB), troponin I (TnI), cardiac myosin binding protein-C (MyBP-C) and L-type calcium (Ca^{2+}) channels (LTCC). These aforementioned proteins regulate the excitation-contraction

coupling (ECC) cycle of the cardiomyocytes and hence the contraction of the heart (Bers, 2002; El-Armouche and Eschenhagen, 2009; Figure 8).

During ECC, Ca^{2+} plays an essential role in cardiac contraction by maintaining high cytosolic Ca^{2+} concentration to activate cross-bridge formation between myofilaments proteins, which in turn develops pressure in the heart chambers and hence provides energy for the ejection of blood (Luo and Anderson, 2013). Ca^{2+} enters the cardiomyocytes via tubular-dependent LTCC and later, with the phosphorylation of PLB at Ser^{16} by PKA, increases sarcoplasmic reticulum Ca-ATPase (SERCA2a) activity, thereby facilitating cytoplasmic Ca^{2+} reuptake into the sarcoplasmic reticulum (SR) lumen. The phosphorylation of PLB, cMyBP-C, and TnI by PKA could be reversed by protein phosphatases (PP) (Figure 8). Phosphatases are discussed in more detail below.
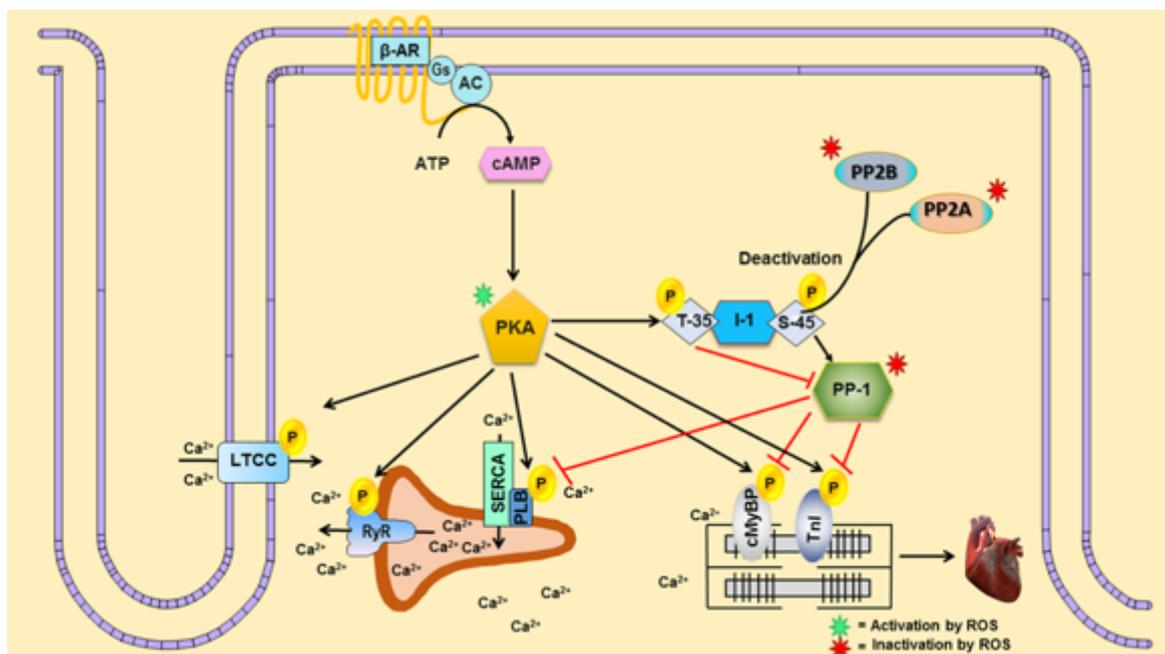
Figure 8 | Recent advances in redox regulation of β–adrenergic signaling pathway in cardiac myocytes. β-adrenergic (**β-AR**) receptors, localized in micro-domains of the sarcolemma formed by **t-tubules**, activate stimulatory G proteins (**Gs**) which stimulate adenyl cyclase (**AC**) to make **cAMP**. **cAMP** then promotes an increase in protein kinase A (**PKA**)–dependent phospholamban (**PLN**) phosphorylation. This in turn promotes increased uptake of calcium ions (Ca^{2+}), via the sarcoplasmic reticulum Ca^{2+} ATPase (**SERCA**), into the sarcoplasmic reticulum (SR) increasing SR Ca^{2+} load, and SR Ca^{2+} release through ryanodine receptors (**RyR2**). In addition, activation of PKA also phosphorylates L-Type Ca^{2+} channel (**LTCC**), troponin I (**TnI**) and cardiac myosin binding protein-C (**MyBP-C**). Moreover, activated **PKA**, also triggers phosphorylation of **I-1** at Threonine-35 to act as an inhibitor of phosphatase **PP-1**, resulting in dephosphorylation of **PLB**, **cMyBP** and **TnI**. On the contrary, **PP-2A** and **PP-2B** deactivates **I-1** by dephosphorylation at Serine-45 and hence activation of **PP-1**. ROS alters the balance between kinase and phosphatase activity by activating and deactivating in a dose-dependent manner respectively and hence complex signaling paradigm with the downstream cardiac proteins (adapted from Bers, 2002).

1.5   Serine/threonine phosphatases

Many physiological functions are influenced by phosphorylation/de-phosphorylation events, such as cell differentiation, cell signaling, gene expression, neuronal activity, mitosis and metabolic functions (see reviews by McCluskey et al., 2002; Virshup and Shenolikar, 2009; Schulz and Wieczorek, 2013). Regulatory processes dictate the balance between two key enzymes; protein kinases, which transfer phosphate from ATP to the protein (phosphorylation), and protein phosphatases, which catalyze the opposite reaction (dephosphorylation). Protein phosphatases are considered to be relatively non-specific enzymes that exist only to reverse the action of protein kinases. In eukaryotic cells, phosphorylation's main targets are three hydroxyl-containing amino acids serine, threonine and tyrosine, and out of these, mainly serine is targeted. While understating the role of phosphatases and kinases, which are almost equal in number in the human genome i.e. 90 vs 107, whereas the number of catalytic subunits of serine/threonine phosphatases is much lower than that of Ser/Thr kinases (40 vs 428; Moorhead et al, 2007). Protein phosphatase families include: protein phosphatase type-1 (PP-1, ~38.5 kDa; Figure 9), type-2 (PP-2), consisting of PP-2A, PP-2B (calcineurin) and PP-2C. Recently, this set has been extended to PP-4, PP-5, PP-6 and PP-7 (Herzig and Neumann, 2000; Shi, 2009). PP-1 and PP-2 are the major constituents of phosphatase activity (~90%). Specifically, PP-1 is ubiquitously expressed in most cardiac cell types, including cardiomyocytes (El-Armouche and Eschenhagen, 2009).

1.5.1   Protein phosphatase 1 (PP-1)

PP-1 is a monomeric 37-kDa protein (Bollen et al., 2010) containing 330 residues. PP-1 plays a leading role in Ser/Thr PP activity (~45%). All isoforms of PP-1 contain thirteen Cys residues that hold two manganese (Mn^{2+}) ions at the center of the structure. Mn^{2+} is an essential element in biological systems and occurs in various oxidation states (+2. +3. +4. +6 and +7). It is also a cofactor for important enzymes and metalloproteins that are necessary for proper functioning (Martinez-Finley et al., 2013). Mammalian cells have three genes which encode four isoforms of PP-1's catalytic subunits PP-1α, PP-1β (or δ), PP-1γ1 & PP-1γ2 (Cohen 1988). Moreover, different isoforms of PP-1 shares similar sequence of percentages - 93% (γ1/γ2), 91% (α/γ1), 89% (α/β), 88% (α/γ2), 87% (γ1/β) and 85% (γ2/β). All aforementioned isoforms of PP-1 have a distinctive tissue distribution and subcellular localization, which assembled to a favored binding to regulatory subunits and perform distinct functions (MacMillan et al., 1999).

PP-1 is highly conserved throughout evolution and is involved in a diverse array of cellular processes including muscle contraction, gene transcription, synaptic plasticity, glycogen metabolism and cell cycle progression (Cyert and Thorner, 1989). In the heart PP-1 has been shown to specifically regulate basal cardiac contractility and feedback to β-adrenergic stimulation. There are two types of PP-1 inhibitors: natural small molecular toxins and protein inhibitors. Various toxins exist inhibiting PP-1 activity, including tautomycin, calyuclin A, microcystin and okadaic acid (OA). However, many endogenous proteins, such as the acid- and heat-stable inhibitor proteins – inhibitor-1 (1-1), inhibitor-2 (I-2), dopamine- and cyclic-AMP-regulated phosphoprotein (DARPP-32) have been shown to inhibit PP-1, leading to changes in localization and activity.

1.5.2 Predicted oxidative modifications of PP-1

ROS are capable of modulating the response of numerous cell-signaling pathways and serve as secondary messengers that control signal transduction by oxidizing cysteines of various kinases and phosphatases (Chiarugi, 2005). Oxidative modifications of PKA have been shown to enhance cardiac contractility via increased phosphorylation of key proteins (Brennan et al., 2006). In general, PKA inhibits PP-1 activity by phosphorylating PP inhibitor-1 (I-1) at Thr^{35} and PP-2B dephosphorylating I-1 at Ser^{45}, acting as a counterbalance (MacLennan and Kranias, 2003; El-Armouche and Eschenhagen, 2009). In contrast, ROS can inhibit the activity of phosphatases and improve the ability of PKA to phosphorylate various downstream cardiac proteins (Figure 8). So far it has been shown that inactivation of PP-2B occurs in a thiol-oxidation-dependent manner (Li et al., 2004).

PP-1 contains highly conserved motifs with putative reactive Cys residues (Cys^{155} and Cys^{158}) in proximity to the active site similar to thioredoxin (CXXC) (Figure 9.A). In the presence of oxidants, the Cys residues are expected to form a disulfide bridges, which results in loss of PP-1 activity (Fetrow. et al., 1999). This putative mechanism will be examined in this thesis.

Interestingly, besides targeting Cys for redox regulation, the PP-1 structure also has binuclear metal ions which reside about 3.3 Å apart at the center of the catalytic subunit. The two metal ions, manganese (Mn^{2+}), are surrounded by four histidines (His), two aspartic acids (Asp), and one asparagine (Asn). On top, exclusively Cys residues (Cys^{62}, Cys^{127}, Cys^{140}, Cys^{155}, Cys^{171}, Cys^{202}, Cys^{245} and Cys^{273}) also lie near the Mn^{2+} ions (Figure 9.B).
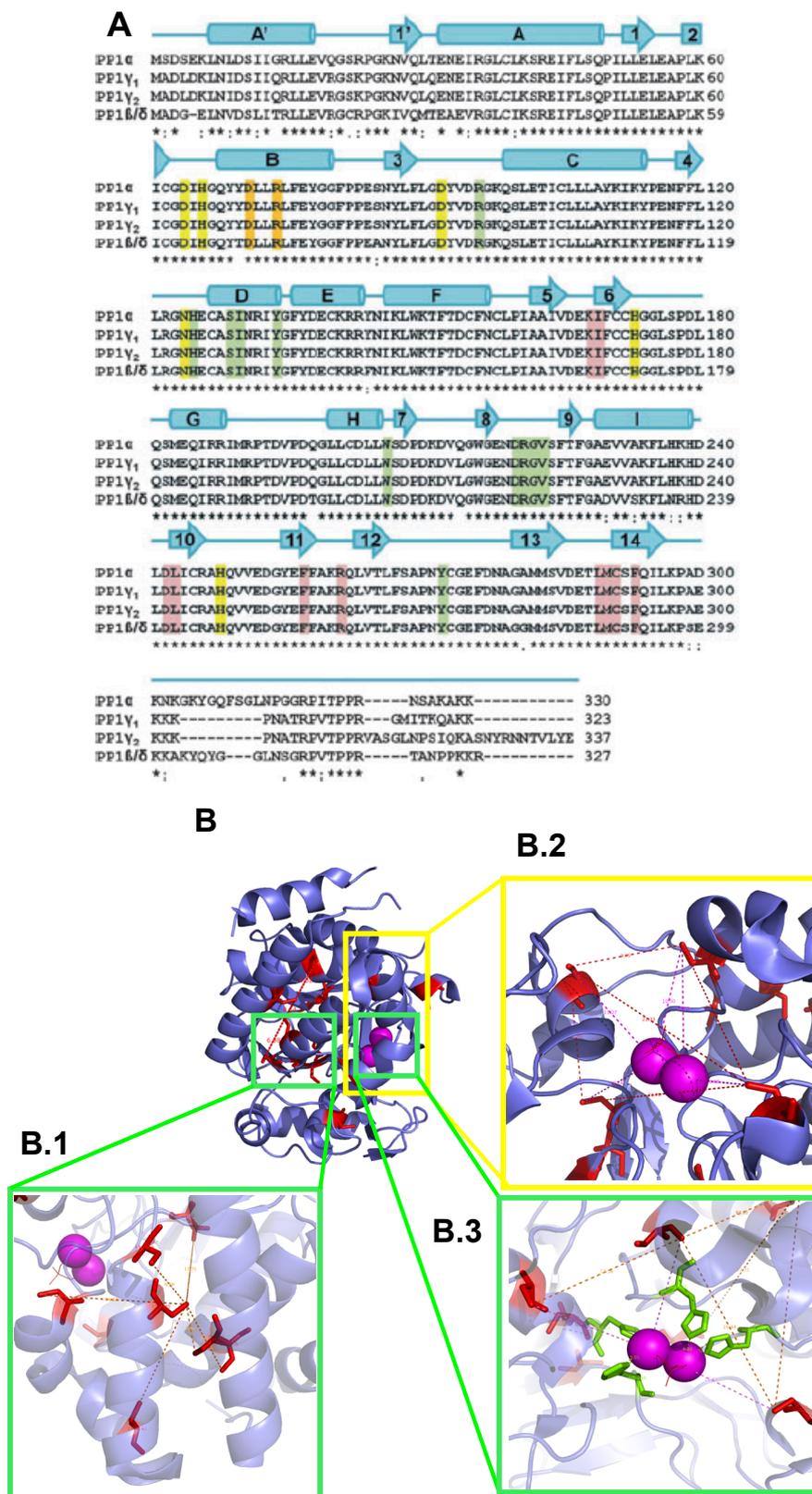
Figure 9 | Sequence and detailed structure analysis of PP-1 (PDB id: 4MOV). (A) A summary of multiple sequence alignment of the three isoforms (α, β and γ-1 and γ-2) of PP-1. The following symbols are used to depict residues: identical (*), conservative (:) and similar (.). Above the sequence,

α-helices are represented as cylinders, β-strands are indicated with arrows, and metal coordinated residues are highlighted in yellow (Peti et al., 2013). (B) Binuclear metal center surrounded by Cys residues in the PP-1 structure, showing the networking Cys residues as red in color, the distance between them as red dotted lines and the rest of the backbone peptide is shown as a blue cartoon (B.1). The two Mn^{2+} ions are shown as pink balls surrounded by close Cys residues (B.2), with four Histidines surrounding the Mn^{2+} ions (B3).

1.6 ER stress and the unfold protein response

Cardiovascular studies have intensively focused on the function of the sarcoplasmic/endoplasmic reticulum (SR/ER) in cardiomyocytes, mainly due to SR's role as a primary source of intracellular Ca^{2+}, which regulates the contraction and relaxation of myofilaments. Currently, it is well accepted that a relationship exists between disturbances in Ca^{2+} handling and heart disease, and thus the regulation of Ca^{2+} is an important pharmaceutical target for treatment of cardiovascular diseases. In addition, there is increasing evidence showing that the ability of protein handling in the SR/ER is also affected by heart disease. This leads to ER stress which has been first described in 1988 in simian cells (Kozutsumi et al., 1988).

A network of membranes, known as cisternae, builds the ER in eukaryotic cells. Membrane and secretory proteins are produced in the ER, then processed, folded, and exported via the Golgi apparatus to the cell membrane or released into the interstitial space. In the ER lumen, balanced protein folding is primarily maintained by levels of calcium, molecular chaperones, protein glycosylation and the redox-status. Perturbations in the balance of accumulation and removal of misfolded/unfolded proteins can lead to physiological/pathological consequences, a condition which is called ER stress (Glembotski, 2007). To overcome this and to restore function back to the cell, a series of events takes place, including the degradation of misfolded proteins, the increase in production of chaperones, and the downregulation of protein translation. This process is known as unfolded protein response (UPR). If UPR is not successful, the cell will undergo apoptosis (Fribley et al., 2009). In the heart, it has also been shown that UPR is activated during I/R; furthermore long-term stresses that lead to cardiac hypertrophy and heart failure (Glembotski, 2008).

ER stress is prominently led by three ER-transmembrane proteins, i.e. inositol-requiring enzyme-1 (IRE-1) (Cox et al., 1993), protein kinase R-like ER kinase (PERK) (Shi et al., 1998) and activating transcription factor 6 (ATF6) (Zhu et al., 1997). These proteins act as the primary proximal effectors of the UPR signaling pathway. When the ER protein folding machinery functions efficiently, the ER luminal domains of IRE-1, PERK and ATF6 resides at the ER-resident chaperone called binding protein (BiP). Upon perturbation of ER protein

folding machinery, aggregation of misfolded proteins begins: BiP dissociates from IRE-1, PERK and ATF6, and attaches to the hydrophobic regions of misfolded proteins in order to support their folding. This translocation of BiP activates the three proximal effectors, which in turn activates the UPR signaling pathway (Bertolotti et al., 2000; Minamino and Kitakaze, 2010; Kimata and Kohno, 2011).

1.6.1 ER-transmembrane proteins (IRE-1, PERK and ATF6)

The IRE-1 gene was first discovered in mammals which encodes a type 1 ER transmembrane protein (Mori et al., 1993). IRE-1 functions as a kinase and as an endoribonuclease. In the UPR, IRE-1 gets detached from BiP due to the accumulation of misfolded proteins in the ER. Autophosphorylation by its kinase activity occurs and dimerization activates IRE-1 leading to the subsequent activation of endoribonuclease activity. IRE-1α is the most important isoform for UPR, it efficiently splices the X-box binding protein 1 mRNA (XBP-1). After the spliced mRNA is translated, the splice variant, i.e. XBP-1s moves to the nucleus and triggers the activation of ER-stress responsive genes, which regulate the protein folding machinery, transportation and protein degradation (Calfon et al., 2002; Figure 10).

PERK is a type 1 ER transmembrane protein kinase and found as a monomer under unstressed conditions (Shi et al., 1998). Without ER stress, the luminal part of the monomeric PERK associates with the ER chaperone BiP. As soon as ER stress is initiated, BiP relocates from PERK to misfolded ER proteins. BiP relocalization permits PERK to dimerize, which facilitates trans-autophosphorylation in a mechanism similar to growth factor receptor activation (Ma et al., 2002). After dimerization and autophosphorylation, PERK is activated, which further phosphorylates and activates eukaryotic translation initiation factor 2α (elF2α) at Ser[51], which in turn prevents the initiation of global translation (Bertolotti et al., 2000). Global translational inhibition decreases the protein-folding load on the ER, aiding in the recovery of ER homeostasis and the amelioration of efficient protein folding machinery (Harding et al., 1999). With respect to translational arrest, elF2α phosphorylation also translates specific mRNAs such as the activating transcription factor 4 (ATF4). This results in increased expression of ATF4, which assists in regulating transcription factors such as C/EBP homologous protein (CHOP). This protein contributes to programmed cell death (Figure 10).

Similar to IRE-1 and PERK regulation, ATF6 is an ER transmembrane protein that, during the unstressed state, exists as a dimer linked by intermolecular SS bridges in the luminal domain and is associated with BiP. During ER stress, protein misfolding increases which

leads to the sequestration of BiP away from the ER-luminal domain of ATF6. In contrast to the other two effectors, which remain in the ER lumen upon dissociation of BiP and disulfide bridge cleavage, a 90 kDa form of ATF6 translocate to the Golgi-complex, where it is cleaved by site-1 and site-2 proteases (S1P and S2P) (Figure 10). The resulting cleaved 50 kDa ATF6 is a cytosolic fragment, which translocate to the nucleus where it can form homodimers or heterodimers with a small group of basic leucine zipper transcription factors which trigger UPR genes expression in the nucleus. Therefore, activated ATF6 is an essential element of the UPR (Glembotski, 2014).
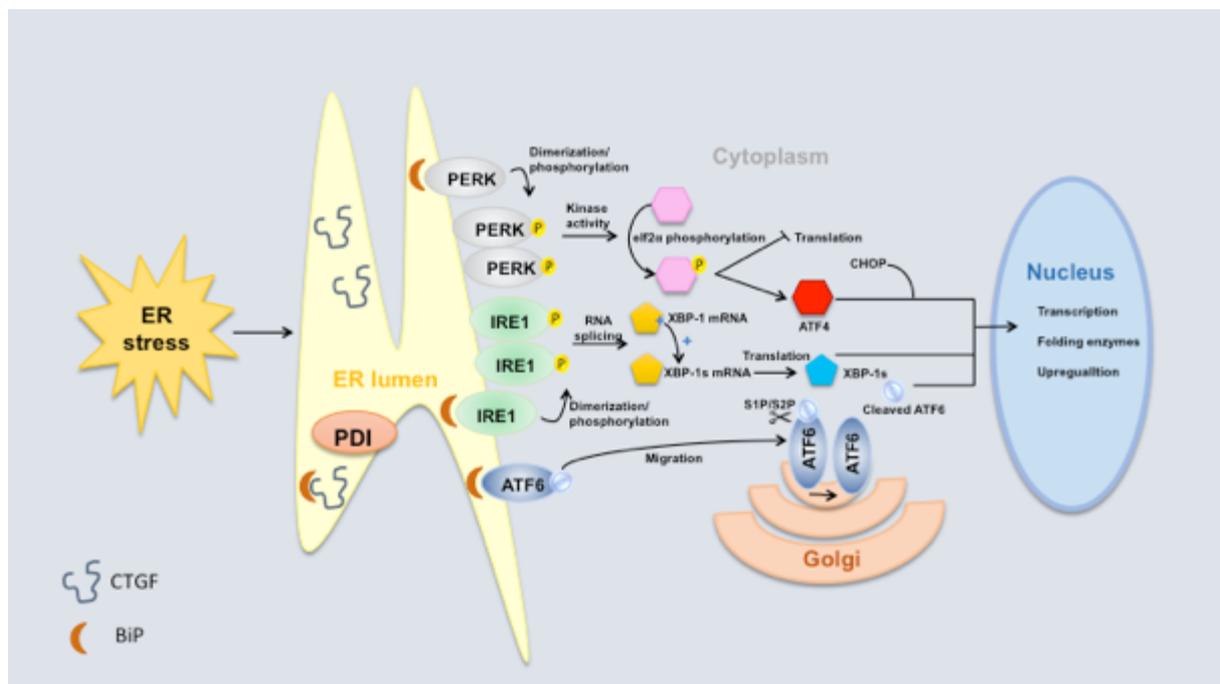
Figure 10 | ER stress signaling pathways in eukaryotic cells. ER stress is triggered by disturbance in nutrient or energy balance of a cell, results in misfolded protein accumulation in the ER and hence activation of the UPR as a surviving process. Three ER transmembrane proteins play an essential role in maintaining the balance between ER stress and UPR: Inositol-requiring enzyme-1 (**IRE-1**), protein kinase R-like ER kinase (**PERK**) and activating transcription factor 6 (**ATF6**)–in combination with the ER chaperone **BiP** (adapted from Groenendyk et al., 2013).

1.7 Connective tissue growth factor

CCN proteins are known to have abundant amounts of Cys (>10%) and a complete conservation of the 38 Cys residues position in the sequence (Bork, 1993). In total, there are six exclusive proteins within the CCN family, which share the same sequence homologies and secondary structure. The CCN family of secreted Cys-rich proteins with similar structure are grouped together and abbreviated according to these proteins: Cys-rich protein 61 (cyr61=CCN1), connective tissue growth factor (CTGF=CCN2) and nephroblastoma overexpressed protein (NOV=CCN3) (Leask and Abraham, 2006). The other three members

of the CCN family are Wnt-1-induced proteins (WISP): WISP-1 (CCN4), WISP-2 (CCN5) and WISP-3 (CCN6) (Rachfal and Brigstock, 2005). CTGF was discovered in 1991. Gary et al. described a platelet-derived growth factor (PDGF)-related mitogen in the medium of human umbilical vein endothelial cells (HUVECs) and termed it CTGF (Bradham DM et al., 1991).

1.7.1 Structural and functional properties of CTGF

CTGF is a 36 kDa matricellular protein of the CCN family containing four distinct structure modules: (1) an insulin-like growth factor binding protein (IGFBP), (2) a von-Willebrand factor type C (vWF-C), (3) a thrombospondin motif (TSP1), and (4) a Cys knot (CT) at the carboxyterminal end (Figure 11.A). Between modules (2) and (3), CTGF has a hinge region which can be cleaved by proteases into two fragments of similar molecular weights.

All four modules are involved in different physiological functions such as cell proliferation, migration, adhesion, differentiation, matrix production and apoptosis. From the pathobiology perspective CTGF is overexpressed in fibrotic lesions, fibrogenesis, cancer, atherosclerosis (Au et al., 2010; Leeuwis et al., 2010; Jacobson and Cunningham, 2012) and is also involved in wound healing, angiogenesis and epithelial-mesenchymal transition (Alfaro et al., 2013; Sonnylal et al., 2013; Liu et al., 2014b).

CTGF contains 39 conserved Cys residues, spread over four modules and can form intra- and inter-disulfide bridges. The IGFBP N-terminal domain contains twelve Cys residues (Hwa et al., 1999). vWF-C, also known as chordin-like Cys-rich (CR) repeats, contains ten Cys residues. The first motif 'Cys^{2}XXCys^{3}XCys^4' lies in the middle and the second motif 'Cys^{8}Cys^{9}XXCys^{10}' lies at the end of the repeat (Bork, 1993). In total, TSP-1 contains six Cys residues and the motif 'CSXTCG' (Tan et al., 2002). The last domain, located at the carboxy-terminal end, is known as the CT module or Cys knot. It has been suggested that the CT module may be involved in dimerization as it serves this function in transforming growth factor-β (TGF-β), nerve growth factor and PDGF (Bork, 1993).

The CT is stable in structure with two SS bridges forming a ring structure and the fifth Cys projecting through the ring to allow the formation of a third SS bridge (Perbal et al., 1998) (Figure 11.B). Within the aqueous environment, this structure enhances the availability of hydrophobic residues in monomers and supports the formation of homo- or heterodimers. To initiate signal transduction through their respective receptors, dimers serve as an active state of the CT bearing growth factors. Individual domains of CTGF show a distinct functions, where the C-terminal arbitrate fibroblast proliferation and the N-terminal domain arbitrates myofibroblast differentiation and collagen synthesis (Grotendorst and Duncan, 2005).
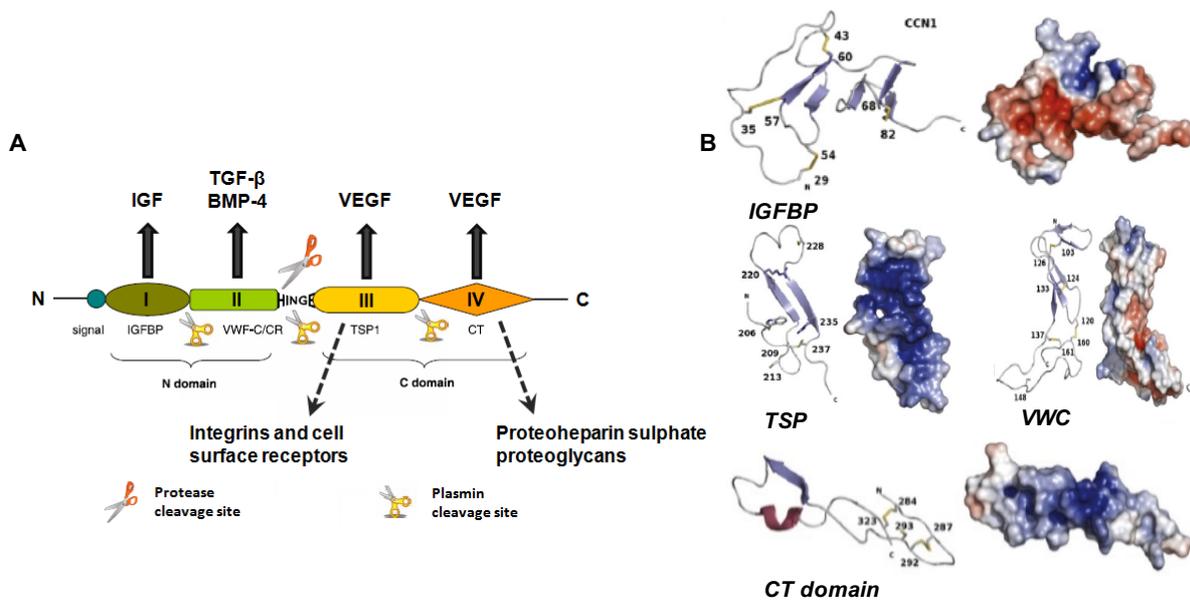
Figure 11 | Schematic structure of CTGF protein. (A) Structural CTGF is composed of four modules: IGFBP, vWF-C, TSP1 and the CT (adapted from Winter et al., 2008). (B) 3D chemical structure with various domains of CTGF showing intra and inter-SS bridges (Holbourn et al., 2008).

1.7.2 Functional aspect of CTGF in heart diseases

Previous studies on CTGF have focused on cardiac fibrosis (Jatho et al., 2015; Ongherth et al., 2015); yet it is still uncertain whether CTGF plays any role in cardiomyocytes. Contrary to studies on the negative impacts of CTGF during fibrogenesis, several reports suggest that CTGF plays a cardio-protective role. This cardio-protective role was shown by Ahmed et al, as CTGF restored phosphokinase signaling by promoting the inhibition of GSK-3β and activating phospho-SMAD2 (Ahmed et al., 2011).

CTGF can also attenuate hypertrophic signaling in cardiac myocytes in response to chronic pressure (Gravning et al., 2013a). The same research group suggested that paracrine regulation of GRK5 activity in cardiomyocytes may contribute to cardio-protective actions of CTGF in heart failure (Gravning et al., 2013b). In adult cardiac myocytes CTGF has also been shown to directly trigger the Akt/GSK-3β signaling pathway, which leads to increasing tolerance to hypoxia and oxidative stress (Moe et al., 2013). One of the studies furthermore describes an antagonizing function of CTGF by using a CTGF monoclonal antibody. The results showed that the antibody may decrease the danger of hypertensive coronary illness in patients (Szabo et al., 2014).

In the context of cellular proliferation, CCN1 and CTGF are known to be positive regulators of growth, whereas CCN3 and CCN5 are negative growth regulators (Brigstock, 1999; Lau and Lam, 1999). In different tissues and organs, CCN1 and CTGF (structurally related, but

functionally distinct multimodular proteins) are expressed during pathological or developmental events. In one of the studies, it was shown that, using *in vitro* computation of biological activities, CCN1 expression triggered a genetic reprogramming of structural, adhesive and angiogenic proteins, whereas CTGF induced aggregation of the extracellular matrix, a primary stage of fibrotic diseases (Chaqour and Goppelt-Struebe, 2006).

Further comparisons by the Park group showed differential expression of CTGF and CCN5 during the process of cardiac remodeling and opposite regulation during the fibrosis and cardiac hypertrophy. In other words, CTGF and CCN5 were shown to be pro-and anti-hypertrophic, respectively. The CT domain is absent in CCN5, which is known to have hypertrophic activity in CTGF. The deletion of the CT domain in CTGF restores hypertrophic function similar to a CCN5-like dominant negative molecule (Yoon et al., 2010). The same group demonstrated that CCN5 expression was significantly decreased in end-stage heart failure samples compared to non-failing heart samples. In addition, both *in vivo* and *in vitro* experimentation showed that CCN5 triggers apoptosis only in myofibroblasts, but surprisingly not in cardiomyocytes or fibroblasts. The authors suggest that CCN5 could reverse cardiac fibrosis (Jeong et al., 2016).

In 2001 it was reported that, in dermal fibroblasts and activated hepatic stellate cells, CTGF protein is quantitatively emitted through the Golgi apparatus and is rapidly degraded in the endosome (Chen et al., 2001). In chondrocytes it was found that the loss of CTGF triggers deformed extracellular matrix (ECM) organization and chondrocyte death, accompanied by increased cellular stress (Hall-Glenn et al., 2013). CTGF and its role in ER stress in the heart has not been fully understood. Therefore, the involvement of CTGF in the ER stress response and UPR signaling pathway was analyzed.

2 Aim of the study

Abnormal phosphorylation levels of cardiac key proteins and elevated reactive oxygen species (ROS) production were shown to contribute to contractile dysfunction and fibrosis in failing hearts. Within this context this thesis aimed to investigate the redox sensitivity of the cytosolic PP1 and the secreted CTGF in cardiomyocytes. Both proteins were demonstrated before to be dysregulated in the diseased heart, however, the influence of a changed redox status on their regulation in cardiomyocytes had been not assessed.

Given that redox sensitivities of cardiac PP-1 and CTGF are involved in contractile dysfunction and fibrosis, which are the main pathomechanisms of cardiac diseases, the thesis addresses the following objectives:

● **Identify the mechanisms of PP-1 oxidation and test whether oxidized PP-1 contributes to the pathophysiology of abnormal protein phosphorylation and myocardial dysfunction in failing myocardium**

More specifically, the following questions were addressed:

1. Does PP-1 activity change, due to cysteine oxidation status or dinuclear metal (Mn^{2+}) ions in response to oxidative stress?

2. Does glutathionylation play any role in the activity of PP-1?

The main experimental procedure consisted of activity assays to identify the PP-1 activity. Mass spectrometry was used, as a method to analyze different Cys-based post-translational modification in PP-1.

● **Test the hypothesis whether redox and ER stress affects CTGF and *vice versa***

In detail, the following questions were addressed:

1. Is CTGF affected by redox and ER stress in cardiomyocytes?

2. Is ER stress and the UPR pathway affected by the expression of CTGF?

We attempted to answer the above questions using a combination of non-reducing gels and incubation of the cells with redox and ER stress eliciting agents. Furthermore, we also used si-RNA techniques to knockdown CTGF expression in cardiomyocytes and assessed the impact on various ER stress markers.

3 Materials & Methods

3.1 Materials

3.1.1 Antibodies

Table 1 | Primary antibodies

| Primary antibody | Dilution | | Source | Type/ Clone | Catalogue No. | Company |
	W.B	IF				
α-actinin	1:2000	1:500	Mouse	Monoclonal/6-11B-1	T6793	Sigma-Aldrich
ATF6	1:500	1:50	Rabbit	Polyclonal	H-280	Santa Cruz
BiP	1:500	-	Rabbit	Monoclonal	C50B12	Cell Signaling
CHOP	1:500	1:3200	Mouse	Monoclonal	L63F7	Cell Signaling
CTGF	1:200	1:50	Goat	Polyclonal/L-20	sc-14939	Santa Cruz
CSQ	1:1000	-	Rabbit	Polyclonal	9102S	Dianova
Phospho cMyBP-C-Ser^{282}	1:5000	-	Rabbit	Polyclonal	ALX-215-057-R050	Enzo Life Science
Phospho-Inhibitor-1-Thr^{35}	1:1000	-	Rabbit	Polyclonal	#2302	Cell Signaling
IRE1-α	1:1000	-	Rabbit	Monoclonal	14C10	Cell Signaling
PDI	1:1000	1:100	Rabbit	Monoclonal	C81H6	Cell Signaling
Phospho PLB-Ser^{16}	1:1000	-	Rabbit	Polyclonal	9102S	Badrilla
PKA RI	1:200	-	Mouse	Monoclonal	610166	BD Transduction Laboratories
PP-1	1:200	-	Mouse	Monoclonal	E-9	Santa Cruz
PP-1 alpha (α)	1:1000	-	Mouse	Polyclonal	C-19	Santa Cruz
SERCA2	1:200	-	Goat	Polyclonal	Sc-8094	Santa Cruz
α-tubulin (~Tubulin)	1:2000	-	Mouse	Monoclonal	T 5168	Sigma-Aldrich

Table 2 | Horseradish peroxidase (HRP)-conjugated secondary antibodies

Secondary antibody	Dilution	Source	Catalogue No.	Company
Goat	1:10000	Donkey	Sc-2020	Santa Cruz
Mouse	1:10000	Rabbit	A9044	Sigma-Aldrich
Rabbit	1:40000	Goat	A9169	Sigma-Aldrich

Table 3 | Fluorophore-conjugated secondary antibodies for immunofluorescence

Secondary antibody	Dilution	Fluorophore	Source	Catalogue No.	Company
Goat	1:300	Cy3	Rabbit	305-165-003	Jackson Immuno Research
Goat	1:100	FITC	Rabbit	305-095-003	Jackson Immuno Research
Goat	1:150	FITC 488	Rabbit	305-095-003	Jackson Immuno Research
Rabbit	1:500	Alexa-fluor 594	Goat	111-475-144	Jackson Immuno Research

3.1.2 Buffers and solution

Table 4 | Composition of the used buffers, solutions, and media

Immunoblotting	
GST-fish buffer (500 ml)	25 ml 1 M Tris (pH 7.4 with HCl) 75 ml 1 M NaCl 2 ml 1 M MgCl$_2$ 50 ml glycerol 5 ml Igepal CA-630 up to 500 ml distilled water
4x SDS-PAGE sample loading buffer (50 ml)	25 ml glycerol 5 ml β-Mercaptoethanol 3.25 g SDS 15 ml 300 mM Tris (pH 6.8 with HCl) 0.125 g bromophenol blue up to 50 ml distilled water
Blocking buffer	10 ml 1 M Tris-HCl pH 7.4 20 ml 0.5 M malemide 20 ml 10% SDS up to 50 ml H$_2$O
2x Non-reducing loading buffer	4 ml 1 M Tris-HCl pH 6.8 1.6 gm SDS 8 ml Glycerol, 87% 4 mg bromophenol blue

	8 ml 0.5 M malemide
2x Reducing loading buffer	4 ml 1 M Tris-HCl pH 6.8 1.6 gm SDS 8 ml Glycerol, 87% 4 mg bromophenol blue 8 ml 0.5 M malemide 0.617 gm DTT
10x TBS buffer (1000 ml)	12.12 g Tris 87.65 g NaCl up to 1000 ml distilled water pH 7.4 with HCl
TBS-T buffer (1000 ml)	1000 ml 10x TBS 1 ml tween 20
5x SDS-PAGE electrophoresis buffer (1000 ml)	15.1 g Tris 94 g glycine 5 g SDS up to 1000 ml distilled water pH 8.3 with KOH
Blotting buffer (1000 ml)	3.02 g Tris 14.4 g glycine 200 ml methanol up to 1000 ml distilled water
12% SDS-polyacrylamide gel (50 ml)	16.5 ml distilled water 20 ml acrylamide rotiphorese gel 30 solution 12.5 ml 1.5 M Tris (pH 8.8 with HCl) 0.5 ml 10% SDS 0.5 ml 10% APS
15% SDS-polyacrylamide gel (50 ml)	11.5 ml distilled water 25 ml acrylamide rotiphorese gel 30 solution 12.5 ml 1.5 M Tris (pH 8.8 with HCl) 0.5 ml 10% SDS 0.5 ml 10% APS 0.02 ml TEMED
5% SDS-polyacrylamide gel (20 ml)	13.6 ml distilled water 3.4 ml acrylamide rotiphorese gel 30 solution 2.5 ml 1 M Tris (pH 6.8 with HCl) 0.2 ml 10% SDS 0.2 ml 10% APS 0.02 ml TEMED
10% SDS (100 ml)	10 g SDS up to 100 ml distilled water
10% APS (10 ml)	1 g APS up to 10 ml distilled water
Ponceau S stain (100 ml)	5 ml glacial acetic acid 0.2 g Ponceau S powder up to 100 ml distilled water

Immunofluorescence (IF)	
4% paraformaldehyde (PFA) (250 ml)	10 g paraformaldehyde 50 µl 10 N NaOH 25 ml 10x PBS up to 250 ml distilled water pH adjusted to 7.0 with HCl
0.05% Triton (50 ml)	250 µl 10x Triton up to 50 ml PBS
1x Roti-Immunoblock (50 ml)	5 ml 10x Roti-Immunoblock up to 50 ml distilled water
Agarose gel electrophoresis	
1% Agarose gel (50 ml)	0.5 g agarose powder 50 ml 1x TAE buffer 2 µl ethidium bromide (10 mg/ml)
50x TAE buffer (1000 ml)	242.28 g Tris 57.1 ml glacial acetic acid 200 ml 0.25 M EDTA (pH 8.0 with NaOH) up to 1000 ml distilled water
Bacterial culture media and plates	
LB medium (1000 ml)	10 g tryptone 5 g yeast extract 10 g NaCl up to 1000 ml distilled water pH 7.0 autoclave
LB agar plates with carbenicillin (1000 ml)	10 g tryptone 5 g yeast extract 10 g NaCl 15 g agar up to 1000 ml distilled water pH 7.0 with NaOH autoclave let cool to about 50°C, then add 1 ml carbenicillin stock (50 mg/ml), then cast as 20 ml/10 cm petri dish
SOB medium (1000 ml)	20 g tryptone 5 g yeast extract 0.5 g NaCl 10 ml 25 mM KCl up to 1000 ml distilled water pH 7.4 autoclave 5 ml autoclaved 2 M MgCl$_2$
SOC medium (100 ml)	1 ml filter-sterilized 2 M glucose up to 100 ml SOB medium

Cell Isolation	
Calcium- and bicarbonate- free Hanks with HEPES (CBFHH) (1000 ml)	40 ml NaCl stock (200 g/l) 10 ml MgSO$_4$*H$_2$O stock (20 g/l) 10 ml KH$_2$PO$_4$ stock (6 g/l) 10 ml Na$_2$HPO$_4$*2H$_2$O stock (5.97 g/l) 10 ml glucose dihydrate stock (100 g/l) 100 ml HEPES stock (47.66 g/l) up to 1000 ml distilled water, sterile by filtration pH 7.4 with NaOH
Heat inactivated FCS (50 ml)	50 ml FCS was incubated in a water bath adjusted to 56°C for 30 minute (min), during which it was shaken gently every 5 min.
Non-cardiomyocyte medium (NCM)	500 ml DMEM GlutaMAX 1 g/l glucose 50 ml heat-inactivated FCS 5 ml P/S (100x)
0.4% Trypan blue (100 ml)	0.4 mg trypan blue 100 ml distilled water
Neo-natal rat cardiomyocyte (NRCM) culture media	
Cardiomyocyte medium (CM)	Minimal Essential Medium (MEM-Earle, 2.2 g / l NaHCO$_3$, without L-glutamine) FCS (inactivated) 10% (v/v) L-glutamine, 1% (v/v) Penicillin / streptomycin, 1% (v/v) BrdU (10 mM) 1% (v/v) freshly added
BrdU (5-bromo-2'-deoxyuridine)	BrdU 10 mM In aqueous solution, sterile filtered. Stable for one week at 4°C.
Other buffers and solution	
BIAM labeling buffer	1 ml 10 mM Tris (pH 6.8 with HCl) 1ml 1% Triton X-100 6.6 ml 100mM Nacl 10 ml 1% SDS up to 100 ml distilled water (Prior to use, add 100 µM BIAM to solution)
Stage A buffer	0.5% (v/v) acetic acid in water
Stage B buffer	80% (v/v) acetonitrile 0.5% (v/v) acetic acid in water
Loading buffer for MS	1% (v/v) acetonitrile 0.1% (v/v) formic acid in water

3.1.3 Chemicals and reagents

Table 5 | List of chemical and reagents

Chemical and Reagents	Manufacturer
Acetic acid (100%)	Carl Roth
Acetonitrile	AppliChem
Agar	Peqlab
Agarose	AppliChem
Ammonium bicarbonate	Sigma-Aldrich
Ammonium persulfate (APS)	AppliChem
Ascorbic acid	AppliChem
Aqua B. Braun	Braun
N-(Biotinoyl)-N'-(Iodoacetyl)Ethylenediamine (BIAM)	Thermo-Scientific
Brefeldin a (BFA)	Sigma-Aldrich
Bromophenol blue	AppliChem
Carbenicillin	Applichem
Control siRNA-A	Santa Cruz
CTGF siRNA	Santa Cruz
Cycloheximide	Santa Cruz
4',6-diamidino-2-phenylindole (DAPI)	Sigma-Aldrich
Diamide	Sigma-Aldrich
Dimethylsulfoxide (DMSO)	Sigma-Aldrich
DMEM Glutamax, 1 g/l glucose, pyruvate	Life Technologies
DMEM Glutamax, 4.5 g/l glucose	Life Technologies
DNA loading buffer (6x)	Thermo-Scientific
DNA molecular weight standard (1 kb DNA ladder)	Thermo Scientific
Dithiothreitol (DTT)	Applichem
Ethanol, absolute	J.T. Baker
Ethidium bromide	Sigma-Aldrich
EvaGreen dye for qPCR	Solis Biodyne
Fetal calf serum (FCS)	Life Technologies
Formaldehyde (37%)	Merck
Formic acid	AppliChem
GeneRuler 1 Kb plus DNA ladder	Thermo-Scientific
Glucose	AppliChem
Glycerol	AppliChem
Glycine	AppliChem
Hydrogen peroxide (H_2O_2) solution	Sigma-Aldrich

4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)	Carl Roth
Igepal CA-630	Sigma-Aldrich
Iodoacetamide (IAA)	Sigma-Aldrich
Isopropanol	Carl Roth
Lipofectamine RNAiMAX transfection reagent	Life Technologies
6x Loading Dye (Agarose gel electrophoresis)	Thermo Scientific
Lumi-Light PLUS Western Blotting Substrate	Roche
Magnesium chloride (MgCl$_2$)	AppliChem
Manganese chloride (MnCl$_2$)	AppliChem
Maleimide	Sigma-Aldrich
β-Mercaptoethanol	AppliChem
Methanol	Carl Roth
MG132	Sigma-Aldrich
Okadaic acid	Enzo life sciences
Paraformaldehyde (PFA)	Sigma-Aldrich
Penicillin/streptomycin (P/S)	Life Technologies
Phosphate-buffered saline (PBS) without Ca^{2+}	Life Technologies
PolyFect transfection reagent	Qiagen
Ponceau S	Sigma-Aldrich
Protein Marker „Roti-Mark Standard."	Carl Roth
Roti-Block (Blocking Reagent) 10X	Carl Roth
Roti-Nanoquant (Bradford reagent)	Carl Roth
Sodium chloride (NaCl)	Sigma-Aldrich
Sodium dodecyl sulfate (SDS)	AppliChem
Streptavidin agarose	Thermo-Scientific
Streptavidin, horseradish peroxidase conjugate	Thermo-Scientific
SuperSignal West Femto	Thermo Fisher Scientific
Bond-Breaker TCEP Solution, Neutral pH	Thermo-Scientific
Tetramethylethylenediamine (TEMED)	Merck
Thapsigargin (TGN)	Calbiochem
Tris ultrapure (Tris base)	AppliChem
Triton X-100	Carl Roth
Trypan blue	Fluka
Trypsin-EDTA 0.05%	Life Technologies
Tunicamycin	Santa Cruz
Tween-20	Carl Roth
Urea	AppliChem

