

Thesis Title : Investigating The Structural and Functional Changes Due to Variations In Redox-Regulating Glutathione Peroxidases and Thioredoxin Reductases.

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Background

Selenium is an essential micronutrient with important functions in human health and several pathophysiological conditions. The biological effects of selenium are largely mediated by selenium-containing proteins (selenoproteins). Selenoproteins represent diverse molecular pathways and biological functions [1]. There are 25 genes encoding selenoproteins in humans [1]. In selenoproteins, the 21st amino acid selenocysteine (Sec, U) is inserted in the nascent polypeptide chain during the process of translational recoding of the UGA stop codon [2]. In addition to the UGA-codon, a cis-acting element in the mRNA, called Sec-insertion sequence (SECIS), binds SBP2 to recruit other proteins, such as eukaryotic elongation factor selenocysteine-tRNA specific, to form the complex responsible for the recoding process [3]. Those peptides or proteins or enzymes containing selenocysteine are called selenopeptides or selenoproteins or selenoenzymes respectively. These selenoproteins play a critical role in antioxidant defense, hormone metabolism, immune responses and muscle development. [1] There are selenoproteins that have incorporated Sec under a precise process requiring the UGA codon, a specified tRNA (Sec tRNA [Ser]Sec), some regulatory proteins, and the SECIS element. Second, there are proteins that contain selenomethionine (SeMet), in addition to Sec. The third class consists of selenium-binding proteins (SBP), which bind Se by some unknown mechanisms [4]. All these selenoproteins are broadly divided into three families such as Glutathione peroxidases (GPXs), Thioredoxin reductases (TRs) and Iodothyronine deiodinases (DIOs). Being an important component of various antioxidant selenoprotein enzymes like glutathione peroxidase (GPX) and thioredoxin reductase (TRXR), plays an important role in combating oxidative stress caused due to excessive generation of ROS (reactive oxygen species). We will be focusing on two protein families Glutathione Peroxidases and Thioredoxin Reductases which have isoforms with selenocysteine and cysteine at the active sites. The presence of selenium improves the redox properties of the protein.



Fig.1 Chemical structure of selenocysteine and cysteine. The two compounds differ by one atom, a selenium atom in selenocysteine and a sulfur atom in cysteine.

These two proteins take place in redox reactions. Glutathione peroxidase (GPx) oxidizes thiols to disulfides with an active site that contains a Sec and thioredoxin reductases (TrxR) feature a selenosulfide bond (GCUG motif) in the active site that reduces disulfides to thiols.[5]

- Glutathione Peroxidase

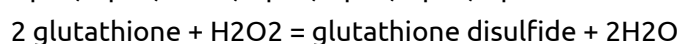
Glutathione acts as an antioxidant, a free radical scavenger and a detoxifying agent. It also acts as a coenzyme for enzymes and it protects against oxygen radicals and toxic compounds. Se- dependent GSH peroxidase is capable of utilizing hydrogen peroxide (H₂O₂) and a variety of organic hydroperoxides as substrates. Glutaredoxins belong to the thioredoxin superfamily of structurally similar thiol-disulfide oxidoreductases catalyzing thiol-disulfide exchange reactions via reversible oxidation of two active-site cysteine residue. The GPX and TRXR gene is conserved in human, chimpanzee, rhesus monkey, dog, cow, rat, chicken, zebrafish, and frog. This family includes various isoforms in humans such as GPX1, GPX2, GPX3, GPX4, GPX5, GPX6, GPX7, GPX8. Of which GPX1, GPX2, GPX3, GPX4 and GPX6 have a sec residue in its active site and GPX5, GPX7 and GPX8 have a cys residue in place of it. The biological functions include cellular response to oxidative stress, spermatogenesis and reduced phospholipid hydroperoxide. The protein belongs to the alpha-beta class. The catalytic site of GSHPx contains a conserved residue which is either a cysteine or, in many eukaryotic GSHPx, a selenocystein. [6]