3.1.4 Kits

Table 6 | List of kits

Kit	Application	Manufacturer
EnzChek Phosphatase Assay Kit	Phosphatase activity assay	Molecular probes
Exprep plasmid SV mini	Miniprep plasmid purification from bacteria	GeneAll
GoTaq green master mix	PCR	Promega
High pure PCR product purification kit	PCR product purification	Roche
5x HOT FIREPOL EvaGreen qPCR Mix Plus	qPCR	Solis Biodyne
Lumi-light western blotting substrate	Chemiluminescence protein blot visualization	Roche
Neonatal Heart Dissociation Kit	Isolation of beating cardiomyocytes	Miltenyi Biotec
ProFluor Ser/Thr PPase Assay Kit	Phosphatase activity assay	Promega
Revert Aid First Strand cDNA Synthesis Kit	RNA reverse transcription into cDNA	Thermo-Scientific
RNeasy	Total RNA isolation	Qiagen

3.1.5 Cells

Table 7 | List of bacterial and mammalian cells

Cells	Description
HSP47 knock out (KO)	Isolated from MEFs
Primary neo-natal rat cardiomyocytes (NRCM)	Isolated weekly from neonatal Wistar rats (1-3 days old)

3.1.6 Primers and recombinant proteins

Table 8 | List of primers used for qPCR and RT-PCR

Gene	Primer	Sequence (5`→ 3`)	Annealing temperature
CTGF	Forward	CCG GGT TAC CAA TGA CAA TA	58°C
	Reverse	CAC ACC CCA CAG AAC TTA GC	
PBGD	Forward	CCT GAA ACT CTG CTT CGC TG	58°C

	Reverse	CTG GAC CAT CTT CTT GCT GAA	
PDI	Forward	CTT CTT CAA GGA CGC AGG GT	58°C
	Reverse	GCG GCC TTC ATC AAA CTT CTT	
XBP-1	Forward	TTA CGA GAG AAA ACT CAT GGG C	58°C
	Reverse	GGG TCC AAC TTG TCC AGA ATG	

Table 9 | List of recombinant protein

Enzyme	Source	Company
PP-1A,active,GST-tagged, human recombinant	Sf9 insect cells	Sigma-SRP5338
PP-1A, His-tagged	E.coli	Sigma-P7937

3.1.7 Laboratory instrument, general material and software

Table 10 | List of instruments

Instrument	Model No.	Manufacturer
Autoclave	VX-150	Systec
Cell counting chamber	Fuchs-Rosenthal bright-line	Marienfeld-Superior
Cell culture incubator	Steri-cult 200 Incubator	Forma Scientific
Cell culture incubator	Labotect Incubator C 200	Labotect
Cell sieve	Cell dissociation sieve - tissue grinder kit (250 µm pore size)	Sigma-Aldrich
Centrifuge bench top	Centrifuge 5804 R	Eppendorf
Centrifuge bench top	Sigma 3K30	Sigma
Centrifuge table top	Tabletop centrifuge 5415 D	Eppendorf
Centrifuge table top	Combi-spin FVL-2400N	Biosan
Centrifuge table top	Centrifuge 5417 R	Eppendorf
Chemiluminescence imaging system	Versa doc MP	Bio-Rad
Double distilled water system	Milli-Q	Millipore
Electric power supply and control	Powerpac	Bio-Rad
Heating block	Thermomixer compact	Eppendorf
Incubator	CFC-free	Sanyo
Inverted fluorescence microscope	Axiovert 200	Zeiss
Inverted fluorescence microscope with climate chamber	Olympus IX 81	Olympus

Inverted microscope	Axiovert S100 TV	Zeiss
Microplate reader	Flex Station 3	MDS Analytical Technologies
Microscope camera	CAM-XM10-T-Camera	Olympus
Nanospray Flex ion-Source	ES071	Thermo-Scientific
pH meter	WTW	Inolab
Pipettes	Pipetman	Gilson
Plate reader	FlexStation3	Molecular Devices
Pump	ME2	Vacuubrand
Q Exactive Plus System	LTQ Orbitrap XL	Thermo-Scientific
Real-Time-PCR-System	TaqMan 7900HT Fast Real-Time-PCR System	Applied Biosystems
Rocker	Diomax 1030	Heidolph
Rotation shaker	Reax 3	Heidolph
Scale	Portable	Sartorius
Shaker	GFL 3016	GLF
Shaker	Vibramax 100	Heidolph
Shaking incubator	Innova 4300	New Baunswick Scientific
Spectrophotometer	Nanodrop 1000	Peqlab
Sonicator	Sonifier B-12	Branson Sonic Power
Temperature control chamber	Certomat	B. Braun
Thermocycler	Mastercycler gradient	Eppendorf
Ultracentrifuge	L8-70M	Beckman
Ultracentrifuge rotor	SW-27	Beckman
Ultra-high performance liquid chromatography unit (UPLC)	Dionix Ultimate 3000	Thermo-Scientific
UV agarose gel imaging system	Gel doc XR	Bio-Rad
Vortexer	VF 2 Vortexer	Janke u. Kunkel IKA Labortechnik
Water bath	2764	Eppendorf
Western blotting setup	Mini-protean tetra cell 4-gel system	Bio-Rad

Table 11 | List of general material

General material	Manufacturer
Acclaim PepMap100 C18 column	Thermo-Scientific
Cell culture dishes	Greiner Bio One
Cell scrapers	Sarstedt
Centrifuge tubes	Beckman
Empore SPE Disks	Sigma-Aldrich (66883-U SUPELCO)
Filter syringes	Sarstedt
Liquid junction emitters	New Objective
Multi-well cell culture plates	Greiner Bio One

Nitrocellulose membrane, Whatman, Protran	GE Healthcare
PCR reaction tubes	Sarstedt
Pipette tips	Sarstedt
Pipette tips with filters	4titude
Protein desalting spin columns	Thermo-Scientific
Reaction tubes (15, 50 ml)	Greiner Bio One
Reaction tubes (0.5, 1.5, 2 ml)	Sarstedt
Reprosil Saphir	Dr. Maisch GmbH
Serological pipettes	Sarstedt
Silica beads	Amerbruch
96 well microtiter plates	Thermo-Scientific
96 well solid black microplates	Corning
Wide opening, serological pipettes	Falcon

Table 12 | List of software

Program	Application	Manufacturer
Citavi	Managing references	Swiss academic software
GraphPad Prism 6.0c	Statistical calculations and graphs drawing	GraphPad
ImageJ 1.51a	Evaluation of fluorescence intensity	National Institutes of Health, USA
Image Lab 5.1	Operating the Versa Doc MP system and for semi-quantification of western blots	Bio-Rad
Peaks 7.0 Search engine	Analysing Mass-Spectrometry's data	Bioinformatics solutions Inc.
PyMOL 0.99rc6-bin-win32	Structural analysis of proteins	Schrödinger, Inc.
SDS 2.4	Operating the TaqMan 7900HT Fast Real-Time-PCR System, and its data analysis	Applied Biosystems
Xcellence pro	Operating the Olympus microscopy system for cell imaging	Olympus

3.2 Methods

3.2.1 Cell culture methods

3.2.1.1 Preparation of cardiomyocytes from neonatal rats

Neonatal rats were sacrificed on postnatal day 0-3 (P0-P3) in accordance with ethical standards. Hearts were extracted using forceps and ventricular tissues were collected in cell culture dishes with ice-cooled Calcium- and bicarbonate- free Hanks with HEPES (CBFHH). Ventricular tissues were cut in half and transferred to a new culture dish with 20 ml of CBFHH and washed one additional time to remove excess blood. The tissues were minced into small pieces (1-2 mm^3) and then transferred via a 10ml pipette tip to a gentle MACS C-tube (up to ~20 neonatal rat hearts).

The following enzymes were prepared from the neonatal heart dissociation kit (for 20 neonatal rat hearts) according to the manufacturer's instructions. Reagent preparation summarized in Table 13:

Table 13 | Reagent preparation

Enzyme mix 1		Enzyme mix 2		
Enzyme P	Buffer X	Buffer Y	Enzyme A	Enzyme D
62.5 µl	2300 µl	25 µl	12.5 µl	100 µl

The enzyme mix 1 was preheated for 5 minutes (min) at 37°C and mixed with enzyme mix 2. 2.5 ml of the enzyme mix was transferred to the C-tube containing the tissues and incubated in an inward position with the cap down for 15 min at 37°C. The C-tube was then attached to the sleeve of a gentle MACS Dissociator and followed by the program named 'h_Tumour_01.' Incubation steps were repeated twice to complete the digestion of tissue. After the completions of the program, C-tubes were removed from the gentleMACS Dissociator and samples were carefully resuspended in 7.5 ml of a non-cardiomyocyte medium (NCM).

To separate the tissue from the suspension, a sieve was moistened (pore size 250 µm) with 3-4 ml of NCM. The cell suspension was transferred through the sieve, collected in a fresh cell culture dish and added to a falcon tube. Cell suspensions were centrifuged at 300 g for 5 min (20°C), and the supernatant was carefully discarded and re-suspended in 4 ml of 1x Phosphate-buffered saline (PBS), homogenized and then filtered.

The percoll gradient method was used to extract and purify only the cardiomyocytes fraction from the heterogeneous cell suspensions (consisting of a mixture of fibroblasts, cardiomyocytes, and erythrocytes). The percoll stock solution was prepared by mixing 40.5 ml of percoll (4°C) with 4.5 ml of 10x PBS. Then, a top and bottom precoll solution (TPS, BPS) was prepared, by mixing 9 ml of percoll stock with 11 ml of 1x PBS and 13 ml percoll stock with 7 ml of 1x PBS respectively. To differentiate between both solutions, 200 µl of phenol red stock solution was added to TPS. Subsequently, 4 ml of TPS was added to two 15 ml reaction tubes, and 3 ml of BPS was slowly added to the bottom while transferring the pipette upward. Next, 2 ml of cell suspension mixed with 1x PBS was slowly added and centrifuged at 750 g for 30 min at 20°C. The percoll gradient (Figure 12) was divided into three different gradients (as per density of tissue), i.e. fibroblasts, cardiomyocytes, and erythrocytes.
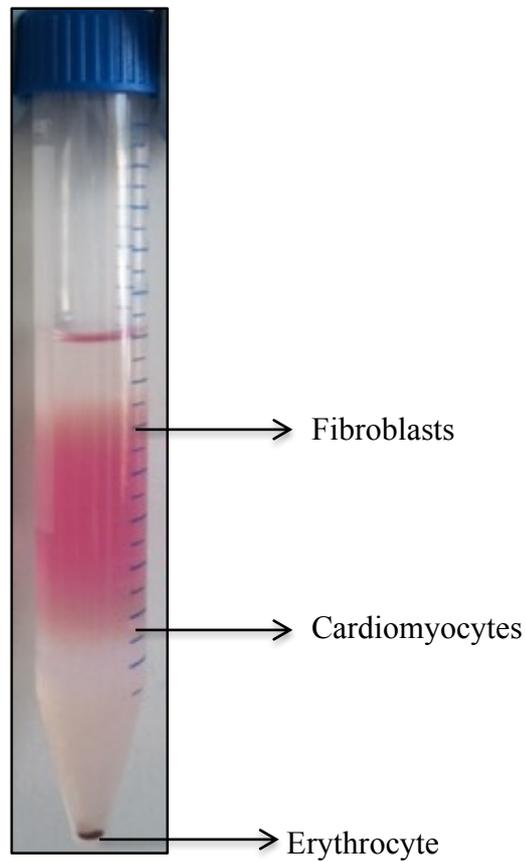
Figure 12 | Percoll gradient with three distinct layers consisting of fibroblasts, cardiomyocytes, and erythrocytes.

The upper 5 ml of the solution composed of fibroblasts was removed, and 2 ml of cardiomyocyte layer was carefully transferred into a new 50 ml tube and washed twice with pure DMEM cardiomyocyte medium (CM) at 300 g for 5 min at 20°C. The supernatant was discarded, and the pellet was resuspended in 10 ml of pre-warmed DMEM CM. The overgrowth of fibroblasts within cardiomyocytes was prevented by the adding 10 mM of

antimetabolite 5'-bromo-2'-deoxyuridine (BrdU) in the cardiomyocytes medium, which selectively inhibited the DNA synthesis and consequently cell proliferation of fibroblasts.

Cells were counted as follows: 10 µl of cell suspension was mixed with 10 µl tryphan blue and transferred to a Neubauer counting chamber. The cell number was counted in all four corner chamber, and the average value was used to quantify the cell number as shown below:

$$Cell\ quantity\ x\ 2\ (dilution)\ x\ 10^4 = cell\ amount/mL$$

Cells were seeded into 1% collagen (diluted in PBS) coated plates. For six or twelve well plates, 1×10^6 or 0.5×10^6 cells/well were added, respectively. The neo-natal rat cardiomyocytes (NRCM) culture was maintained at 37°C in 5% CO_2 in a humidified incubator. The medium was exchanged after 48 hours (h).

3.2.1.2 Immunofluorescence of NRCM cells

After the specific treatment of cells, the medium was aspirated, and the cells were fixed with 4% paraformaldehyde (PFA) for 15 min at room temperature (RT). The cells were then washed twice with PBS and incubated for 5 min with 0.05% Triton solution to permeabilize the cell membranes. Cells were washed twice with 1X PBS, before being incubated with 1x Roti-Immunoblock for 1 h at RT. Next, the cells were incubated overnight in the dark at 4°C with PBS containing the primary antibody against the protein of interest. Cells were then washed twice with 1x PBS and incubated overnight at 4°C in the dark with the specific fluorophore-conjugated secondary antibodies and DAPI to stain the nuclei (see Table 4 for respective dilutions). Finally, the cells were washed twice with PBS and images were taken using inverted fluorescence microscopy (Olympus).

3.2.1.3 Live cell imaging

The experiments were performed in a 6 well plate with cells incubated with 2 ml of medium. The cells were incubated with 10^2 µM, 10^3 µM and 10^4 µM of H_2O_2 for 90 min in the climate chamber of the inverse fluorescence microscope (Olympus), keeping the ideal enviornment at 37°C, 5% CO_2, 57.37% lamp intensity and the exposure time of 20 ms. A 40x objective and a bright-field filter were used to capture the video. The time-lapse recording was setup at one frame every 20 min for a total duration of 24 h, with the first frame taken at 0 sec. The change in the morphology of cells was further analyzed with Image J.

3.2.1.4 Gene knockdown via siRNA transfection

After seeding NRCMs (day 1), the first transfection was performed after 19-20 h (day 2). For the transfection, 2 different siRNA constructs were used: a siRNA, which does not bind to mammalian mRNA to serve as a control (siRNA ctrl) and a second siRNA construct that binds to the mRNA of CTGF (siRNA ctgf) and blocks its translation. For each well (6 well plate) transfection, RNAi duplex-Lipofectamine RNAiMAX complexes were first produced as follows:

(1) 9.9 µl siRNA (22nM) was gently mixed with 900 µl Opti-MEM (OM).

(2) In parallel, 9.9 µl Lipofectamine RNAiMAX was mixed with 900 µl OM.

(3) Both solutions were mixed and incubated for 20 min at RT.

For the transfection, the cells were washed twice with warm PBS. Then 600 µl of 37°C warm OM was added together with 400 µl of transfection solution in each well for 6 h at 37°C. Later the cells were washed twice with warm PBS and then incubated with fresh CM (day 2). The second transfection was performed 24 h after the first transfection as described above (day 3). The next medium change was conducted after 24 h (day 4). The next day (24 h later) the cells were used for particular experiments (Figure 13).
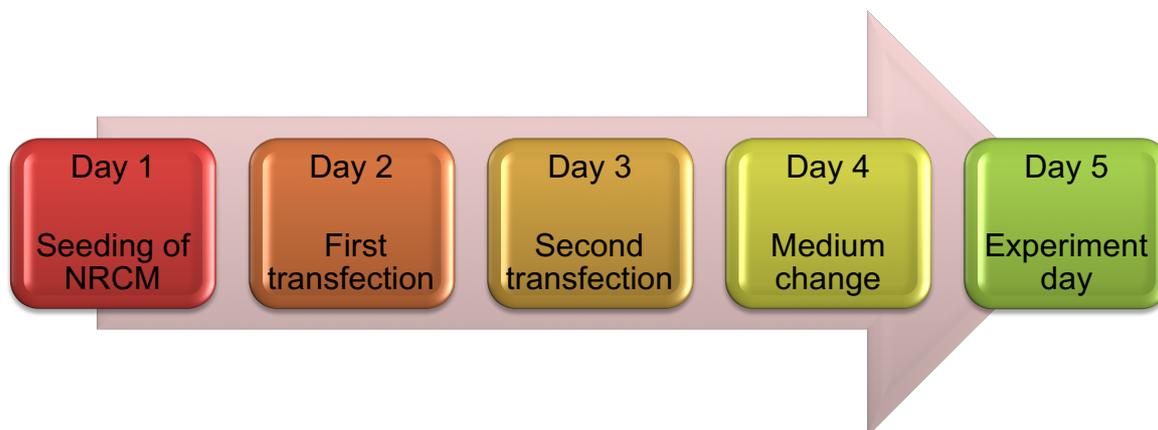
Figure 13 | Timeline for siRNA transfection.

3.2.2 Protein biochemical methods

3.2.2.1 Preparation of samples for western blotting

NRCMs were kept at 37°C and 5% CO_2 in a humidified incubator for 3-4 days with one medium exchange. On the day of the experiments, the cells were incubated with respective reagents for the definite period under controlled conditions. For protein analysis, the cells were lysed with ice-cold GST-fish lysis buffer and afterwards centrifuged at 13000 g for 10

min at 4°C to remove the cell debris. The supernatant was mixed with 4x sample loading buffer. Subsequently, the samples were incubated at 95°C for 5 min on a heating block and then gently centrifuged.

For reducing and non-reducing gel, the cells were lysed with a blocking buffer containing malemide, which blocks the free thiol groups. The cells were later mixed with a 2x loading buffer (including malemide) with and without, DTT respectively. To keep the disulfide bridges intact, non-reduced samples were not heated, whereas reduced samples were heated at 95°C for 5 min.

3.2.2.2 Protein quantification of samples via Bradford assay

To measure protein concentration, a colorimetric protein assay was used, which was based on a shift in the absorbance maximum when dye associates with proteins. A standard curve was established with BSA dilutions of known concentrations. 200 µl of 1x Roti-Nanoquant solution was mixed with 1 µl of the protein sample (diluted 1:150) and incubated for 5 min at RT. Together with the standard curve, the absorbance of each sample was detected at 595 nm with the microplate reader Flexstation3, and the protein concentrations were calculated by linear regression analysis.

3.2.2.3 Protein separation by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and detection by immunoblot

The electrophoresis for the immunoblotting was carried out in 12-15% SDS-PAGE. Protein samples from Section 3.2.2.1 were separated at 200 V on polyacrylamide gels. They were then blotted onto a nitrocellulose membrane for 1 h at 100 V. The membranes were blocked for 1 h with 1x Roti-Block, washed with the TBST and incubated overnight with a primary antibody at 4°C. The following day, the membrane was washed three times for 10 min with TBST and incubated with the secondary antibody for 1 h at RT. At the end, the membrane was washed again three times for 10 min with TBST. For protein-antibody complex detection, west-dura extended duration substrate was used, and for imaging a chemiluminescence imager equipped with the software Quantity one.

3.2.2.4 BIAM labeling assay

The N-(Biotinoyl)-N-(Iodoacetyl) Ethylenediamine (BIAM) labeling assay was performed to confirm Cys oxidation status change in response to ROS. Briefly, NRCMs were incubated at 37°C for 15 min in a NRCM medium containing 10^2 µM H_2O_2. Then the cells were washed with PBS and lysed with a BIAM labeling buffer. The control samples were treated without a

BIAM labeling buffer. Debris was removed by centrifugation at 13000 g for 10 min, and supernatants were further incubated at 37°C for 30 min. Excess BIAM was withdrawn from the lysates using protein desalting columns. The lysates were aliquoted into input and output samples. Output lysates were mixed with 50 µl streptavidin beads and incubated overnight on a shaker at 4°C. The next day the beads were washed thrice with a labeling buffer and the sample was cooked with loading buffer at 95°C for 5 min. Both input and output samples were then loaded for immunoblotting (Figure 14) (see Section 3.2.2.3).
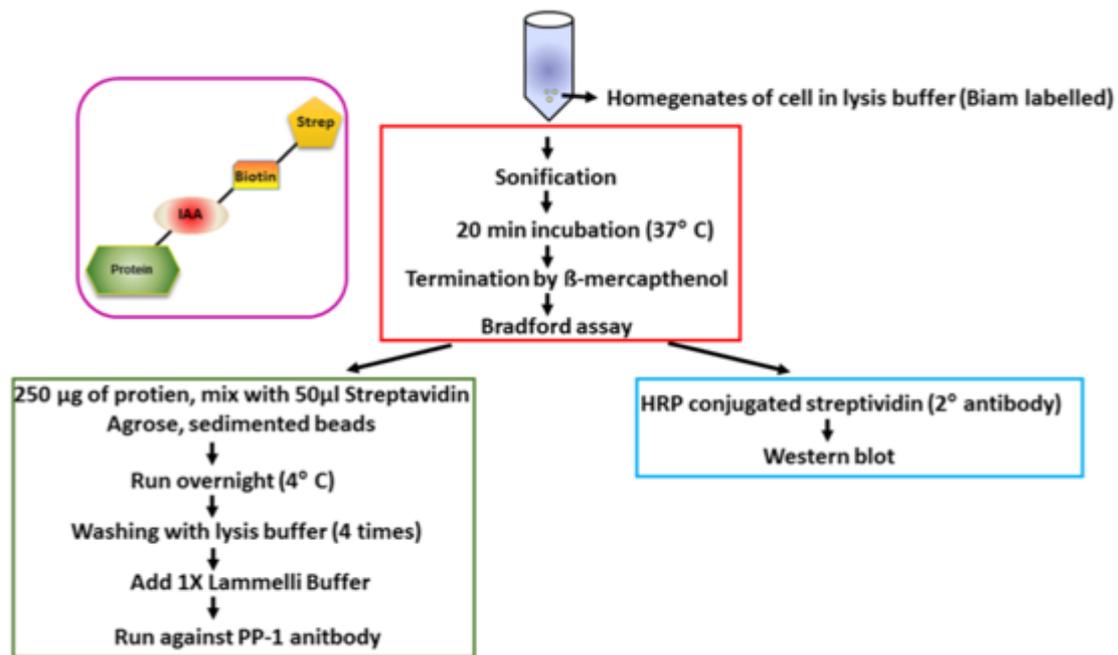
Figure 14 | General assay format for the BIAM labeling assay

3.2.2.5 Phosphatase activity assay

Total PP activity was measured using the EnzChek Phosphatase Assay Kit. Human heart tissue homogenates, NRCM and whole heart mouse tissue were generated in a passive lysis buffer (20 mM Tris-HCl pH 7.5, 1 mM Na_2EDTA, 150 mM NaCl, 1 mM EGTA, 1% Triton and protease inhibitor). Afterwards the protein content (20 µg total protein) was measured using a Pierce BCA Protein Assay Kit. The 100 µM 6,8-difluoro-4-methylumbelliferyl phosphate (DiFMUP) substrate was mixed with a 100 mM reaction buffer (sodium acetate pH 5.5), which was then added to the protein and incubated at RT for 15 min. Fluorescence was measured using an excitation wavelength of 360 nm and an emission wavelength detection at 460 nm on a Flexstation 3 (Molecular Devices; Figure 15). The fluorescence values were used as read out to calculate the PP activity.
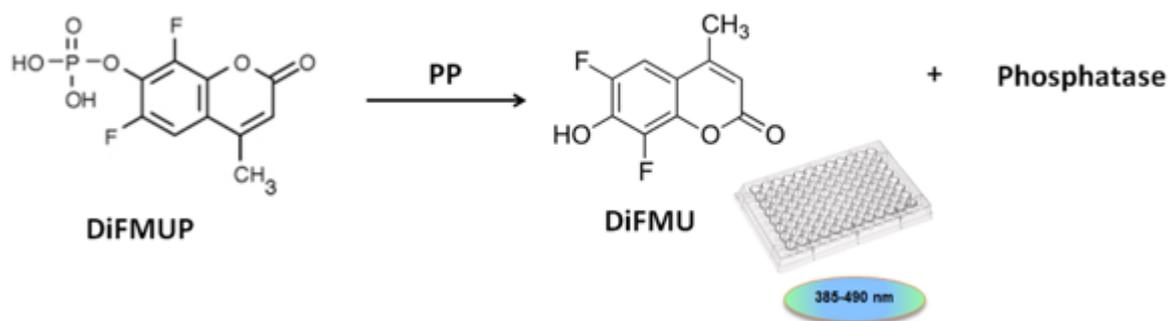
Figure 15 | Chromogenic reactions in the EnzChek Phosphatase Assay Kit. In the presence of reaction buffer, PP cleave the DiFMUP substrate, converting into phosphatase and hydrolyzed DiFMU (6, 8-Difluoro-7-Hydroxy-4-Methylumbelliferone) as a fluorogenic product (excitation/emission =385/490 nm).

For calculating the PP-1 activity, a recombinant protein (rPP-1) was used as a source of phosphatases and a commercial Promega PP activity assay. The phosphate activity was measured in 96 well formats and involved 'add, mix and read' steps (Table 14). The assay started with a reaction of rPP-1 with a reaction buffer including phosphorylated bisamide rhodamine 110 peptide (R110 substrate) and control 7-Amino-4-methylcoumarin (AMC substrate). The latter serves as a control for compounds that inhibit the protease. In this context, both R110 and AMC substrate were non-fluorescent. However, after the phosphatase reaction, the addition of a protease solution stopped the reaction. Then both substrates were digested, producing highly fluorescent R110 and AMC. A phosphate solution was prepared including $MgCl_2$ and $MnCl_2$, and the assay was initiated by adding a 25 µl substrate solution. The reaction was continued for 10 min, and then a protease solution was added for 90 min. To stabilize the reaction, 25 µl of stabilizer solution was added. The R110 signal was measured using an excitation wavelength of 485 nm and an emission wavelength at 530 nm. The AMC signal was measured using excitation wavelength of 360 nm and emission wavelength at 460 nm. The ratio between R110 and AMC signal was used to calculate the PP-1 activity.

Table 14 | General assay format for 96 well plates used in Promega PP assay

Phosphatase solution	Reaction buffer (µl)	Control (µl)
Reaction buffer	5	5
PP-1	0	10
$MgCl_2$	1	1
$MnCl_2$	3.125	3.125
H_2O	15.9	5.9
Total	25	25

Substrate solution	
5x Reaction buffer (µl)	200
R11 substrate (µl)	1
AMC substrate (µl)	1
H_2O (µl)	798
Total (ml)	1
Protease solution	
5x Termination buffer (µl)	200
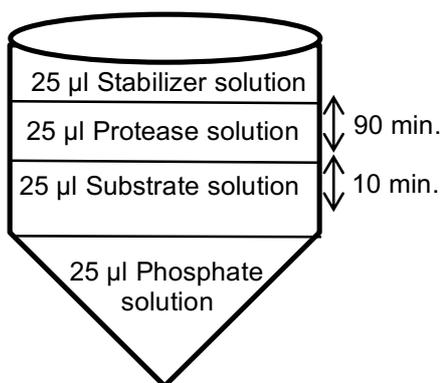
Protease Reagent (µl)	20
Okadaic acid (300 µM)	10
H_2O (µl)	770
Total (ml)	1
Stabilizer solution	
5x Termination buffer (µl)	200
Stabilizer reagent (µl)	1
H_2O (µl)	799
Total (ml)	1

Figure (funnel diagram):
- 25 µl Stabilizer solution
- 25 µl Protease solution — 90 min.
- 25 µl Substrate solution — 10 min.
- 25 µl Phosphate solution

3.2.3 Molecular biological methods

3.2.3.1 RNA isolation

NRCM were grown to 80% confluence, washed once with PBS and total RNA was extracted using the RNeasy mini kit, according to the supplier's procedure with an additional homogenization step using a QIA shredder. Afterwards the RNA concentration was determined using a Nanodrop 1000 device. Samples were stored at -80°C.

3.2.3.2 cDNA synthesis

cDNA synthesis was performed using Revert Aid First cDNA Synthesis Kit from 1 µg RNA (extracted from NRCM at step 3.2.3.1) according to manufacturer's protocol. Samples were stored at -20°C.

3.2.3.3 Quantitative real-time polymerase chain reaction (qRT-PCR)

qRT-PCR was performed to detect the expression level of various genes in NRCM. As the housekeeping gene, PBGD was used for normalization. In general, qPCR was performed using 5x HOT FIREPOL EvaGreen qPCR Mix Plus Kit as per manufacturer's protocol. Primers for each gene were diluted to 10 µM, and cDNA samples were diluted to 1:20 with RNAse free water. For each sample four replicates were performed with a master mix for each gene, which was prepared as shown in Table 15:

Table 15 | Master Mix for qRT-PCR

Reagent	Volume
Primer for (10 pmol/µL)	1 µL
Primer rev (10 pmol/µL)	1 µL
Template DNA /Standard DNA (1:20)	1 µL
RNase-free water	13 µL
5x EvaGreen qPCR mix	4 µL

Initially, 1 µl of the diluted cDNA was added per well (Micro-Amp optical reaction 384-well plate), and mixed with 19 µl of the master mix. Analyzes were carried out with TaqMan 7900HT Fast Real-Time-PCR System and as per the following program (Table 16):

Table 16 | qPCR Program

Cycle	Temperature	Time	Number of Cycles
Initial Denaturation	95°C	15 min	
Denaturation	95°C	15 s	
Annealing	60°C	20 s	30
Elongation	72°C	40 s	
Dissociation curve	95°C	15 s	
	60°C	15 s	
	95°C	15 s	

In the end, threshold cycle values (C_T) were calculated for each gene. Finally, to calculate gene expression, ΔΔct value was used by the following formula:

$$2^{-\Delta\Delta ct} = 2^{(ctSample - ctHousekeeping\ gene\ in\ sample) - (ctControl - ctHousekeeping\ gene\ in\ control)}$$

3.2.4 Analytical methods

3.2.4.1 Differentiation of disulfide bridges assisted by protein structural analysis with Pymol

Pymol 0.99rc6-bin-win32 was used to generate structural plots. A Pymol script file was prepared that enabled a prior selection of several residues and their labeling either as colored sticks or balls. Another feature of the Pymol program was the measurement of molecular distances that are summarized in the cross-correlation Table 21. The red dotted lines indicate the distances that could be measured (Figure 9) and that are also shown in

Table 21 for every reasonable combination of two Cys residues. Gnerally, the molecular distance for forming a bridge lies within 3 Å. However, small structural changes could also induce other proximities since most of the Cys residues are in about 6 Å distance (Figure 9).

3.2.4.2 Performing the oxidative stress experiment and sample preparations for the identification of various PTMs in PP-1

The experimental design was simplified by incubating GST-tagged PP-1A (supplied in 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 10 mM glutathione, 0.1 mM EDTA, 0.25 mM DTT, 0.1 mM PMSF, and 25% glycerol) in 50mM TRIS pH 7.5 buffer with the following four treatments: (1) without Mn^{2+}, without H_2O_2, (2) without Mn^{2+}, 500 µM H_2O_2 (15 min), (3) 0.1 mM Mn^{2+}, without H_2O_2, and (4) 0.1 mM Mn^{2+}, 500 µM H_2O_2 (15 min).

After the oxidation, 10 mM (final concentration) of iodoacetamide (IAA) were immediately added to the samples to block free Cys residues within 20 min. Then 4% SDS solution was added to obtain a final SDS concentration of 1% and immediately afterwards the samples were subjected to a filter-aided sample preparation (FASP) (Wisniewski et al., 2009). The buffer exchange was performed twice for 8 M Urea and twice for 50 mM ammonium bicarbonate (ABC). This was followed by an overnight protein digestion at 37°C using a digestion buffer (1 µg trypsin/100 ng with 50 mM ABC in 100 µl total volume). The membrane was not allowed to dry during the digestion.

The above experiment was repeated with the 100 ng of the His-tagged recombinant PP-1 alpha protein for each of the above conditions. The advantage of using this protein was that the reducing reagents were omitted from the buffers. Hence, the reactions can be performed under "real" oxidative conditions, and the FASP digestions were performed as described in above FASP protocol.

3.2.4.3 Peptide purification via stage tips

After 18 h the digest was spun through the filter and then subsequently washed with 50 µl 50 mM ABC buffer, 50 µl 0.5 M NaCl and 100 µl Stage A buffer. All fractions were collected in the microspin tube. Samples were then desalted according to the stage tip protocol (Rappsilber et al., 2007). Three discs of the desalting material (Empore SPE Disks; C18-Octadecyl, diameter 47 mm) were prepared in yellow pipette tips and activated with 50 µl methanol, washed with 50 µl Stage B buffer and then equilibrated with 100 µl Stage A buffer. After loading the sample, the stage tips were washed with another 100 µl Stage A buffer and

either stored until measurement or directly eluted with two times 40 µl Stage B buffer into 96 well microtiter plates.

3.2.4.4 LC-MS/MS analysis

For the experimental design with GST-tagged PP-1A, measurements were performed on a Q Exactive Plus interfaced with an ultra-high performance liquid chromatography (UPLC) unit and a Nanospray Flex Ion-Source Ion Source. Peptides were loaded onto a C18 PepMap100 column (300 µm internal diameter (i.d) x 5mm length) with 5 µM particle size and 100 Å pore width using the capillary pump of the UPLC. The loading pump was operated at a flow rate of 25 µl/min with the loading buffer (1% acetonitrile (v/v), 0.1% formic acid (v/v) in water). For shorter separation, the nano-LC pump unit was operated at 0.5 µl/min and delivered a linear binary gradient from 5% to 44% buffer B (80% acetonitrile (v/v) in 0.1% formic acid in water) in 28,53 min or 31.02 min, respectively. It was followed by a wash-out at 99% buffer B for 4 min and re-equilibration to 1% buffer for 10 min or 5 min, respectively. Data acquisition started at 2 min when the concentration of buffer B had increased by 5% from the starting conditions. The total acquisition time was 48.53 min or 47.52 min, respectively. The gradient times were adjusted to the dead volume of the pre-column 1 or pre-column 2 connections. The in-house columns were packed directly into liquid junction emitters (100 µm i.d, tip 10+/- 1 µm) with 2.4 µm Reprosil Saphir fused silica beads. The length of the columns varied between 12 cm and 15 cm.

Ionization power of the source was 2.6 keV, and the MS spectra was recorded in 45 min with the following parameters: the full MS scan was 200 m/z with a resolution of 70,000; an automatic gain control (AGC) target value of 3×10^6 total ion counts with maximal ion injection time of 250 ms; the mass spectra were recorded in the profile mode within a mass range of 300-2500 m/z. All precursor ions with a charge state between two and six were chosen for fragmentation, applying an isolation window of 2 m/z if they met the intensity threshold of $6,7 \times 10^3$. This threshold is calculated from the AGC target value of 10^5 and the underfill ratio of 1%. To achieve this target value, a maximal injection time of 150 ms for any precursor was allowed. The MS/MS spectra were recorded with the following parameters: the resolution was 17,500 at 200 m/z. All selected Precursor ions underwent higher-energy collisional dissociation (HCD) fragmentation with a normalized energy of 30. MSMS spectra were recorded in the centroid mode within the mass range of 200-2000 m/z. All precursors being fragmented were dynamically excluded for 30sec within 10 ppm mass accuracy for a second fragmentation event in order to increase the sensitivity for low abundance precursors.

For the repeated experiment with the His-tagged PP-1, the experimental design for the measurement was altered again: the measurement time was now 60 min and the system was operated in the one-pre-column setup. Measurements were performed on a Q Exactive Plus System, interfaced with FlexSource Electrospray Ion Source. The spray voltage was 3.3 keV using a direct junction emitter (stainless steel emitter) with a capillary temperature of 250°C. No extra gas supply was used for sheath gas, auxiliary gas or spare gas. Data acquisition was performed in the positive ion mode. MS Spectra were recorded with the following parameters: resolution at 200 m/z was 70,000, AGC target value $3x10^6$, max injection time 160 ms, and all MS spectra were recorded in profile mode within a mass range of 200-2000 m/z. All precursor ions with a charge state between 2 and 6 were chosen for fragmentation, applying an isolation window of 2 m/z if they met the intensity threshold of $6,7x10^3$. The threshold was calculated from the AGC target value of $1x10^5$ and the underfill ratio of 1% and underwent HCD fragmentation with a stepped energy fragmentation scheme. This scheme included three fragmentations at the normalized fragmentation energies 30, 35 and 40 and the subsequent overlay of the obtained spectra to a single spectrum. The MSMS spectra were recorded with the following parameters: resolution at 200 m/z 17,500 and max injection time 150 ms. All MSMS spectra were recorded in the centroid mode within the mass range of 200-2000 m/z. Finally, all precursors within 10 ppm mass accuracy eluting within a time frame of 30sec were excluded from fragmentation for a second time to increase the sensitivity for low abundance precursors.

3.2.4.5 Data analysis with Peaks 7.0 software

Spectra were searched with the Peaks 7.0 search engine. Briefly, regular redox-modifications were examined, and a search strategy for the identification of disulfide-linked spectra from a known/identified protein was established. For the former, the following parameters were used: the database was UniProt human (July,7th, 2015) comprising 68605 proteins. Variable modifications in the case of GST-tagged PP-1 used were Carbamidomethylation (c, +57.02), Cysteine oxidation to cysteic acid (c, +47.98), Cysteinyl C (c, +119.00), Deamidation (NQ) (d, +0.98), Disulfide Bridge unpaired fragmentation (d, -2.02), Dehydroalanine C (d, -33.99), Deoxidation (M) (d, +31.99), Glutathione disulfide (g, +305.07), Hydroxylation (h, +15.99), Oxidation (M) (o, +15.99), Oxidation (HW) (o, +15.99), Oxidation or Hydroxylation (C) (o, +15.99), Persulfide C (p, +31.97) and Phosphorylation (STY) (p, +79.97). The parent mass tolerance allowed was set to 10 ppm and fragment mass tolerance was set to 0.02 Da. The enzyme specificity used was Trypsin, missing cleavages were limited to four, but cutting before Proline was authorized in contrast to the standard

Trypsin settings. Variable modifications permitted for the search were five. Extraction of data points was set to the top 100 peaks for each spectrum.

Variable modifications in the case of His-tagged PP-1 used were: Acetylation (N-term) (a, +42.01), Arginine oxidation to glutamic semialdehyde (a, -43.05), Carbamidomethylation (c, +57.02), Deamidation (NQ) (d, +0.98), Disulfide CID breakage (d, +33.99), Dehydroalanine (d, -33.99), Dehydration (d, -18.01), Dihydroxy (d, +31.99), Hydroxylation (h, +15.99), internal disulfide bond (i, -1.01), internal disulfide bond unpaired fragmentation (i, -2.02), Methyl ester (m, +14.02), Oxidation (M) (o, +15.99), Oxidation (HW) (o, +15.99), Oxidation or Hydroxylation (C) (o, +15.99), Proline oxidation to pyroglutamic acid (p, +13.98), Sulfone C (s, +47.98) and S-Persulfide C (s, +31.97). Otherwise the same conditions were used as with the GST-tagged protein.
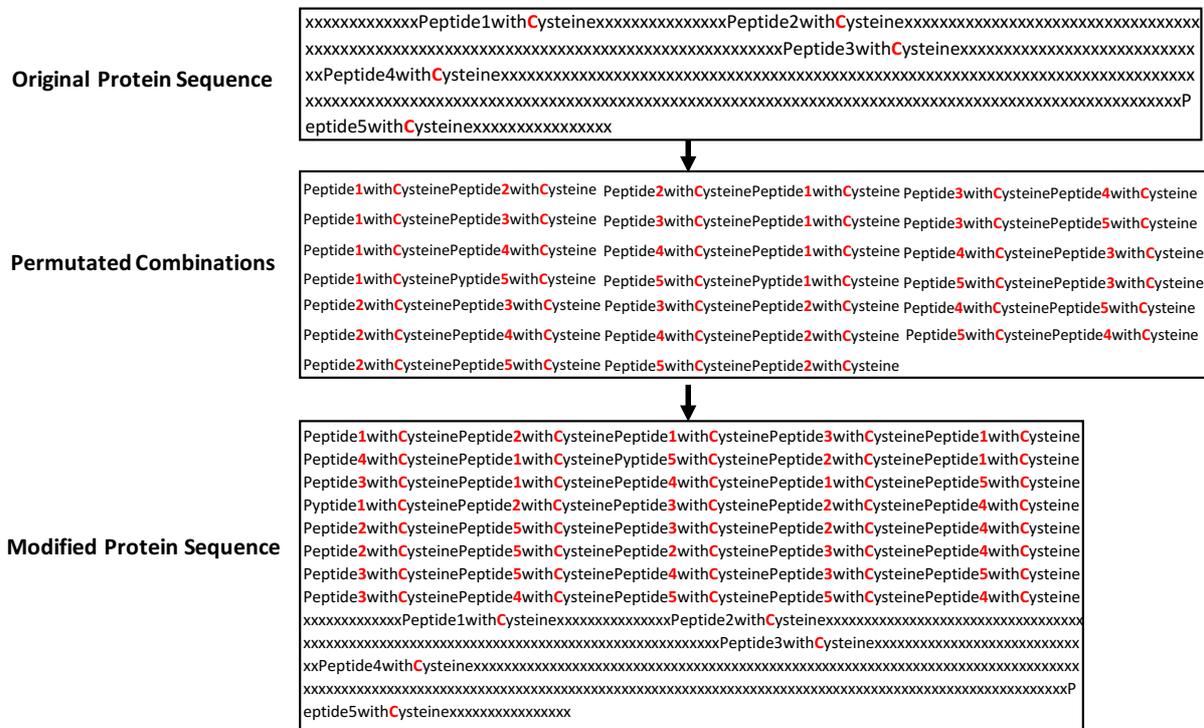
Figure 16 | Scheme is showing the generation of permutated Cys-peptide database. The top box shows the original protein sequence with the Cys-containing peptides indicated within any other sequence x. The Cys residues are highlighted in bold red. In the middle box, the Cys-containing peptides were excised and permutated with each other. In the bottom box, all combinations were reassembled with the original protein sequence to form the new database entry.

Since a database search from a linear protein database does not allow the simultaneous detection of two structurally close but sequence-wise distant peptides, an automatic search strategy for this data was created. For the identification of disulfide-linked peptides, a new linearized database entry was created that contained two peptides combinations in a permutation. An artificial disulfide-protein was incorporated to the normal human database in order to perform the search with the normal search engine. This protein allowed the detection

of the intact disulfide-linked peptides from a non-reduced sample, considering them as longer peptides with a missed cleavage in the middle either as AB or BA combination. The method used to detect these peptides is described in detail in Figure 16 (above).
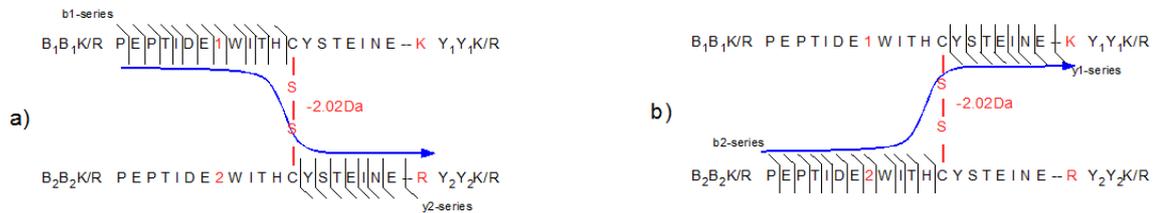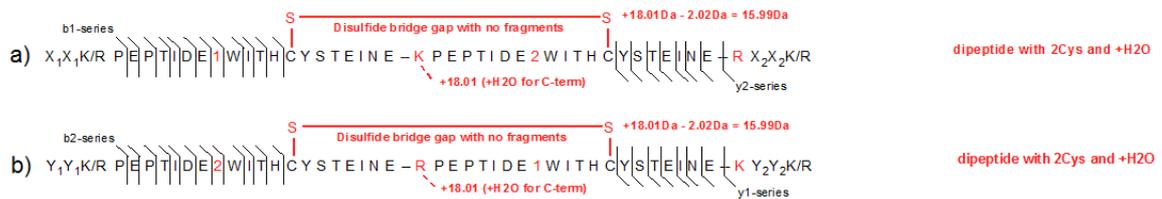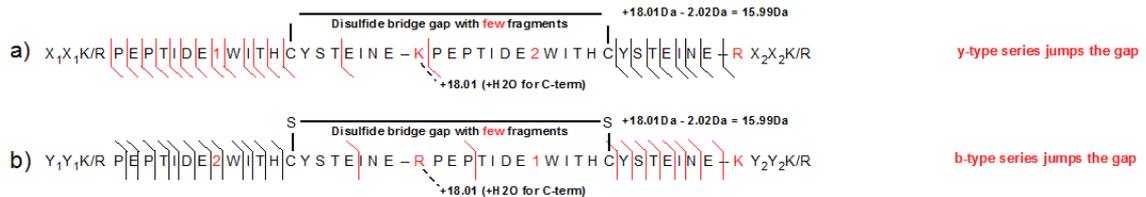
Figure 17 | A robust strategy behind the identification of disulfide bridges. Point 1) brings out the drawbacks searching disulfide-link spectra in a normal linear database when the peptides are distant in the protein sequence. Fragmentation of one peptide might be favored and then it jumps over the disulfide-link. Point 2) and 3), illustrated how a novel solution is applying permutated combination of the Cys peptides in the database and how further identification works. Hereby case a) represents the order of Peptide1Peptide2 and case b) the reverse order Peptide2Peptide1 for the spectrum annotation.

From previous studies on cross-linked peptides, often the b-type or the y-type ion series breaks at the site of crosslinking so that a dipeptide is identified by a partial series from the one and a partial series from the other peptide. There are also studies about branched peptides, which observed that the ion series might jump from one peptide to the other over the site of interaction (e.g. Hsiao et al., 2009a). Measurements on the QE favors the generation of y-type ion series. In order to unequivocally identify two disulfide linked peptides, they need to show fragmented ions in the spectrum that argue for this "jumping the gap"-mechanism. When searching the data on behalf of the permutated database, the spectra have to match both the intact molecular weight and the fragmentation pattern. For these peptides, two main characteristics were taken into account: the loss of two hydrogen (-2.02 a.m.u.) Da because of the disulfide bridge, and the hydroxylation of the free C-terminus of the second peptide (+18.01 a.m.u.), that is now buried in the sequence of the permutated

database. This provides a C-terminal modification (e.g. positioned at the K or R for a trypsin digestion) of +15.99 a.m.u. (equal to oxidation). In order to evaluate whether spectra were correctly annotated, the search was conducted both for the position of the +15.99 a.m.u modification as well as for the occurrence of large molecular weight fractions that complemented either the y-type or the b-type ion series (Figure 17).

3.2.5 Statistical analysis

Data are reported as the mean of n=2 or mean ± SEM of n>3. Experiments with n=2 are showing the solid data as second independent experiments were performed to confirm the finding. Statistical analyses were performed with GraphPad Prism 6. Multiple group comparisons were calculated by one or two-way ANOVA, followed by Bonferroni's multiple comparison tests. Comparison of two group experiments was performed using a t-Test. The significance definitions are *$p<0.05$, **$p<0.01$, ***$p<0.001$ and ****$p<0.0001$ vs. control.

In the case of mass spectroscopy experiments, samples were injected twice in the instruments. Only one out of two experiments give us consistent results along with the theory. Spectral count is different for every Cys site and every modification.

4 Results

4.1 Physiological effect of redox regulation in cardiac myocytes

To initially investigate the effect of oxidative stress on cardiac myocytes, a very simple experiment was established by making live cell imaging of NRCM with increasing concentrations of H_2O_2 to force oxidative stress. NRCM was incubated at 37°C until it was fully mature and beating. A movie was recorded with one frame taken every 20 min for 24 h (with the first frame taken at 0 sec), with control (0 µM), 10^2 µM, 10^3 µM and 10^4 µM of H_2O_2 (movie available on CD; Figure 18). Without H_2O_2, the NRCM cell contraction was, as expected, normal and its hold same with exposure to the physiological dose of 10^2 µM H_2O_2. With higher concentration doses of 10^3 µM, reduced contraction of cells was observed and at 10^4 µM cell death was observed. After video recording of the cultured NRCM, a matlab based script according to Huebsch et al. was used to generate a heat map of the mean contraction of the contractile regions for each given H_2O_2 concentrations (Huebsch et al., 2015; Figure 18.A). The same script was afterwards used to quantify the contraction velocity and the relaxation velocity of the myocytes at each H_2O_2 concentration. From this values the relaxation-contraction ratios were calculated to give a measure of myocyte vitality.

A
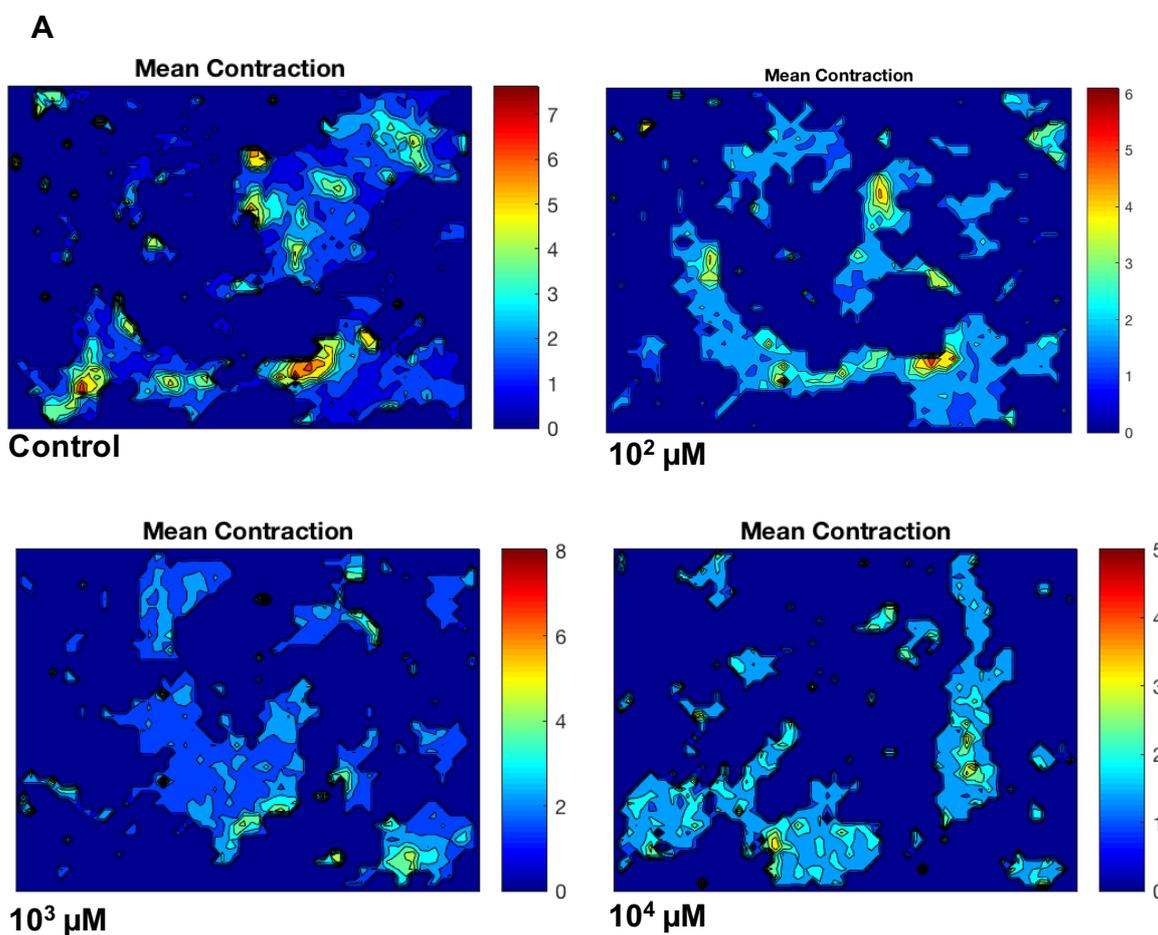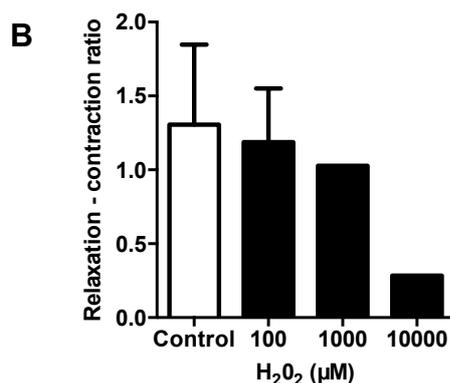
Figure 18 | Analysis of NRCM relaxation-contraction ratio exposed to oxidative stress. (A) Heat map depicting the mean of the time-averaged magnitude of every motion (myocyte contraction). A center of mean contraction is defined as a region from which contractions (inward motion) and relaxation (outward motion) are maximized over the time-averaged course of the movie. (B) Quantitative analysis representing ratio of relaxation to contraction. Values are given as mean ± SEM, n=2-4.

Quantitative analysis of the ratio between contraction to relaxation, suggested that ratios remains similar with 10^2 µM H_2O_2, and gradually decreased at higher concentrations of H_2O_2 (Figure 18.B).

4.2 Redox regulation of PP-1 in the heart

4.2.1 Importance of PP-1 in physiological functioning of NRCM

4.2.1.1 Investigation of phosphatase activity in human heart samples, NRCM, and whole heart mouse tissue

To calculate Phosphatase activity, two different kits were used. For human heart samples, NRCM and whole heart tissue Enzchek kit, and for recombinant protein the Promega phosphatase activity assay were used (Section 3.2.2.5).
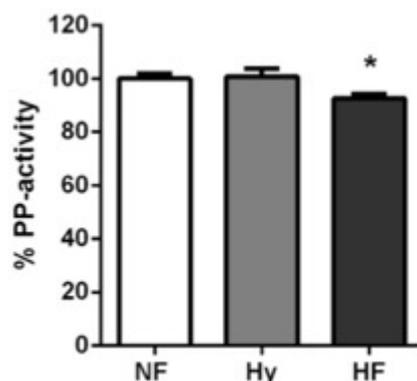
Figure 19 | Phosphatase activities in human NF, Hy and HF hearts. Total phosphatase activities of human heart homogenates (20 µg total protein); non-failing (NF), hypertrophy (Hy) and heart failure(HF) patients were measured using EnzChek Phosphatase Assay Kit. Values are given as

mean ± SEM, NF=5, Hy=15 and HF=23. Statistical comparison was performed by t-test, *p< 0.05 vs. NF.

To differentiate between different types of phosphatases, i.e. PP-1 and PP-2A, OA as phosphatase inhibitor, was used in the Enzchek kit assay. Total phosphatase activity in heart failure (HF) patients was 7.38% lower as compared to non-failing (NF) and hypertrophy (Hy) samples (Figure 19). The inhibitory activity of 10 and 100 nM of OA was expected to inhibit mainly PP-2A and PP-1 activity respectively. In NRCM and total heart tissue from mice, PP-1 contributes to approximately 10% of total PP-activity (Figure 20). Heart samples were kind gift from Dr. Thomas H. Fischer, Clinic for Cardiology and Pneumology, Goettingen.
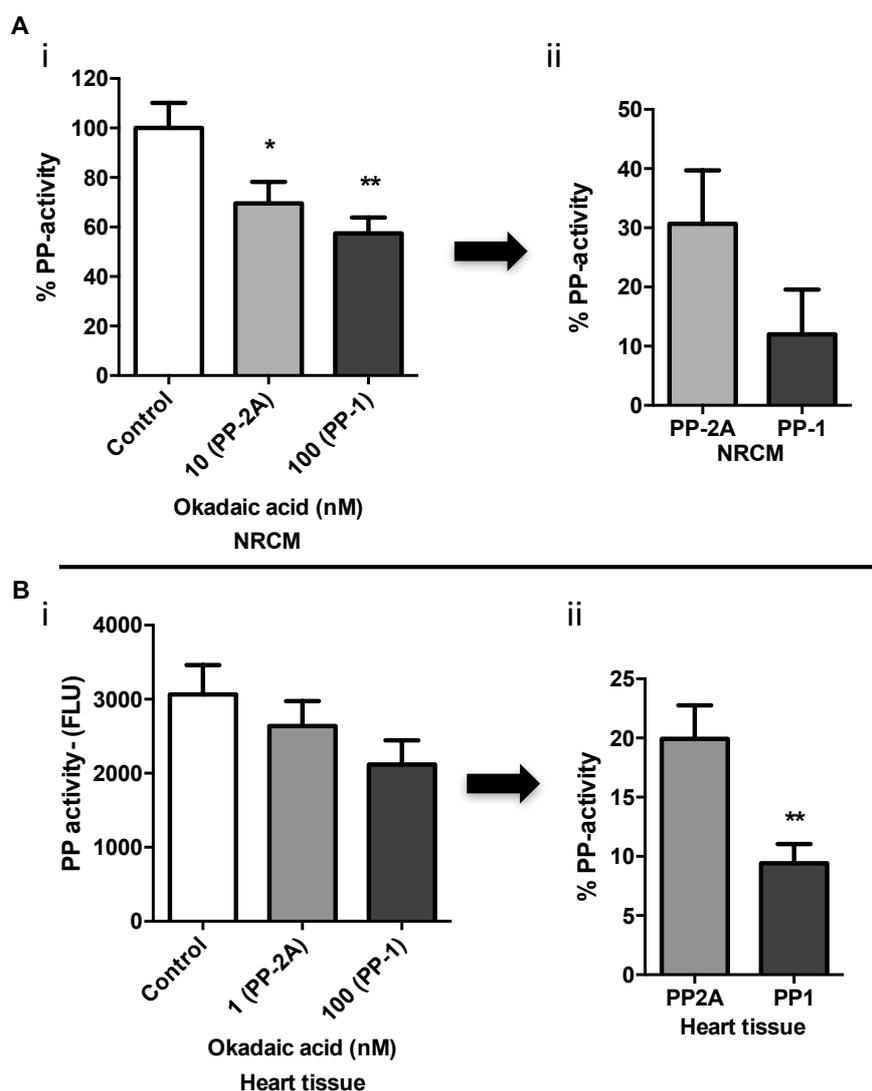
Figure 20 | Phosphatase activity in NRCM and whole heart tissue of mouse. Total phosphatase activities depicting percentage of PP-2A and PP-1 activity in (A) NRCM and (B) whole heart tissue of mouse. Activity was measured using ProFluor Ser/Thr PPase Assay in the presence of 10 nM (PP-2A) and 100 nM (PP-1) OA. Panel A, B – Figure ii represents the %PP-activity after calculations as per following formula {PP-2A = [Control- 10(PP-2A)]/Control; PP-1 = [10(PP-2A)- 100(PP-1)]/Control}. Values are given as mean ± SEM, n=3-6. Statistical comparison was performed

by t-tests, *p<0.05, **p<0.01 vs. control (Panel A, B – Figure i), **p<0.01 vs PP-2A (Panel A, B – Figure ii).

4.2.1.2 Impact of oxidative stress on phosphatase activity in NRCM and rPP-1

To test the effect of oxidative stress on PP-1 activity, in the presence of Mn^{2+} buffer, the experiments were conducted using either recombinant PP-1 alpha (rPP-1-GST tagged) *in vitro* or in living neonatal rat cardiomyocyte (NRCM) treated with the oxidizing agent H_2O_2. Results show that increasing concentrations of H_2O_2 induced a significant reduction in recombinant PP-1 activity (Figure 21.A); with a maximum effect of H_2O_2 after 10-30 min incubation time (Figure 21.B). Consistently, the reducing agent Tris-(2-carboxyethyl) phosphine (TCEP), when added to recombinant PP-1, reversed H_2O_2-induced PP-1 inactivation (Figure 21.C). However, when NRCM was treated with H_2O_2, the net decrease in PP-activity reached around 25%, indicating that not only PP-1 activity was affected by oxidation (Figure 22).
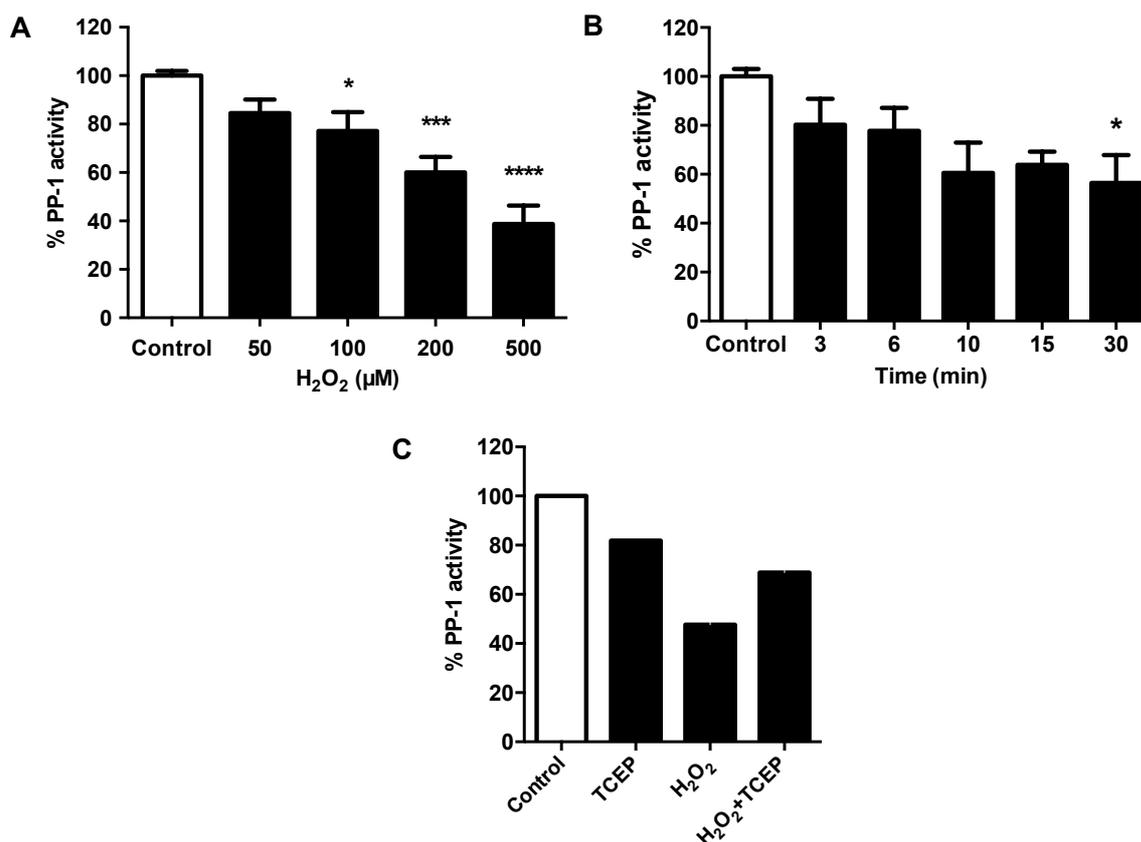
Figure 21 | Phosphatase activity in rPP-1. (A) Activity of recombinant-PP-1 (rPP-1) incubated with H_2O_2 (0, 50, 100, 200, 500 µM) for 10 min. Values are given as mean ± SEM, n=3-4. (B) The activity of rPP-1 incubated with H_2O_2 (200 µM) for the indicated time period. Values are given as mean ± SEM, n=2-4. (C) Reversibility of rPP-1 activity, when incubated with H_2O_2 (200 µM, 15 min) followed by TCEP (100 mM) for 5 min. Values are given as mean, n=2. Statistical comparison was performed by one-way ANOVA, and post hoc correction with Bonferroni's multiple comparison tests, *p<0.05, ***p<0.001, ****p<0.0001 vs. control.
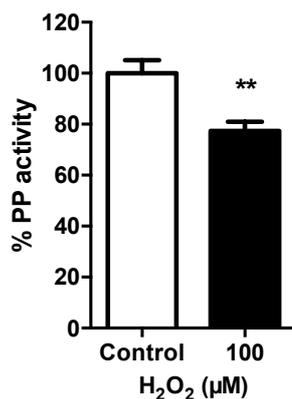
Figure 22 | Phosphatase activities in NRCM. Total phosphatase activity in NRCM incubated with H_2O_2 (100 µM) for 3 min was measured using EnzChek Phosphatase Assay Kit. Values are given as mean ± SEM, n=5. Statistical comparison was performed by t-test, **p<0.01 vs. control.

4.2.1.3 Impact of oxidative stress on cross talk between PKA and PP-1 signaling pathways

Regarding the impact on downstream targets, immunoblotting revealed that the phosphorylation status of classical PP-1 downstream target proteins such as phospholamban (PLB) and cardiac myosin binding protein-C (cMyBP-C) were differentially affected by H_2O_2, indicating a complex layer of regulation of both redox-sensitive kinases and phosphatases (Figure 24.A). Whereas the phosphorylation status at Thr^{35} of protein phosphatase inhibitor-1 (I-1), a crosstalk protein between protein kinase A and PP-1 signaling showed a bell-shaped phosphorylation response with a maximal peak at 100 µM (Figure 23).
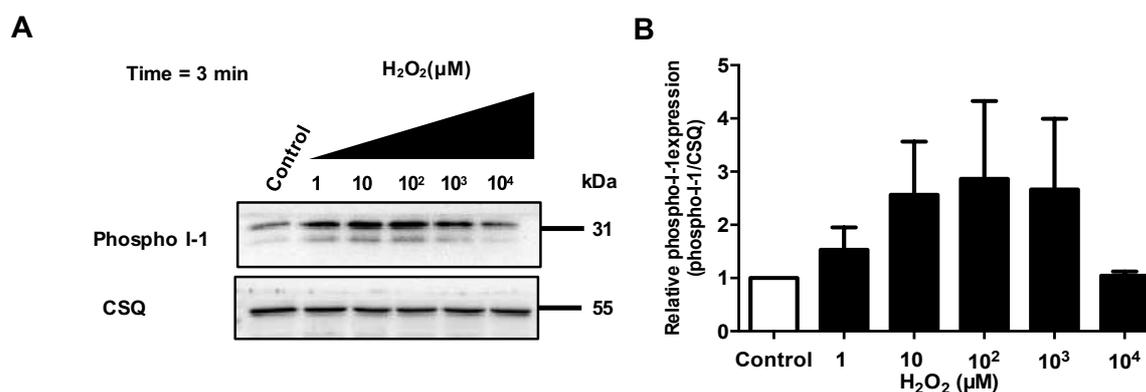
Figure 23 | Phospho I-1 phosphorylation after H_2O_2 treatment. (A) Immunoblot and (B) quantification of concentration dependency of H_2O_2 mediated phospho I-1 expression in NRCM. The analysis was normalized to CSQ, and the change in phospho I-1 expression was estimated relative to the control. Values are given as mean ± SEM, n=3. Statistical comparisons were performed by one-way ANOVA, and post hoc correction with Bonferroni's multiple comparison tests, *p< 0.05 vs. control.

There was no effect of H_2O_2 at physiological concentration on PLB phosphorylation at Ser16 (p-PLB-Ser^{16}) or cMyBPC phosphorylation at Ser^{282} (p-cMBPC-Ser^{282}) after 3 min (Figure 24.C). Time-dependent experiments, however, showed a remarkable decrease in PLB-Ser^{16}

phosphorylation after 10 min. In contrast, cMyBPC-Ser^{282} phosphorylation was bell-shaped, peaking after 10 min of H_2O_2 incubation (Figure 24.B).
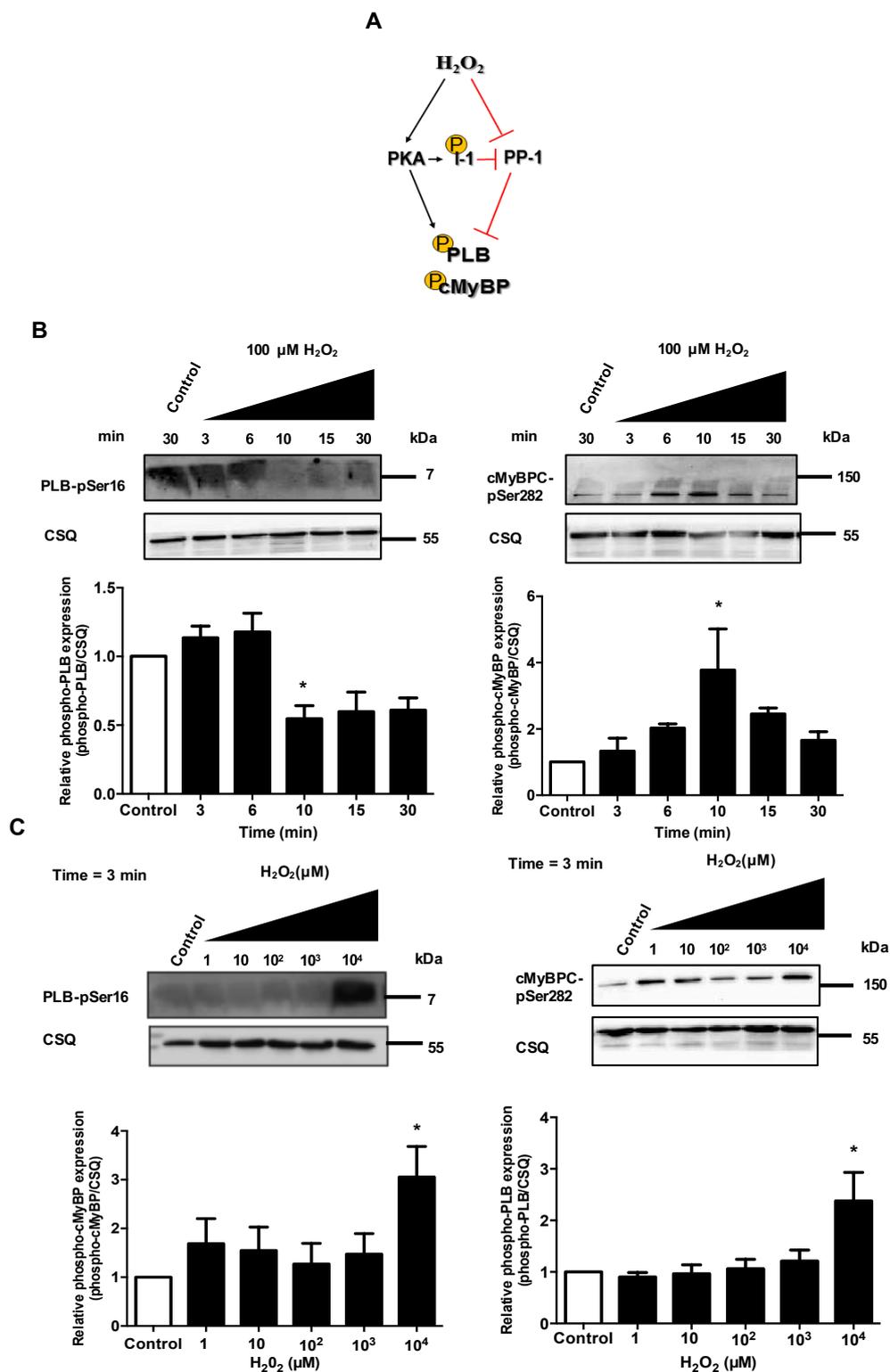
Figure 24 | Putative mechanism of PKA activation and PP-1 inactivation by H_2O_2. (A) The mechanism is showing the summary of activating and inhibitory effect of H_2O_2 on PKA and PP-1 respectively, and hence changes in phosphorylation of downstream cardiac protein, PLB and cMyBPC. Immunoblot and quantification of (B) time course and (C) concentration dependency of H_2O_2 mediated PLB-pSer^{16} and cMyBPC-pSer^{282} expression in NRCM. The analysis was normalized to

CSQ, and the change in PLB-pSer[16] and cMyBPC-pSer[282] expression was estimated relative to the control Values are given as mean ± SEM, n= 3. Statistical comparisons were performed by one-way ANOVA, and post hoc correction with Bonferroni's multiple comparison tests, *$p < 0.05$ vs. control.

4.2.2 Identification of redox-sensitive Cys in PP-1

4.2.2.1 Investigating the disulfide bridges in phosphatases via immunoblotting

A PKA regulatory subunit-1 (PKA RI) dimer formation in NRCM was established to identify any disulfide bridges within the PP-1 protein. In the presence of maleimide NRCM were then treated with increasing concentrations of oxidizing agents, i.e. diamide and H_2O_2. Afterwards the sample was processed on a gel under non-reducing conditions which formed a RI subunit of PKA and resulted in an SDS-resistant dimer.
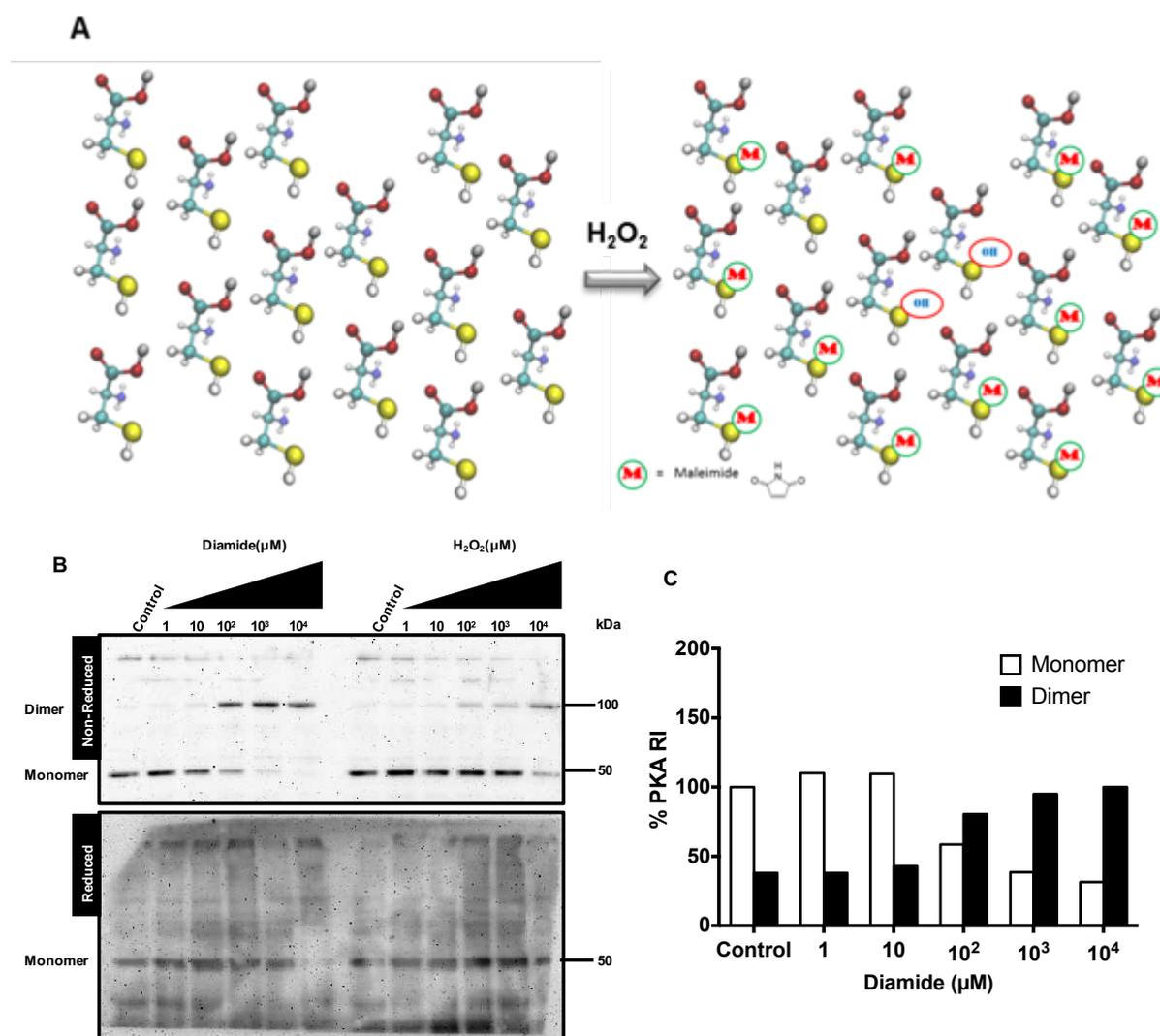
Figure 25 | Effect of increasing concentration of diamide and H_2O_2 (15 min) on NRCMs under non-reducing and reducing conditions in PKA. (A) Mechanism depicting the blocking of free thiols with maleimide during the oxidation process in the protein (B) Immunoblots probed with anti-PKA RI and (C) densitometric analysis showing the percentage of PKA-RI that exists as monomer or dimer. Values are given as mean, n=2.

This dimer formation was no longer present when samples were run on reduced gel (inclusive of DTT). This data shows a shift of bands from 50 kDa to 100 kDa, was due to intermolecular disulfide bridges formations. disulfide bridge formation was dose dependent, since more than 50% of PKA RI in NRCM becoming dimeric after 15 min with 100 µM, in contrast to the treatment of H_2O_2, same observation was observed at 10^4 µM (Figure 25.B). The bar chart shows the quantitative data of two experiments, when treated with diamide, indicating the percentage of PKA-RI having monomer and dimer formation (Figure 25.C).

The same samples were run which were used to establish PKA-RI dimer formation. This step allowed us to identify any inter-molecular disulfide bridge formations in PP-1. Figure 26 shows that with dose dependence of diamide, PP-1 expression decreased, and no shift in the gel was observed as per PKA immunoblots. The bar chart shows the quantitative data of two experiments, when treated with diamide, indicating the decreasing expression of PP-1 (Figure 26.B). However, interestingly a dimer formation was observed at 70 kDa both in control and increased concentrations of diamide, which were less expressed at 10^3 µM diamide and eventually disappeared at 10^4 µM diamide.
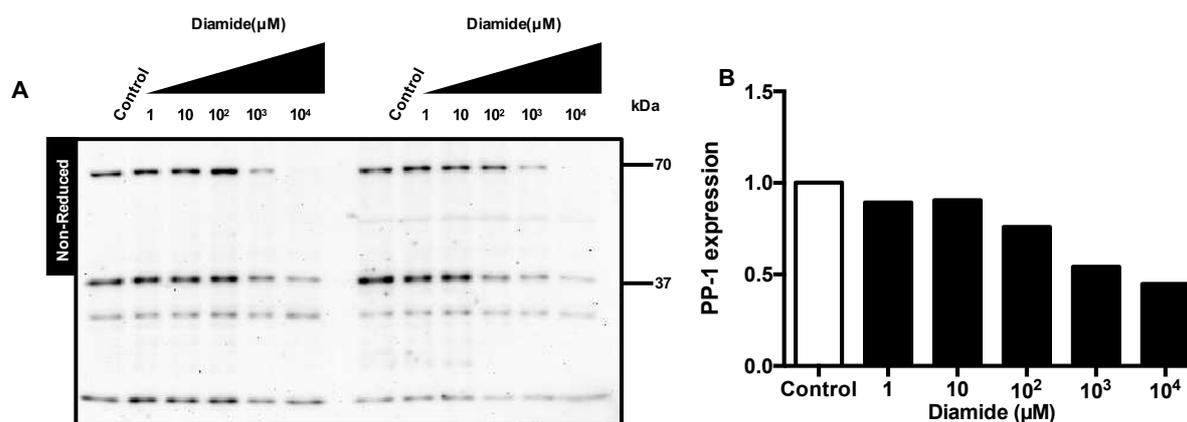
Figure 26 | Effect of increasing concentrations of diamide (15 min) on NRCMs under non-reducing condition probed with PP-1 (catalytic subunit). (A) Immunoblot representing the decreasing expression of PP-1 (37 kda) and a dimer formation at 70 kDa, which eventually disappears at 10^4 µM diamide. (B) Densitometric analysis showing the decreasing expression of PP-1. Values are given as mean, n=2.

PP-2A was examined more closely to check for disulfide bridge formation within the family of phosphatases. Some literature has suggested that PP-2A might also have redox-sensitive Cys. Therefore, the same samples were run on non-reduced gel with PP-2A antibody. In the end, it was discovered that no shift in gel occurred. However, at 10^4 µM diamide, less expression of PP-2A was observed which concurs with the findings of PP-1 (Figure 27).
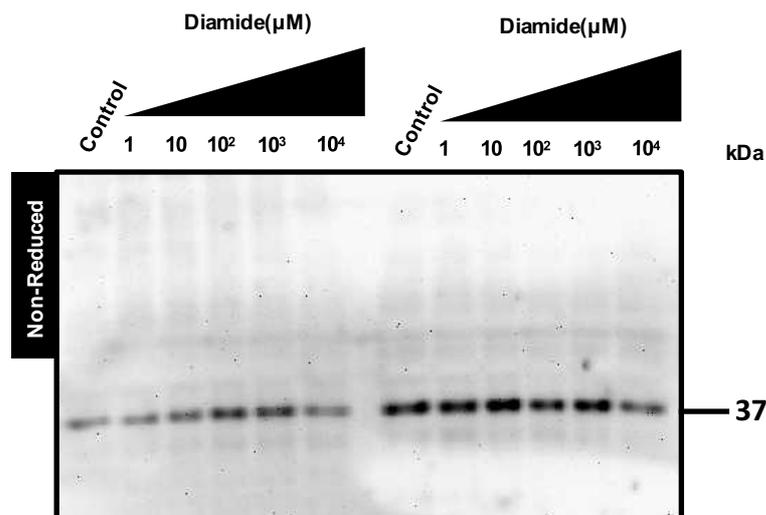
Figure 27 | Effect of increasing concentration of diamide (15 min) on NRCMs under non-reducing conditions probed with anti-PP-2A. Immunoblot representing the reduction of expression of PP-2A (37 kda) at 10^4 µM diamide. Similar results were obtained in a second independent experiment.

While running blots for PP-1, PP-2A and PKA under non-reducing and reducing conditions, an interesting finding was discovered regarding SERCA2a. Without DTT as the reducing agent, a band shift was observed in the non-reducing state with respect to SERCA2a, which was not visible in PP-1 and PP-2A blots. Hence, in conclusion, SERCA2a, which has redox-sensitive Cys, appears to lead to a band shift, whereas phosphatases do not (Figure 28).
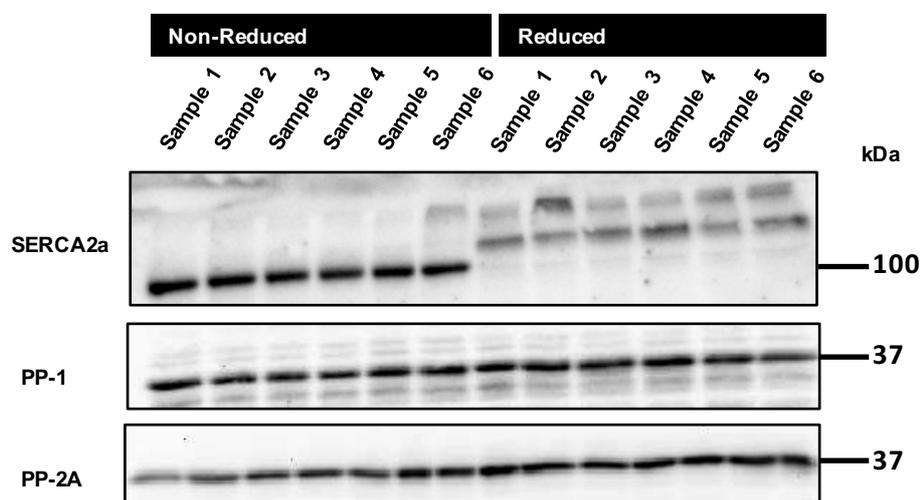
Figure 28 | Cell lysates from NRCM analyzed under non-reducing and reducing conditions probed with anti-SERCA2a, anti-PP-1 (catalytic subunit) and anti-PP-2A. Immunoblots representing a shift in band with respect to SERCA2a, which is not visible for PP-1 and PP-2A. Similar results were obtained in a second independent experiment.

To further confirm that PP-1 Cys-oxidation status changes in response to ROS, a cell permeable, biotinylated iodoacetamide (BIAM) was applied to label free Cys thiol groups

following exposure of NRCM cells to oxidizing agent H_2O_2. For HRP conjugated streptavidin immunoblots, results showed that by the addition of H_2O_2, unexpectedly there was little difference between control and treated cells. However, in comparison to pH 6.5, a visible change occurred with the sample treated with pH 8.5, after BIAM and H_2O_2 were added. No change was observed in the pulldown of PP-1 expression all above-mentioned conditions (Figure 29).
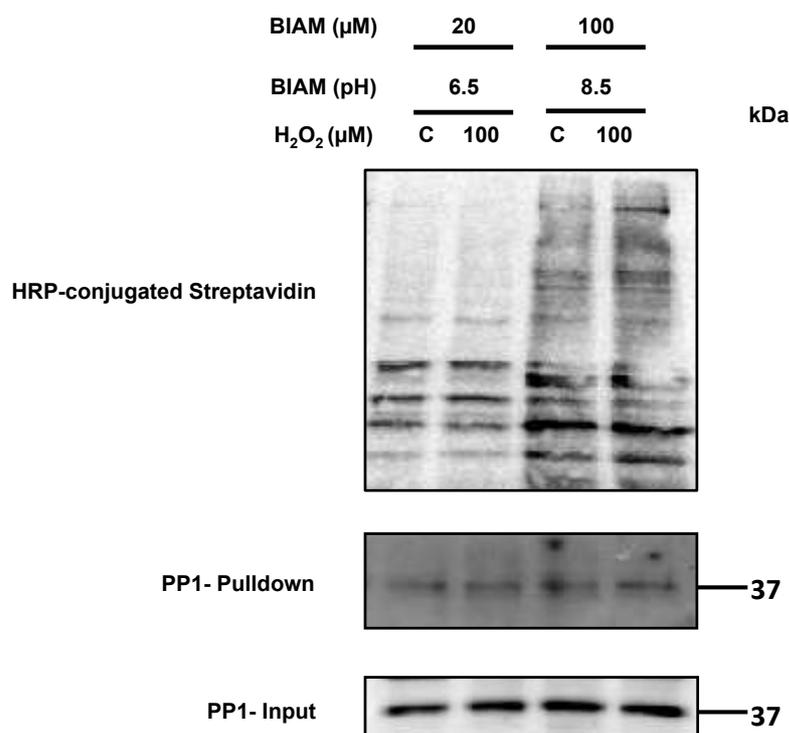
Figure 29 | Identification of oxidized proteins due to redox active Cys. NRCM were treated with 0 μM (Control) and 100 μM H_2O_2 for 15 min, followed by an incubation with BIAM (20 μM - 6.5 pH and 100 μM - 8.5 pH) for 30 min and protein-BIAM adducts were purified using streptavidin pull-down and then subjected to Western blot analysis using PP-1 antibodies in BIAM labeling buffer. Similar results were obtained in a second independent experiment.

4.2.2.2 Analysis of disulfide bridges from bioinformatics tools in PP-1

To calculate pK_a values, PROPKA 3.1 (Søndergaard et al., 2011) was used, to semi-quantitatively estimate pK_a values of all the Cys residues of PP-1A. It is empirical pK_a predictors, based on the better physical description of the desolvation and dielectric response for the protein. Table 18 shows that out of thirteen Cys residues, only two (Cys^{155} and Cys^{171}) had the lowest pK_a i.e. 8.01 and 8.97 respectively.

Table 18 | Analysis of pKa of PP-1A (PDB id: 4MOV) by PROPKA 3.1. The representative table represents the pKa of all Cys in the PP-1α. Cys[155] and Cys[171] are susceptible to oxidation because of pK_a near to 8.

Pos.	pKa
CYS 39 A	18.69
CYS 62 A	12.64
CYS 105 A	12.94
CYS 127 A	10.12
CYS 140 A	13.30
CYS 155 A	8.01
CYS 158 A	10.65
CYS 171 A	8.97
CYS 172 A	12.59
CYS 202 A	11.56
CYS 245 A	18.55
CYS 273 A	9.76
CYS 291 A	11.94

The Cysteine Oxidation Prediction Algorithm (COPA) is an algorithm used to predict oxidation-susceptible sites, which can further help a protein's Cys residues susceptible to redox-mediated regulation and identify possible enzyme catalytic sites with reactive cysteine thiols (Sanchez et al. 2008). Table 17 demonstrates that Cys 39, 155, 171 and 245 were susceptible to oxidation by sulfur distance, and that the oxidation status was reduced. Also, Cys[127] and Cys[273] are vulnerable to oxidation due to a low pK_a and a reduced oxidation state.

Table 17 | Analysis of oxidation status of all Cys by COPA. Representative table represents the oxidation status, pK_a and various other predictions, whose full form are as follow: Oxidation Status Codes: RED – Reduced, DSB - Disulfide Bond, OXI – Oxidized; Prediction Codes: SOD - Susceptible to Oxidation by Sulfur Distance, SEP - Susceptible to Oxidation by Exposure and pKa, NOS - Not Oxidation Susceptible, N/P - No Prediction.

SEQUENCE POSITION	SEQUENCE	OXIDATION STATUS	PREDICTION	_	ASA	pKa
39	QLTENEIRGLCLKSREIFLSQ	RED	SOD		0	13.15
62	LLELEAPLKICGDIHGQYYDL	RED	NOS		0.07	12.33
105	DRGKQSLETICLLLAYKIKYP	RED	NOS		1.65	12
127	NFFLLRGNHECASINRIYGFY	RED	SEP		4.87	5.96
140	INRIYGFYDECKRRYNIKLWK	RED	NOS		0	10.6
155	NIKLWKTFTDCFNCLPIAAIV	RED	SOD		0.26	9.78
158	LWKTFTDCFNCLPIAAIVDEK	RED	NOS		0	8.52
171	IAAIVDEKIFCCHGGLSPDLQ	RED	SOD		0	11.98
172	AAIVDEKIFCCHGGLSPDLQS	RED	NOS		0	12.72
202	PTDVPDQGLLCDLLWSDPDKD	RED	NOS		0.21	10.73
245	FLHKHDLDLICRAHQVVEDGY	RED	SOD		0	14.28
273	LVTLFSAPNYCGEFDNAGAMM	RED	SEP		17	8.1
291	AMMSVDETLMCSFQILKPA--	RED	NOS		4.45	9.67

The third prediction software for Cys oxidation that was used was DiANNA (version 1.1), a software program composed of an artificial neural network located on a web server. The software provides information on various Cys oxidation states and disulfide connectivity of proteins. The amino acid sequence of PP-1α was analyzed in DiANNA with the help of the support vector machine (SVM) with spectrum kernel. SVM predicts whether a Cys is reduced or involved in a disulfide bridge or bound to the metallic ligand. The results in Table 19 show that only Cys[273] obtained a score of 1, an indication that the compound is highly likely to both oxidize and form disulfide bridges. (Ferre and Clote, 2005).

Table 19 | Analysis of disulfide oxidation state prediction of PP-1A by DiANNA 1.1 web. It is a neural network, which takes the input of evolutionary information of symmetric window centered on each Cys. It tells us a score of a Cys oxidation state prediction with the score of 1. The program provides a score for a Cys oxidation state prediction, with a score of 1 indicating high susceptibility to oxidation and a strong likelihood to form disulfide bridge.

Sequence Position	Cysteine neighborhood window	Score
39	EIRGLCLKSRE	0.0
62	APLKICGDIHG	0.0
105	SLETICLLLAY	0.0
127	RGNHECASINR	0.0
140	GFYDECKRRYN	3e-05
155	KTFTDCFNCLP	0.0
158	TDCFNCLPIAA	0.0
171	DEKIFCCHGGL	0.0
172	EKIFCCHGGLS	0.0
202	DQGLLCDLLWS	0.0
245	DLDLICRAHQV	0.0
273	SAPNYCGEFDN	1.0
291	DETLMCSFQIL	0.0

Furthermore, Cys S-glutathionylaton (GSH) sites from PP-1 A (PDB id: 3N5U) were also verified in human species with the dbGSH database. The database integrates all experimentally verified S-glutathionylated peptides from research articles using a text mining approach. The Figure 30 refers to the identified Cys, i.e. 140, 158 and 245 which were S-glutathionylated (Chiang et al., 2012). The top part of the figure shows the proposed S-glutathionylation sites in the context of solvent accessibility and protein secondary structure. Both Cys[140] and Cys[245] were located at the end or beginning of a helical structure, respectively, and at a solvent accessible area. The predicted Cys[158] was found in the middle of a helical structure and hence was not solvent accessible. The lower part of the picture shows two consensus motifs for probable glutathionylation sites. It is easily visible that the acidic amino acid residues Asp (D) and Glu (E) are in close proximity.
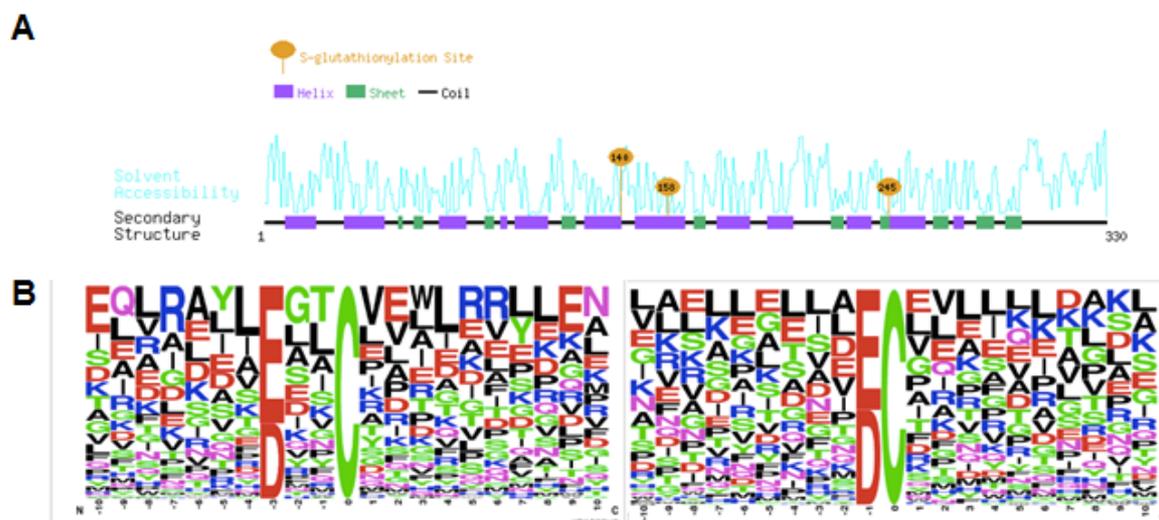
Figure 30 | Graphical representation of S-glutathionylation sites analyzed by the dbGSH database in PP-1. (A) An overview of protein S-glutathionylation sites with functional and structural information of human PP-1 (PDB id: 3N5U) showing Cys^{140}, Cys^{158} and Cys^{245} to be S-glutathionylated. (B) Substrate motifs are showing a conserved region for Cys^{140} and Cys^{245}.

4.2.3 Analysis via Mass Spectrometry

The reason for beginning the analyses with mass spectrometry (MS) was to prove the existence of the predicted disulfide bridges. The Pymol software for measuring the molecular distances from the non-oxidized protein structure did not predict any intra-molecular disulfide bridges. The immunoblots, on the other hand, were able to show band shifts which represented inter-disulfide bridges (Section 4.2.2.1), however, it is unclear whether these disulfide bridges are artifacts. MS is the only method that maps all PTMs and sites in a single experiment without *a priori* knowledge.

4.2.3.1 Investigating the PTMs in GST-tagged PP-1

According to the computational prediction (Section 4.2.2.2), possible Cys residues were identified, which could be oxidized and may be susceptible to disulfide bridge formation. The experiment was performed with four different conditions (1: $-Mn^{2+}$ $-H_2O_2$; 2: $-Mn^{2+}$ $+H_2O_2$; 3: $+Mn^{2+}$ $-H_2O_2$; 4: $+Mn^{2+}$ $+H_2O_2$) so that one can draw some conclusions about the protective effect of Mn^{2+} and the oxidative effect of high H_2O_2 conditions. In addition to the common PTMs, a new database search strategy was created to unequivocally identify disulfide linked peptides from non-reduced and proteolytic digested samples. Although in principle many different PTMs could be formed under oxidative stress, the main emphasis was given on the identification of (i) free cysteines (which were protected by alkylation with IAA), (ii) glutathionylated Cys, (iii) potential disulfides being indicated by an unheterolytic cleavage of the disulfide link forming one persulfide and one dehydroalanine peptide, and (iv) sulfone

oxidized cysteines (which is the highest oxidatitive form of cysteines). The experiment was performed as described in Section 3.2.4. The H_2O_2 (500 µM) concentration used in the experiment would more than out-compete the protective effect of Mn^{2+} ions in the buffer.

The data was generated with LC-MS/MS using an Ultimate 3000 chromatography system coupled to a Q-Exactive Plus system. The sample was loaded onto the precolumn and eluted in reverse to the separation column. Data acquisition was performed with a top ten-centroid mode method. It was then searched with Peaks 7.0 with semi-Trypsin specificity because some long nonspecific fragments could have been undetectable. With this method, a longer fragment was identified that had a non-specific cleavage site comprising Cys^{273} and Cys^{291} but starting at Ser^{268}. However, the two short peptides that included Cys^{39} and Cys^{127} were not detectable (Appendix 1) under all condition, so in conclusion, they might be involved in some intra-molecular interaction or unexpected modification.

The search included all relevant Cys modifications, namely dehydroalanine, persulfide, sulfenic acid, sulfonic acid, glutathionylation, internal disulfide bridges on one peptide (but which could also be represented by a combination of the first two modifications), and carbamidomethylation. Furthermore, all other types of amino acid oxidations were included: phosphorylations of Ser, Thr and Tyr, N-terminal protein acetylation and side reactions such as pyrolation of Gln and Pyro Asn and deamidations of Glu and Asp.

Glutathionylation in the non-protective buffer was observed under harsh oxidative stress (Figure 31.B) for the three Cys residues Cys^{140}, Cys^{245} with good spectra and Cys^{202} with a lower quality spectrum. Many Cys residues were observed with dehydroalanine and persulfide modifications only under these experimental conditions, which suggest that these Cys residues form disulfide bridges. The over-oxidation of the Cys residues was also displayed. Figure 31 provides a sequence summary of the four distinct buffer conditions. The above-mentioned modified Cys residues are highlighted with green or gray boxes, respectively.

A) - Mn²⁺, - H₂O₂

B) - Mn²⁺, + H₂O₂

Persulfide/ Dehydroalanine Generated by MSMS fragmentation of Disulfides

Glutathion peptides
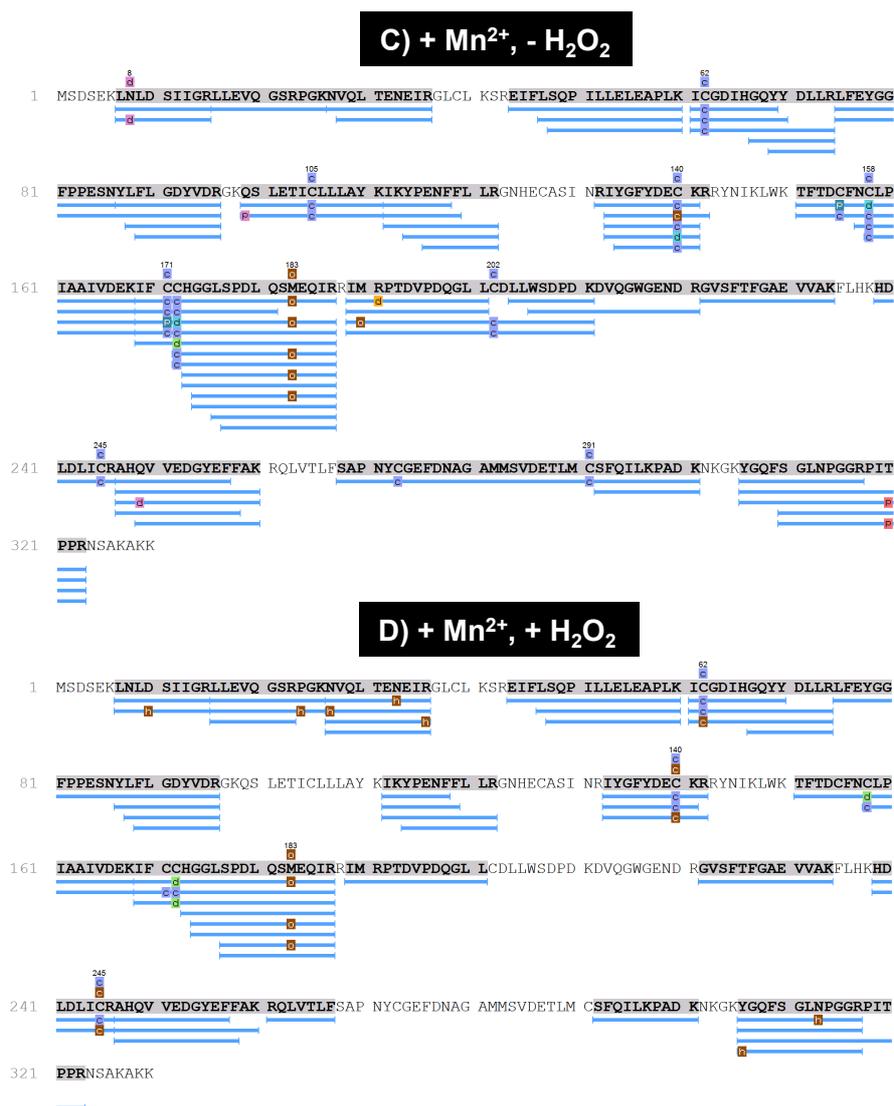
Figure 31 | *In vitro*-assays for MS readout using the GST-tagged PP-1 protein. All sequences which were successfully identified by Peaks 7.0 software are depicted by a blue bar below the sequence. All PTMs, their abbreviations and their color code are shown on the left. With respect to four different conditions, output PTM was generated with specifically (B) showing glutathionylated Cys^{140}, Cys^{202} and Cys^{245} in the gray and persulfide/dehydroalanine generated by MSMS fragmentation of disulfide in a green box with respective spectra.

The spectrum shown for Cys[140] (Figure 32) shows that Cys underwent glutathionylation. Furthermore, the ion tables and the error bar plots are shown as they were extracted from Peaks 7.0 software. The remaining spectra for Cys[202] and Cys[245] is shown in Appendix 3. The table in Figure 32 summarizes the following ions: on the left side of the peptide sequence are Immonium ions, b-type ions, b-H_2O ions, b-NH_3 ions, a-type ions and B (2+) ions. On the right side of the peptide sequence are y t-type ions, y-H_2O ions, y-NH_3 ions and y (2+) ions. The error plot below shows all fragment ions within 0.02 Da mass deviations that were allowed for the search. Since almost the entire ion series was covered, the correct identification could be made with confidence.

#	Immonium	b	b-H2O	b-NH3	a	b (2+)	Seq	y	y-H2O	y-NH3	y (2+)	#
1	86.10	114.09	96.08	97.06	86.10	57.55	I					9
2	136.07	277.15	259.14	260.13	249.16	139.08	Y	1329.46	1311.47	1312.47	665.24	8
3	30.03	334.17	316.17	317.15	306.18	167.59	G	1166.40	1148.40	1149.39	583.71	7
4	120.08	481.25	463.23	464.22	453.25	241.12	F	1109.40	1091.38	1092.36	555.19	6
5	136.08	644.31	626.30	627.28	616.31	322.65	Y	962.31	944.32	945.30	481.66	5
6	88.04	759.34	741.32	742.31	731.34	380.17	D	799.26	781.24	782.23	400.13	4
7	102.06	888.38	870.37	871.35	860.38	444.69	E	684.24	666.22	667.21	342.62	3
8	381.09	1296.46	1278.44	1279.43	1268.46	648.73	C(+305.07)	555.19	537.18	538.16	278.10	2
9	101.11						K	147.11	129.10	130.09	74.06	1

Figure 32 | Spectra and positions of the glutathione-modified Cys[140]. The top shows the spectrum with y-type ions in red and b-type ions in blue. The ion table below displays the exact masses of each of the ions and indicates the site of the PTM. In the structure, Cys residues are highlighted in red, glutathione-modified in cyan, and indicated by an arrow.

In order to describe the different solvent accessibilities and the reactivity of the diverse Cys residues, the above-mentioned modifications were quantified using the spectral count approach. The data from the GST-tagged PP-1 for the four experimental conditions demonstrated that the Cys residues are subtyped in 2-3 groups (Figure 33). Overall, free Cys spectral counts were higher in number in both conditions $-Mn^{2+}$ $-H_2O_2$ and $+Mn^{2+}$ $-H_2O_2$. persulfide/disulfide bridge and sulfone formation was higher when $-Mn^{2+}$ $+H_2O_2$ was

incubated. In contrast, the presence of additional Mn^{2+} ions protected the Cys residues from further oxidation. Three Cys residues also formed glutathionylation.

The PTMs of the 13 Cys residues were quite different from one another. Data for Cys^{155} and Cys^{158} as well as Cys^{171} and Cys^{172} has to be interpreted pairwise because they most likely form an internal disulfide bridge upon sample preparation. Mn^{2+} ions in the buffer protect them completely from sulfone formation. Disulfide bridges are observed under all conditions, but to a lesser extent when no H_2O_2 is applied. Apparently, the presence of the Mn^{2+} does not alter this effect. For instance, Cys^{155} and Cys^{158}, disulfide formation dominated in the condition with $-Mn^{2+}$ $+H_2O_2$ and no free Cys in the 4th condition with $+Mn^{2+}$ $+H_2O_2$: 50% of persulfide/disulfide bridge and 50% free Cys or 50% of persulfide/disulfide bridge and 40% free Cys, respectively. However, spectral counting results demonstrated, that Cys^{158} formed 10% of sulfone Cys in the condition with $-Mn^{2+}$ $+H_2O_2$ which hints at the fact, that it might be free under some circumstances while Cys^{155} is more protected. Cys^{171}/Cys^{172} showed a similar trend but about 20-25% of persulfide/disulfide bridge formation under condition 2. When no H_2O_2 treatment was performed, spectral count for Cys^{171} and Cys^{172} showed 62-70% of free Cys and only the rest to be persulfide/ disulfide bridges, which can be explained by the disfavored orientation of the sulfhydryl groups in the peptide. Around 58-62% of persulfide/ disulfide bridge spectral count was discovered in the condition with $-Mn^{2+}$ $+H_2O_2$ and was reduced to 12-20% in the condition with $+Mn^{2+}$ $-H_2O_2$ and rest free Cys.

Cys^{273} and Cys^{291} lie at the outermost region of the structure show a complete 100% persulfide/disulfide bridge spectral count in the condition with $-Mn^{2+}$ $+H_2O_2$. This result is reversed with 100% free Cys in the condition with $+Mn^{2+}$ $-H_2O_2$. No spectra were found for the last condition with $+Mn^{2+}$ $+H_2O_2$.

For Cys^{140}, Cys^{202} and Cys^{245} glutathionylation (in gray) was detected, that apparently competes with the disulfide formation; and further also showed 62%, 47% and 50% of sulfone spectra counts, respectively, with the condition $-Mn^{2+}$ $+H_2O_2$. Cys140 also forms sulfone with Mn^{2+} in the buffer. However, in Cys^{202} and Cys^{245}, no sulfone formation was observed with the condition $+Mn^{2+}$ $-H_2O_2$. In Cys^{140} and Cys^{245}, 35% and 50% of Cys residues formed sulfone, respectively, with the condition $+Mn^{2+}$ $+H_2O_2$ and no spectral count was identified for Cys^{202} for the latter condition. Mn^{2+} completely protected Cys^{62} and Cys^{105}. In the condition with $-Mn^{2+}$ $+H_2O_2$, around 33% and 68% of sulfone formations were identified in the two aforementioned Cys, respectively. In the last condition, almost 35% sulfone formations were identified in Cys^{62} and however no spectra were identified for the Cys^{105}.

Figure 33 | Quantification of the amount of free, persulfide/disulfide and sulfonic acid -Cys residues by spectral counting. Columns show the percentage distribution of all the four modifications. Yellow represent sulfone formation, grey represent glutathione formation, green represent persulfide/disulfide formation and blue represent free Cys residues.

In order to verify the results from persulfide/dehydroalanine detection and unequivocally identify the disulfide-linked peptides within the data set, a new data search strategy was

introduced. Though the fact that other software packages are available such as StavroX (Gotze et al., 2012), SearchXlinks (Wefing et al., 2006), XlinkX (Liu et al., 2015) or several commercial software from the mass spectrometry vendors e.g. the PepFinder Software from ThermoFischer Scientific. The results were compared with the StavroX software, but the quality of the identified spectra was not satisfying, since the program annotated the peptide fragments just from the free N-and C-termini of the two peptides (data not shown). An interesting question would be, if a more robust strategy might be able to detect the higher order b-type or y-type fragments. Similar to the observation with sumolated peptides (Hsiao et al., 2009b), that the ion series jumped from one to the other peptides for branched species. Based on this idea a search database was generated, as was described in detail in the Section 3.2.4.5. In the at end, tall linear peptides were identified. The actually disulfide-linked region would not be identified by fragmented ions, but high molecular fragments were observed in the second peptide after jumping the linkage. Hence, this behavior for some of the following spectra was observed and incorporated into the database to optimize the search strategy. The strategy worked well for a single or few known proteins but a general strategy for the understanding of the disulfide network of an entire proteome is still needs to be developed. Two example spectrums are shown in Figure 34. The modification in the middle of the peptide is calculated as follows:

Water (free C-terminus) – Hydrogen = Jump over Disulfide bridge

18.01 Da – 2.02 Da = 15.99 Da

Table 20 summarizes the observed disulfide spectra from the four experimental conditions in a reactivity scheme. The previously described Cys^{155} and Cys^{158} are located in a distance that is suitable for inter-disulfide bridge formation. The olive-green squares being identified under all conditions are $Cys^{155}Cys^{158}$ and $Cys^{171}Cys^{172}$ that are placed on the same Trypsin derived peptide and hence could be generated from sample preparation. Interestingly, the – Mn^{2+} +H_2O_2 condition resulted in a significant increase in the occurrence of disulfide peptides, which is concurrent with our prior observation of the persulfides/dehydroalanines. The light green squares were generated by a weaker spectrum. However, only Cys residues in the upper left part of the figure involved with a strong emphasis of the Cys^{39} and Cys^{127}, as they were not identified as non-modified peptides in these samples. For Cys^{127}-Cys^{140} a disulfide link was found under all conditions except for the last one. The same holds true for the Cys^{39}-Cys^{127} link. Without the addition of Mn^{2+}, a Cys^{127}-Cys^{245} disulfide link was also observed. Disulfide exchange might be in concurrence with the protection of the Mn^{2+}. Many disulfide peptides were positively identified and were in proximity within the 3D structure (Cys^{140} XL Cys^{39}, Cys^{105} XL Cys^{39}, Cys^{140} XL Cys^{127}, Cys^{140} XL $Cys^{154}Cys^{158}$ and Cys^{127} XL

Cys^{154}Cys^{158}). The common basis for this mass spectrometry identification is that at least one of the two ion series, y-type or b-type, has to "jump the gap" of the cysteine that spans between the two peptides. Note, that at high concentrations of proteins or peptides at neutral pH, a so-called disulfide scrambling can take place which rearranges disulfide formations from a partially denatured protein or in the peptide solution. To avoid these very acidic digestion conditions, one possible alternative could be in combination with pepsin. Figure 34 represents spectra and ion table of Cys^{39} XL Cys^{127} and Cys^{127} XL Cys^{140} showing disulfide bridge formation. The remaining corresponding spectra are shown in the Appendix 4.

Table 20 | Cross-reactivity scheme for disulfide linkages in GST-tagged PP-1A. Spectra generated with GST-tagged PP-1, showing disulfide bridges pattern in all four conditions.

A) - Mn^{2+}, - H$_2$O$_2$

	Cys 39	Cys 62	Cys 105	Cys 127	Cys 140	Cys 155	Cys 158	Cys 171	Cys 172	Cys 202	Cys 245	Cys 273	Cys 291	Mn^{2+}
Cys 39	▨			A.1										
Cys 62		▨												
Cys 105			▨											
Cys 127	A.1			▨	A.2				A.3					
Cys 140				A.2	▨									
Cys 155						▨	(green)							
Cys 158						(green)	▨							
Cys 171								▨						
Cys 172									▨					
Cys 202										▨				
Cys 245				A.3							▨			
Cys 273												▨		
Cys 291													▨	
Mn^{2+}														▨

B) - Mn^{2+}, + H$_2$O$_2$

	Cys 39	Cys 62	Cys 105	Cys 127	Cys 140	Cys 155	Cys 158	Cys 171	Cys 172	Cys 202	Cys 245	Cys 273	Cys 291	Mn^{2+}
Cys 39	▨	B.1	B.2	B.3	B.4	B.5				B.6				
Cys 62	B.1	▨												
Cys 105	B.2		▨											
Cys 127	B.3			▨	B.7	B.9					B.8			
Cys 140	B.4			B.7	▨	B.10								
Cys 155	B.5			B.9	B.10	▨	(green)							
Cys 158						(green)	▨							
Cys 171								▨	(green)					
Cys 172								(green)	▨					
Cys 202	B.6									▨				
Cys 245				B.8							▨			
Cys 273												▨		
Cys 291													▨	
Mn^{2+}														▨

C) + Mn^{2+}, - H$_2$O$_2$

	Cys 39	Cys 62	Cys 105	Cys 127	Cys 140	Cys 155	Cys 158	Cys 171	Cys 172	Cys 202	Cys 245	Cys 273	Cys 291	Mn^{2+}
Cys 39	▨			C.1										
Cys 62		▨												
Cys 105			▨											
Cys 127	C.1			▨	C.2									
Cys 140				C.2	▨									
Cys 155						▨	(green)							
Cys 158						(green)	▨							
Cys 171								▨	(green)					
Cys 172								(green)	▨					
Cys 202										▨				
Cys 245											▨			
Cys 273												▨		
Cys 291													▨	
Mn^{2+}														▨

D) + Mn^{2+}, + H$_2$O$_2$

	Cys 39	Cys 62	Cys 105	Cys 127	Cys 140	Cys 155	Cys 158	Cys 171	Cys 172	Cys 202	Cys 245	Cys 273	Cys 291	Mn^{2+}
Cys 39	▨													
Cys 62		▨												
Cys 105			▨											
Cys 127				▨										
Cys 140					▨									
Cys 155						▨	(green)							
Cys 158						(green)	▨							
Cys 171								▨	(green)					
Cys 172								(green)	▨					
Cys 202										▨				
Cys 245											▨			
Cys 273												▨		
Cys 291													▨	
Mn^{2+}														▨

.

Figure 34 | Disulfide-peptide conjugates (spectrum and fragment table) of (A) Cys[39] and Cys[140] (B) Cys[127] and Cys[140]. In the structure plot, the two involved Cys are indicated with green color.

Results from Figure 26 proposed the native inter-disulfide bridges forming a dimer at 70 kDa, which were then analyzed by the mass spectrometry approach. Only one inter-disulfide bridge with Cys[127] was discovered. However, it was only discovered in the condition $-Mn^{2+}$ $+H_2O_2$, which contradicted the results from the immunoblot. It even showed inter-disulfide bridge under control conditions. Figure 35 represents the protein surface of PP-1 (PDB id: 4MOV), with Cys residues in red (Cys[127], Cys[273] and Cys[39]) at the surface. These residues are prone to oxidation and might form inter-disulfide bridges with other proteins. Also, the structure nicely shows the entry point for manganese ions in green and pink (Histidines and Mn^{2+}).

Pymol and the PDB structure 4MOV were used to map all the possible distances of Cys residues with each other and the Mn^{2+} ions to understand the data obtained from the MS/MS fragmentation experiments (Table 21). The color-coding ranges from the heavy blue (shortest distance) to white (longest distance). The approximate color-coding distribution is shown on the right side of the table. The solid black boxes indicate the two possible Cys networks over closer interaction, showing, that the protein could be seen as divided into two halves. The Cys residues at the rim carry another color code: green boxes indicate Cys with

persulfide/dehydroalanine modification as a strong indicator for a cleaved disulfide bridge (only in the $-Mn^{2+}$ $+H_2O_2$ condition, compare supplemental information) and in grey for glutathione-modified sites. Cys^{202} was found within both above mentioned modifications. Mn^{2+} ions are indicated in pink in the structure. The values are given in Å.

#	Immonium	b	b-H2O	b (2+)	Seq	y	y-H2O	y (2+)	#
1	30.03	58.03	40.02	29.51	G				20
2	87.06	172.07	154.06	86.54	N	2140.94	2122.93	1070.97	19
3	110.07	309.13	291.12	155.07	H	2026.89	2008.88	1013.95	18
4	102.06	438.17	420.16	219.59	E	1889.83	1871.82	945.42	17
5	76.02	541.18	523.17	271.09	C	1760.76	1742.78	880.90	16
6	44.05	612.22	594.21	306.61	A	1657.78	1639.77	829.39	15
7	60.04	699.25	681.24	350.13	S	1586.75	1568.73	793.87	14
8	86.10	812.34	794.33	406.67	I	1499.71	1481.70	750.36	13
9	87.06	926.38	908.37	463.69	N	1386.63	1368.62	693.81	12
10	129.11	1082.48	1064.47	541.74	R	1272.59	1254.58	636.79	11
11	30.03	1139.50	1121.49	570.25	G	1116.49	1098.47	558.74	10
12	87.06	1253.54	1235.53	627.27	N	1059.46	1041.45	530.23	9
13	126.07	1406.60	1388.59	703.80	H(+15.99)	945.42	927.41	473.21	8
14	102.06	1535.64	1517.63	768.32	E	792.37	774.36	396.68	7
15	76.02	1638.65	1620.64	819.83	C	663.32	645.31	332.16	6
16	44.05	1709.69	1691.68	855.34	A	560.32	542.30	280.66	5
17	60.04	1796.72	1778.71	898.86	S	489.28	471.27	245.14	4
18	86.10	1909.80	1891.79	955.40	I	402.25	384.24	201.62	3
19	87.06	2023.85	2005.84	1012.42	N	289.16	271.15	145.08	2
20	129.11				R	175.12	157.11	88.06	1

Figure 35 | Spectrum and ion table for the inter-disulfide bridges with Cys[127]. Surface structure is showing the outer bound Cys residues- 127, 273 and 39, which could be prone to oxidation.

Table 21 | Distance measurements [Å] of the Cys residues with each other and Mn^{2+}. The PDB file 4MOV contains structural information of the PP-1A under non-oxidative conditions and was used in Pymol to measure all cysteine distances with each other and the Mn^{2+}.

	Cys 39	Cys 62	Cys 105	Cys 127	Cys 140	Cys 155	Cys 158	Cys 171	Cys 172	Cys 202	Cys 245	Cys 273	Cys 291	Mn^{2+}		colorcode
Cys 39		19,68	6,97	15,72	8,68	4,54	16,54	24,20	22,15	23,69	25,46	23,86	33,78	19,92		30,00
Cys 62	19,68		6,82	18,44	22,31	17,76	10,41	8,60	7,78	19,53	6,91	18,04	13,11	11,82		27,00
Cys 105	6,97	6,82		18,96	8,66	9,65	16,64	24,81	23,01	7,04	25,08	21,18	30,20	20,19		24,00
Cys 127	15,72	18,44	18,96		10,72	14,07	13,94	21,41	15,82	12,56	23,80	16,44	28,94	11,00		21,00
Cys 140	8,68	22,31	8,66	10,72		11,64	16,65	27,56	23,75	23,16	27,53	18,62	33,79	17,45		18,00
Cys 155	4,54	17,76	9,65	14,07	11,64		6,98	20,33	18,59	20,82	22,60	23,76	27,07	17,75		15,00
Cys 158	16,54	10,41	16,64	13,94	16,65	6,98		20,98	19,30	12,02	23,67	27,99	29,58	19,72		12,00
Cys 171	24,20	8,60	24,81	21,41	27,56	20,33	20,98		5,96	19,42	4,57	24,22	9,08	15,24		10,00
Cys 172	22,15	7,78	23,01	15,82	23,75	18,59	19,30	5,96		13,56	6,43	19,11	15,03	19,24		9,00
Cys 202	23,69	19,53	7,04	12,56	23,16	20,82	12,02	19,42	13,56		19,87	21,33	26,59	12,41		8,00
Cys 245	25,46	6,91	25,08	23,80	27,53	22,60	23,67	4,57	6,43	19,87		21,25	7,15	14,37		7,00
Cys 273	23,86	18,04	21,18	16,44	18,62	23,76	27,99	24,22	19,11	21,33	21,25		26,60	9,08		6,00
Cys 291	33,78	13,11	30,20	28,94	33,79	27,07	29,58	9,08	15,03	26,59	7,15	26,60		20,86		5,00
Mn^{2+}	19,92	11,82	20,19	11,00	17,45	17,75	19,72	15,24	19,24	12,41	14,37	9,08	20,86			4,00

The first idea was that some Cys residues should be protected from even higher oxidative reaction by formation of internal disulfide bridges presumably those that are in contact with the Mn^{2+} ions. This hypothesis has to be followed up in a second experiment, where glutathione protection mechanism is taken away from the sample.

4.2.3.2 Investigating PTMs in His-tagged PP-1

In order to verify whether glutathionylation is involved in the formation of transient disulfide links, a His-tagged PP-1 was used to identify various PTMs using the same methods as discussed in the previous section. The spectra from four conditions demonstrated that hyper-oxidation of Cys to sulfonic acid upon H_2O_2 treatment was partially prevented at $Cys^{155}Cys^{158}$ and $Cys^{171}Cys^{172}$ and Cys^{62} and Cys^{105} if Mn^{2+} was added to the buffer. Figure 36.B shows that all Cys residues, including Cys^{127}, were detected at the highest oxidation state. Only Cys^{39} was not identified by the digestion. This shows that the oxidative stress is much more harmful to the protein in the absence of GSSH and GST. The missing Cys^{39} was identified as the sole disulfide bridging cysteine in this set of experiments. The spectrum of the Cys^{39} XL $Cys^{155}Cys^{158}$ linked peptide is shown in Figure 37.

Figure 36 | *In vitro*-assays for MS readout using the His-tagged PP-1 (data based on analysis from Peaks7.0 software). All PTMs, their abbreviations and their color code are shown on the left. With respect to four different conditions, output PTM was generated with specifically (B) showing sulfone formation at various Cys residues in the yellow box with respective bar chart and spectra.

B) −Mn^{2+} +H$_2$O$_2$

Cys^{39} XL Cys^{155} Cys^{158}

#	b	b-H2O	b (2+)	Seq	y	y-H2O	y (2+)	#
1	58.03	40.02	29.51	G				23
2	171.11	153.10	86.06	L	2521.17	2503.16	1261.08	22
3	272.11	254.09	136.55	C(-2.02)	2408.08	2390.07	1204.54	21
4	385.19	367.18	193.09	L	2307.09	2289.08	1154.05	20
5	513.28	495.27	257.14	K	2194.01	2176.00	1097.50	19
6	614.33	596.32	307.67	T	2065.91	2047.90	1033.46	18
7	761.40	743.39	381.20	F	1964.86	1946.85	982.93	17
8	862.45	844.44	431.72	T	1817.80	1799.79	909.40	16
9	977.47	959.46	489.24	D	1716.75	1698.74	858.87	15
10	1112.48	1094.45	556.73	C(+31.97)	1601.72	1583.71	801.36	14
11	1259.52	1241.51	630.26	F	1466.74	1448.73	733.87	13
12	1373.57	1355.56	687.28	N	1319.67	1301.66	660.34	12
13	1510.57	1492.56	755.78	C(+33.99)	1205.63	1187.62	603.33	11
14	1623.65	1605.64	812.33	L	1068.63	1050.62	534.81	10
15	1720.70	1702.69	860.85	P	955.55	937.54	478.27	9
16	1833.79	1815.78	917.39	I	858.49	840.48	429.75	8
17	1904.82	1886.81	952.91	A	745.41	727.40	373.17	7
18	1975.86	1957.85	988.43	A	674.38	656.36	337.69	6
19	2088.95	2070.93	1044.97	I	603.33	585.32	302.17	5
20	2188.01	2170.00	1094.51	V	490.25	472.24	245.63	4
21	2303.04	2285.03	1152.02	D	391.18	373.17	196.09	3
22	2432.08	2414.07	1216.54	E	276.16	258.15	138.58	2
23				K	147.11	129.10	74.06	1

Figure 37 | Cys^{39} XL Cys^{155}Cys^{158} disulfide-peptide conjugates (spectrum and fragment table) is the disulfide link induced in the His-tagged PP-1. In the structure plot, the two involved Cys are indicated in green. They are located in a local proximity that makes the formation of a disulfide bridge.

Finally, the amount of free and hyper-oxidized Cys residues (sulfonic acid) was quantified by extracting the label free quantification (LFQ) of each peptide using PEAKS 7.0 software. The color code of Table 22 ranges from green (lowest value) to red (highest value). The amount of free Cys residues is highest with +Mn^{2+} −H$_2$O$_2$ in the buffer and slightly lower with −Mn^{2+} − H$_2$O$_2$ in the buffer, which suggests that oxidation could also occur in Cys residues exposed to the air. Cys^{105} and Cys^{245} have interesting values: the lowest values were observed as expected by treatment with H$_2$O$_2$ and no protective Mn^{2+} present. However, for one Cys the opposite was observed, however it was not displayed in the sequence. In accordance with these observations, Table 22 shows the highest values for the formation of sulfonic acid, when no protective Mn^{2+} ions counteracted the H$_2$O$_2$ treatment. From a structural perspective, Cys^{105} is the furthest distance from the Mn^{2+} binding centrum and also completely shielded by the His residues that cage the two Mn^{2+} ions. Cys^{140} is almost equally prone to form sulfonic acid as it is close to the Mn^{2+} binding centrum.

Table 22 | Possible Cys residues that can undergo sulfonation in His-tagged PP-1. The color code is from green for lowest value to red for the highest value. Dark green labeled boxes=free cysteine; Orange labeled boxes=Sulfonic acid (SO_3).

Peptide	Cys	Free Cys				Color Code
		no Mn + no H_2O_2	no Mn + with H_2O_2	with Mn + no H_2O_2	with Mn + with H_2O_2	
ICGDIHGQYYDLLR	Cys62	1,36E+06	2,73E+05	2,89E+06	8,04E+05	1,60E+07
QSLETICLLLAYK	Cys105	6,01E+06	4,85E+05	1,60E+07	2,09E+06	8,50E+06
GKQSLETICLLLAYK	Cys105	3,13E+06	1,00E+03	2,07E+06	1,00E+03	5,00E+06
GNHECASINR	Cys127	6,10E+04	2,68E+05	1,00E+03	1,00E+03	1,00E+06
IYGFYDECKRR	Cys140					5,00E+05
TFTDCFNCLPIAAIVDEK.I	Cys155Cys158	3,02E+06	1,00E+03	3,61E+06	2,64E+05	1,00E+05
IFCCHGGLSPDLQSM(+15.99)EQIR	Cys171CYs172	1,32E+06	1,00E+03	4,60E+06	9,20E+05	5,00E+04
IFCCHGGLSPDLQSMEQIRR	Cys171Cys172	9,19E+05	1,00E+03	5,96E+06	2,27E+05	1,00E+04
IM(+15.99)RPTDVPDQGLLCDLLWSDPDK	Cys202					5,00E+03
FLHKHDLDLICR	Cys245	3,98E+06	1,44E+05	3,49E+06	1,00E+03	2,00E+03
HDLDLICR	Cys245	1,93E+06	1,00E+03	1,16E+07	8,37E+05	1,00E+03

Peptide	Cys	Sulfone Cys			
		no Mn + no H_2O_2	no Mn + with H_2O_2	with Mn + no H_2O_2	with Mn + with H_2O_2
ICGDIHGQYYDLLR	Cys62	1,00E+03	1,09E+06	1,00E+03	1,00E+03
QSLETICLLLAYK	Cys105	7,10E+04	8,40E+06	1,00E+03	1,46E+05
GKQSLETICLLLAYK	Cys105	1,00E+03	3,73E+06	1,00E+03	3,67E+04
GNHECASINR	Cys127				
IYGFYDECKRR	Cys140	1,00E+03	7,30E+06	1,82E+05	2,13E+05
TFTDCFNCLPIAAIVDEK.I	Cys155Cys158	1,00E+03	2,41E+05	6,40E+04	1,00E+03
IFCCHGGLSPDLQSM(+15.99)EQIR	Cys171CYs172	1,00E+03	8,27E+05	1,00E+03	1,00E+03
IFCCHGGLSPDLQSMEQIRR	Cys171Cys172				
IM(+15.99)RPTDVPDQGLLCDLLWSDPDK	Cys202	1,00E+03	2,96E+05	1,00E+03	1,00E+03
FLHKHDLDLICR	Cys245	1,00E+03	1,01E+06	1,00E+03	1,00E+03
HDLDLICR	Cys245	1,00E+03	1,98E+06	4,17E+05	5,51E+05

4.2.3.3 Oxidation of other amino acids in the vicinity of Mn^{2+} ion in GST / His-tagged PP-1

The X-ray structure of PP-1 has revealed four Histidines (His) close to dinuclear metal ions. While finding the PTM in both, GST and His-tagged PP-1, surprisingly His[248] was solely identified to be oxidized in the GST-tagged protein experiment. This might suggest that it is more solvent exposed as the other His residues and probably not involved in the binding of the Mn^{2+} ions. An unexpected result was found in the His-tagged protein experiment, where His[248] was unaffected while His[125] was oxidized with buffer B ($-Mn^{2+}$ $-H_2O_2$). The spectra are shown in Figure 38.

Tyr[272] can be oxidized, however it is only 4.4 Å from the Mn^{2+} ion and 3.68 Å from His[66], hence do not have a protective effect. The oxidizable His[248] is only 8.55 Å from the Mn^{2+} ion (Figure 39). The sample containing GSH and GST resulted in surprising oxidations in the presence of additional Mn^{2+}. Fairly consistent mono-oxidation occurred under all conditions except in buffer condition B ($-Mn^{2+}$ $+H_2O_2$).

B) –Mn²⁺ +H₂O₂

A. His²⁴⁸

#	b	b-H2O	b (2+)	Seq	y	y-H2O	y (2+)	#
1	72.04	54.03	36.52	A				14
2	225.10	207.09	113.05	H(+15.99)	1584.73	1566.72	792.87	13
3	353.16	335.14	177.08	Q	1431.68	1413.67	716.34	12
4	452.22	434.21	226.61	V	1303.62	1285.61	652.31	11
5	551.29	533.28	276.15	V	1204.54	1186.54	602.78	10
6	680.34	662.33	340.67	E	1105.48	1087.46	553.24	9
7	795.36	777.35	398.18	D	976.44	958.43	488.72	8
8	852.39	834.37	426.69	G	861.41	843.40	431.21	7
9	1015.45	997.44	508.22	Y	804.39	786.38	402.70	6
10	1144.49	1126.48	572.75	E	641.32	623.32	321.16	5
11	1291.56	1273.55	646.28	F	512.28	494.28	256.64	4
12	1438.63	1420.62	719.81	F	365.22	347.21	183.11	3
13	1509.67	1491.65	755.33	A	218.15	200.14	109.57	2
14				K	147.11	129.10	74.06	1

B. His¹²⁵

#	b	b-H2O	b (2+)	Seq	y	y-H2O	y (2+)	#
1	58.03	40.02	29.51	G				10
2	172.07	154.06	86.54	N	1107.45	1089.44	554.22	9
3	325.13	307.12	163.06	H(+15.99)	993.41	975.39	497.20	8
4	454.17	436.16	227.58	E	840.35	822.34	420.68	7
5	605.16	587.15	303.08	C(+47.98)	711.31	693.30	356.15	6
6	676.20	658.19	338.60	A	560.32	542.30	280.66	5
7	763.23	745.22	382.12	S	489.28	471.27	245.14	4
8	876.32	858.31	438.66	I	402.25	384.24	201.62	3
9	990.36	972.35	495.68	N	289.16	271.15	145.08	2
10				R	175.12	157.11	88.06	1

Figure 38 | Spectra and ion table of oxidized Histidine (His²⁴⁸/His¹²⁵) caging around the dinuclear Mn²⁺ ions. In the structure of PP-1, the two Mn²⁺ ions are caged by the four His residues (His⁶⁶, His¹²⁵, His¹⁷³ and His²⁴⁸). Spectra under H₂O₂ treatment represent show mono-oxidized His²⁴⁸ and His¹²⁵ in the (A) GST and (B) His-tagged- PP-1, respectively.

A

#	b	b-H2O	b (2+)	Seq	y	y-H2O	y (2+)	#
1	180.07	162.05	90.53	Y(+15.99)				18
2	237.09	219.08	119.04	G	1750.93	1732.92	875.98	17
3	365.14	347.14	183.07	Q	1693.91	1675.90	847.46	16
4	512.21	494.20	256.61	F	1565.86	1547.84	783.43	15
5	599.25	581.24	300.12	S	1418.78	1400.78	709.89	14
6	656.27	638.26	328.63	G	1331.75	1313.74	666.38	13
7	769.35	751.34	385.15	L	1274.73	1256.72	637.87	12
8	883.39	865.38	442.20	N	1161.64	1143.64	581.32	11
9	980.45	962.44	490.72	P	1047.61	1029.60	524.30	10
10	1037.47	1019.46	519.23	G	950.55	932.54	475.78	9
11	1094.49	1076.48	547.75	G	893.53	875.52	447.27	8
12	1250.59	1232.58	625.80	R	836.51	818.50	418.76	7
13	1347.64	1329.63	674.32	P	680.41	662.38	340.71	6
14	1460.73	1442.72	730.86	I	583.35	565.35	292.13	5
15	1561.78	1543.77	781.39	T	470.27	452.26	235.64	4
16	1658.83	1640.82	829.91	P	369.23	351.18	185.11	3
17	1755.88	1737.87	878.44	P	272.17	254.11	136.59	2
18				R	175.12	157.11	88.06	1

B

#	b	b-H2O	b (2+)	Seq	y	y-H2O	y (2+)	#
1	196.06	178.05	98.53	Y(+31.99)				18
2	253.08	235.07	127.04	G	1750.93	1732.92	875.97	17
3	381.14	363.13	191.07	Q	1693.91	1675.90	847.46	16
4	528.21	510.20	264.60	F	1565.86	1547.84	783.43	15
5	615.24	597.23	308.12	S	1418.79	1400.78	709.89	14
6	672.26	654.25	336.63	G	1331.75	1313.74	666.38	13
7	785.35	767.34	393.17	L	1274.73	1256.72	637.87	12
8	899.39	881.38	450.19	N	1161.65	1143.64	581.33	11
9	996.44	978.43	498.72	P	1047.60	1029.60	524.30	10
10	1053.46	1035.45	527.23	G	950.55	932.54	475.78	9
11	1110.49	1092.48	555.74	G	893.53	875.52	447.27	8
12	1266.59	1248.58	633.79	R	836.51	818.50	418.76	7
13	1363.64	1345.63	682.32	P	680.41	662.37	340.70	6
14	1476.72	1458.71	738.86	I	583.36	565.35	292.18	5
15	1577.77	1559.76	789.39	T	470.28	452.26	235.64	4
16	1674.82	1656.81	837.91	P	369.22	351.18	185.11	3
17	1771.88	1753.87	886.44	P	272.17	254.16	136.59	2
18				R	175.12	157.11	88.06	1

Figure 39 | Spectra and ion table of oxidized Tyrosine (Tyr²⁷²) close to Cys²⁷³ in the (A) GST and (B) His-tagged PP-1 respectively.

4.3 Regulation of CTGF under stress conditions in cardiomyocytes

4.3.1 Expression of CTGF in physiological functioning of the heart

Human non-failing (NF), ischemic (ICM), and dilated cardiomyopathy (DCM) heart samples were used to examine the expression of CTGF in heart disease. Immunoblotting analysis demonstrated that CTGF expression was significantly higher in DCM samples as compared to NF samples (Figure 40.A). In ICM samples, CTGF expression was slightly but not significantly increased compared to NF samples (Figure 40.B).
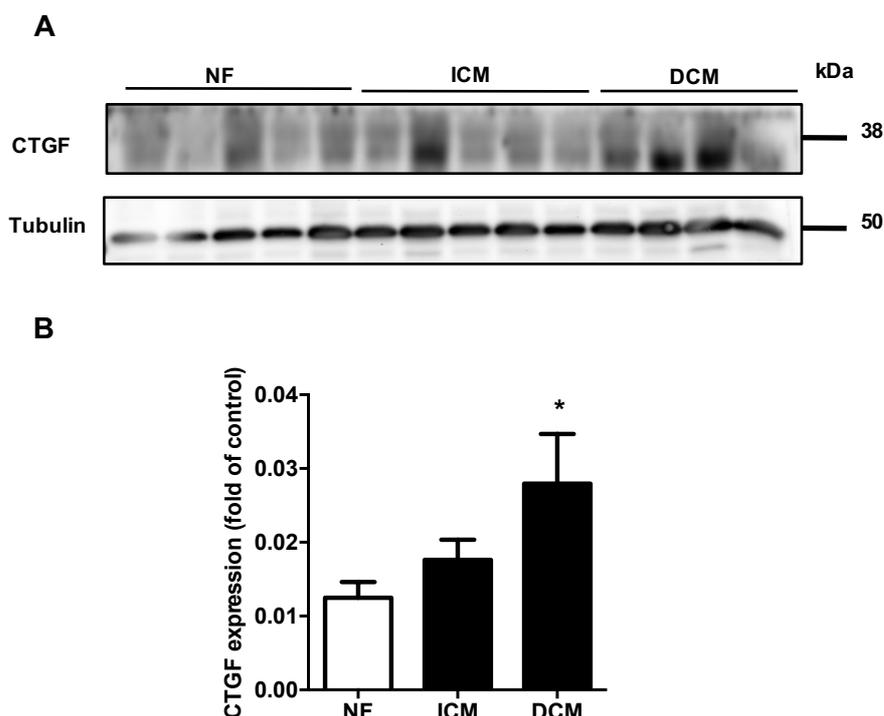
Figure 40 | Higher protein expression of CTGF in end-stage human heart failure. (A) Immunoblot and (B) relative analysis of CTGF expression in non-failing (NF) hearts, end-stage heart failure ischemic cardiomyopathy (ICM) and dilated cardiomyopathy (DCM). Analysis was normalized to Tubulin, and the change in CTGF expression was estimated relative to the control. Values are given as mean ± SEM, NF=5, ICM=5 and DCM=4. Statistical comparison was performed by one-way ANOVA, and post hoc correction with Bonferroni's multiple comparison tests, *$p<0.05$ vs. NF. The heart samples were kindly provided by Dr. Thomas H. Fischer, Clinic for Cardiology and Pneumology, Goettingen.

4.3.2 Influence of oxidative and ER stress on CTGF expression in NRCM

The influence of the oxidative environment on CTGF expression was subsequently analyzed in NRCM with 100 µM H_2O_2 for short incubation times of up to 30 min (time intervals of 3, 6, 10, 15 and 30 min) and longer incubation times of up to 6 h (time interval of 2, 4 and 6 h). In both settings, the last time point was kept as control time point. The relative analysis of

immunoblots (Figure 41) revealed that no significant change in CTGF levels occurred after H_2O_2 treatment.
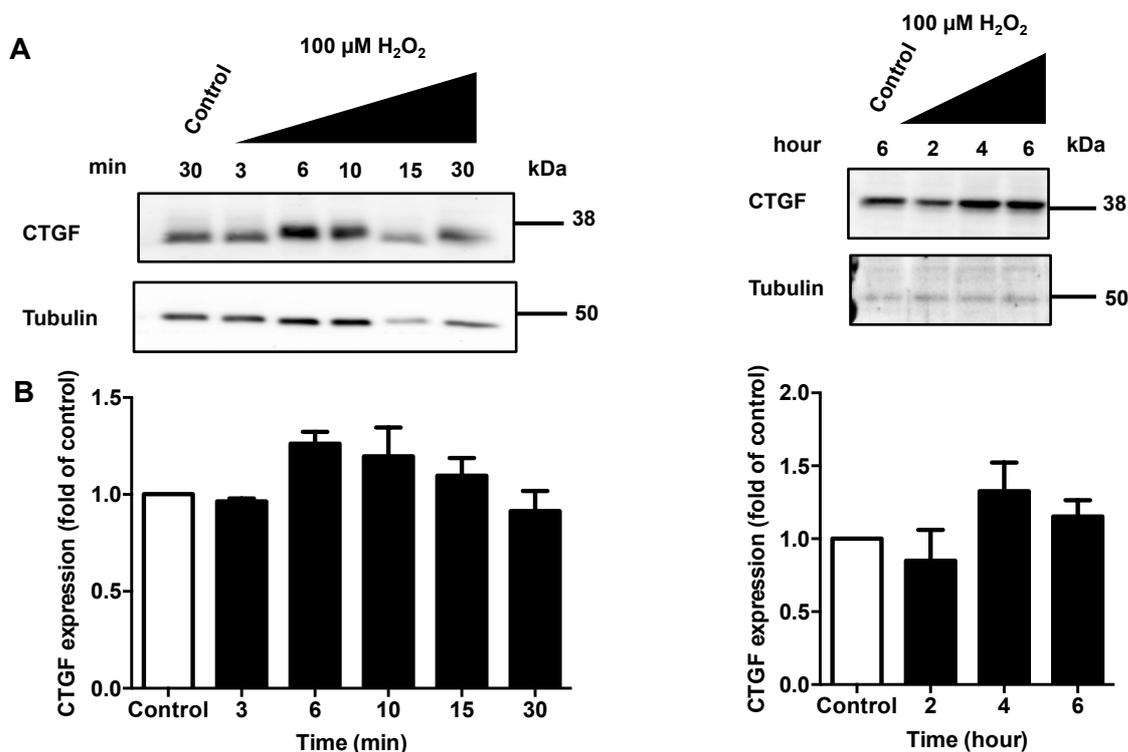
Figure 41 | Protein expression of CTGF in cardiomyocytes exposed to H_2O_2. (A) Immunoblot and (B) relative analysis of CTGF in neonatal rat cardiomyocytes (NRCM) exposed to 100 µM H_2O_2 at different time points. Analysis was normalized to Tubulin, and the change in CTGF expression was estimated relative to the control. Values are given as mean ± SEM, n=3-5. Statistical comparisons were performed by one-way ANOVA, and post hoc correction with Bonferroni's multiple comparison tests, *$p<0.05$ vs. control.

To determine the effect of reducing conditions on CTGF, the NRCMs were treated with 50 mM DTT for up to 30 min (time interval with 3, 6, 10, 15 and 30 min). In the respective immunoblot analysis, CTGF showed a time-dependent staircase pattern. This likely reflects different structural forms of CTGF in the cells, with the lower band having a molecular weight of 36 kDa and the top band with a molecular weight of 38 kDa in non-reducing SDS-PAGE. Furthermore, by the addition of the reducing agent in SDS-PAGE sample buffer (reducing condition), the staircase pattern was no longer present supporting the hypothesis of in cell reduction of CTGF by DTT (Figure 42.A). In addition, the concentration-dependent effect of DTT and diamide on CTGF in a non-reducing condition was analyzed. The results demonstrated that the staircase pattern of CTGF was dependent on the DTT concentration. The oxidizing agent diamide was without effect (Figure 42.B)

A

B
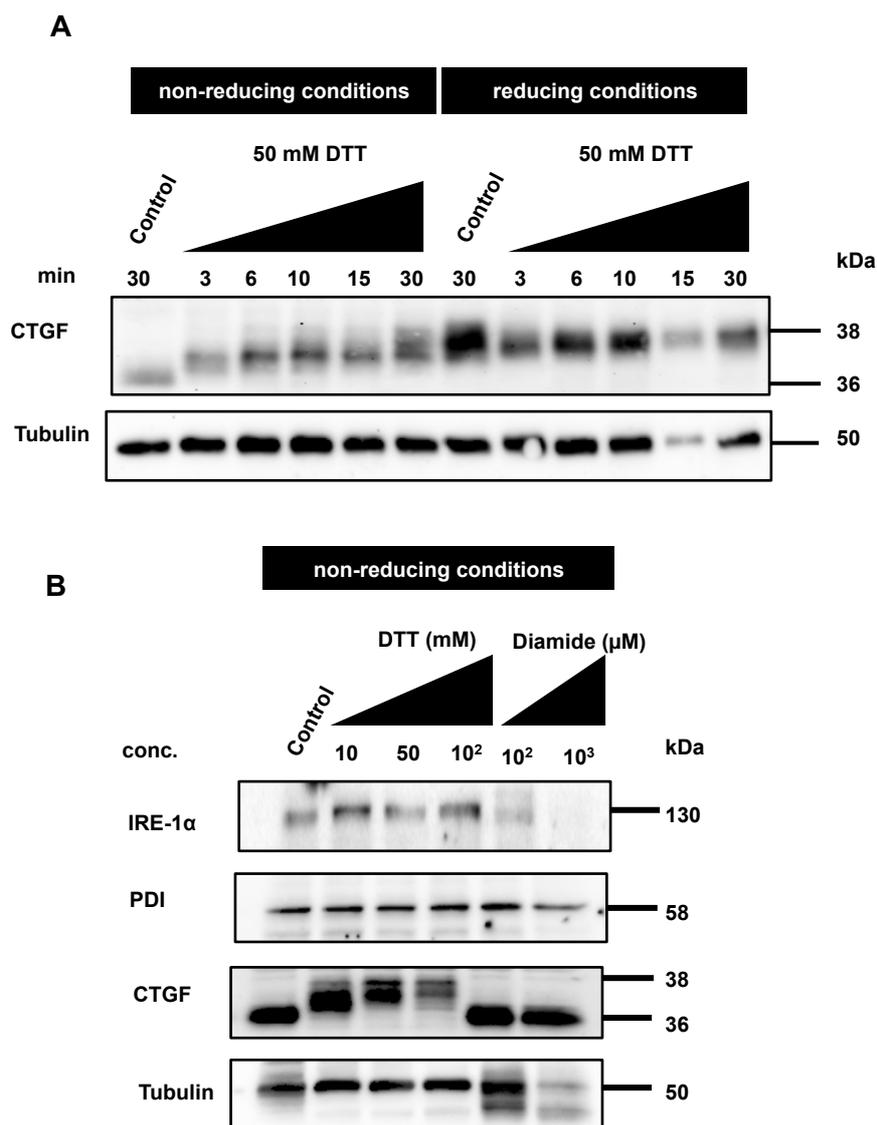
Figure 42 | Protein expression of CTGF in cardiomyocytes exposed to DTT and diamide. (A)
Immunoblot of CTGF in NRCM exposed to 50 mM DTT for different time periods analyzed under non-
reducing and reducing conditions. Tubulin is shown as loading control. (B) Immunoblot analysis of the
ER stress markers IRE-1α and PDI and CTGF in NRCM exposed to increasing concentrations of DTT
and diamide under non-reducing conditions. Tubulin is shown as loading control, n=3.

Further, the intracellular Ca^{2+}-depleting agent thapsigargin (TGN) was studied as it has been
described to induce ER stress similar to DTT (Zhang et al., 2010). As shown in Figure 43,
CTGF expression was significantly increased after 6 h when NRCM were treated with 3 µM
TGN. Under the used conditions the ER stress markers PDI and IRE1-α were only up-
regulated by trend.
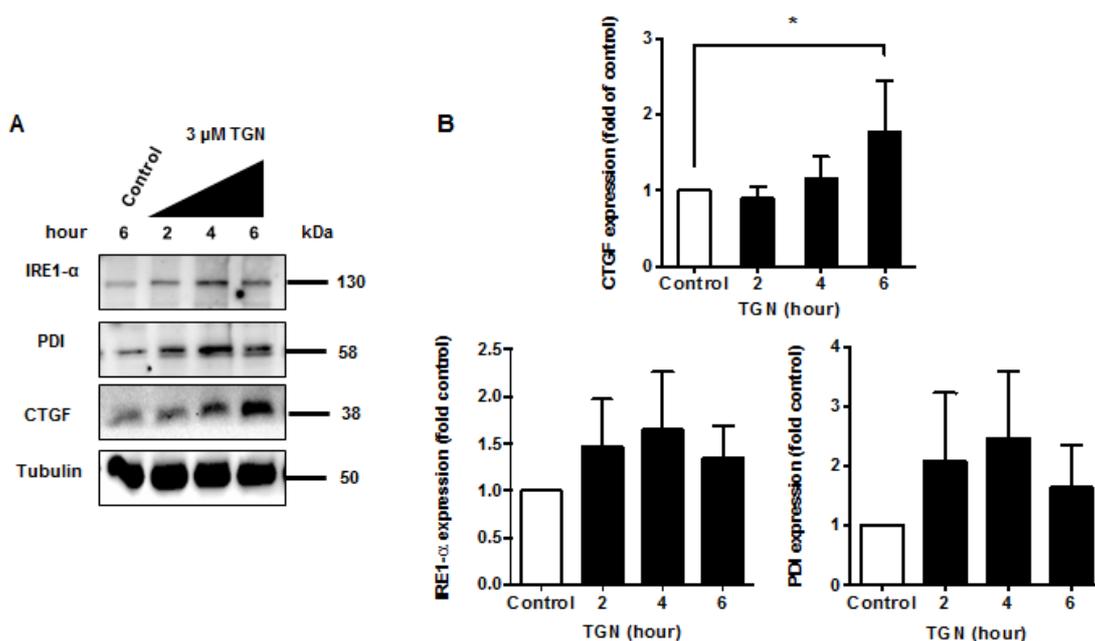
Figure 43 | Protein expression of CTGF in NRCM exposed to TGN. (A) Immunoblot and (B) relative analysis of CTGF, IRE1-α, and PDI in NRCM exposed to 3 µM thapsigargin (TGN) for the indicated time periods. Analysis was normalized to Tubulin, and the change in CTGF expression was estimated relative to the control. Values are given as mean ± SEM, n=3-5. Statistical comparisons were performed by one-way ANOVA, and post hoc correction with Bonferroni's multiple comparison tests, *$p<0.05$ vs. control.

Aside from DTT and TGN, tunicamycin (Tm) has been demonstrated to induce ER stress (Kaufman, 1999). Therefore, the NRCMs were treated with Tm for different time periods and with various concentrations and CTGF was again analyzed by immunoblot. However, under neither conditions a change in CTGF expression as well as in the ER stress marker IRE1-α could be observed (Figure 44).

B

Time = 1 hour   Tm (µg/ml)

conc.   Control   2.5 5 10   kDa

CTGF   38

Tubulin   50
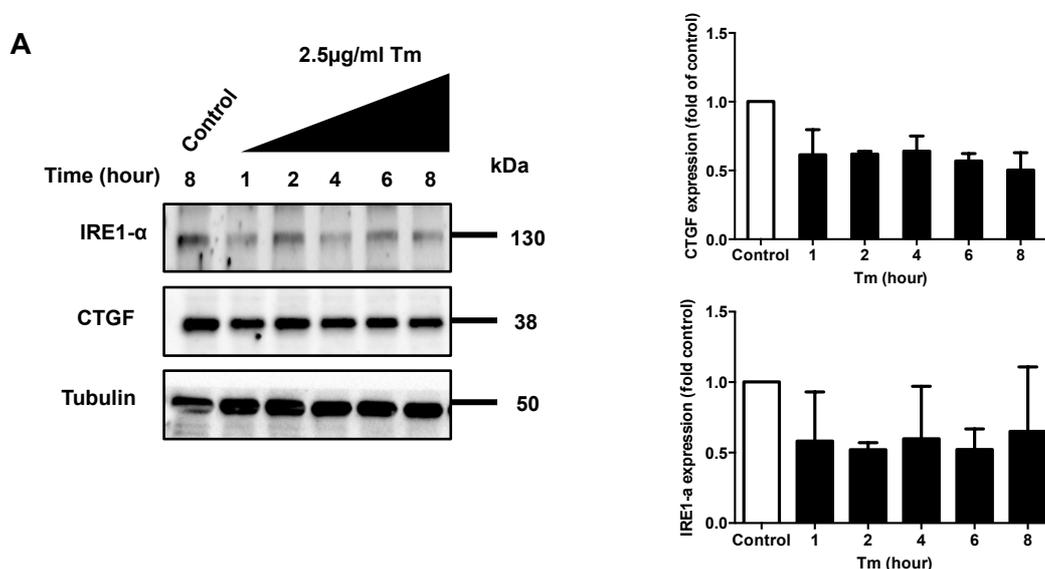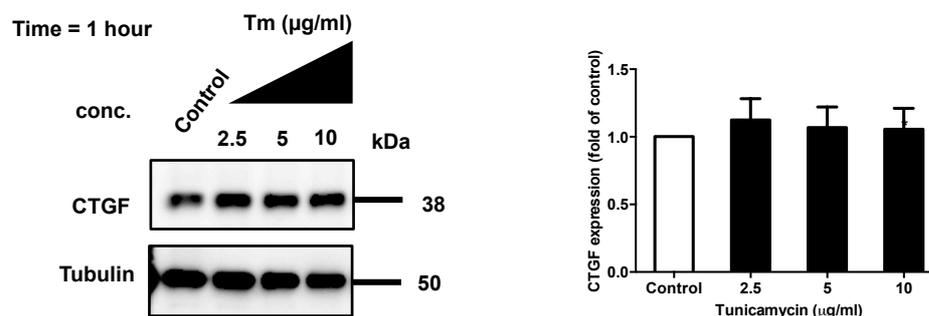
Figure 44 | CTGF protein expression in NRCM exposed to Tm. (A) Immunoblot and relative analysis of CTGF and IRE1-α in NRCM exposed to (A) 2.5 µg/ml Tm for different time periods. Values are given as mean ± SEM, n=4. (B) treated with different concentrations (2.5, 5 and 10 µg/ml) for 1 hr. Values are given as mean ± SEM, n=3. Analysis was normalized to Tubulin, and the change in CTGF expression was estimated relative to the control. Statistical comparisons were performed by one-way ANOVA, and post hoc correction with Bonferroni's multiple comparison tests, *p<0.05 vs. control.

4.3.3 Impact of heat shock response and chaperone/heat shock protein on CTGF expression in NRCM

As ER stress induced by DTT and TGN influenced CTGF in its oxidative state and expression, respectively, the effect of other stressors was investigated next. To analyze the influence of the heat shock protein hsp47, CTGF expression was analyzed in control mouse embryonic fibroblasts (MEF) and hsp47 knockout mouse embryonic fibroblasts. In this experiment, CTGF was found to be less expressed in hsp47 MEF compared to control cells. Next, the NRCM were incubated at 42°C for 1h with a subsequent recovery phase for different time periods. CTGF was found to be up-regulated 2 h after the heat shock (Figure 45).
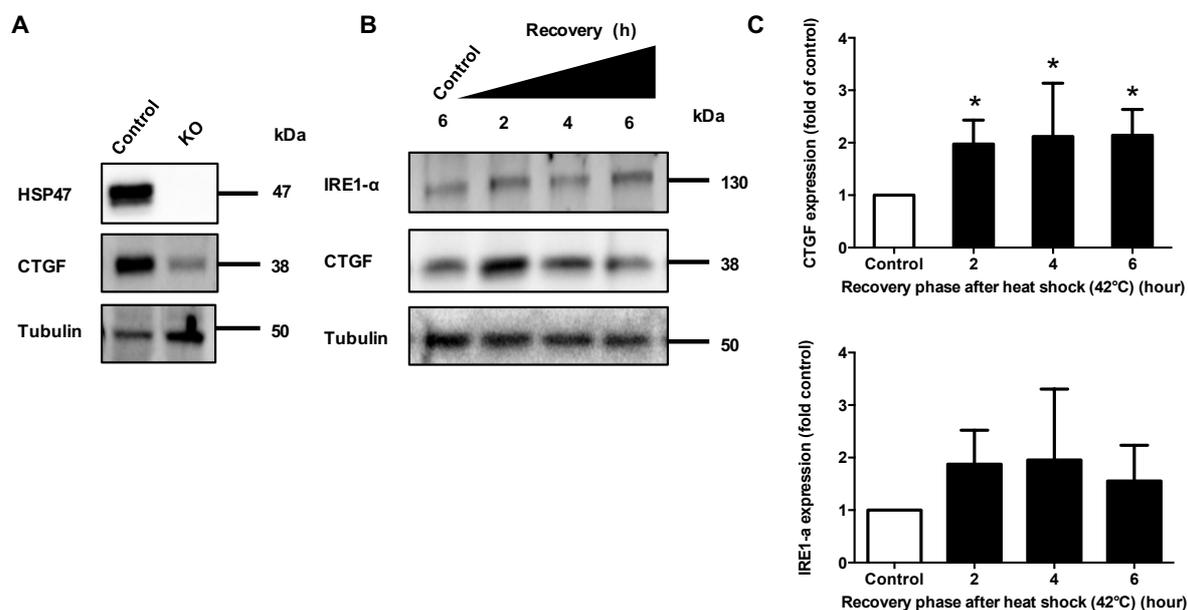
Figure 45 | Effect of heat shock on the expression of CTGF. (A) Protein expression of CTGF, HSP47 and Tubulin in control and HSP47 knockout mouse embryonic fibroblasts, n=1. (B) Immunoblot and (C) relative analysis of CTGF, IRE1-α and Tubulin as a loading control in cell extracts from NRCM after heat shock at 42°C for 1 h and recovery phases of 2-6 h. Values are given as mean ± SEM, n=5-6. Statistical comparisons were performed by one-way ANOVA, and post hoc correction with Bonferroni's multiple comparison tests, *p<0.05 vs. control.

4.3.4 Impact of pH, MG132 and BFA on CTGF expression in NRCM

To further examine the effects of the pH of the culture medium on CTGF expression, the NRCMs were cultivated under different pH conditions (pH 8, 7, 7.5 and 6) for 1 h. However, no effect was detectable (Figure 46.A). Moreover, inhibition of the proteasome with different concentrations of MG132 was also without effect (Figure 46.B). And finally blockade of the transport from the ER to the Golgi apparatus by brefeldin A (BFA) showed no effect (Figure 46.C).
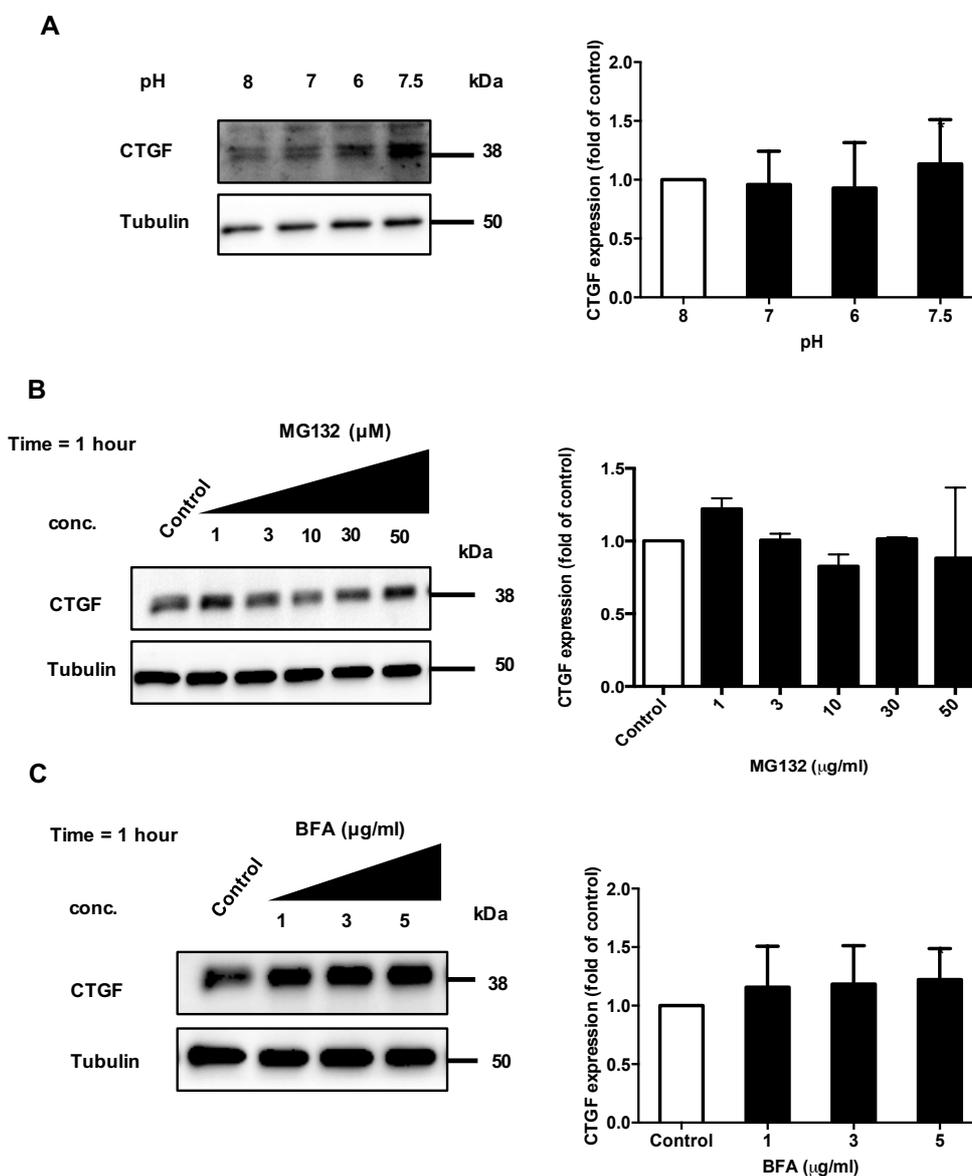
Figure 46 | The effect of pH, MG132 and BFA treatment on CTGF expression in NRCM. Immunoblot and relative analysis of CTGF in NRCM exposed to (A) Different pH over 1 h. Value are given as mean ± SEM, n=6. (B) Concentration-dependent of MG132 over 1 h. Value are given as mean ± SEM, n=3 (C) Concentration-dependent of BFA over 1 h. Value are given as mean ± SEM, n=3. Tubulin is shown as loading control. Statistical comparisons were performed by one-way ANOVA, and post hoc correction with Bonferroni's multiple comparison tests, *p<0.05 vs. control.

4.3.5 Impact of siRNA-CTGF knockdown on NRCM

As CTGF was demonstrated to be regulated by cellular stressors like DTT and TGN, in the next step the effect of CTGF expression on the ER stress response was analyzed. Therefore, CTGF expression was reduced by a siRNA approach. Immunofluorescence analysis demonstrated that the knockdown of CTGF appeared to lead to a translocation of ATF6 to the nucleus (Figure 47).

Moreover, the knockdown of CTGF led to a significant downregulation of the ER stress markers IRE-1α, PDI, and BiP (Figure 48). Only CHOP was not changed in its expression.

Next, the splicing of the XBP1 mRNA was analyzed by PCR which had been demonstrated to occur during the unfolded protein response (Wang et al., 2014). In these experiments, the knockdown of CTGF resulted in the amplification of two PCR products representing the unspliced (US) and spliced (S) XBP1 mRNA (Figure 49).

To validate, the immunoblot data, the expression of ER markers at mRNA level was examined. As shown in Figure 50, with successful knockdown of CTGF at mRNA level, other ER stress markers were not changed.
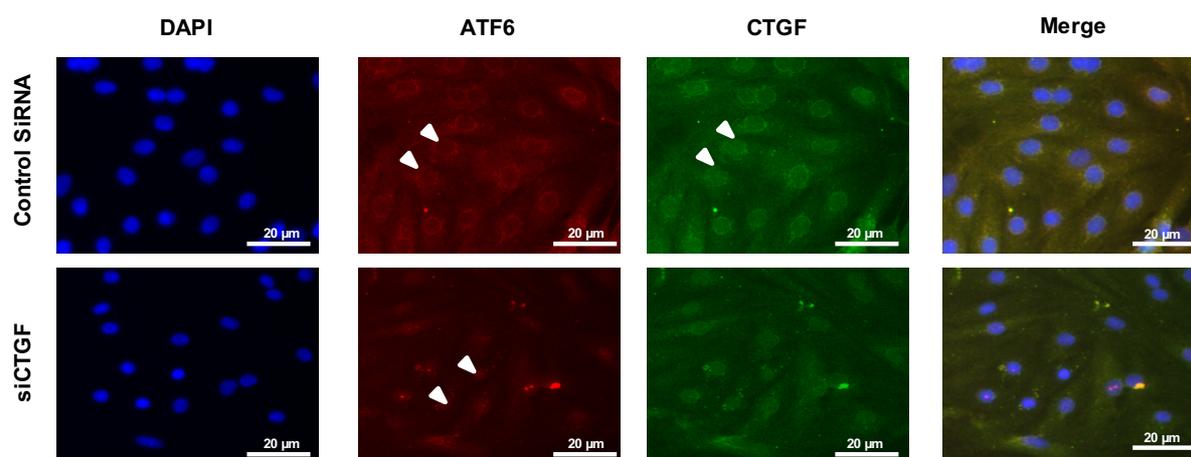
Figure 47 | Knockdown of CTGF in NRCM and ATF6 localization. siRNA was used to partially knockdown CTGF in NRCM. In the control siRNA, ATF6 was localized at endomembranes (arrow heads), whereas in the cells treated with the siRNA, ATF6 was moved to the nucleus (arrow heads). Immunofluorescence staining was performed to detect CTGF (green), ATF6 (red) and the nuclei were stained with DAPI (blue), n=2, Scale bar: 20 µm.

A

B
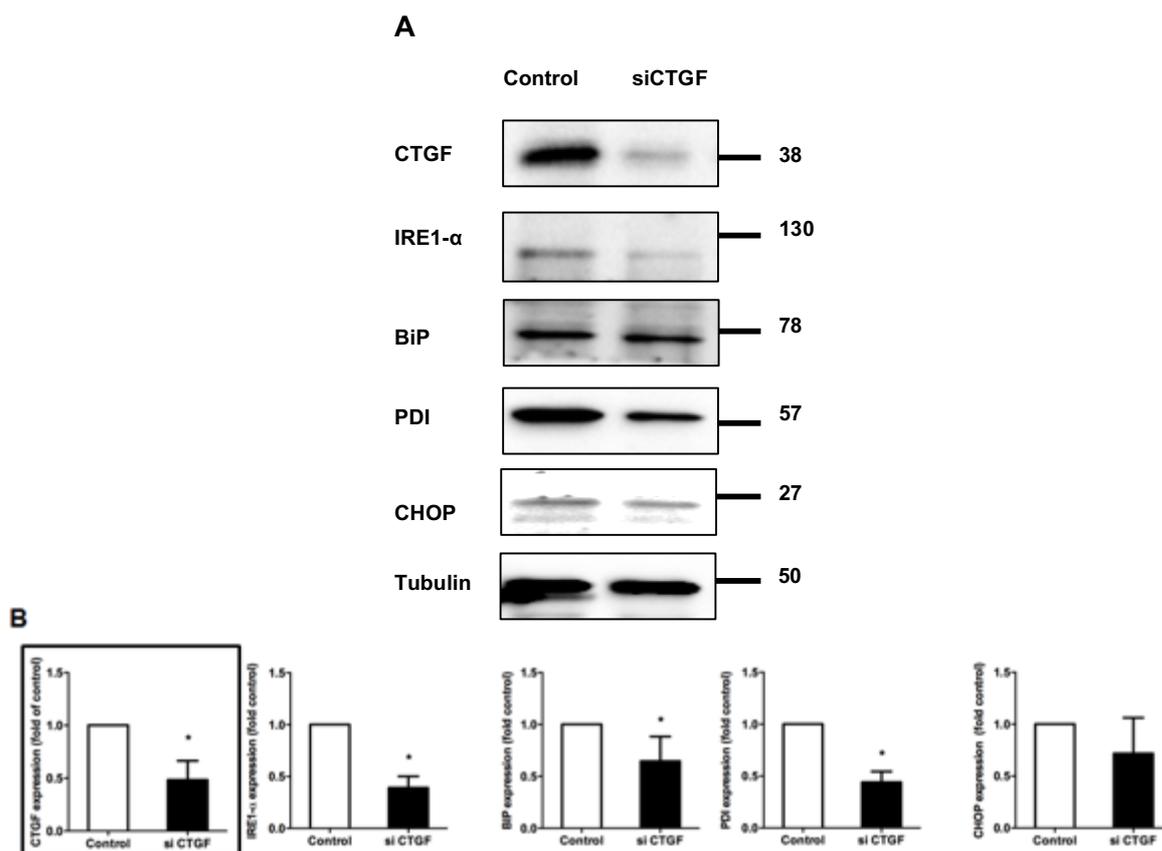
Figure 48 | Partial knockdown of CTGF in NRCM and various ER stress markers at the protein level. (A) Immunoblot and (B) relative analysis of CTGF, IRE1-α, BIP, PDI and CHOP (markers of ER stress) on siCTGF-partial knockdown NRCM. Values are given as mean ± SEM, n=4-7. Statistical comparisons were performed by an unpaired t-test, *p<0.05 vs. control.
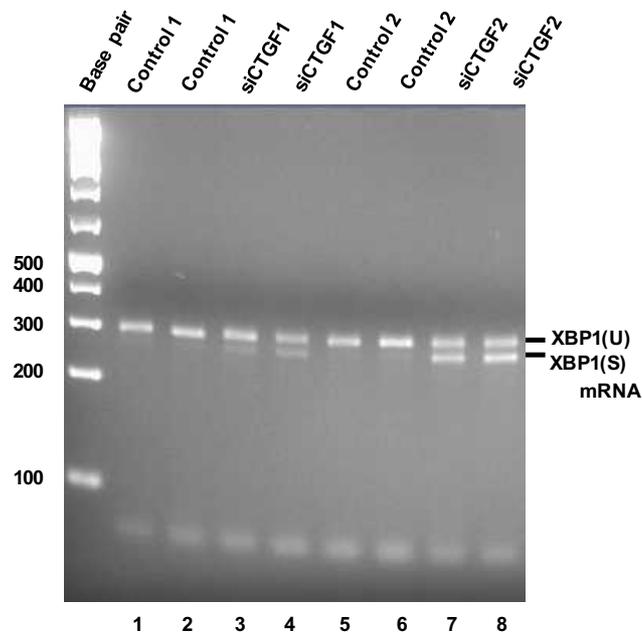
Figure 49 | Knockdown of CTGF in NRCM with XBP1 splicing. XBP1 mRNA was detected by RT-PCR. XBP1US was observed as a 289 bp band, and XBP1S was observed as a 263 bp band on siCTGF-partial knockdown NRCM, n=2.
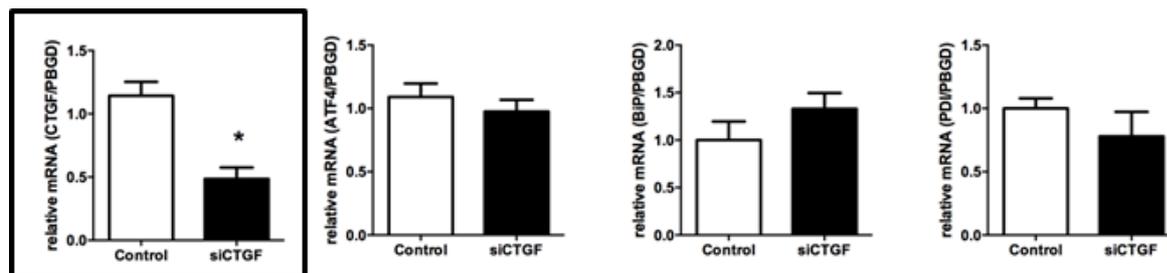
Figure 50 | Partial knockdown of CTGF in NRCM and various ER stress markers at the transcription level. mRNA level of CTGF, ATF4, PDI, and BiP was assessed by qPCR in control and CTGF knockdown NRCM. The values are given as mean ± SEM, n=4. Statistical comparisons were performed by a paired t-test, *p<0.05 vs. control.

5 Discussion

In spite of significant improvements in the treatment of cardiovascular diseases, heart failure remains a prevalent problem. Heart failure describes a critical state in which the heart is not able to maintain normal cardiac output, and thus blood supply to the organs is diminished. The leading causes of heart failure include arrhythmia, cardiomyopathy, hypertension, myocardial infarction and ischemic heart disease (Drazner, 2011; Minicucci et al., 2011; Yancy et al., 2013; Diez, 2014; Lip et al., 2016; Marti-Carvajal and Kwong, 2016). For decades variations in the phosphorylation and redox status of various proteins have been characterized in different areas of heart diseases, however this has unfortunately not resulted in many new therapeutic treatments. Both abnormal phosphorylation states of essential cardiac proteins and elevated ROS production contribute to contractile dysfunction and fibrosis in failing hearts. Cys residues are subjected to oxidation, however the interplay between oxidation and downstream phosphorylation events remains unclear. Despite its important role in the cardiovascular system whether redox changes to PP-1 affect the regulation of its targets is not fully understood. In this study, mass spectrometry was used to explore the oxidation state and various PTMs of PP-1. In addition to irreversible modifications, such as sulfonic acid formation of Cys residues, mass spectrometry may work as a robust strategy to identify transient modifications such as disulfide bridge formation and glutathionylation of PP-1.

The effect of oxidative stress is, however, not restricted to the cytoplasm of a cell. Also other compartments are affected by increased oxidative stress. With that respect, the endoplasmic reticulum (ER) is of special interest as oxidative stress can induce ER stress leading to an accumulation of unfolded transmembrane and secreted proteins. This results in the unfolded protein response which itself is a reactive oxygen species producing mechanism (Santos CX et al., 2009). Therefore, the secreted protein CTGF, which is strongly induced in heart failure, was studied as a target of oxidative changes in the ER and as a potential regulator of ER stress.

5.1 H_2O_2 influences the movement and morphology of cardiac cells

Exogenous H_2O_2 application is a valuable tool for altering oxidant-dependent signaling in cardiac tissue and cells. For maintenance and efficient physiological functioning of the cardiovascular system, it has been well established that levels of endogenous H_2O_2 need to be lower and that higher levels are associated with various diseases (Halliwell et al., 2000; Gough and Cotter, 2011). Recently it has become clear that H_2O_2 is not exclusively

associated with deleterious functions but also mediates normal physiological processes (Schroder and Eaton, 2008). The mechanisms by which oxidation alters the heart's contractile properties resulting in myocardial remodeling is not entirely understood.

To investigate the possibility that H_2O_2 exogenously regulates cardiac function, NRCMs were treated with different concentrations of H_2O_2 for 24 h and a time-lapse movie was recorded. Heat maps were generated from the movies showing the spots of myocyte contraction as mean of the time-averaged magnitude of every motion. For that a matlab based script was used, according to Huebsch et al, which also quantified the contraction velocity and the relaxation velocity of the myocytes at each H_2O_2 concentration (Huebsch et al., 2015). From this values the relaxation-contraction ratios were calculated to give a measure of myocyte vitality. A resolution of 0.6442 µm/pixel (20× objective) was used and dead floating cells were tried to filter with the script build-in cleaning mechanism. The script first determines spatiotemporal information about motion vector direction and intensity of every beating cardiac myocyte which will then be translated into the results seen in Figure 18. Results demonstrated that a concentration of 10^4 µM H_2O_2 significantly damaged NRCM vitality. However, NRCMs were resistant to lower concentrations 100 or 10^3 µM of H_2O_2 (Figure 18). During disease states when ROS is elevated cardiac function is compromised altering calcium homeostasis and negatively affecting myofilament proteins responsible for mediating contractile functioning. The reciprocal synergy between ROS and calcium signaling has been shown to modify various calcium channels, pumps, and exchangers (Görlach et al., 2015). Lower levels of endogenous H_2O_2 are essential for normal physiological functioning and signaling, whereas higher levels are associated with altered cell morphology in cardiac myocytes (Schroder and Eaton, 2008). Another study showed that acute treatment of 100 µM H_2O_2 for 45 min in NRCM, triggers MAP kinase activation which could further be stopped by catalase (Sabri et al., 1998). In addition, treating the cardiomyocytes or fibroblast with 0-200 µM H_2O_2 for 30 min induces MAPK, and while the cells are remaining healthy, some downstream phosphorylation targets were downregulated by EGF receptor inhibitors (Purdom and Chen, 2005). Interestingly, another group revealed that different concentrations of H_2O_2 showed a different effect in rat cardiomyocytes over the period of 24 h, such that 10-30 µM triggered protein synthesis, 200 µM induced apoptosis and 300-1000 µM triggered both apoptosis and necrosis (Kwon et al., 2003). In summary, exogenous application of H_2O_2 to tissues or cells in the range of 0.1-1.0 mM over several hours will not lead to the death of cells but rather triggers downstream signaling pathways.

5.2 Oxidative stress influences cardiac β-adrenergic signaling pathway and PP-1 activity

According to the enzymatic activities, there are two enzyme subclasses within the family, PP-1, and PP-2, which further consists of PP-2A and PP-2B (calcineurin) and PP-2C (Ingebritsen and Cohen, 1983). In the cardiovascular system PP-1, PP-2A and PP-2B are responsible for 90% of protein phosphatase activity (El-Armouche and Eschenhagen, 2009). Neumann et al first identified increased PP-1 activity as a hallmark of cardiovascular disease (Neumann et al., 1997). Almost one decade ago it was established that both *in vitro* and *in vivo* PP-1 activity is inhibited by H_2O_2 (3 mM) treatment and further that it could be reversed *in vitro* by thiol-oxidant *N*-acetyl-cysteine (NAC) and reduced glutathione (GSH). Moreover, it was found that NAC pretreatment protected from H_2O_2 triggered PP-1 inactivation, eradicating H_2O_2 triggered eIF2α phosphorylation and protein synthesis inhibition (O'Loghlen et al., 2003). In the following year, it was also shown in rat hippocampal and SHSY5Y human neuroblastoma cells, that there was a decrease in the phosphorylation of I-2 and PP-1 activity due to H_2O_2 incubation; the authors proposed that oxidative stress-induced activation of Cdk5 led to I-2 phosphorylation, preventing its inhibitory effect on PP-1 (Zambrano et al., 2004). How oxidative stress and β-adrenergic signaling pathways converge via PP-1 in cardiac cells was analyzed. To assess PP activity two assays were utilized: EnzChek kit assay (DiFMUP as substrate; Figure 15) and ProFluor Ser/Thr PPase Assay (R110 as substrate; Table 14).

Using the ProFluor Ser/Thr PPase assay, PP-1 and PP-2A activity was calculated by using a selective inhibitor, i.e. OA in NRCM and whole heart tissue of mice (Figure 20). Using this assay, findings are in line with previous work, demonstrating a 10% contribution of PP-1 in both species. Structure-function analysis suggests that PP-1 activity may be regulated by two redox-sensitive Cys residing in proximity to its active site (Fetrow. et al., 1999). To further validate whether PP-1 activity is oxidant-dependent, a recombinant PP-1-GST tagged peptide was used with the abovementioned kit. PP-1 activity was inhibited by the exogenous addition of H_2O_2 in both a concentration and time-dependent manner (Figure 21.A-B). Furthermore, the reversibility of the inhibition of PP-1 activity was elucidated via the reducing agent (TCEP) (Figure 21.C). The EnzChek kit assay was used to calculate total phosphatase activity in human non-failing (NF), ischemic cardiomyopathy (ICM) and dilated cardiomyopathy (DCM) donor hearts. Interestingly, the total phosphate activity was lower (~7%) in DCM as compared to ICM and NF (Figure 19). Consequently, using the same kit, in NRCM the inhibition of total PP activity was identified by 25%, in the presence of 100 µM H_2O_2 as exogenous oxidant (Figure 22). In parallel recently, Santos et al, demonstrated that,

recombinant PP-1 was also concentration-dependently inactivated by H_2O_2. However, thiol reductant such as DTT, glutathione and Cys did not restore PP-1 activity as removal of excess of H_2O_2 by catalase treatment. But with a one-electron reductant, ascorbate, efficiently reversed PP-1 inactivation (Santos et al., 2016).

The PP-1-inhibtory subunit I-1 is strongly expressed in the cytosol of cardiomyocytes, and thus plays a primary role in phosphorylation feedback loops and expedites crosstalk between phosphatases and kinases. During β-adrenergic stimulation, I-1 was activated via PKA, consequently inhibition of PP-1, which leads to the formation of a positive feedback loop augmenting the phosphorylation of several cardiac substrates such as PLB, RyR2, cMyBPC and TnI (El-Armouche et al., 2003; Heijman et al., 2013). Also, many cardiac proteins are dephosphorylated by PP-1 and PP-2A, for example PP-1 triggers the dephosphorylation of TnI at Ser[23/24] (Solaro and Kobayashi, 2011). The results in NRCM demonstrated a novel impact of oxidative stress on various cardiac proteins playing a vital role in the functioning of the β-adrenergic signaling pathway (Figure 24). The phosphorylation status of protein phosphatase inhibitor-1 (I-1), a crosstalk protein between PKA and PP-1 signaling, showed a bell-shaped phosphorylation response with a maximal peak at 100 µM H_2O_2 (Figure 23). This data is in line with results from the Eaton group, i.e. the oxidant-induced bell-shaped PKA phosphorylation was highest at 100 µM H_2O_2 (5 min) in adult rat ventricular myocytes; after which there was a loss of phosphorylation (Brennan et al., 2006). However, no effect on phosphorylation of both PLB-Ser[16] and cMyBP-C-Ser[282] was observed in concentration dependent manner (Figure 24.C). For PLB-Ser[16], phosphorylation was decreased after 10 min incubation with 100 µM H_2O_2, whereas phosphorylation of cMyBP-C-Ser[282] showed a bell-shaped curve with the highest peak at 10 min (Figure 24.B). One possible explanation could be that PP-1 activity was decreased after 10 min (Figure 21.A) and that might suggest for the cMyBP-Ser[282] phosphorylation to be high after 10 min. Nevertheless, it is surprising that PLB-Ser[16] phosphorylation is decreased after 10 min. If H_2O_2 is incubated longer, it is possible that it has more time to phosphorylate and activate I-1 via PKA oxidant dependent activation (Brennan et al., 2006), which will inhibit PP-1 more strongly and therefore increase phosphorylation at PLB-Ser[16]. However, on the other hand, a study done in cardiomyocytes directed NOX2 transgenic mouse model, Ang II-stimulated NOX2-ROS production increased cardiac contractile performance by augmenting SERCA activity driven by enhanced PLB phosphorylation and led to faster contraction and relaxation. Further, it was established that PP-1 activity was strongly inhibited in Ang II-treated transgenic mice, which is in line with the mechanism where NOX2 inactivates PP-1, permitting for an increase in PLB phosphorylation. Still, the spatial interrelationship between NOX2, PP-1 and PLB or how PP-1 is inhibited by NOX2, remains elusive at this stage. One possible suggestion could be that,

NOX2 inhibits PP-1 locally at the junctional SR and this then transduces the signal to PLB at the network SR (Zhang et al., 2015).

In summary, these results suggest that the activity of PP-1 is inhibited in the presence of oxidation and that it can be reversed in the presence of the reducing agents. Our findings also demonstrated that the expression of PLB-Ser[16] and cMyBP-C-Ser[282] were differentially affected by H_2O_2, indicating a complex layer of regulation of both redox-sensitive kinases and phosphatases. Results from failing human samples show that PP activity is diminished in failing myocardium. The pathway described in this study could therefore be novel possibility with which to treat cardiovascular disease.

5.3 Identification of redox-sensitive Cysteine residues in PP-1 via immunoblotting

In 2006, it was demonstrated that the regulatory subunit of type I PKA contain redox-sensitive Cys residues With respect to the availability of cellular H_2O_2, this leads to the two RI subunits of the tetrameric holoenzyme to form inter-protein disulfide bridges (Brennan et al., 2006). The same experiment with H_2O_2 and diamide was successfully replicated in NRCM. In addition, samples were also treated with maleimide, which reacts with free thiol groups and further stops oxidative reactions or disulfide bridge formation. Interestingly, using similar protocols in NRCM samples, PP-1 immunoblots show the formation of a dimer at 70 kDa (Figure 26) suggesting that inter-molecular disulfide bridges may be formed in PP-1 in its native state. These results are similar to those obtained from the Brautigan group which showed that purified PP-1 from rabbit skeletal muscle formed a 70 kDa polypeptide as a potential dimer of the 38 kDa monomer (Brautigan and Shriner, 1989). Surprisingly, Cys[127] was also identified to form inter-disulfide bridges as one of the PTMs using mass spectrometry (Figure 35). One of the limitation of these findings is that although the dimer formation was identified from control samples up to 10^4 μM diamide, including 37 kDa. However, mass spectrometry results indicated that only Cys[127] was identified to form inter-disulfide bridges and only with condition $-Mn^{2+}$ and $+H_2O_2$, in contrast to the immunoblot data. In addition to this reduced expression of PP-1 was identified with increasing concentration of diamide at 37 kDa. An explanation for the above-mentioned result may be that the epitope of the antibody is less available after a dose of 10^3 and 10^4 μM diamide.

In 2007, it was discovered in rat cerebral cortex, that inhibition of PP-2A activity was associated with the formation of intramolecular disulfide bridges (Foley et al., 2007). With the same above-mentioned samples, band shifts were also checked and immunoblots showed

that the same results as PP-1 but no intramolecular disulfide bridge formation in PP-2A (Figure 27).

As SERCA in previous experiments with HPLC-ESI (electrospray ionization)-MS/MS (tandem MS) showed two Cys residues (Cys[344] and Cys[349]) forming internal disulfide bridges (Sharov et al., 2006). To some extent the formation of an internal disulfide bridge in SERCA2a could be demonstrated by immunoblotting of shifted bands (Figure 28). One conclusion from the observation of a shift to lower size when running the NRCM samples under non-reducing conditions is that SERCA2a is forming an internal disulfide bridge. However, under the above-mentioned conditions for PP-1, no such shift was observed therefore contradicting the idea of internal disulfide bridges formation in PP-1. Similar results were observed for PP-2A.

A biotin-conjugated iodoacetamide (BIAM) labeling assay was used to investigate whether PP-1 Cys-oxidation changes in response to ROS. From the results, one conclusion is that, free Cys residues are mostly protected from oxidations at very acidic pH and performing the assay at slightly acidic or slightly basic conditions does not protect the Cys residues from being oxidized. These results suggest that, under the chosen conditions, the Cys residues of PP-1 are not susceptible to oxidation by H_2O_2 (Figure 29). However, the 'Biotin-switch assay' should be used as an indirect method to check for oxidized Cys in proteins as an alternative to the BIAM labeling assay (Forrester et al., 2009).

In summary, immunoblots demonstrated the PKA's dimerization and SERCA's band shift, thus representing Cys oxidations in the respective protein. With respect to PP-1, results suggest the dimerization formation at 70 kDa, however a band shift was not observed. Section 5.5.6, illustrates the prediction software results, which concur with above mentioned findings.

5.4 Known oxidative state of Cysteine in PP-1

The recent X-ray structure of oxidized PP-1γ (PDB id: 4UT3; Figure 51) reveals that only two Cys residues Cys[127] and Cys[273] are oxidized. Cys[273] resides on the outskirt of the structure, might be one of the reasons, for not identifying any connection towards disulfide bridges. In contrast, since Cys[127] lies in the vicinity of various other Cys, it is expected that it interchanges disulfide bridges with the network around it in the, those disulfide links were identified with mass spectrometry (see Figure 31). The same results were identified in the recent crystallographic studies, stating that Cys[127] and Cys[273], were often oxidized to a sulfenic derivative in electron density maps (Santos et al., 2016).
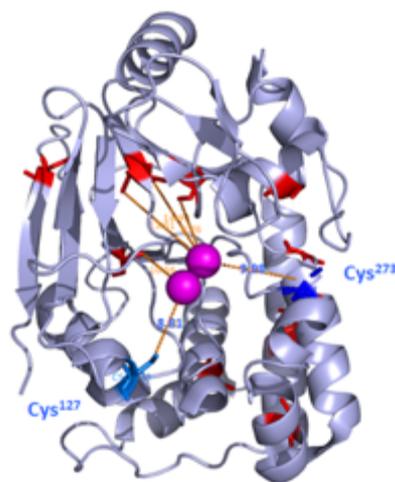
Figure 51 | The X-ray structure of PP-1γ (PDB id: 4UT3) in its oxidized state. The two blue Cys residues (Cys[127] and Cys[273]) are oxidized in the presence of H_2O_2. The distances between oxidized Cys and Mn^{2+} are measured as follows: Cys[127]-Mn^{2+} = 8.81 Å; Cys[273]-Mn^{2+} = 9.98 Å.

5.5 Impact of redox stress on PP-1 detected by mass spectroscopy

The varied chemistry of Cys residues is due to the electronic structure of its thiol group, resulting in multiple oxidation states (-2 to +6) and redox modifications (sulfenylation, -SOH; sulfinylation, $-SO_2H$; sulfonylation, $-SO_3H$; glutathionylation, -SSG; SS formation, etc.). All of which contribute to various signaling pathways. For example, it has been well established that tyrosine phosphatases are sensitive to oxidation by ROS, especially H_2O_2, acts as an intracellular second messenger in the cells (den Hertog et al., 2005; Tonks, 2005). In the following sub-sections, the key focus will be to discuss the structure of PP-1 with respect to its various PTMs, Mn^{2+} role, networking of disulfide bridges and predictive computational methods.

5.5.1 Redox-response of GST-tagged PP-1 involves glutathionylation of Cys: 140, 202 and 245

PTP-1B is the most studied redox-sensitive signaling protein which consists of reversible oxidized Cys involving Cys[215] to be glutathionylated (Barrett et al., 1999). Though H_2O_2 is known to be a secondary messenger, excessive concentrations may also damage various amino acids (Berlett and Stadtman, 1997). Cys residues are first oxidized to sulfenic acid, which is reducible, however they may be further oxidized to irreversible sulfinic and sulfonic acid. Another possible pathway could involve the protective pathway by reacting with glutathione to form a reversible disulfide link, which prevents excessive oxidation. S-glutathionylation is reversible and so protects and modifies structures/functions of proteins.

Thus it can consequently regulate signaling pathways to maintain cellular homeostasis (Grek et al., 2013).

In the course of the experiments, it should be considered that, PP-1 is tagged with glutathione S-transferase (GST), and supplied with 10mM of excess glutathione (GSH) in the buffer for the stability of the protein. In this thesis, results (Figure 32) have demonstrated that in GST-tagged PP-1, glutathionylation can be induced at outer-bound Cys residues (i.e. Cys^{140}, Cys^{202} and Cys^{245}) only as a consequence of oxidative stress. Also, with Cys^{202}, both complete glutathionylation and persulfide modifications were identified (Figure 33). Altogether, the findings of the mass spectrometry experiments provide strong evidence that Cys residues in PP-1 are prone to oxidation. Therefore, glutathionylation in PP-1 could be a fast response to oxidative stress that protects the protein from further damage. A major limitation of this finding is that the same experiments were not performed with different concentrations of GSH in the presence of the GST-tagged PP-1 and with different concentrations of GSH in the presence of a non-tagged protein. Therefore, it cannot be unequivocally concluded from the data that glutathionylation occurs. Also, the non-detectable Cys peptides generated with LC-MS/MS were believed to be involved with disulfide bridge formation, but they were not detectable. Therefore, these Cys residues may be altered by a hitherto unknown modification (Append 1).

Additional experiments are necessary to repeat the above findings intracellularly. This is critical for illustrating the glutathionylation state of PP-1, as GSH is present in large amounts (~10mM) in cells and reduces various oxidizing agents, including H_2O_2. Furthermore, it would be necessary to identify which modifications occur *in situ*, such as: sulfenic acid (Cys-SOH), intra-disulfide (Cys-SS) and/or mixed disulfide (Cys-SSG). Nonetheless, the above results indicate that that the action of GSH to modify Cys-SOH into Cys-SSG in the intracellular context to form glutathione mixed disulfide links (PP-1-SSG), is the first reaction to oxidative stress. The results also demonstrated that the activity could be recovered with TCEP or by thioltransferase (see also Figure 21).

5.5.2 Mn^{2+} in external buffer plays a protective role for Cysteine oxidation

Nearly two decades ago, it was shown that PP-1 activity mainly relies on the di-nuclear metal center for catalysis, rather than Cys-redox modifications (Egloff et al., 1995; Goldberg et al., 1995). Later it was demonstrated by Zhang et al in a cardiomyocyte-targeted NOX2-transgenic mouse model that elevated NOX2 activity modulates cardiomyocytes SR Ca^{2+} uptake and contractile function. Which further, increased PLB phosphorylation in both heart

tissues and cardiomyocyte cells, which correlated with the inhibition of PP-1 activity. This mechanism was additionally confirmed with PKA inhibitors blocking Ang II-mediated enhancement of contractile function in transgenic myocytes. (Zhang et al., 2015). The same group later also showed that PP-1 inhibition is mainly redox-regulated by the metal center and not by the Cys oxidation. In particular, NOX4 mediate the inhibition of PP-1 resulting in enhanced eIF2α phosphorylation and increase in ATF4 levels increase cell survival during protein misfolding stress and is strongly protective against acute cardiac or kidney injury (Santos et al., 2016).

A theoretical study on the reaction mechanisms of PP-1 has demonstrated a novel mechanism behind the different oxidation states of the Mn^{2+} at the catalytic center. Based on the high-resolution crystal structure, it was shown that the different oxidation states of Mn ion (i.e. Mn^{3+}-Mn^{2+} and Mn^{3+}-Mn^{3+}) can shorten the bond lengths between the metal ions by 0.15 Å, which triggers the energy barriers (Zhang et al., 2013). In parallel, recently Santos et al., demonstrated that soaking of PP-1 crystals with H_2O_2 did not alter the general structural features of the active site, however a contraction of the average metal coordination sphere by 0.12 Å compared to ascorbate-treated crystals (Santos et al., 2016).

The active center is placed in proximity to Cys^{127}, Cys^{273}, Cys^{155} and Cys^{158}. Half of the Cys network, including Cys^{39}, Cys^{105}, Cys^{127}, Cys^{140}, Cys^{155} and Cys^{158}, are in proximity to the metal ions and hence could be protected from oxidation. The other half is situated further from the metal ions and hence will not be protected. When the formation of disulfide bridges in our assay was observed, this separation into two halves quickly became visible (see also the two black boxes in Table 21). In addition, it was identified that extra Mn^{2+} ions in the buffer took reduced redox stress so that fewer disulfide bridges were formed as compared to the $-Mn^{2+}$ +H_2O_2 conditions. The same was observed for other Cys-based PTM in PP-1. This result was obtained under harsh, non-physiological conditions (Table 20.C), but it could also be possible under higher H_2O_2 concentrations in the cellular context. Furthermore, the Cys sulfone levels were used as a readout of the damaging effect of H_2O_2 (Table 22). Additional Mn^{2+} in the buffer protected all Cys residues from over-oxidation, and therefore concluded that under physiological conditions, the two Mn^{2+} bound in the complex could provide protection from oxidation.

5.5.3 Network of disulfide bridges might play a protective role in maintenance of GST-tagged PP-1 activity under redox-stress

In this thesis, the role of various PTM including intra-molecular disulfide bridge formation and gluthathionylation were assessed during oxidative stress. Oxidative stress has been shown

to affect the activity of the protein. This inhibition can also be reversed with the treatment of TCEP (Figure 21). S-Gluthathionylation was shown to be a fast response to a harsh oxidative treatment, but this modification may have deleterious effects if prolonged. Therefore, the formation of the disulfide bridges from the Cys residues, not in proximity to one another, may play a role in maintaining structural integrity of PP-1. Hence the structure of PP-1 could be highly dynamic, however the formation of transient intra-molecular disulfide bridges cannot be identified using typically employed approaches, such as non-reducing immunoblots. Nevertheless, these modifications may be identified using with mass spectrometry.

This approach was used in LC-MSMS experiments under four conditions: including and excluding both Mn^{2+} and H_2O_2 in the buffer solutions. A curious result that was identified under all conditions was that Cys^{155} and Cys^{158}; Cys^{171} and Cys^{172} formed disulfide bridges. The direct proximity of Cys^{171} and Cys^{172} supports this behavior, and the disulfide bridge remains intact. This is an expected result because they are always placed on one peptide when the protein was digested with Trypsin. On the other hand, Trypsin digestion is performed at almost neutral pH, which can lead to artifact formation of disulfide bridges. The observation was because generally the detection of disulfide linked peptides is in only enabled by H_2O_2 treatment. Artifact formations did not play a major role in this experiment. Cys^{39} and Cys^{127} were not detected as free peptide spectra, which allows us to conclude that they play a major role in disulfide networking.

When a normal search was performed with all the Cys modifications, the formation of persulfides and/or dehydroalanines was observed for the following Cys residues: Cys^{62}, Cys^{105}, Cys^{155}, Cys^{202} and Cys^{291}, that must be formed under CID conditions from either disulfide bridges or any other type of disulfide-link. In conclusion, oxidative stress might induce disulfide bridge formations that freeze structural changes in the protein. Altogether, seven disulfide bridges were identified as true ones (Cys^{140} XL Cys^{39}, Cys^{105} XL Cys^{39}, Cys^{140} XL Cys^{127}, Cys^{140} XL $Cys^{154}Cys^{158}$ and Cys^{127} XL $Cys^{154}Cys^{158}$). However, none of them were found to be in the appropriate proximity to form native disulfide bridges. In order to form disulfide bridges, Cys residues need more flexibility of the protein to come closer to each other. Three more disulfide bridge spectra were weaker and could be hence artifacts from the database search (Cys^{39} XL Cys^{62}, Cys^{39} XL Cys^{127} and Cys^{140} XL $Cys^{154}Cys^{158}$), since the spectra do not contain enough information about the second peptide. Also from a structural point of view, these disulfide bridges are not very likely to form because the Cys residues are too far away from each other so that this could be explained by disulfide scrambling. On the other hand, not all Cys residues are involved in disulfide links when harsh oxidative stress is applied to the protein. Although experiments were performed under slightly acidic conditions,

the results from all four conditions provide sufficient information to conclude that true disulfide links were formed. Also, disulfide shuffling upon digestion with Trypsin might have also played a role.

With the aid of pymol and PDB id: 4MOV, all possible distances were measured in Ängström between all thirteen Cys. The distance between all the Cys and Mn^{2+} ions in PP-1 was measured as well. Table 21, arguments for the Cys which are closer to Mn^{2+} ions might be protected from oxidation. Figure 52.A shows close proximities of Cys^{273}, Cys^{202} and Cys^{127}. According to the distance measurements of the structure determined under non-oxidative conditions, one should also see protection for Cys^{245} and Cys^{62} (Table 21). The latter are close proximity to $Cys^{155}Cys^{158}$ which forms a disulfide bridges under all conditions (as detected in Figure 52.B). Cys^{155} being in the center vicinity of all the Cys residues, it appears to be an ideal Cys to form disulfide bridges with other Cys. The same holds true for Cys^{245} (Figure 52.C) which shows a second network between Cys^{291}, Cys^{62}, Cys^{245}, Cys^{171} and Cys^{172}. $Cys^{171}Cys^{172}$ could form a disulfide under all conditions. The two potential networks based on the proximities are indicated in black boxes in Table 21. To conclude, the experiments do not answer the question to which extent the Mn^{2+} ions are protective against the oxidation, since the H_2O_2 levels are quite artificially high. As stated above, Cys^{273} and Cys^{291} are too far away from the active center and not protected by the Mn^{2+} ion, either. However, a truncated peptide was identified starting from Ser^{168} being completely reduced without H_2O_2, and a persulfide formation at Cys^{291} when H_2O_2 was applied.

Over two decades ago, site-directed mutants of the catalytic subunit of rabbit muscle PP-1 were generated, as their activity is highly susceptible to inactivation by sulfhydryl reagents. In an experiment, they had mutated the following Cys residues: 39, 62, 171, 202, and 273 from Cys to Ser. All six mutants were active; and so did not depend on the mechanism of a cysteinyl-phosphate intermediate (Zhang et al., 1994). In the same study, interestingly, $C^{273}S$ mutant, this Cys is closest to the C-terminus, shows a similar activity to the wild-type recombinant enzyme, which suggests that that portion of C-terminus of PP-1 can be cleaved without loss of activity (Cohen, 1989; Bollen and Stalmans, 1992). Recent study confirming the abovementioned findings that, PP-1 $Cys^{127/273}Ser$ double variant mutant exhibited the same catalytic activity as wild type PP-1 and responded identically to H_2O_2 treatment (Santos et al., 2016).

In the future, it would be interesting to conduct mutagenesis studies involving the replacement of redox-sensitive Cys residues in PP-1. For instance, Cys^{127} would be a convincing link between disulfide bridges and PP-1 activity. It is also possible that an amino

acid switch could alter the PP-1 structure, leading to total inactivation of PP-1 and disrupting the β-adrenergic signaling pathway.
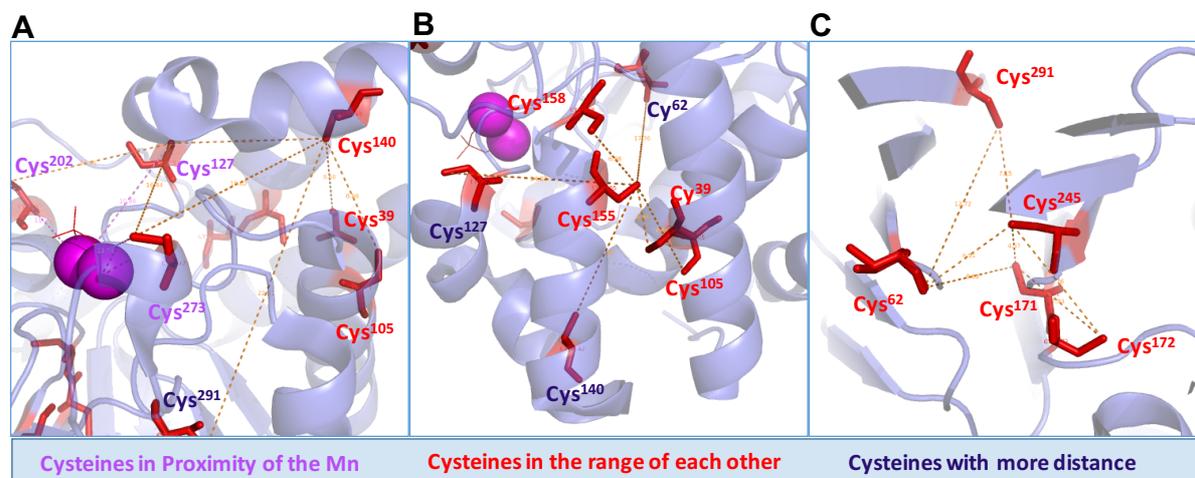
Figure 52 | Proximities of several Cys residues forming a network. Red Cys have a very high proximity, and blue Cys residues are in the range of 14 Å. (A) The cartoon representation shows close proximities towards the Mn^{2+} for Cys^{273}, Cys^{202} and Cys^{127}. The distance measurements of the structure determined under non-oxidative conditions, shows that Cys^{245} and Cys^{62} are protected (Table 21). (B) The figure shows the network of closer related Cys residues around Cys^{155}. $Cys^{155}Cys^{158}$ can form a disulfide under all conditions and might interact with Cys^{39} and Cys^{105}. On the other hand, Cys^{140} and Cys^{127} are further away. With Cys^{155} in the center vicinity of all the Cys residues, it could be ideal for forming disulfide bridges with all other Cys. (C) The same holds true for Cys^{245} which shows a second network between Cys^{291}, Cys^{62}, Cys^{245}, Cys^{171} and Cys^{172}. $Cys^{171}Cys^{172}$ would also form a disulfide bridge under all conditions.

5.5.4 Redox response of His-tagged PP-1 involves sulfone formation

From the Peaks results, the first observation from the His-tagged PP-1A protein was that a large number of sulfone was formed at all Cys residues when H_2O_2 was applied, but no persulfides were formed at all. Furthermore, other amino acids were oxidized, which was not present in the GST-tagged PP-1A. This confirms the protective mechanism of GST in the buffer for all other amino acids, but also clearly shows that glutathione in GST-tagged PP-1 is protecting the protein from further oxidation and preventing the Cys from being oxidized up to the sulfone state, a mechanism missing completely in the His-tagged PP-1.

Similar to GST-tagged PP-1, the free peptides with Cys^{39} were not visible, but some oxidized peptides comprised Cys^{127}. Cys^{39} is the only disulfide bridge discovered close to $Cys^{155}Cys^{158}$ in the condition with non Mn^{2+} and with H_2O_2. To conclude, the fast formation of sulfones of all the other Cys prevented more disulfide bridge formations. The distance table also shows the by far lowest distance of only 4.54 Å between Cys^{39} and Cys^{155}, which argues for the fast formation of a disulfide bridges as a protection mechanism even more strongly.

When samples were free from Mn^{2+} and H_2O_2, all Cys were free, and carbamidomethylation was possible. Surprisingly, when Mn^{2+} were not present in the buffer, the addition of H_2O_2 led to the formation of sulfonic acid of all Cys residues, whereas in the presence of Mn^{2+} ions this was not the case.

5.5.5 Do Histidine residues cage the dinuclear Mn^{2+} ions to shield them from Cysteine residues?

The X-ray structure of PP-1 has revealed the six residues which coordinate the Mn^{2+} ions (Asp^{64}, His^{66}, Asp^{92}, Asn^{124}, His^{173} and His^{248}). His^{66} is found next to one Mn^{2+} ion, His^{248} is next to the other Mn^{2+} ion, which excludes His^{125} from the unity sequence of the ligands amino acids. It has been well established that His^{125} is required for catalysis and that it is not a ligand to both metal ions, but that it is within the range of 5 Å (Lohse et al., 1995). However, site-directed mutagenesis of His^{125} been shown to have considerable effect on its catalytic activity as well as on the stability of the protein, which opens the question for the role of His.
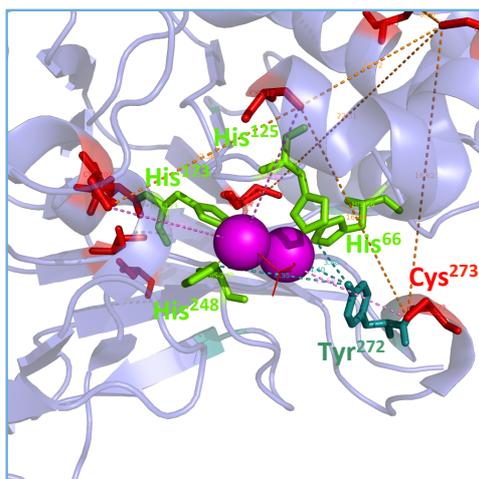
Figure 53 | His caging Mn^{2+} and Tyr^{272} arrangement correlated to Cys network His arrangement around the dinuclear Mn^{2+} ions. In the structure of PP-1, two Mn^{2+} ions are surrounded by the four His residues (His^{66}, His^{125}, His^{173} and His^{248}). Tyr^{272} is close by Mn^{2+} ions and gets oxidized.

Di-nuclear Mn^{2+} ions are surrounded by four His (His^{66}, His^{125}, His^{173} and His^{248}). Interestingly, in this study, His^{125} was discovered to be oxidized in the spectral data in the condition with Mn^{2+} and with H_2O_2 in His-tagged PP-1, while His^{248} was discovered to be oxidized in the GST-tagged protein (Figure 53). This lead to a conclusion that first Mn^{2+} is getting oxidized, followed by His. Cys residues might behave independently from this because they are shielded by the His residues from the protected distance of the metal ions. Mn^{2+} ions do not protect Cys from oxidation unless a certain order of oxidation (first Mn^{2+} and

later His) occurs as a protection mechanism. One possible conclusion could be that first Mn^{2+} gets oxidized and then His but that somehow Mn^{2+} ions fail to protect Cys from oxidation.

In addition, Tyr^{272} gets oxidized in the condition with non Mn^{2+} ions and with H_2O_2, which resides next to the Cys^{273} and also in close proximity of dinuclear Mn^{2+} ions. One suggestion for this oxidation could be that Tyr aids in the oxidation of both metal ions and Cys^{273} by transferring the electrons to them or vice versa. More experimental data are needed to prove this interesting finding.

5.5.6 Disulfide bridges determination in PP-1 using predictive computational methods and its correlation with mass spectrometry data

Cys is known as a tripotic acid in which the pK_a of the thiol group has been identified to be 8.2 (Tajc et al., 2004). Thiol groups are considered to be mild acids, where the pK_a value can be altered due to the protein microenvironment. In conclusion, the pK_a value will play a significant role, whether a thiol group will be reactive enough to oxidize, and hence to form disulfide bridges with a neighboring thiol group. In total, four different prediction software programs were used to identify the probability for disulfide bridges in PP-1 with corresponding PDB id or sequence information, namely PROPKA 3.1, COPA, DiANNA and dbGSH database.

PROPKA 3.1 software identified Cys^{155} and Cys^{171} as the Cys with the lowest pK_a. The corresponding thiol groups containing Cys can be highly susceptible to oxidation and can later form disulfide bonds. Mass spectrometry analysis found those Cys residues to be involved in disulfide bridges in all times, but this correlates also with its simplified detection on just one Tryptic peptide. In contrast, COPA, an algorithm based disulfide bridges predictor software, identified Cys 39, 127, 155, 171, 245 and 273 to be redox sensitive. This software categorizes different Cys residues into oxidation susceptible and oxidation-non-susceptible and further classified three points for prediction of thiol oxidation susceptibility i.e. distance to the nearest Cys sulfur atom, solvent accessibility, and pK_a. Interestingly, both Cys^{39} and Cys^{127} were also not identified as free Cys peptides under all buffer conditions, and to be involved in disulfide formation. Cys^{245} is known as one target to glutathionylation. Furthermore, DiANNA software predicted mainly Cys^{273} to be in oxidation state with the score of 1. Also, from a structural point of view, PP-1 contains dinuclear Mn^{2+} in the pocket of the catalytic subunit, and Cys^{273} and Cys^{291} were quite distant from it and hence could not be protected from oxidation by Mn^{2+} ions. In the mass spectrometry analysis, initially these two Cys residues were not detected at all, but a repeated search in aid with a semi-trypsin

activity that allows one non-specific cleavage, it was possible to detect a truncated peptide starting at Ser^{168}, where both Cys residues were non-oxidized in non Mn^{2+} and with H_2O_2 and hence must be protected. Under harsh oxidative conditions, Cys^{291} was also identified as a persulfide, which indicated that it might form disulfide bridge or mixed disulfide bridge with glutathione. Another prediction software/database dbGSH proposed that Cys^{140}, Cys^{158} and Cys^{245} could be S-glutathionylated, which could be correlated with the MS results that demonstrated Cys^{140}, Cys^{202} and Cys^{245} to be S-glutathionylated (discussed in detail in Section 5.5.1).

5.6 Proposed mechanism for protection of PP-1 from irreversible loss of activity

In the native structure of PP-1, there is no disulfide bridge formation and no Cys residues are known to play any role in redox modified signaling mechanism. In this thesis, a mechanism for the redox modification of PP-1 is purposed (Figure 54). At this point, it is uncertain which part of the mechanism described below may be responsible for the redox-based activity of PP-1, but it seems clear that the GST activity and GSH at high concentrations actively protects the Cys residues from the formation of an irreversible state of sulfonic acid, which further leads to a protective mechanism that involves formation of various disulfide bridges. However, external addition of Mn^{2+} in the buffer also leads to the novel protection of Cys and His from oxidation.

Figure 54 | A proposed mechanism showing the vital role of glutathione, His, Cys and Mn^{2+} ions in the activity of PP-1. With the aid of two His-tagged and GST-tagged rPP-1, glutathione

protects the Cys from the irreversible (sulfone) modification by forming a strong disulfide-network. Dinuclear Mn^{2+} ions at the center pocket of the catalytic unit, also act as protecting agent by lowering the Cys oxidations and decrease in disulfide bridges formation. Histidine surrounding the Mn^{2+} also protects the metal ions from further oxidation, by allowing themselves to get oxidized first. PP-1 activity could be recovered by TCEP, if Cys are still in a reversible stage, i.e. disulfide bridges.

This novel observation sheds light on the mechanism of PP-1 as a redox sensor. As highlighted in Figure 55, the presence of glutathione leads to the oxidant induced inactivation of PP-1 via transient intra-molecular disulfide bridge formation. It is possible that PP-1 might first form mixed-disulfide bridges via S-glutathionylation, which could be reverted back to the original structure in the presence of GST. This work also highlights, how GSH plays a protective role by not allowing Cys to form an irreversible sulfonic acid state, whereas the disulfide bridges between Cys^{39} and $Cys^{155}Cys^{158}$ are prominent after the sulfenic acid formation.

Figure 55 | Cys redox sensor in PP-1. The proposed mechanism of S-glutathionylation of PP-1 to protect against extreme oxidation, and to make PP-1 a novel regulatory model for redox regulation.

5.7 Clinical importance of redox-modified PP-1

Peroxide stress and the consequent formation of sulfenic acid and further transient disulfide bridges is believed to be a common mechanism in disease formation, e.g. of the brain, or the blood circulation system, i.e. in the development of high blood pressure/hypertension (Rybka et al., 2011). Presently, very little research has been undertaken examining the role of PP-1

oxidation specifically in context of cardiovascular research (Zhang et al., 2015; Santos et al., 2016).

PP-1 is found as a target to oxidative stress in the brain, forming transient disulfide bridges (Foley et al., 2016), but the exact mechanism and the extent of the disulfide formation have not been determined. Repeated psychological stress might increase disulfide formation and the inhibition of the four non-Peridoxin-like proteins in the brain which directly increases the vulnerability for disturbed glutamate neurotransmission. In this context, thioredoxin was also mentioned as a marker protein in the early development of the disease. In particular in schizophrenia, a disturbed antioxidant defense system in combination with increased formation of reactive oxygen species plays a crucial role (Wu et al., 2013).

In conclusion, these results describe in detail a potential role of disulfide bridges and PTMs of the PP-1 alpha as a protection mechanism to maintain the activity of the protein under oxidative stress conditions in the context of heart disease, which may hold clinical and therapeutic implications.

5.8 Regulation of CTGF in response to ER stress

The number of cardiomyocytes usually decreases during HF (Nakano et al., 2012). Although the cause of this loss has not been fully clarified, it is known that cardiomyocytes are exposed to many different stressors in HF (Richardson et al., 1996; Chien, 1999). Among the stressors, mechanical stress is considered to induce growth response in the overloaded myocardium by the release of the growth promoting factors, such as Ang II, endothelin-1 and TGF-β (Ruwhof and van der Laarse, 2000). Besides mechanical stress, cardiomyocytes are also constantly exposed to oxidative stress (Santos et al., 2011).

In the first part of this thesis, a mechanism was suggested depicting the S-glutathionylation of PP-1 that could further lead to intra- and inter- disulfide bond formation and protect the protein from irreversible state of sulfonic acid. After establishing PP-1 a well-defined role in folding mechanisms, it will be really interesting to identify, which component of the cell plays role in the folding of the protein and maintains the hemostasis of the cell. To answer that question, one of the classic and little known secretory proteins called as CTGF was targeted. Various cellular functions, including processing of secreted proteins, calcium storage and folding of proteins, are controlled by ER and disruption to the ER function leads to ER stress. In the following sections of the discussion, the main focus is to identify whether the ER and CTGF have any co-relationship in maintaining the physiological balance of the cell.

The in this thesis presented data demonstrates that an increase in oxidative stress as elicited by the application of H_2O_2 led to a slight increase of intracellular CTGF levels, which were however not significant. Trends toward an up-regulation of CTGF were observed 6 min and 4 h after H_2O_2 addition (Figure 41.A). The long-term up-regulation of CTGF under stress conditions was further confirmed by the conduction of heat-shock experiments. These results are in accordance with previous findings of up-regulation of CTGF under stress conditions, in particular mechanical stress (Chudgar et al., 2006). These findings therefore indicate that CTGF up-regulation may be an initial response to different kinds of stressors in cardiomyocytes.

It has previously been shown that ER stress can be initiated by chemicals such as dithiothreitol (DTT), thapsigargin (TGN) and tunicamycin (Tm) which change the redox balance, Ca^{2+} homeostasis and protein glycosylation state in the ER, respectively, as well as brefeldin A, which inhibits transportation of proteins from the ER to the Golgi complex (Kozutsumi et al., 1988; Kaufman, 1999; Breckenridge et al., 2003; Merksamer et al., 2008). When these chemicals were applied to the cells, the ER protein folding machinery deteriorates. The aggregation of malfunctioned, misfolded proteins is a hallmark signal of ER stress (Chang et al., 1987). In this thesis, results obtained from cells exposed to DTT demonstrated that CTGF may form an intramolecular disulfide bridge as demonstrated with immunoblots showing molecular weight shifts from between 36 and 38 kDa under non-reducing and reducing SDS-PAGE conditions (Figure 42).

Furthermore, results also demonstrated that when NRCM were treated with TGN, it induced ER stress by emptying intracellular Ca^{2+} stores, which led to an up regulation of CTGF after 6 h (Figure 43). To determine if glycosylation was essential for rat CTGF localization and secretion, NRCM was also incubated with Tm. In contrast to TGN, incubation of NRCM with Tm did not affect CTGF expression. These results are perhaps not surprising as in rat CTGF, no N-linked glycosylation site has been identified. Thus, N-linked glycosylation could be excluded from a process influencing CTGF expression and secretion.

A liver study showed that hsp47, TGF-β1 and CTGF are involved in the pathogenesis of hepatic fibrosis infected by *Schistosoma japonicum*, and that downregulation of hsp47 in a hepatic mouse model of schistosomiasis led to downregulation of CTGF (Huang et al., 2014). The expression of CTGF in hsp47 knockout mouse embryonic fibroblasts was analyzed and results demonstrated that in these cells CTGF expression was markedly downregulated supporting a link between the collagen processing hsp47 and CTGF (Figure 45).

5.8.1 No evidence for the impact of pH, MG132 and BFA on CTGF expression in NRCM

In human airway smooth muscle cells, it has been demonstrated that extracellular acidification of pH 6.3 can trigger the CTGF expression and that this plays a major role in the formation of ECM proteins and necessary for airway remodeling via the GPR68-$G_{q/11}$-IP_3-Ca^{2+} signaling (Ichimonji et al., 2010). Within the cardiovascular field the impact of pH has not been studied in relation to CTGF expression. To assess the effects of pH on CTGF expression, NRCM were exposed to a wide range of pH conditions. Results demonstrated that in NRCM, different pH conditions had no major influence on CTGF expression (Figure 46.A). Next, MG132 (a potent, membrane-permeable proteasome inhibitor) was used to assess the correlation between proteasome degradation and CTGF expression in NRCM. CTGF showed no concentration dependent change in expression with MG132 treatment; indicating that CTGF, under these conditions, does not appear to have a role in proteasome degradation (Figure 46.B). In 2002, it was demonstrated that BFA-induced Golgi disruption blocks CTGF secretion (Chen et al., 2001). However, in this study no change was observed in the expression of CTGF with increasing concentrations of BFA in NRCM (Figure 46.C).

5.8.2 Knockdown of CTGF affects ER stress markers in NRCM

During I/R, all sources of oxygen and energy substrates are diminished in the myocardium, leading to an increased production of ROS. This eventually triggers the UPR signaling pathway. In this context, it has been reported that an increased expression of UPR-related genes in cardiomyocytes occurs such as BiP, XBP-1, and PDI, which have been identified following myocardial infarction in mouse and human hearts (Thuerauf et al., 2006; Severino et al., 2007). As a consequence, it could result in perturbation of ER oxidative balance and Ca^{2+} homeostasis, and hence the loss of cardiac function and ultimately apoptosis (Scarabelli and Gottlieb, 2004). More than two decades ago the concept of ER stress was identified and recently its role in cardiovascular functioning has been increasingly recognized (Kozutsumi et al., 1988). Various external and physiological changes such as ischemia, heat, hypoxia, glucose and metabolic starvations are strong inducers of the ER stress signaling pathway (Toth et al., 2007).

In this thesis, different stressors were used to analyze their effect on CTGF and its role in ER stress signaling pathway in cardiomyocytes. The obtained results demonstrate that certain factors inducing ER stress can increase CTGF expression. However, whether CTGF can

affect ER stress was not clear. Therefore, the regulation of various ER stress related proteins and their dependence on CTGF were examined.

When the unfolded protein response (UPR) is initiated various UPR signaling pathways are activated. One such pathway involves the transmembrane activating transcription factor 6 (ATF6) which is sequentially cleaved by site-1 protease (S1P) and S2P inside the Golgi (Ye et al., 2000). This allows the cytosolic fragment of ATF6 to enter the nucleus. In this study, the putative roles of CTGF and ATF6 in NRCM were investigated by siRNA-mediated silencing of CTGF *in vitro.* Immunofluorescence analysis demonstrated that siCTGF-transfected cells had a lower CTGF and ATF6 expression (mainly in the nucleus) than siControl-transfected cells (Figure 47). In a previous study, it was shown that when ATF6 is conditionally active in cardiomyocytes, as achieved by a transgenic mouse line, and upon *in vivo* ischemic injury, ATF6 triggers cytoprotective ER stress proteins involving BiP and GRP94, which can function to reduce ischemic injury (Martindale et al., 2006). Moreover, in a mouse model of pressure overload hypertrophy, ATF6 has been suggested to play an adaptive role (Lynch et al., 2012).

Next, the relationship between CTGF and the third UPR activation pathway was analyzed, which is governed by the dimerization and autophosphorylation of IRE1-α. Under basal non-stress conditions, IRE1-α is inactive, but upon stress, there is a conformational alteration of IRE1-α induced by its phosphorylation, which exposes a ribonuclease capacity that removes an intron from XBP1 mRNA (XBP1US). A recent study from Lyons group has demonstrated that deletion of CTGF can induce cellular stress and plays a protective role in the survival of chondrocytes (Hall-Glenn et al., 2013). Another recent study demonstrated that, an adenovirus (Ad5-CMV-CCN2) mediated gene transfer induced ER stress and UPR in primary hepatic stellate cells and hepatocytes (Borkham-Kamphorst et al., 2016). Results have demonstrated the partial knockdown of CTGF in NRCM, leads to significant downregulation of the ER stress markers IRE-1α, PDI, and BiP (Figure 48), which is in line with research as discussed above. However, the same results were not established at the transcription level, as ER stress markers expressions were almost the same (Figure 50). The length of XBP1S was observed to be 263 base pairs, and the US variant had 289 base pairs for the siCTGF partial knockdown and conclusive of proteins expression data from the partial knockdown of CTGF in NRCM (Figure 49). In conclusion, the data suggest that either CTGF is governing the expression of ER stress markers or vice versa.

5.9 Role of secretory cells with respect to ER stress

Different cells respond in various ways to ER stress which leads to the activation of UPR by the secretory apparatus. The purpose of UPR is to increase the competence of a cell to carry out protein secretion and protect the cell from ER stress (Ron and Walter, 2007). Few publications exist on the relationship between UPR, ER stress and the secretory pathway, and how secretory proteins relate to the survival of the cell during ER stress. The targeting of CTGF proteins in cardiomyocytes and their role in UPR represents a unique aspect of this study. Previously this has only been examined in chondrocytes and hepatocytes (Hall-Glenn et al., 2013; Wu et al., 2015).

Mammalian cells, including antibody-secreting plasma cells and insulin secreting pancreatic β-cells, are involved in maintaining the balance between ER competence to the protein folding demand and have large fluxes in their secretory loads (Moore and Hollien, 2012). The first work done to understand the relationship between secretory cells and the UPR was done in plasma cell differentiation. Here it was shown that XBP-1 plays an important factor in this process, as without it cells undergo apoptosis (Iwakoshi et al., 2003). Furthermore, it was previously discovered that during early B-cell development there is an IRE1-α deficiency (Zhang et al., 2005). In contrast to plasma cells, deletion or mutation of Perk or perturbation of the elF2α phosphorylation site induces β-cell deficiency, deregulation of glucose metabolism and early-onset diabetes (Harding et al., 2001; Zhang et al., 2002). Also, deletion of XBP-1 in β-cells damages secretion, insulin processing and *in vivo* proliferation (Lee et al., 2011). Similarly, lack in any of the three primary signaling pathways in the liver negotiates the response to acute ER stress, inducing suppression of metabolic gene expression, which is regulated by up-regulation of CHOP (Rutkowski et al., 2008). Furthermore, osteoblasts also highly express PERK, ATF4 and XBP1 (Clauss et al., 1993; Zhang et al., 2002; Saito et al., 2011) and both PERK and IRE1-α (Murakami et al., 2009; Saito et al., 2011) are activated during differentiation. The triggering of UPR in osteoblasts is likely to be involved in bone morphogenetic protein 2 (Bmp2) signaling, which is required for osteoblast differentiation and bone formation. Thorough research has been done in various secretory cell types, but there is much to be learned about the UPR in specific tissues and organs.

5.10 Higher expression of CTGF in the end stage heart failure

In the left ventricular tissue CTGF expression was significantly up-regulated in DCM and showed a trend toward elevation in ICM heart samples (Figure 40). These results are in accordance with previous studies demonstrating that CTGF is up-regulated in both animal and human models of HF (Daniels et al., 2009). In the context of HF and cardiac

hypertrophy, downstream effectors of $G_{\alpha q}$-coupled receptors for endothelin-1, norepinephrine and Ang II regulate CTGF expression (Kemp et al., 2004). However, the role of CTGF as a marker for fibrosis or triggering protein for interstitial fibrosis of the heart remains to be fully elucidated. Interestingly, in the heart and other organs, CTGF's modular domain structure influences the binding and consequent signaling of TGF-β which is known to be a regulator of fibrillar collagen gene transcription (Abreu et al., 2002).

Figure 56 represents a summary of all the results related to the effect of various stressors on CTGF expression. Red boxes, including changes in BFA, MG132 and pH demonstrated no effect on CTGF expression. However, green boxes including oxidizing agents, ER stress reducing agents, ER stress markers, heat shock, HSP47 and end stage heart failure, showed changes on the CTGF expression. The CTGF data argue for an interconnection of CTGF and ER stress, as ER stress modulates CTGF and *vice versa*, CTGF expression modulates proteins of the ER stress cascade.
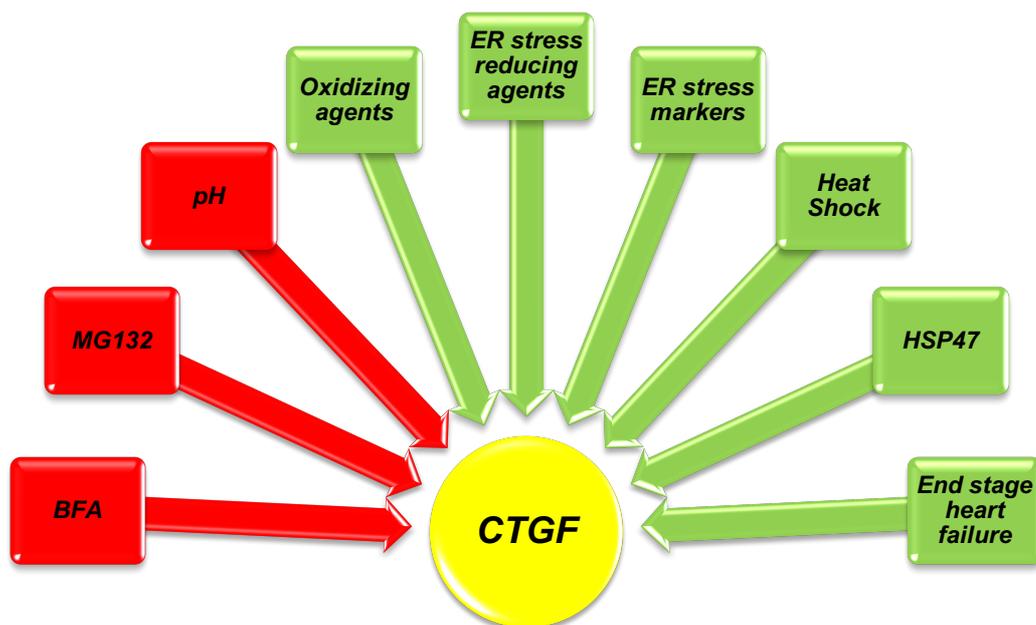
Figure 56 | Summary of all effectors on CTGF. Cartoon picture representing all the stressors used during the experiments. Boxes in red show no effect on CTGF expression, whereas green boxes represent with effect on CTGF expression.

6 Appendix

6.1 Spectrum covered by MS

Append 1 | Peak results indicating the Cys peptides that were not detected with red boxes. No detection might point at an unknown modification or involvement in disulfide bridges.

```
  1  MSDSEK LNLD SIIGRLLEVQ GSRPGKNVQL TENEIR GLCL KGLCLKGLCL K ICGDIHGQY YDLLR GLCLK QSLETICLLL

 81  AYK GLCL K GN HECASINRGL CLK YGFYDE CK GLCLK TFT DCFNCLPIAA IVDEK GLCLK IFCCHGGLSP DLQSMEQIR G

161  LCLK PTDVPD QGLLCDLLWS DPDK GLCLK H DLDLICR GLC LKQLVTLFSA PNYCGEFDNA GAMMSVDETL MCSFQILKQL

241  VTLFSAPNYC GEFDNAGAMM SVDETLMCSF QILKGLCLKQ LVTLFSAPNY CGEFDNAGAM MSVDETLMCS FQILKICGDI

321  HGQYYDLLRQ LVTLFSAPNY CGEFDNAGAM MSVDETLMCS FQILKQSLET ICLLLAYKQL VTLFSAPNYC GEFDNAGAMM

401  SVDETLMCSF QILKGNHECA SINRQLVTLF SAPNYCGEFD NAGAMMSVDE TLMCSFQILK IYGFYDECKQ LVTLFSAPNY

481  CGEFDNAGAM MSVDETLMCS FQILKTFTDC FNCLPIAAIV DEKQLVTLFS APNYCGEFDN AGAMMSVDET LMCSFQILKI

561  FCCHGGLSPD LQSMEQIRQL VTLFSAPNYC GEFDNAGAMM SVDETLMCSF QILKPTDVPD QGLLCDLLWS DPDKQLVTLF

641  SAPNYCGEFD NAGAMMSVDE TLMCSFQILK HDLDLICRQL VTLFSAPNYC GEFDNAGAMM SVDETLMCSF QILKQLVTLF

721  SAPNYCGEFD NAGAMMSVDE TLMCSFQILK SR EIFLSQPI LLELEAPLK I CGDIHGQYYD LLRGLCLKIC GDIHGQYYDL

801  LRICGDIHGQ YYDLLRICGD IHGQYYDLLR QSLETICLLL AYKICGDIHG QYYDLLRGNH ECASINRICG DIHGQYYDLL

881  RIYGFYDECK ICGDIHGQYY DLLRTFTDCF NCLPIAAIVD EKICGDIHGQ YYDLLRIFCC HGGLSPDLQS MEQIRICGDI

961  HGQYYDLLRP TDVPDQGLLC DLLWSDPDKI CGDIHGQYYD LLRHDLDLIC RICGDIHGQY YDLLRQLVTL FSAPNYCGEF

1041 DNAGAMMSVD ETLMCSFQIL K LFEYGGFPP ESNYLFLGDY VDR GKQSLET ICLLLAYKGL CLKQSLETIC LLLAYKICGD

1121 IHGQYYDLLR QSLETICLLL AYKQSLETIC LLLAYKQSLE TICLLLAYKG NHECASINRQ SLETICLLLA YKIYGFYDEC

1201 KQSLETICLL LAYKTFTDCF NCLPIAAIVD EKQSLETICL LLAYKIFCCH GGLSPDLQSM EQIRQSLETI CLLLAYKPTD

1281 VPDQGLLCDL LWSDPDKQSL ETICLLLAYK HDLDLICRQS LETICLLLAY KQLVTLFSAP NYCGEFDNAG AMMSVDETLM

1361 CSFQILK IKY PENFFLLR GN HECASINRGL CLKGNHECAS INRICGDIHG QYYDLLRGNH ECASINRQSL ETICLLLAYK

1441 GNHECASINR GNHECASINR GNHECASINR IYGFYDECKG NHECASINR T FTDCFNCLPI AAIVDEKGNH ECASINRIFC

1521 CHGGLSPDLQ SMEQIRGNHE CASINRPTDV PDQGLLCDLL WSDPDKGNHE CASINR HDLD LICRGNHECA SINR QLVTLF

1601 SAPNYCGEFD NAGAMMSVDE TLMCSFQILK IYGFYDECKG LCLKIYGFYD ECKICGDIHG QYYDLLRIYG FYDECKQSLE

1681 TICLLLAYKI YGFYDECKGN HECASINRIY GFYDECKIYG FYDECK IYGF YDECKTFTDC FNCLPIAAIV DEK IYGFYDE

1761 CKIFCCHGGL SPDLQSMEQI RIYGFYDECK PTDVPDQGLL CDLLWSDPDK IYGFYDECKH DLDLICRIYG FYDECKQLVT

1841 LFSAPNYCGE FDNAGAMMSV DETLMCSFQI LKRRYNIKLW KTFTDCFNCL PIAAIVDEKG LCLKTFTDCF NCLPIAAIVD

1921 EKICGDIHGQ YYDLLRTFTD CFNCLPIAAI VDEKQSLETI CLLLAYKTFT DCFNCLPIAA IVDEKGNHEC ASINRTFTDC

2001 FNCLPIAAIV DEKIYGFYDE CKTFTDCFNC LPIAAIVDEK TFTDCFNCLP IAAIVDEKTF TDCFNCLPIA AIVDEKIFCC

2081 HGGLSPDLQS MEQIRTFTDC FNCLPIAAIV DEKPTDVPDQ GLLCDLLWSD PDKTFTDCFN CLPIAAIVDE KHDLDLICRT

2161 FTDCFNCLPI AAIVDEKQLV TLFSAPNYCG EFDNAGAMMS VDETLMCSFQ ILKIFCCHGG LSPDLQSMEQ IRGLCLKIFC

2241 CHGGLSPDLQ SMEQIRICGD IHGQYYDLLR IFCCHGGLSP DLQSMEQIRQ SLETICLLLA YKIFCCHGGL SPDLQSMEQI

2321 RGNHECASIN RIFCCHGGLS PDLQSMEQIR IFCCHGGLSP DLQSMEQIRT FTDCFNCLPI AAIVDEKIFCC

2401 HGGLSPDLQS MEQIRIFCCH GGLSPDLQSM EQIRIFCCHG GLSPDLQSME QIRPTDVPDQ GLLCDLLWSD PDKIFCCHGG

2481 LSPDLQSMEQ IRHDLDLICR IFCCHGGLSP DLQSMEQIRQ LVTLFSAPNY CGEFDNAGAM MSVDETLMCS FQILKR IMRP

2561 TDVPDQGLLC DLLWSDPDK G LCLKPTDVPD QGLLCDLLWS DPDKICGDIH GQYYDLLRPT DVPDQGLLCD LLWSDPDKQS

2641 LETICLLLAY KPTDVPDQGL LCDLLWSDPD KGNHECASIN RPTDVPDQGL LCDLLWSDPD KIYGFYDECK PTDVPDQGLL

2721 CDLLWSDPDK TFTDCFNCLP IAAIVDEKPT DVPDQGLLCD LLWSDPDKIF CCHGGLSPDL QSMEQIRPTD VPDQGLLCDL

2801 LWSDPDKPTD VPDQGLLCDL LWSDPDKPTD VPDQGLLCDL LWSDPDKHDL DLICRPTDVP DQGLLCDLLW SDPDKQLVTL

2881 FSAPNYCGEF DNAGAMMSVD ETLMCSFQIL K PTDVPDQGL LCDLLWSDPD KDVQGWGEND RGVSFTFGAE VVAKFLHKHD

2961 LDLICR GLCL KHDLDLICRI CGDIHGQYYD LLRHDLDLIC RQSLETICLL LAYKHDLDLI CRGNHECASI NRHDLDLICR

3041 IYGFYDECKH DLDLICRTFT DCFNCLPIAA IVDEKHDLDL ICRIFCCHGG LSPDLQSMEQ IRHDLDLICR PTDVPDQGLL

3121 CDLLWSDPDK HDLDLICRHD LDLICRHDLD LICRQLVTLF SAPNYCGEFD NAGAMMSVDE TLMCSFQILK AHQVVEDGYE

3201 FFAK RQLVTL FSAPNYCGEF DNAGAMMSVD ETLMCSFQIL KGLCLKQLVT LFSAPNYCGE FDNAGAMMSV DETLMCSFQI

3281 LKICGDIHGQ YYDLLRQLVT LFSAPNYCGE FDNAGAMMSV DETLMCSFQI LKQSLETICL LLAYKQLVTL FSAPNYCGEF

3361 DNAGAMMSVD ETLMCSFQIL KGNHECASIN RQLVTLFSAP NYCGEFDNAG AMMSVDETLM CSFQILKIYG FYDECKQLVT

3441 LFSAPNYCGE FDNAGAMMSV DETLMCSFQI LKTFTDCFNC LPIAAIVDEK QLVTLFSAPN YCGEFDNAGA MMSVDETLMC

3521 SFQILKIFCC HGGLSPDLQS MEQIRQLVTL FSAPNYCGEF DNAGAMMSVD ETLMCSFQIL KPTDVPDQGL LCDLLWSDPD

3601 KQLVTLFSAP NYCGEFDNAG AMMSVDETLM CSFQILKHDL DLICRQLVTL FSAPNYCGEF DNAGAMMSVD ETLMCSFQIL

3681 KQLVTLFSAP NYCGEFDNAG AMMSVDETLM CSFQILKPAD KNK GKYGQFS GLNPGGRPIT PPR NSAKAKK
```

A) $-Mn^{2+}$ $-H_2O_2$

Legend:

- Acetylation (K) (+42.01)
- Acetylation (N-term) (+42.01)
- Carbamidomethylation (+57.02)
- Cysteine ribosylation (+132.04)
- Cysteine mercaptoethanol (+76.00)
- Deamidation (NQ) (+0.98)
- Dimethylation (+28.03)
- Dehydration (-18.01)
- Dihydroxy (+31.99)
- Dehydration (-18.01)
- Formylation (+27.99)
- Glycidamide adduct (+87.03)
- Hydroxylation (+15.99)
- internal disulfide bond unpaired fragmentation (-2.02)
- internal disulfide bond (-1.01)
- Methylation (+14.02)
- N-Succinimidyl-2-morpholine acetate (+127.06)
- Oxidation (M) (+15.99)
- Oxidation (HW) (+15.99)
- O-Diethylphosphorylation (+136.03)
- Phosphorylation (STY) (+79.97)
- Pyro-glu from Q (-17.03)
- Replacement of 2 protons by calcium (+37.95)
- Replacement of 2 protons by iron (+53.92)
- Replacement of proton with ammonium ion (+17.03)
- Sulphone C (+47.98)
- Sodium adduct (+21.98)
- 3-sulfanylpropenoyl (+88.00)
- Deamidation (NQ) (+0.98), Dimethylation (+28.03)
- Dimethylation (+28.03), Dehydration (-18.01)
- Methylation (+14.02), Hydroxylation (+15.99)
- Mutation

B) $-Mn^{2+}$ $+H_2O_2$

Legend (modifications):
- Acetylation (K) (+42.01)
- Acetylation (Protein N-term) (+42.01)
- Acetylation (N-term) (+42.01)
- Carbamidomethylation (+57.02)
- Cysteine ribosylation (+132.04)
- Carbamylation (+43.01)
- Carboxylation (DKW) (+43.99)
- Carboxylation (DKW) (+43.99)
- Deamidation (NQ) (+0.98)
- Dimethylation (+28.03)
- Dehydration (-18.01)
- Dehydration (-18.01)
- Dehydroalanine (C) (-33.99)
- Dihydroxy (+31.99)
- Formylation (+27.99)
- Glutathione disulfide (+305.07)
- HexNAcetylation (C) (+203.08)
- Hydroxylation (+15.99)
- internal disulfide bond unpaired fragmentation (-2.02)
- internal disulfide bond (-1.01)
- Methylation (+14.02)
- Oxidation (M) (+15.99)
- Oxidation (MW) (+15.99)
- Phosphorylation (STY) (+79.97)
- Proline oxidation to pyroglutamic acid (+13.98)
- Sulphone C (+47.98)
- Thio-HexNAcetylation (C) (+187.09)
- Deamidation (NQ) (+0.98), Dimethylation (+28.03)
- Mutation

```
1     MSDSEKLNLD SIIGRLLEVQ GSRPGKNVQL TENEIRGLCL KGLCLXGLCL KICGDIHGQY YDLLRGLCLK QSLETICLLL

81    AYXGLCLKGN HECASINRGL XIYGFYDE CKGLCLXKTFT DCFNCLPIAA IVDEKGLCLK IFCCHGGLSP DLQSMEQIRG

161   LCLKPTDVPD QGLLCDLLWS DPDKGLCLKH DLDLICRGLC LKQLVTLFSA PNYCGEFDNA GAMMSVDETL MCSFQILKQL

241   VTLFSAPNYC GEFDNAGAMM SVDETLMCSF QILKGLCLKQ LVTLFSAPNY CGEFDNAGAM MSVDETLMCS FQILKICGDI

321   HGQYYDLLRQ LVTLFSAPNY CGEFDNAGAM MSVDETLMCS FQILKQSLET ICLLLAYKQL VTLFSAPNYC GEFDNAGAMM

401   SVDETLMCSF QILKGNHECA SINRQLVTLF SAPNYCGEFD NAGAMMSVDE TLMCSFQILK IYGFYDECKQ LVTLFSAPNY

481   CGEFDNAGAM MSVDETLMCS FQILKTFTDC FNCLPIAAIV DEKQLVTLFS APNYCGEFDN AGAMMSVDET LMCSFQILKI

561   FCCHGGLSPD LQSMEQIRQL VTLFSAPNYC GEFDNAGAMM SVDETLMCSF QILKGLCLKQ LVTLFSAPNY CGEFDNAGAM

641   SAPNYCGEFD NAGAMMSVDE TLMCSFQILK HDLDLICRQL VTLFSAPNYC GEFDNAGAMM SVDETLMCSF QILKQLVTLF

721   SAPNYCGEFD NAGAMMSVDE TLMCSFQILK SREIFLSQPI LLELEAPLKI CGDIHGQYYD LLRGLCLKIC GDIHGQYYDL

801   LRICGDIHGQ YYDLLRICGD IHGQYYDLLR QSLETICLLL AYKICGDIHG QYYDLLRGNH ECASINRICG DIHGQYYDLL

881   RIYGFYDECK ICGDIHGQYY DLLRTFTDCF NCLPIAAIVD EKICGDIHGQ YYDLLRIFCC HGGLSPDLQS MEQIRICGDI

961   HGQYYDLLRP TDVPDQGLLC DLLWSDPDKI CGDIHGQYYD LLRHDLDLIC RICGDIHGQY YDLLRQLVTL FSAPNYCGEF

1041  DNAGAMMSVD ETLMCSFQIL KLFEYGGFPP ESNYLFLGDY VDRGKQSLET ICLLLAYKGL CLKQSLETIC LLLAYKICGD

1121  IHGQYYDLLR QSLETICLLL AYKQSLETIC LLLAYKQSLE TICLLLAYKG NHECASINRQ SLETICLLLA YKIYGFYDEC

1201  KQSLETICLL LAYKTFTDCF NCLPIAAIVD EKQSLETICL LLAYKIFCCH GGLSPDLQSM EQIRQSLETI CLLLAYKPTD

1281  VPDQGLLCDL LWSDPDKQSL ETICLLVTLF HDLDLICRQS LETICLLLAY KQLVTLFSAP NYCGEFDNAG AMMSVDETLM

1361  CSFQILKIKY PENFFLLRGN HECASINRGL CLKGNHECAS INRICGDIHG QYYDLLRGNH ECASINRQSL ETICLLLAYK

1441  GNHECASINR GNHECASINR GNHECASINR IYGFYDECKG NHECASINRT FTDCFNCLPI AAIVDEKGNH ECASINRIFC

1521  CHGGLSPDLQ SMEQIRGNHE CASINRPTDV PDQGLLCDLL WSDPDKGNHE CASINHDLD LICRGNHECA SINRLVTLF

1601  SAPNYCGEFD NAGAMMSVDE TLMCSFQILK IYGFYDECKG LCLKIYGFYD ECKICGDIHG QYYDLLRIYG FYDECKQSLE

1681  TICLLLAYKI YGFYDECKGN HECASINRIY GFYDECKIYG FYDECKTFTD CFNCLPIAAIV DEKICGDIHG QYYDLLRIYG FYDE

1761  CKIFCCHGGL SPDLQSMEQI RIYGFYDECK PTDVPDQGLL CDLLWSDPDK IYGFYDECKH DLDLICRIYG FYDECKQLVT

1841  LFSAPNYCGE FDNAGAMMSV DETLMCSFQI LKRRYNIKLW KTFTDCFNCL PIAAIVDEKG LCLKTFTDCF NCLPIAAIVD

1921  EKICGDIHGQ YYDLLRTFTD CFNCLPIAAI VDEKQSLETI CLLLAYKTFT DCFNCLPIAA IVDEKGNHEC ASINRTFTDC

2001  FNCLPIAAIV DEKIYGFYDE CKTFTDCFNC LPIAAIVDEK TFTDCFNCLP IAAIVDEKTF TDCFNCLPIA AIVDEKIFCC

2081  HGGLSPDLQS MEQIRTFTDC FNCLPIAAIV DEKPTDVPDQ GLLCDLLWSD PDKTFTDCFN CLPIAAIVDE KHDLDLICRT

2161  FTDCFNCLPI AAIVDEKQLV TLFSAPNYCG EFDNAGAMMS VDETLMCSFQ ILKIFCCHGG LSPDLQSMEQ IRGLCLKIFC

2241  CHGGLSPDLQ SMEQIRICGD IHGQYYDLLR IFCCHGGLSP DLQSMEQIRQ SLETICLLLA YKIFCCHGGL SPDLQSMEQI

2321  RGNHECASIN RIFCCHGGLS PDLQSMEQIR IYGFYDECKI FCCHGGLSPD LQSMEQIRTF TDCFNCLPIA AIVDEKIFCC

2401  HGGLSPDLQS MEQIRIFCCH GGLSPDLQSM EQIRIFCCHG GLSPDLQSME QIRPTDVPDQ GLLCDLLWSD PDKIFCCHGG

2481  LSPDLQSMEQ IRHDLDLICR IFCCHGGLSP DLQSMEQIRQ LVTLFSAPNY CGEFDNAGAM MSVDETLMCS FQILKRIMRP

2561  TDVPDQGLLC DLLWSDPDKG LCLKTDVPD QGLLCDLLWS DPDKICGDIH GQYYDLLRPT DVPDQGLLCD LLWSDPDKQS

2641  LETICLLLAY KPTDVPDQGL LCDLLWSDPD KGNHECASIN RPTDVPDQGL LCDLLWSDPD KIYGFYDECK PTDVPDQGLL

2721  CDLLWSDPDK TFTDCFNCLP IAAIVDEKPT DVPDQGLLCD LLWSDPDKIF CCHGGLSPDL QSMEQIRPTD VPDQGLLCDL

2801  LWSDPDKPTD VPDQGLLCDL LWSDPDKPTD VPDQGLLCDL LWSDPDKHDL DLICRPTDVP DQGLLCDLLW SDPDKQLVTL

2881  FSAPNYCGEF DNAGAMMSVD ETLMCSFQIL KPTDVPDQGL LCDLLWSDPD KDVQGWGEND GVSFTFGAE VVAKFLSHKHD

2961  LDLICRGLCL KHDLDLICRI CGDIHGQYYD LLRHDLDLIC RQSLETICLL LAYKHDLDLI CRGNHECASI NRHDLDLICR

3041  IYGFYDECKH DLDLICRTFT DCFNCLPIAA IVDEKHDLDL ICRIFCCHGG LSPDLQSMEQ IRHDLDLICR PTDVPDQGLL

3121  CDLLWSDPDK HDLDLICRHD LDLICRHDLD LICRQLVTLF SAPNYCGEFD NAGAMMSVDE TLMCSFQILK AHQVVEDGYE

3201  FFAKRQLVTL FSAPNYCGEF DNAGAMMSVD ETLMCSFQIL KGLCLKQLVT LFSAPNYCGE FDNAGAMMSV DETLMCSFQI

3281  LKICGDIHGQ YYDLLRQLVT LFSAPNYCGE FDNAGAMMSV DETLMCSFQI LKQSLETICL LLAYKQLVTL FSAPNYCGEF

3361  DNAGAMMSVD ETLMCSFQIL KGNHECASIN RQLVTLFSAP NYCGEFDNAG AMMSVDETLM CSFQILKIYG FYDECKQLVT

3441  LFSAPNYCGE FDNAGAMMSV DETLMCSFQI LKTFTDCFNC LPIAAIVDEK QLVTLFSAPN YCGEFDNAGA MMSVDETLMC

3521  SFQILKIFCC HGGLSPDLQS MEQIRQLVTL FSAPNYCGEF DNAGAMMSVD ETLMCSFQIL KPTDVPDQGL LCDLLWSDPD

3601  KQLVTLFSAP NYCGEFDNAG AMMSVDETLM CSFQILKHDL DLICRQLVTL FSAPNYCGEF DNAGAMMSVD ETLMCSFQIL

3681  KQLVTLFSAP NYCGEFDNAG AMMSVDETLM CSFQILKPAD KNKGKYGQFS GLNPGGRPIT PPRNSAKAKK
```

1　MSDSEK**LNLD SIIGRLLEVQ GSRPGKNVQL TENEIR**GLCL KGLCLKGLCL K**ICGDIHGQY YDLLR**GLCLK **QSLETICLLL**

Carbamidomethylation (+57.02)
Deamidation (NQ) (+0.98)
Disulfide Bridge unpaired fragmentation (-2.02)
Dehydroalanine C (-33.99)
Disulfide bridge Trypsin Cleavage (+15.99)
Oxidation (M) (+15.99)
Oxidation or Hydroxylation (C) (+15.99)
Persulfide C (+31.97)
Phosphorylation (STY) (+79.97)
Pyro-glu from Q (-17.03)

81　A**YKGLCLKGN HECASINR**GL CLK**IYGFYDE CK**GLCLK**TFT DCFNCLPIAA IVDEK**GLCLK **IFCCHGGLSP DLQSMEQIR**GL

C) +Mn²⁺−H₂O₂

161　LCLKPTDVPD QGLLCD**LLWS DPDK**GLCLK**H DLDLICR**GLC LKQLVTLFSA PNYCGEFDNA GAMMSVDETL MCSFQILKQL
241　VTLFSAPNYC GEFDNAGAMM SVDETLMCSF QILKGLCLKQ LVTLFSAPNY CGEFDNAGAM MSVDETLMCS FQILKICGDI
321　HGQYYDLLRQ LVTLFSAPNY CGEFDNAGAM MSVDETLMCS FQILKQSLET ICLLLAYKQL VTLFSAPNYC GEFDNAGAMM
401　SVDETLMCSF QILKGNHECA SINRQLVTLF SAPNYCGEFD NAGAMMSVDE TLMCSFQILK IYGFYDECKQ LVTLFSAPNY
481　CGEFDNAGAM MSVDETLMCS FQILKTFTDC FNCLPIAAIV DEKQLVTLFS APNYCGEFDN AGAMMSVDET LMCSFQILKI
561　FCCHGGLSPD LQSMEQIRQL VTLFSAPNYC GEFDNAGAMM SVDETLMCSF QILKICGDLL DPDKQLVTLF
641　SAPNYCGEFD NAGAMMSVDE TLMCSFQILK HDLDLICRQL VTLFSAPNYC GEFDNAGAMM SVDETLMCSF QILKQLVTLF
721　SAPNYCGEFD NAGAMMSVDE TLMCSFQILK SR**EIFLSQPI LLELEAPLK**I CGDIHGQYYD LLRGLCLKIC GDIHGQYYDL
801　LRICGDIHGQ YYDLLRICGD IHGQYYDLLR QSLETICLLL AYKICGDIHG QYYDLLRGNH ECASINRICG DIHGQYYDLL
881　**RIYGFYDECK** ICGDIHGQYY DLLRTFTDCF NCLPIAAIVD EKICGDIHGQ YYDLLRIFCC HGGLSPDLQS MEQIRICGDI
961　HGQYYDLLRP TDVPDQGLLC DLLWSDPDKI CGDIHGQYYD LLRHDLDLIC RICGDIHGQY YDLLRQLVTL FSAPNYCGEF
1041　DNAGAMMSVD ETLMCSFQIL K**LFEYGGFPP ESNYLFLGDY VDR**GKQSLET ICLLLAYKGL CLKQSLETIC LLLAYKICGD
1121　IHGQYYDLLR QSLETICLLL AYKQSLETIC LLLAYKQSLE TICLLLAYKG NHECASINRQ SLETICLLLA YKIYGFYDEC
1201　KQSLETICLL LAYKTFTDCF NCLPIAAIVD EKQSLETICL LLAYKIFCCH GGLSPDLQSM EQIRQSLETI CLLLAYKPTD
1281　VPDQGLLCDL LWSDPDKQSL ETICLLLAYK HDLDLICRQS LETICLLLAY KQLVTLFSAP NYCGEFDNAG AMMSVDETLM
1361　CSFQILK**IKY PENFFLLR**GN HECASINRGL CLKGNHECAS INRICGDIHG QYYDLLRGNH ECASINRQSL ETICLLLAYK
1441　GNHECASINR GNHECASINR GNHECASINR **IYGFYDECKG NHECASINR**T FTDCFNCLPI AAIVDEKGNH ECASINRIFC
1521　CHGGLSPDLQ SMEQIRGNHE CASINRPTDV PDQGLLCDLL WSDPDKGNHE CASINRHDLD LICRGNHECA SINRQLVTLF
1601　SAPNYCGEFD NAGAMMSVDE TLMCSFQILK IYGFYDECKG LCLKIYGFYD ECKICGDIHG QYYDLLRIYG FYDECKQSLE
1681　TICLLLAYKI YGFYDECKGN HECASINRIY GFYDECKIYG FYDECKTFTC FNCLPIAAIV DEKIYGFYDE
1761　CKIFCCHGGL SPDLQSMEQI RIYGFYDECK PTDVPDQGLL CDLLWSDPDK IYGFYDECKH DLDLICRIYG FYDECKQLVT
1841　LFSAPNYCGE FDNAGAMMSV DETLMCSFQI LKRRYNIKLW KTFTDCFNCL PIAAIVDEKG LCLKTFTDCF NCLPIAAIVD
1921　EKICGDIHGQ YYDLLRTFTD CFNCLPIAAI VDEKQSLETI CLLLAYKTFT DCFNCLPIAA IVDEKGNHEC ASINRTFTDC
2001　FNCLPIAAIV DEKIYGFYDE CKTFTDCFNC LPIAAIVDEK TFTDCFNCLP IAAIVDEKTF TDCFNCLPIA AIVDEKIFCC
2081　HGGLSPDLQS MEQIRTFTDC FNCLPIAAIV DEKPTDVPDQ GLLCDLLWSD PDKTFTDCFN CLPIAAIVDE KHDLDLICRT
2161　FTDCFNCLPI AAIVDEKQLV TLFSAPNYCG EFDNAGAMMS VDETLMCSFQ ILKIFCCHGG LSPDLQSMEQ IRGLCLKIFC
2241　CHGGLSPDLQ SMEQIRICGD IHGQYYDLLR IFCCHGGLSP DLQSMEQIRQ SLETICLLLA YKIFCCHGGL SPDLQSMEQI
2321　RGNHECASIN RIFCCHGGLS PDLQSMEQIR IYGFYDECKI FCCHGGLSPD LQSMEQIRTF TDCFNCLPIA AIVDEKIFCC
2401　HGGLSPDLQS MEQIRIFCCH GGLSPDLQSM EQIRFCCHG GLSPDLQSME QIRPTDVPDQ GLLCDLLWSD PDKIFCCHGG
2481　LSPDLQSMEQ IRHDLDLICR IFCCHGGLSP DLQSMEQIRQ LVTLFSAPNY CGEFDNAGAM MSVDETLMCS FQILKR**IMRP**

2561　**TDVPDQGLLC DLLWSDPDK**G LCLKPTDVPD QGLLCDLLWS DPDKICGDIH GQYYDLLRPT DVPDQGLLCD LLWSDPDKQS
2641　LETICLLLAY KPTDVPDQGL LCDLLWSDPD KGNHECASIN RPTDVPDQGL LCDLLWSDPD KIYGFYDECK PTDVPDQGLL
2721　CDLLWSDPDK TFTDCFNCLP IAAIVDEKPT DVPDQGLLCD LLWSDPDKIF CCHGGLSPDL QSMEQIRPTD VPDQGLLCDL
2801　LWSDPDKPTD VPDQGLLCDL LWSDPDKPTD VPDQGLLCDL LWSDPDKHDL DLICRPTDVP DQGLLCDLLW SDPDKQLVTL
2881　FSAPNYCGEF DNAGAMMSVD ETLMCSFQIL KPTDVPDQGL LCDLL**WSDPD KDVQGWGEND RGVSFTTGAE VVAK**FLHKHD
2961　LDLICRGLCL KHDLDLICRI CGDIHGQYYD LLRHDLDLIC RQSLETICLL LAYKHDLDLI CRGNHECASI NRHDLDLICR
3041　IYGFYDECKH DLDLICRTFT DCFNCLPIAA IVDEKHDLDL ICRIFCCHGG LSPDLQSMEQ IRHDLDLICR PTDVPDQGLL
3121　CDLLWSDPDK HDLDLICRHD LDLICRHDLD LICRQLVTLF SAPNYCGEFD NAGAMMSVDE TLMCSFQILK **AHQVVEDGYE**

3201　**FFAK**RQLVTL FSAPNYCGEF DNAGAMMSVD ETLMCSFQIL KGLCLKQLVT LFSAPNYCGE FDNAGAMMSV DETLMCSFQI
3281　LKICGDIHGQ YYDLLRQLVT LFSAPNYCGE FDNAGAMMSV DETLMCSFQI LKQSLETICL LLAYKQLVTL FSAPNYCGEF
3361　DNAGAMMSVD ETLMCSFQIL KGNHECASIN RQLVTLFSAP NYCGEFDNAG AMMSVDETLM CSFQILKIYG FYDECKQLVT
3441　LFSAPNYCGE FDNAGAMMSV DETLMCSFQI LKTFTDCFNC LPIAAIVDEK QLVTLFSAPN YCGEFDNAGA MMSVDETLMC
3521　SFQILKIFCC HGGLSPDLQS MEQIRQLVTL FSAPNYCGEF DNAGAMMSVD ETLMCSFQIL KPTDVPDQGL LCDLLWSDPD
3601　KQLVTLFSAP NYCGEFDNAG AMMSVDETLM CSFQILKHDL DLICRQLVTL FSAPNYCGEF DNAGAMMSVD ETLMCSFQIL
3681　KQLVTLF**SAP NYCGEFDNAG AMMSVDETLM CSFQILKPAD K**NKGK**YGQFS GLNPGGRPIT PPR**NSAKAKK

```
  1  MSDSEK LNLD SIIGRLLEVQ GSRPGKNVQL TENEIR GLCL KGLCLKGLCL K ICGDIHGQY YDLLR GLCLK QSLETICLLL

 81  AYKGLCLKGN HECASINRGL CLK IYGFYDE CK GLCLK TFT DCFNCLPIAA IVDEK GLCLK IFCCHGGLSP DLQSMEQIR
```

D) +Mn^{2+} +H$_2$O$_2$

```
161  LCLKPTDVPD QGLLCDLLWS DPDKGLCLK H DLDLICR GLC LKQLVTLFSA PNYCGEFDNA GAMMSVDETL MCSFQILKQL

241  VTLFSAPNYC GEFDNAGAMM SVDETLMCSF QILKGLCLKQ LVTLFSAPNY CGEFDNAGAM MSVDETLMCS FQILKICGDI
321  HGQYYDLLRQ LVTLFSAPNY CGEFDNAGAM MSVDETLMCS FQILKQSLET ICLLLAYKQL VTLFSAPNYC GEFDNAGAMM
401  SVDETLMCSF QILKGNHECA SINRQLVTLF SAPNYCGEFD NAGAMMSVDE TLMCSFQILK IYGFYDECKQ LVTLFSAPNY
481  CGEFDNAGAM MSVDETLMCS FQILKTFTDC FNCLPIAAIV DEKQLVTLFS APNYCGEFDN AGAMMSVDET LMCSFQILKI
561  FCCHGGLSPD LQSMEQIRQL VTLFSAPNYC GEFDNAGAMM SVDETLMCSF QILKPTDVPD QGLLCDLLWS DPDKQLVTLF
641  SAPNYCGEFD NAGAMMSVDE TLMCSFQILK HDLDLICRQL VTLFSAPNYC GEFDNAGAMM SVDETLMCSF QILKQLVTLF
721  SAPNYCGEFD NAGAMMSVDE TLMCSFQILK SR EIFLSQPI LLELEAPLK I CGDIHGQYYD LLRGLCLKIC GDIHGQYYDL
801  LRICGDIHGQ YYDLLRICGD IHGQYYDLLR QSLETICLLL AYKICGDIHG QYYDLLRGNH ECASINRICG DIHGQYYDLL
881  RIYGFYDECK ICGDIHGQYY DLLRTFTDCF NCLPIAAIVD EKICGDIHGQ YYDLLRIFCC HGGLSPDLQS MEQIRICGDI
961  HGQYYDLLRP TDVPDQGLLC DLLWSDPDKI CGDIHGQYYD LLRHDLDLIC RICGDIHGQY YDLLRQLVTL FSAPNYCGEF
1041 DNAGAMMSVD ETLMCSFQIL K LFEYGGFPP ESNYLFLGDY VDR GKQSLET ICLLLAYKGL CLKQSLETIC LLLAYKICGD
1121 IHGQYYDLLR QSLETICLLL AYKQSLETIC LLLAYKQSLE TICLLLAYKG NHECASINRQ SLETICLLLA YKIYGFYDEC
1201 KQSLETICLL LAYKTFTDCF NCLPIAAIVD EKQSLETICL LLAYKIFCCH GGLSPDLQSM EQIRQSLETI CLLLAYKPTD
1281 VPDQGLLCDL LWSDPDKQSL ETICLLLAYK HDLDLICRQS LETICLLLAY KQLVTLFSAP NYCGEFDNAG AMMSVDETLM
1361 CSFQILK IKY PENFFLLR GN HECASINRGL CLKGNHECAS INRICGDIHG QYYDLLRGNH ECASINRQSL ETICLLLAYK
1441 GNHECASINR GNHECASINR GNHECASINR IYGFYDECKG NHECASINRT FTDCFNCLPI AAIVDEKGNH ECASINRIFC
1521 CHGGLSPDLQ SMEQIRGNHE CASINRPTDV PDQGLLCDLL WSDPDKGNHE CASINRHDLD LICRGNHECA SINRQLVTLF
1601 SAPNYCGEFD NAGAMMSVDE TLMCSFQILK IYGFYDECKG LCLKIYGFYD ECKICGDIHG QYYDLLRIYG FYDECKQSLE
1681 TICLLLAYKI YGFYDECKGN HECASINRIY GFYDECKIYG FYDECKTFTD CFNCLPIAAIV DEKIYGFYDE
1761 CKIFCCHGGL SPDLQSMEQI RIYGFYDECK PTDVPDQGLL CDLLWSDPDK IYGFYDECKH DLDLICRIYG FYDECKQLVT
1841 LFSAPNYCGE FDNAGAMMSV DETLMCSFQI LKRRYNIKLW KTFTDCFNCL PIAAIVDEKG LCLKTFTDCF NCLPIAAIVD
1921 EKICGDIHGQ YYDLLRTFTD CFNCLPIAAI VDEKQSLETI CLLLAYKTFT DCFNCLPIAA IVDEKGNHEC ASINRTFTDC
2001 FNCLPIAAIV DEKIYGFYDE CKTFTDCFNC LPIAAIVDEK TFTDCFNCLP IAAIVDEKTF TDCFNCLPIA AIVDEKIFCC
2081 HGGLSPDLQS MEQIRTFTDC FNCLPIAAIV DEKPTDVPDQ GLLCDLLWSD PDKTFTDCFN CLPIAAIVDE KHDLDLICRT
2161 FTDCFNCLPI AAIVDEKQLV TLFSAPNYCG EFDNAGAMMS VDETLMCSFQ ILKIFCCHGG LSPDLQSMEQ IRGLCLKIFC
2241 CHGGLSPDLQ SMEQIRICGD IHGQYYDLLR IFCCHGGLSP DLQSMEQIRQ SLETICLLLA YKIFCCHGGL SPDLQSMEQI
2321 RGNHECASIN RIFCCHGGLS PDLQSMEQIR IYGFYDECKI FCCHGGLSPD LQSMEQIRTF TDCFNCLPIA AIVDEKIFCC
2401 HGGLSPDLQS MEQIRIFCCH GGLSPDLQSM EQIRIFCCHG GLSPDLQSME QIRPTDVPDQ GLLCDLLWSD PDKIFCCHGG
2481 LSPDLQSMEQ IRHDLDLICR IFCCHGGLSP DLQSMEQIRQ LVTLFSAPNY CGEFDNAGAM MSVDETLMCS FQILKRIMRP
2561 TDVPDQGLLC DLLWSDPDKG LCLKPTDVPD QGLLCDLLWS DPDKICGDIH GQYYDLLRPT DVPDQGLLCD LLWSDPDKQS
2641 LETICLLLAY KPTDVPDQGL LCDLLWSDPD KGNHECASIN RPTDVPDQGL LCDLLWSDPD KIYGFYDECK PTDVPDQGLL
2721 CDLLWSDPDK TFTDCFNCLP IAAIVDEKPT DVPDQGLLCD LLWSDPDKIF CCHGGLSPDL QSMEQIRPTD VPDQGLLCDL
2801 LWSDPDKPTD VPDQGLLCDL LWSDPDKPTD VPDQGLLCDL LWSDPDKHDL DLICRPTDVP DQGLLCDLLW SDPDKQLVTL
2881 FSAPNYCGEF DNAGAMMSVD ETLMCSFQIL KPTDVPDQGL LCDLLWSDPD KDVQGWGEND R GVSFTFGAE VVAK FLHKHD
2961 LDLICRGLCL KHDLDLICRI CGDIHGQYYD LLRHDLDLIC RQSLETICLL LAYKHDLDLI CRGNHECASI NRHDLDLICR
3041 IYGFYDECKH DLDLICRTFT DCFNCLPIAA IVDEKHDLDL ICRIFCCHGG LSPDLQSMEQ IRHDLDLICR PTDVPDQGLL
3121 CDLLWSDPDK HDLDLICRHD LDLICRHDLD LICRQLVTLF SAPNYCGEFD NAGAMMSVDE TLMCSFQILK AHQVVEDGYE
3201 FFAK RQLVTL FSAPNYCGEF DNAGAMMSVD ETLMCSFQIL KGLCLKQLVT LFSAPNYCGE FDNAGAMMSV DETLMCSFQI
3281 LKICGDIHGQ YYDLLRQLVT LFSAPNYCGE FDNAGAMMSV DETLMCSFQI LKQSLETICL LLAYKQLVTL FSAPNYCGEF
3361 DNAGAMMSVD ETLMCSFQIL KGNHECASIN RQLVTLFSAP NYCGEFDNAG AMMSVDETLM CSFQILKIYG FYDECKQLVT
3441 LFSAPNYCGE FDNAGAMMSV DETLMCSFQI LKTFTDCFNC LPIAAIVDEK QLVTLFSAPN YCGEFDNAGA MMSVDETLMC
3521 SFQILKIFCC HGGLSPDLQS MEQIRQLVTL FSAPNYCGEF DNAGAMMSVD ETLMCSFQIL KPTDVPDQGL LCDLLWSDPD
3601 KQLVTLFSAP NYCGEFDNAG AMMSVDETLM CSFQILKHDL DLICRQLVTL FSAPNYCGEF DNAGAMMSVD ETLMCSFQIL
3681 KQLVTLFSAP NYCGEFDNAG AMMSVDETLM CSFQILKPAD KNKGK YGQFS GLNPGGRPIT PPR NSAKAKK
```

Legend:
- Acetylation (K) (+42.01)
- Carbamidomethylation (+57.02)
- Dehydration (-18.01)
- Dihydroxy (+31.99)
- Hydroxylation (+15.99)
- Internal disulfide bond unpaired fragmentation (-2.02)
- Lysine oxidation to aminoadipic semialdehyde (-1.03)
- Methylation (+14.02)
- Oxidation (M) (+15.99)
- Oxidation to nitro (+44.99)
- Sulphone C (+47.98)
- Deamidation (NQ) (+0.98), Dimethylation (+28.03)
- Mutation

Append 2 | The new scrambled PP-1 sequence is displayed with the potential disulfide peptides boxed in red. The red boxes indicate the peptides of interest. Database search strategy for disulfide-linked peptides under four different conditions as discussed in Figure 31.

Cys²⁰²

Im R PT D V PD QG L L C D L L W S D P D K

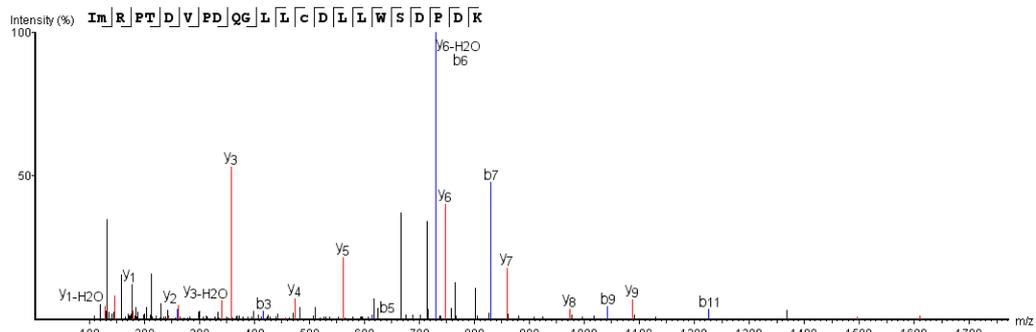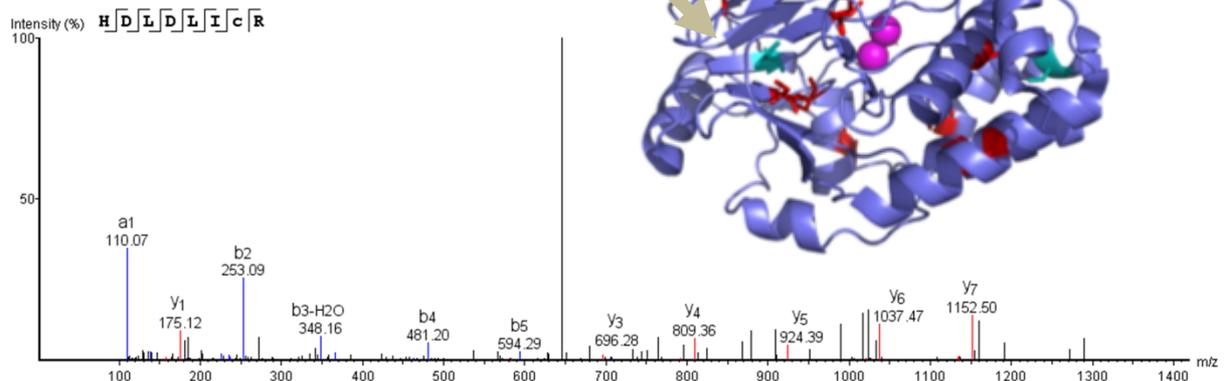| #	b	b-H2O	b (2+)	Seq	y	y-H2O	y (2+)	#
1	114.09	96.08	57.55	I				23
2	261.13	243.12	131.06	M(+15.99)	2835.26	2817.25	1418.13	22
3	417.23	399.22	209.11	R	2688.23	2670.22	1344.61	21
4	514.28	496.27	257.64	P	2532.13	2514.12	1266.56	20
5	615.32	597.32	308.16	T	2435.07	2417.06	1218.04	19
6	730.35	712.35	365.68	D	2334.03	2316.02	1167.51	18
7	829.42	811.41	415.21	V	2219.00	2200.99	1110.00	17
8	926.48	908.47	463.74	P	2119.93	2101.92	1060.47	16
9	1041.49	1023.49	521.25	D	2022.88	2004.87	1011.94	15
10	1169.56	1151.55	585.28	Q	1907.85	1889.84	954.43	14
11	1226.57	1208.57	613.79	G	1779.79	1761.78	890.40	13
12	1339.67	1321.66	670.33	L	1722.77	1704.76	861.89	12
13	1452.75	1434.74	726.88	L	1609.67	1591.68	805.34	11
14	1860.83	1842.82	930.91	C(+305.07)	1496.58	1478.59	748.80	10
15	1975.86	1957.85	988.43	D	1088.52	1070.52	544.76	9
16	2088.94	2070.93	1044.97	L	973.50	955.49	487.25	8
17	2202.02	2184.01	1101.51	L	860.41	842.40	430.71	7
18	2388.10	2370.09	1194.55	W	747.32	729.32	374.17	6
19	2475.14	2457.13	1238.07	S	561.25	543.24	281.13	5
20	2590.16	2572.15	1295.58	D	474.22	456.21	237.61	4
21	2687.22	2669.21	1344.11	P	359.19	341.14	180.10	3
22	2802.24	2784.23	1401.62	D	262.14	244.09	131.57	2
23				K	147.11	129.10	74.06	1

Cys²⁴⁵

H D L D L I C R

#	Immonium	b	b-H2O	b-NH3	a	b (2+)	Seq	y	y-H2O	y-NH3	y (2+)	#
1	110.07	138.07	120.06	121.04	110.07	69.53	H					8
2	88.04	253.09	235.08	236.07	225.10	127.05	D	1152.50	1134.48	1135.46	576.75	7
3	86.10	366.17	348.16	349.15	338.18	183.59	L	1037.47	1019.46	1020.46	519.24	6
4	88.04	481.20	463.19	464.18	453.21	241.10	D	924.39	906.38	907.36	462.70	5
5	86.10	594.29	576.28	577.26	566.29	297.64	L	809.36	791.35	792.36	405.18	4
6	86.10	707.37	689.36	690.35	679.38	354.19	I	696.28	678.27	679.25	348.64	3
7	381.09	1115.45	1097.44	1098.42	1087.46	558.23	C(+305.07)	583.19	565.19	566.17	292.10	2
8	129.11						R	175.12	157.11	158.09	88.06	1

Append 3 | Spectra and positions of the glutathione modified Cys²⁰² and Cys²⁴⁵.

6.2 Summary of all disulfide bridges

A) $-Mn^{2+}$ $-H_2O_2$

1) Cys^{39} XL Cys^{127}

#	b	b-H2O	b (2+)	Seq	y
1	58.03	40.02	29.51	G	
2	172.07	154.06	86.54	N	1573.76
3	309.13	291.12	155.07	H	1459.71
4	438.17	420.16	219.59	E	1322.64
5	541.18	523.17	271.09	C	1193.61
6	612.22	594.21	306.61	A	1090.60
7	699.25	681.24	350.13	S	1019.57
8	812.34	794.33	406.67	I	932.53
9	942.37	924.36	471.69	N(+15.99)	819.45
10	1098.48	1080.46	549.74	R	689.41
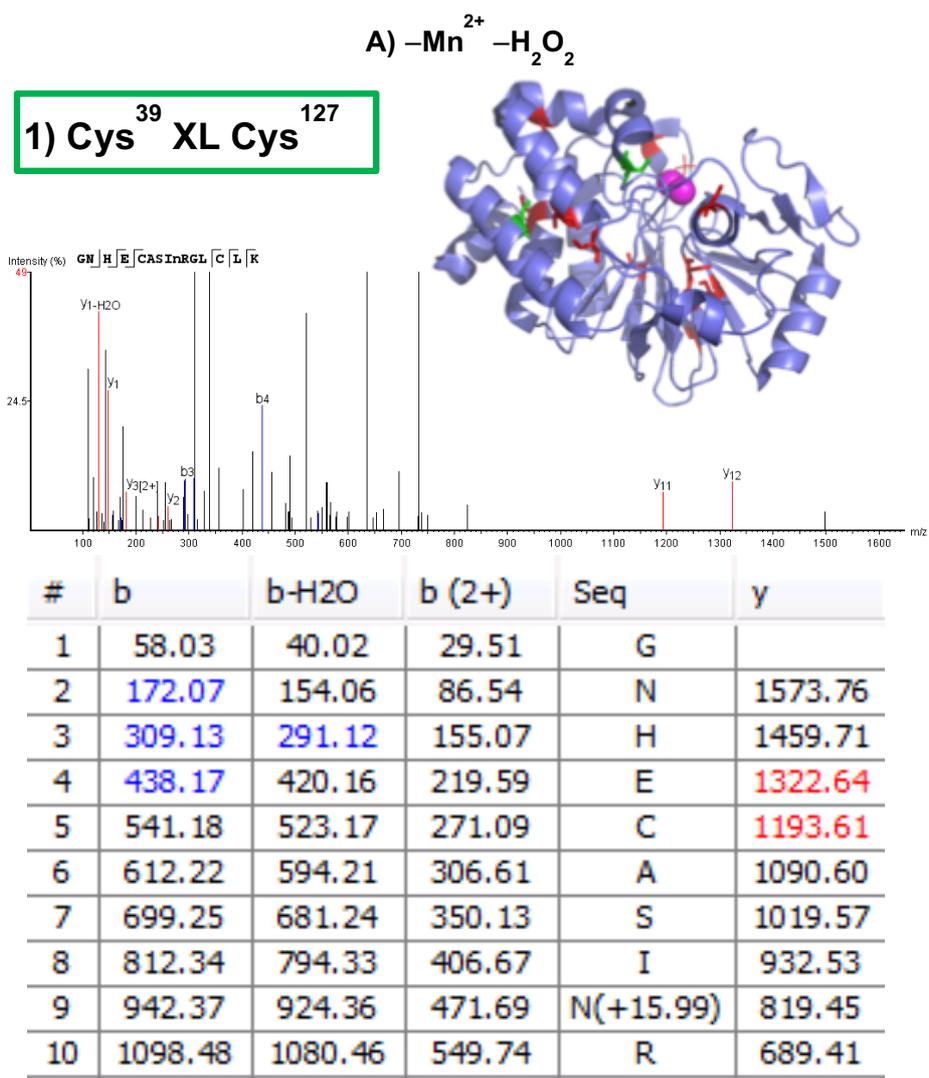
2) Cys^{127} XL Cys^{140}
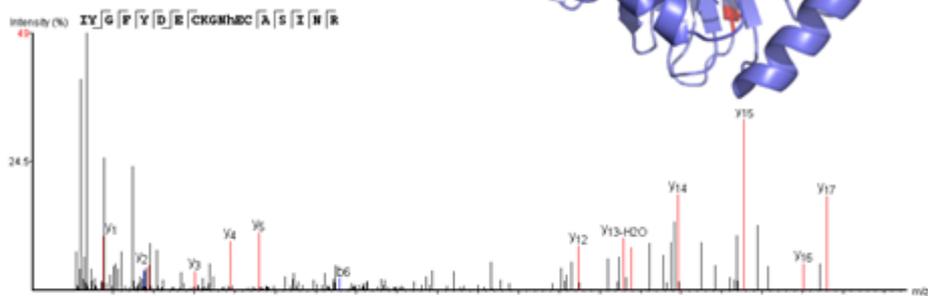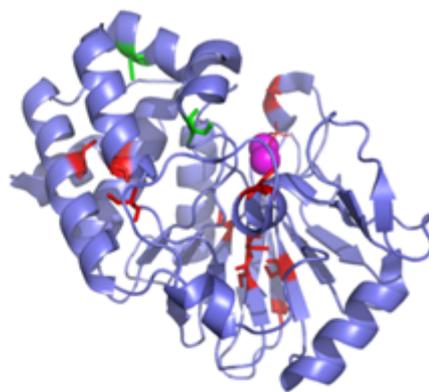
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1	114.09	96.08	57.55	I				19
2	277.15	259.14	139.08	Y	2121.88	2103.86	1061.44	18
3	334.18	316.17	167.59	G	1958.81	1940.80	979.91	17
4	481.25	463.23	241.12	F	1901.79	1883.78	951.40	16
5	644.31	626.30	322.65	Y	1754.72	1736.71	877.86	15
6	759.35	741.32	380.17	D	1591.65	1573.65	796.33	14
7	888.38	870.37	444.69	E	1476.63	1458.60	738.82	13
8	991.39	973.38	496.19	C	1347.59	1329.58	674.29	12
9	1119.48	1101.47	560.24	K	1244.58	1226.57	622.79	11
10	1176.50	1158.49	588.75	G	1116.49	1098.47	558.74	10
11	1290.55	1272.54	645.77	N	1059.46	1041.45	530.23	9
12	1443.60	1425.59	722.30	H(+15.99)	945.42	927.41	473.21	8
13	1572.64	1554.63	786.82	E	792.37	774.36	396.68	7
14	1675.65	1657.64	838.33	C	663.32	645.31	332.16	6
15	1746.69	1728.68	873.84	A	560.31	542.30	280.66	5
16	1833.72	1815.71	917.36	S	489.28	471.27	245.14	4
17	1946.81	1928.79	973.90	I	402.24	384.24	201.62	3
18	2060.85	2042.84	1030.92	N	289.16	271.15	145.08	2
19				R	175.12	157.11	88.06	1

3) Cys^{127} XL Cys^{245}
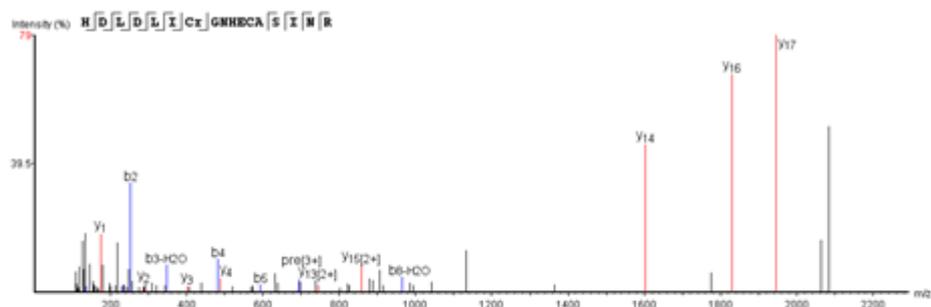
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1	138.06	120.06	69.53	H				18
2	253.09	235.08	127.05	D	1944.89	1926.89	972.95	17
3	366.18	348.16	183.59	L	1829.87	1811.86	915.44	16
4	481.20	463.19	241.10	D	1716.79	1698.78	858.90	15
5	594.28	576.28	297.64	L	1601.74	1583.75	801.38	14
6	707.37	689.31	354.19	I	1488.68	1470.67	744.84	13
7	810.38	792.37	405.69	C	1375.60	1357.58	688.30	12
8	982.48	964.44	491.74	R(+15.99)	1272.59	1254.58	636.79	11
9	1039.50	1021.49	520.25	G	1100.49	1082.48	550.75	10
10	1153.54	1135.53	577.27	N	1043.47	1025.46	522.23	9
11	1290.60	1272.59	645.80	H	929.43	911.42	465.21	8
12	1419.64	1401.63	710.32	E	792.37	774.36	396.68	7
13	1522.65	1504.64	761.83	C	663.32	645.31	332.16	6
14	1593.69	1575.68	797.35	A	560.32	542.30	280.66	5
15	1680.72	1662.71	840.86	S	489.27	471.27	245.14	4
16	1793.81	1775.80	897.40	I	402.24	384.24	201.62	3
17	1907.85	1889.84	954.42	N	289.16	271.15	145.08	2
18				R	175.12	157.11	88.06	1

B) $-Mn^{2+} +H_2O_2$

1) Cys^{39} XL Cys^{62}
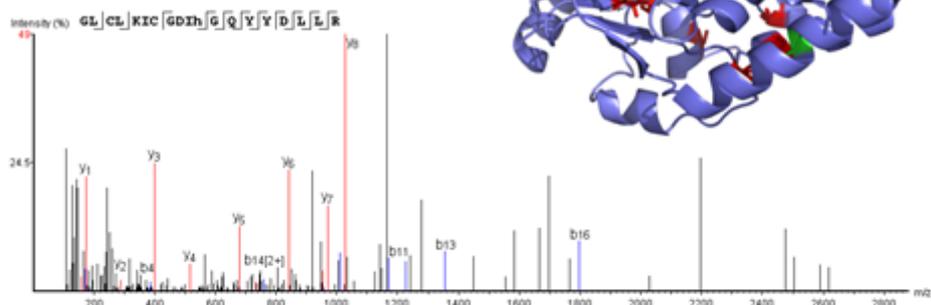
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2	171.11	153.10	86.06	L	2139.07	2121.06	1070.04	18
3	274.12	256.11	137.56	C	2025.99	2007.98	1013.49	17
4	387.20	369.20	194.10	L	1922.98	1904.97	961.99	16
5	515.30	497.29	258.15	K	1809.90	1791.88	905.45	15
6	628.39	610.38	314.69	I	1681.80	1663.79	841.40	14
7	731.39	713.38	366.20	C	1568.72	1550.71	784.86	13
8	788.42	770.41	394.71	G	1465.71	1447.70	733.37	12
9	903.44	885.43	452.22	D	1408.69	1390.68	704.84	11
10	1016.53	998.52	508.76	I	1293.66	1275.65	647.33	10
11	1169.58	1151.57	585.29	H(+15.99)	1180.57	1162.56	590.79	9
12	1226.60	1208.59	613.80	G	1027.52	1009.51	514.26	8
13	1354.65	1336.65	677.83	Q	970.49	952.48	485.75	7
14	1517.72	1499.71	759.37	Y	842.44	824.43	421.72	6
15	1680.79	1662.78	840.89	Y	679.38	661.37	340.19	5
16	1795.81	1777.80	898.41	D	516.31	498.30	258.66	4
17	1908.90	1890.89	954.95	L	401.29	383.28	201.14	3
18	2021.98	2003.97	1011.47	L	288.20	270.19	144.60	2
19				R	175.12	157.11	88.06	1

2) Cys³⁹ XL Cys¹⁰⁵
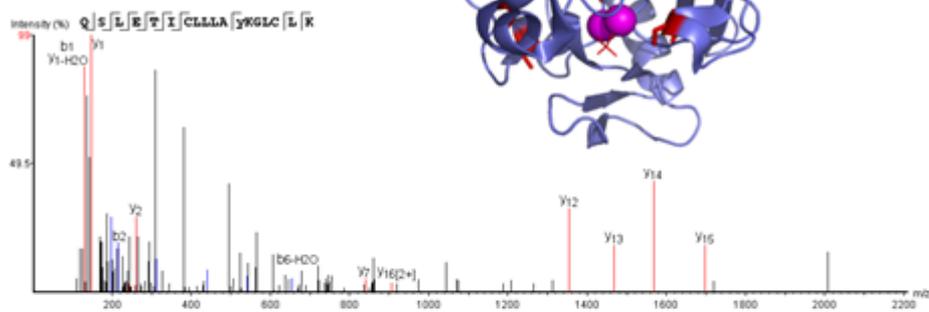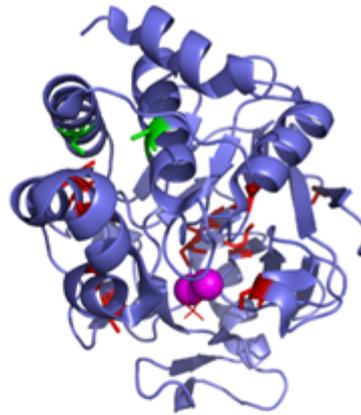
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2	216.10	198.09	108.55	S	1897.05	1879.04	949.03	17
3	329.18	311.17	165.09	L	1810.02	1792.01	905.50	16
4	458.23	440.21	229.61	E	1696.93	1678.93	848.97	15
5	559.27	541.26	280.14	T	1567.89	1549.88	784.45	14
6	672.36	654.34	336.68	I	1466.85	1448.84	733.92	13
7	775.37	757.36	388.18	C	1353.75	1335.75	677.38	12
8	888.45	870.44	444.73	L	1250.75	1232.74	625.88	11
9	1001.53	983.52	501.27	L	1137.67	1119.66	569.33	10
10	1114.62	1096.61	557.81	L	1024.59	1006.58	512.79	9
11	1185.66	1167.64	593.33	A	911.50	893.49	456.25	8
12	1364.71	1346.70	682.86	Y(+15.99)	840.47	822.45	420.73	7
13	1492.81	1474.80	746.90	K	661.41	643.40	331.20	6
14	1549.83	1531.82	775.41	G	533.31	515.30	267.16	5
15	1662.91	1644.90	831.96	L	476.29	458.28	238.65	4
16	1765.92	1747.91	883.46	C	363.21	345.20	182.10	3
17	1879.01	1861.00	940.00	L	260.20	242.18	130.60	2
18				K	147.11	129.10	74.06	1

3) Cys³⁹ XL Cys¹²⁷
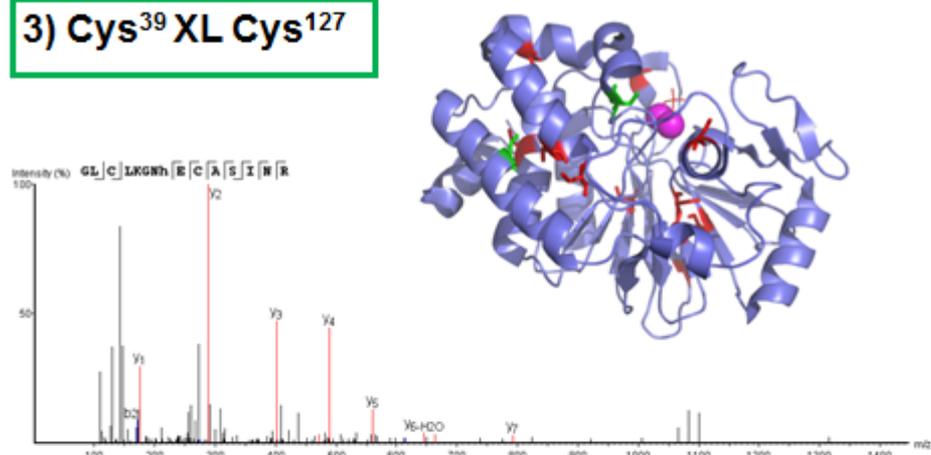
#	b	b-H2O	b (2+)	Seq	y	y-H2O	y (2+)	#
1	58.03	40.02	29.51	G				15
2	171.11	153.10	86.06	L	1573.76	1555.75	787.38	14
3	274.12	256.11	137.56	C	1460.67	1442.66	730.84	13
4	387.21	369.20	194.10	L	1357.66	1339.65	679.33	12
5	515.30	497.29	258.15	K	1244.58	1226.57	622.79	11
6	572.32	554.31	286.66	G	1116.49	1098.47	558.74	10
7	686.37	668.36	343.68	N	1059.46	1041.45	530.23	9
8	839.42	821.41	420.21	H(+15.99)	945.42	927.41	473.21	8
9	968.46	950.45	484.73	E	792.37	774.36	396.68	7
10	1071.47	1053.46	536.24	C	663.32	645.31	332.16	6
11	1142.51	1124.50	571.75	A	560.31	542.30	280.66	5
12	1229.54	1211.53	615.27	S	489.28	471.27	245.14	4
13	1342.62	1324.61	671.81	I	402.24	384.24	201.62	3
14	1456.67	1438.66	728.83	N	289.16	271.15	145.08	2
15				R	175.12	157.11	88.06	1

4) Cys³⁹ XL Cys¹⁴⁰
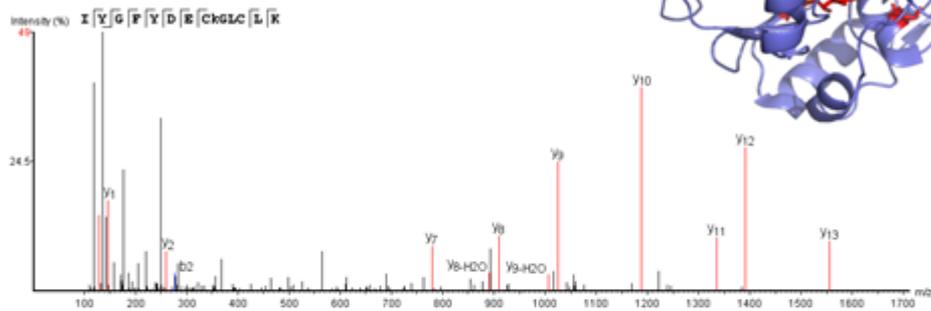
#	b	b-H2O	b (2+)	Seq	y	y-H2O	y (2+)	#
1	114.09	96.08	57.55	I				14
2	277.15	259.14	139.08	Y	1554.70	1536.69	777.85	13
3	334.18	316.17	167.59	G	1391.62	1373.62	696.32	12
4	481.25	463.23	241.12	F	1334.61	1316.60	667.81	11
5	644.31	626.30	322.65	Y	1187.54	1169.53	594.27	10
6	759.34	741.32	380.17	D	1024.48	1006.47	512.74	9
7	888.38	870.37	444.69	E	909.45	891.45	455.23	8
8	991.39	973.38	496.19	C	780.41	762.40	390.71	7
9	1135.48	1117.47	568.24	K(+15.99)	677.40	659.39	339.20	6
10	1192.50	1174.49	596.75	G	533.31	515.30	267.16	5
11	1305.58	1287.57	653.29	L	476.29	458.28	238.65	4
12	1408.59	1390.58	704.80	C	363.21	345.20	182.10	3
13	1521.68	1503.67	761.34	L	260.20	242.19	130.60	2
14				K	147.11	129.10	74.06	1

5) Cys³⁹ XL Cys¹⁵⁵Cys¹⁵⁸
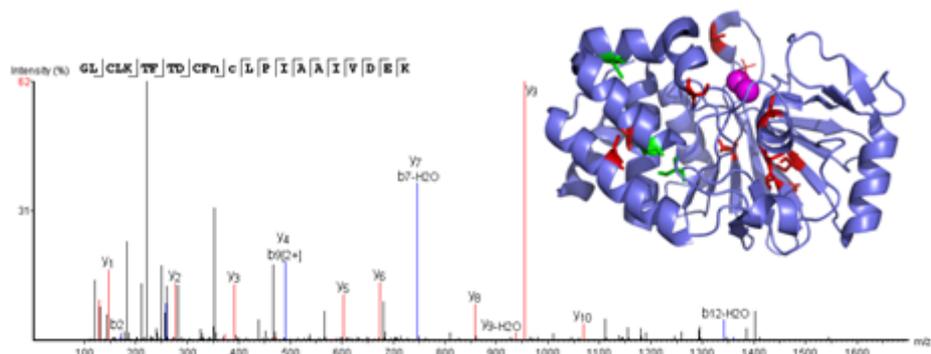
#	b	b-H2O	b (2+)	Seq	y	y-H2O	y (2+)	#
1	58.03	40.02	29.51	G				23
2	171.11	153.10	86.06	L	2521.20	2503.19	1261.10	22
3	274.12	256.11	137.56	C	2408.12	2390.11	1204.56	21
4	387.21	369.20	194.10	L	2305.11	2287.10	1153.05	20
5	515.30	497.29	258.14	K	2192.02	2174.01	1096.51	19
6	616.35	598.34	308.67	T	2063.93	2045.92	1032.46	18
7	763.42	745.40	382.21	F	1962.88	1944.87	981.94	17
8	864.47	846.45	432.73	T	1815.81	1797.80	908.41	16
9	979.49	961.48	490.25	D	1714.77	1696.76	857.88	15
10	1082.50	1064.49	541.75	C	1599.74	1581.73	800.37	14
11	1229.57	1211.56	615.28	F	1496.73	1478.72	748.87	13
12	1359.60	1341.59	680.30	N(+15.99)	1349.66	1331.65	675.33	12
13	1510.60	1492.59	755.80	C(+47.98)	1219.62	1201.61	610.31	11
14	1623.69	1605.68	812.34	L	1068.63	1050.62	534.81	10
15	1720.74	1702.73	860.87	P	955.54	937.54	478.27	9
16	1833.82	1815.81	917.41	I	858.49	840.48	429.75	8
17	1904.86	1886.85	952.93	A	745.40	727.40	373.20	7
18	1975.90	1957.89	988.45	A	674.37	656.36	337.69	6
19	2088.98	2070.97	1044.99	I	603.33	585.33	302.17	5
20	2188.05	2170.04	1094.52	V	490.25	472.24	245.63	4
21	2303.08	2285.07	1152.04	D	391.18	373.17	196.09	3
22	2432.12	2414.11	1216.56	E	276.15	258.14	138.58	2
23				K	147.11	129.10	74.06	1

6) Cys³⁹ XL Cys²⁰²
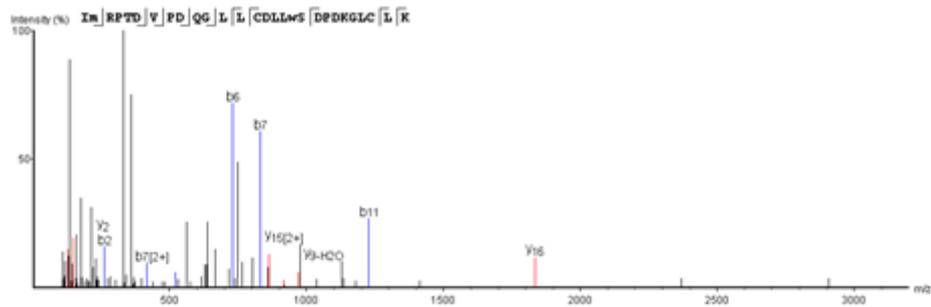
#	b	b+H2O	b (2+)	Seq	y	y+H2O	y (2+)	#
1	114.09	96.08	57.55	I				28
2	261.13	243.12	131.06	M(+15.99)	3060.48	3042.47	1530.74	27
3	417.23	399.22	209.11	R	2913.45	2895.44	1457.22	26
4	514.28	496.27	257.64	P	2757.35	2739.34	1379.17	25
5	615.33	597.32	308.16	T	2660.29	2642.28	1330.65	24
6	730.35	712.35	365.68	D	2559.25	2541.24	1280.12	23
7	829.42	811.41	415.21	V	2444.22	2426.21	1222.61	22
8	926.48	908.47	463.74	P	2345.15	2327.14	1173.08	21
9	1041.50	1023.49	521.25	D	2248.10	2230.09	1124.55	20
10	1169.56	1151.55	585.28	Q	2133.07	2115.06	1067.04	19
11	1226.57	1208.57	613.79	G	2005.01	1987.00	1003.01	18
12	1339.67	1321.66	670.33	L	1947.99	1929.98	974.50	17
13	1452.75	1434.74	726.88	L	1834.89	1816.90	917.95	16
14	1555.76	1537.75	778.38	C	1721.82	1703.81	861.41	15
15	1670.79	1652.78	835.89	D	1618.81	1600.80	809.91	14
16	1783.87	1765.86	892.44	L	1503.79	1485.78	752.39	13
17	1896.96	1878.95	948.98	L	1390.70	1372.69	695.85	12
18	2099.03	2081.02	1050.02	W(+15.99)	1277.62	1259.61	639.31	11
19	2186.06	2168.05	1093.53	S	1075.55	1057.53	538.27	10
20	2301.09	2283.08	1151.04	D	988.51	970.52	494.76	9
21	2398.14	2380.13	1199.57	P	873.49	855.48	437.24	8
22	2513.17	2495.16	1257.08	D	776.43	758.42	388.72	7
23	2641.26	2623.25	1321.13	K	661.41	643.40	331.20	6
24	2698.29	2680.28	1349.64	G	533.31	515.30	267.16	5
25	2811.37	2793.36	1406.18	L	476.29	458.28	238.65	4
26	2914.38	2896.37	1457.69	C	363.21	345.20	182.10	3
27	3027.46	3009.45	1514.23	L	260.20	242.19	130.60	2
28				K	147.11	129.10	74.06	1

7) Cys¹²⁷ XL Cys¹⁴⁰
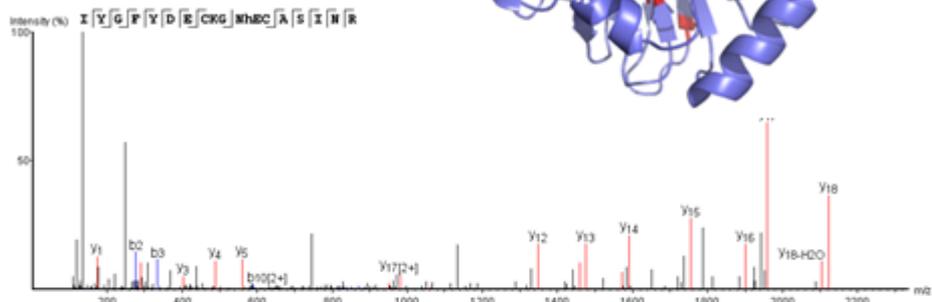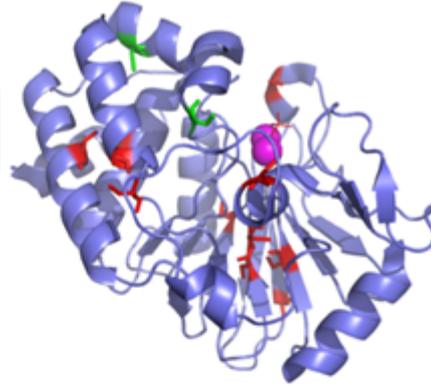
#	b	b+H2O	b (2+)	Seq	y	y+H2O	y (2+)	#
1	114.09	96.08	57.55	I				19
2	277.15	259.14	139.09	Y	2121.86	2103.86	1061.43	18
3	334.18	316.17	167.59	G	1958.80	1940.80	979.91	17
4	481.25	463.23	241.12	F	1901.79	1883.78	951.40	16
5	644.31	626.30	322.65	Y	1754.71	1736.71	877.86	15
6	759.34	741.32	380.17	D	1591.66	1573.63	796.33	14
7	888.38	870.35	444.69	E	1476.63	1458.61	738.82	13
8	991.39	973.38	496.19	C	1347.60	1329.58	674.29	12
9	1119.48	1101.47	560.24	K	1244.58	1226.57	622.79	11
10	1176.50	1158.49	588.77	G	1116.49	1098.47	558.74	10
11	1290.55	1272.54	645.77	N	1059.46	1041.45	530.23	9
12	1443.60	1425.59	722.30	H(+15.99)	945.42	927.41	473.21	8
13	1572.64	1554.63	786.82	E	792.37	774.36	396.68	7
14	1675.65	1657.64	838.35	C	663.32	645.31	332.16	6
15	1746.69	1728.68	873.84	A	560.31	542.30	280.66	5
16	1833.72	1815.71	917.36	S	489.28	471.27	245.14	4
17	1946.81	1928.79	973.90	I	402.24	384.24	201.62	3
18	2060.85	2042.84	1030.92	N	289.16	271.15	145.08	2
19				R	175.12	157.11	88.06	1

8) Cys^{127} XL Cys^{245}
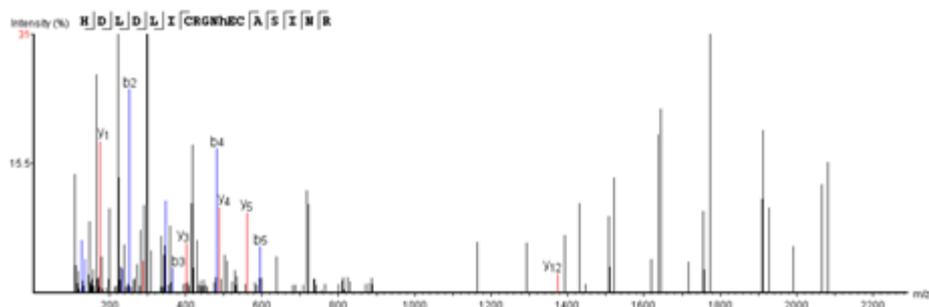
#	b	b-H2O	b (2+)	Seq	y	y-H2O	y (2+)	#
1	138.07	120.06	69.53	H				18
2	253.09	235.08	127.05	D	1944.90	1926.89	972.95	17
3	366.18	348.16	183.59	L	1829.87	1811.86	915.44	16
4	481.20	463.19	241.10	D	1716.79	1698.78	858.90	15
5	594.29	576.28	297.64	L	1601.76	1583.75	801.38	14
6	707.37	689.36	354.19	I	1488.68	1470.67	744.84	13
7	810.38	792.37	405.69	C	1375.60	1357.58	688.30	12
8	966.48	948.47	483.74	R	1272.59	1254.58	636.79	11
9	1023.50	1005.49	512.25	G	1116.49	1098.47	558.74	10
10	1137.55	1119.54	569.27	N	1059.46	1041.45	530.23	9
11	1290.60	1272.59	645.80	H(+15.99)	945.42	927.41	473.21	8
12	1419.64	1401.63	710.32	E	792.37	774.36	396.68	7
13	1522.65	1504.64	761.83	C	663.32	645.31	332.16	6
14	1593.69	1575.68	797.35	A	560.31	542.30	280.66	5
15	1680.72	1662.71	840.86	S	489.28	471.27	245.14	4
16	1793.81	1775.80	897.40	I	402.24	384.24	201.62	3
17	1907.85	1889.84	954.42	N	289.16	271.15	145.08	2
18				R	175.12	157.11	88.06	1

9) Cys^{127} XL Cys^{155}Cys^{158}

GNH|E|cASInRTFTDCFNC|L|PI|A|A|I|V|D|E|K
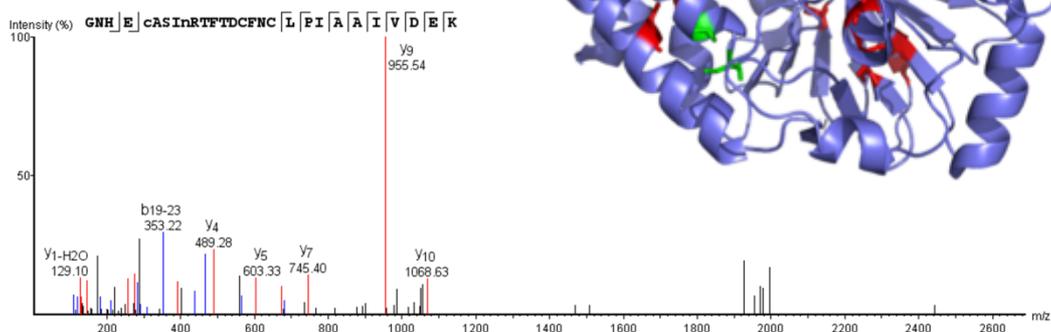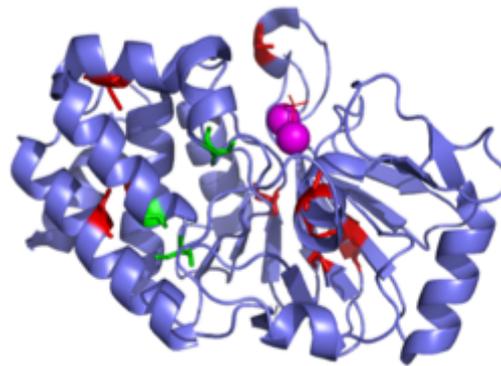
#	Immonium	b	b-H2O	b-NH3	a	b (2+)	Seq	y	y-H2O	y-NH3	y (2+)	#
1	30.03	58.03	40.02	41.00	30.03	29.51	G					28
2	87.06	172.07	154.06	155.05	144.08	86.54	N	3088.38	3070.37	3071.35	1544.69	27
3	110.07	309.13	291.12	292.10	281.14	155.07	H	2974.34	2956.33	2957.31	1487.67	26
4	102.06	438.17	420.16	421.15	410.18	219.59	E	2837.28	2819.27	2820.25	1419.14	25
5	124.01	589.17	571.16	572.14	561.17	295.08	C(+47.98)	2708.24	2690.23	2691.21	1354.62	24
6	44.05	660.20	642.19	643.18	632.21	330.60	A	2557.24	2539.23	2540.22	1279.12	23
7	60.04	747.24	729.23	730.21	719.24	374.12	S	2486.21	2468.19	2469.18	1243.60	22
8	86.10	860.32	842.31	843.29	832.33	430.66	I	2399.17	2381.16	2382.15	1200.09	21
9	103.05	990.36	972.35	973.33	962.36	495.68	N(+15.99)	2286.09	2268.08	2269.06	1143.54	20
10	129.10	1146.46	1128.45	1129.43	1118.46	573.73	R	2156.05	2138.04	2139.02	1078.53	19
11	74.06	1247.51	1229.50	1230.48	1219.51	624.25	T	1999.95	1981.94	1982.92	1000.48	18
12	120.08	1394.58	1376.57	1377.55	1366.58	697.79	F	1898.90	1880.89	1881.88	949.95	17
13	74.06	1495.62	1477.61	1478.60	1467.63	748.31	T	1751.83	1733.82	1734.81	876.42	16
14	88.04	1610.65	1592.64	1593.62	1582.66	805.83	D	1650.79	1632.78	1633.76	825.89	15
15	76.02	1713.66	1695.65	1696.63	1685.66	857.33	C	1535.76	1517.75	1518.73	768.38	14
16	120.08	1860.73	1842.72	1843.70	1832.73	930.86	F	1432.75	1414.74	1415.72	716.88	13
17	87.06	1974.77	1956.76	1957.74	1946.78	987.89	N	1285.68	1267.67	1268.66	643.34	12
18	76.02	2077.78	2059.77	2060.75	2049.79	1039.39	C	1171.64	1153.63	1154.61	586.32	11
19	86.10	2190.86	2172.85	2173.84	2162.87	1095.93	L	1068.63	1050.62	1051.60	534.81	10
20	70.07	2287.92	2269.91	2270.89	2259.92	1144.46	P	955.54	937.54	938.52	478.27	9
21	86.10	2401.00	2382.99	2383.97	2373.01	1201.00	I	858.49	840.48	841.47	429.75	8
22	44.05	2472.04	2454.03	2455.01	2444.04	1236.52	A	745.40	727.40	728.38	373.20	7
23	44.05	2543.08	2525.06	2526.05	2515.08	1272.04	A	674.37	656.36	657.34	337.69	6
24	86.10	2656.16	2638.15	2639.13	2628.16	1328.58	I	603.33	585.32	586.31	302.17	5
25	72.08	2755.23	2737.22	2738.20	2727.23	1378.11	V	490.25	472.24	473.22	245.63	4
26	88.04	2870.25	2852.24	2853.23	2842.26	1435.63	D	391.18	373.17	374.16	196.09	3
27	102.06	2999.30	2981.29	2982.27	2971.30	1500.15	E	276.15	258.14	259.13	138.58	2
28	101.11						K	147.11	129.10	130.09	74.06	1

10) Cys^{140} XL Cys^{155}Cys^{158}
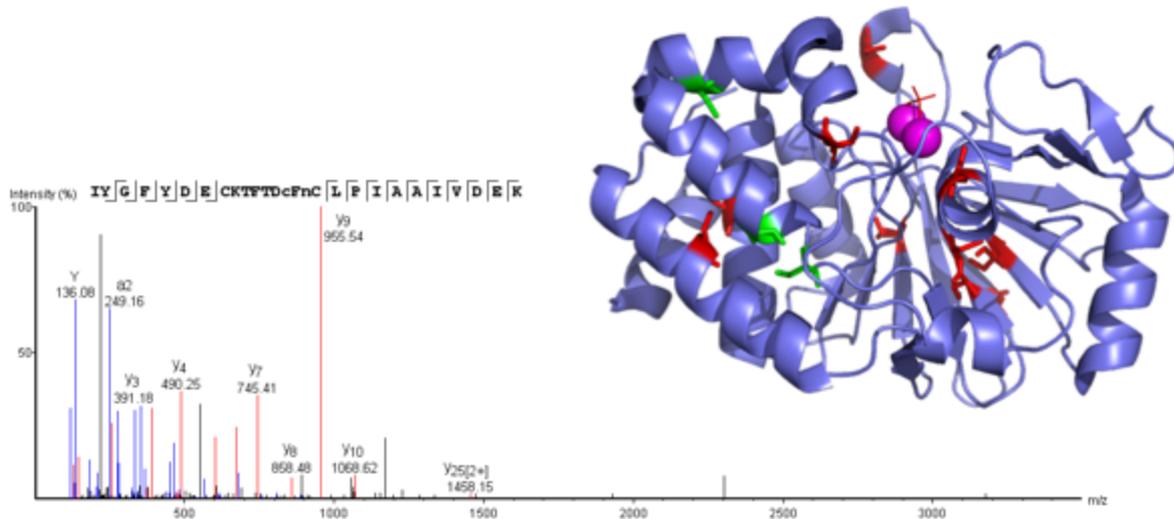
#	Immonium	b	b-H2O	b-NH3	a	b (2+)	Seq	y	y-H2O	y-NH3	y (2+)	#
1	86.10	114.09	96.08	97.06	86.10	57.55	I					27
2	136.08	277.15	259.14	260.13	249.16	139.08	Y	3078.36	3060.35	3061.33	1539.68	26
3	30.03	334.17	316.17	317.15	306.18	167.59	G	2915.29	2897.28	2898.27	1458.15	25
4	120.08	481.24	463.23	464.21	453.25	241.12	F	2858.27	2840.26	2841.24	1429.64	24
5	136.08	644.31	626.30	627.28	616.31	322.65	Y	2711.20	2693.19	2694.18	1356.10	23
6	88.04	759.33	741.32	742.31	731.34	380.17	D	2548.14	2530.13	2531.11	1274.57	22
7	102.06	888.38	870.37	871.35	860.38	444.69	E	2433.11	2415.10	2416.09	1217.06	21
8	76.02	991.39	973.38	974.36	963.39	496.19	C	2304.07	2286.06	2287.04	1152.54	20
9	101.11	1119.48	1101.47	1102.46	1091.49	560.24	K	2201.06	2183.05	2184.03	1101.03	19
10	74.06	1220.53	1202.52	1203.50	1192.53	610.76	T	2072.97	2054.96	2055.94	1036.98	18
11	120.08	1367.60	1349.59	1350.57	1339.60	684.30	F	1971.92	1953.91	1954.89	986.46	17
12	74.06	1468.65	1450.64	1451.62	1440.65	734.82	T	1824.85	1806.84	1807.82	912.93	16
13	88.04	1583.67	1565.66	1566.65	1555.68	792.34	D	1723.80	1705.79	1706.78	862.40	15
14	133.04	1743.70	1725.69	1726.68	1715.71	872.35	C(+57.02)	1608.78	1590.77	1591.75	804.89	14
15	120.08	1890.77	1872.76	1873.74	1862.78	945.89	F	1448.75	1430.73	1431.72	724.87	13
16	103.05	2020.81	2002.80	2003.78	1992.81	1010.90	N(+15.99)	1301.68	1283.67	1284.65	651.34	12
17	76.02	2123.82	2105.81	2106.79	2095.82	1062.41	C	1171.64	1153.63	1154.61	586.32	11
18	86.10	2236.90	2218.89	2219.88	2208.91	1118.95	L	1068.62	1050.62	1051.60	534.81	10
19	70.07	2333.96	2315.95	2316.93	2305.96	1167.48	P	955.54	937.54	938.52	478.28	9
20	86.10	2447.04	2429.03	2430.01	2419.04	1224.02	I	858.48	840.48	841.47	429.75	8
21	44.05	2518.08	2500.07	2501.05	2490.08	1259.54	A	745.41	727.40	728.38	373.20	7
22	44.05	2589.11	2571.10	2572.09	2561.12	1295.06	A	674.37	656.36	657.34	337.69	6
23	86.10	2702.20	2684.19	2685.17	2674.20	1351.60	I	603.33	585.32	586.31	302.17	5
24	72.08	2801.27	2783.26	2784.24	2773.27	1401.13	V	490.25	472.24	473.22	245.63	4
25	88.04	2916.29	2898.28	2899.27	2888.30	1458.65	D	391.18	373.17	374.16	196.09	3
26	102.06	3045.34	3027.33	3028.31	3017.34	1523.17	E	276.16	258.14	259.13	138.58	2
27	101.11						K	147.11	129.10	130.09	74.06	1

C) +Mn^{2+} -H$_2$O$_2$

1) Cys^{39} XL Cys^{127}
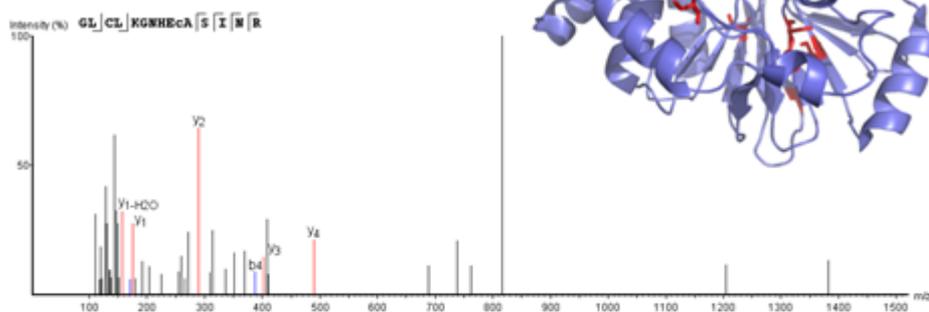
#	b	b+H2O	b (2+)	Seq	y	y-H2O	y (2+)	#
1	58.03	40.02	29.51	G				15
2	171.11	153.10	86.06	L	1573.76	1555.75	787.38	14
3	274.12	256.11	137.56	C	1460.67	1442.66	730.84	13
4	387.20	369.20	194.10	L	1357.66	1339.65	679.33	12
5	515.30	497.29	258.15	K	1244.58	1226.57	622.79	11
6	572.32	554.31	286.66	G	1116.49	1098.47	558.74	10
7	686.37	668.36	343.68	N	1059.46	1041.45	530.23	9
8	823.42	805.41	412.21	H	945.42	927.41	473.21	8
9	952.47	934.46	476.73	E	808.36	790.35	404.68	7
10	1071.47	1053.46	536.24	C(+15.99)	679.32	661.31	340.16	6
11	1142.51	1124.50	571.75	A	560.32	542.30	280.66	5
12	1229.54	1211.53	615.27	S	489.28	471.27	245.14	4
13	1342.62	1324.61	671.81	I	402.25	384.24	201.62	3
14	1456.67	1438.66	728.83	N	289.16	271.15	145.08	2
15				R	175.12	157.08	88.06	1

2) Cys¹²⁷ XL Cys¹⁴⁰
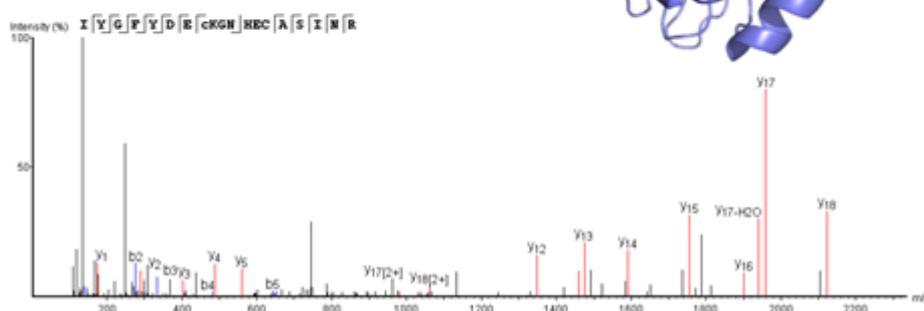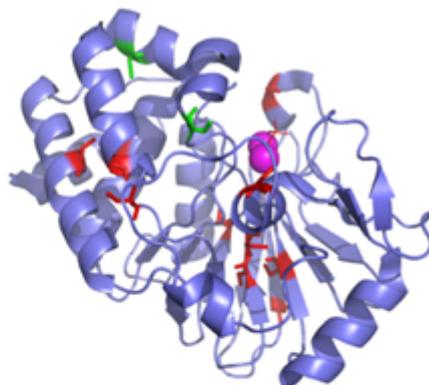
#	b	b-H2O	b (2+)	Seq	y	y-H2O	y (2+)	#
1	114.09	96.08	57.55	I				19
2	277.15	259.14	139.09	Y	2121.87	2103.86	1061.44	18
3	334.17	316.17	167.59	G	1958.81	1940.78	979.91	17
4	481.20	463.23	241.12	F	1901.78	1883.78	951.40	16
5	644.29	626.30	322.65	Y	1754.70	1736.71	877.86	15
6	759.34	741.32	380.17	D	1591.64	1573.65	796.33	14
7	888.38	870.37	444.69	E	1476.63	1458.61	738.82	13
8	1007.38	989.37	504.19	C(+15.99)	1347.58	1329.58	674.29	12
9	1135.48	1117.47	568.24	K	1228.59	1210.57	614.79	11
10	1192.50	1174.49	596.75	G	1100.49	1082.48	550.75	10
11	1306.54	1288.53	653.79	N	1043.47	1025.46	522.23	9
12	1443.60	1425.59	722.30	H	929.43	911.42	465.21	8
13	1572.64	1554.63	786.82	E	792.37	774.36	396.68	7
14	1675.65	1657.64	838.33	C	663.32	645.31	332.16	6
15	1746.69	1728.68	873.84	A	560.31	542.30	280.66	5
16	1833.72	1815.71	917.36	S	489.28	471.27	245.14	4
17	1946.81	1928.79	973.90	I	402.24	384.24	201.62	3
18	2060.85	2042.84	1030.92	N	289.16	271.15	145.08	2
19				R	175.12	157.11	88.06	1

Append 4 | All disulfide-peptide conjugates are shown in their spectrum and fragment table. In the structure plot, the two Cys involved are indicated in green.

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Zhu, C., Johansen, F.E., and Prywes, R. (1997). Interaction of ATF6 and serum response factor. Molecular and Cellular Biology *17*, 4957-4966.

8 Curriculum vitae

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Experience

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Education

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Justus-Liebig-Universität, Giessen, Germany

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Skills

Language skills	English: Fluent in speech, writing and reading (IELTS band score =7.0)
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Extra qualification	Vice president (external relations), AIESEC Giessen, Germany
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Posters presentation

Singh S., Saadatmand A.R., Vettel C., El-Armouche A. (2014). Redox regulation of protein phosphates -1 in cardiomyocytes. Poster presentation at the 80th Annual Meeting of the German Society for Experimental and Clinical Pharmacology and Toxicology (DGPT), Hannover, Germany.

Simranjit Singh, Susanne Lutz (2015). Endoplasmic reticulum stress and oxidative stress – A mixed blessing for connective tissue growth factor in cardiomyocytes. Poster presentation at the Stress Proteins in Growth, Development & Disease, Gordon Research Conference, Tuscany, Italy.