Uniprot ID	Tissue Distribution	Cellular Localization	Biological Function
GPX1 P07203	Highly distributed in the lungs, kidney, red blood cells and liver	Cytosol Mitochondria	Reduces hydrogen peroxides in the cytoplasm. Modulator of the insulin signaling pathway. Acts in an antiapoptotic manner which can support tumor cell survival
GPX2 P18283	Gastrointestinal tract, endothelial cells	Cytosol	Inhibits inflammation-induced carcinogenesis, but also promotes the growth of some cancers like bladder cancer
GPX3 P22352	Kidney, lung, heart, muscle.	Plasma	Acts as a tumor suppressor in many cancers including lung cancer. Deficiency facilitates platelet aggregation
GPX4 P36969	Testis and spermatozoa, kidney, spleen, pancreas, heart, brain	Cytosol, Mitochondria, Plasma	Reduces hydroperoxides from phospholipids. Key regulator of ferroptosis pathway. Modulator of a rare genetic disorder called SSMD.
GPX5 O75715	Testis, spermatozoa, liver, kidney	Epididymis	Deficiency, together with GPX4, decreases male fertility
GPX6 P59796	Embryos and adult	-	Reduces the motor defects found in Huntington's disease
GPX7 Q96SL4	Endoplasmic reticulum	-	Mild glutathione peroxidase activity. Contributes to oxidative protein folding in the ER.
GPX8 Q8TED1	Endoplasmic reticulum	-	Mild glutathione peroxidase activity. Contributes to oxidative protein folding in the ER.
TRXR1 Q16881	Ovary, spleen, heart, liver, kidney and pancreas	Cytoplasm, Nucleus	Reduces disulfideprotein thioredoxin (Trx) to its dithiol-containing form. Involved in the regulation of cellular redox reactions, growth and differentiation
TRXR2 Q9NNW7	Highly expressed in the prostate, ovary, liver, testis, uterus, colon and small intestine.	Mitochondria	Involved in the control of reactive oxygen species levels and the regulation of mitochondrial redox homeostasis.
TRXR3 Q86VQ6	Testis	Cytoplasm, Nucleus, ER	Displays thioredoxin reductase, glutaredoxin and glutathione reductase activities. Catalyzes disulfide bond isomerization.

Table.1 Biological significance of glutathione peroxidase and thioredoxin reductase

- Reaction Mechanism Followed By Glutathione Peroxidase Isoforms

Gpx1, Gpx2, GPx3, Gpx5, Gpx6, Gpx7, Gpx8 follow a reaction as below



Step 3 - GS-SeR + GSH \rightarrow GS-SG + RSeH

Step 4 - Glutathione reductase then reduces the oxidized glutathione to complete the cycle :

GSH represents reduced monomeric glutathione. The mechanism involves oxidation of the selenol of a selenocysteine residue by hydrogen peroxide. This process gives the derivative with a selenenic acid (RSeOH) group. The selenenic acid is then converted back to the selenol by a two step process that begins with reaction with GSH to form the GS-SeR (selenenyl sulfide) and water. A second GSH molecule reduces the GS-SeR intermediate back to the selenol, releasing GS-SG as the by-product.

- GPX4 catalyzes the following reaction:

2 glutathione + lipid-hydroperoxide \rightarrow glutathione disulfide + lipid-alcohol + H₂O

This reaction occurs at the selenocysteine within the catalytic center of GPX4. During the catalytic cycle of GPX4, the active selenol (-SeH) is oxidized by peroxides to selenenic acid (-SeOH), which is then reduced with glutathione (GSH) to an intermediate selenodisulfide (-Se-SG). GPX4 is eventually reactivated by a second glutathione molecule, releasing glutathione disulfide (GS-SG).

Ferroptosis is a recently recognized type of programmed cell death and plays an important role in cancer biology and therapies.

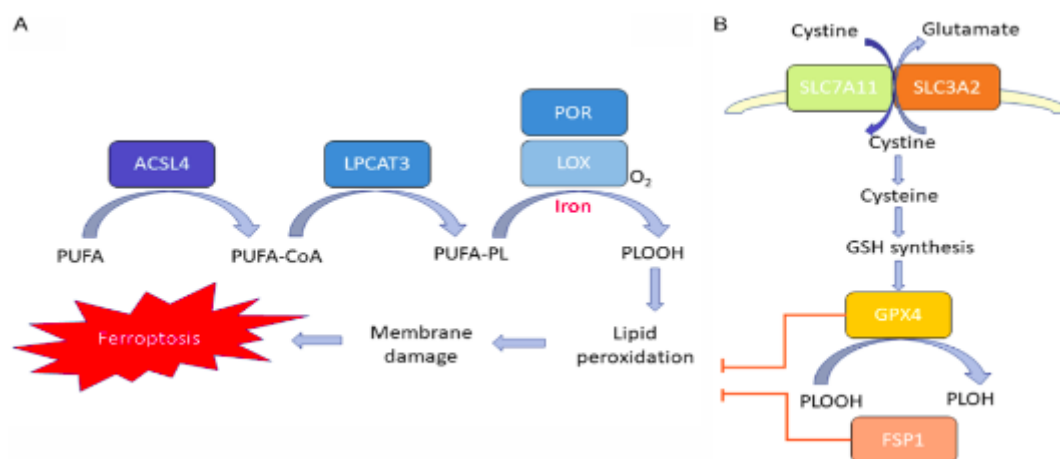


Fig.2 A. Lipid peroxidation pathways. ACSL4 (acyl-CoA synthetase long-chain family member 4) catalyzes the ligation of long-chain PUFAs (phospholipids (PLs) containing polyunsaturated fatty acids (PUFAs) with CoA, and LPCAT3 (lysophosphatidylcholine acyltransferase 3) promotes the esterification and incorporation of these products into membrane phospholipids (PL). [8] PUFA-containing PL is oxidized by iron-dependent enzymes LOX (lipoxygenases) or POR (cytochrome P450 oxidoreductase), resulting in lipid peroxidation, membrane damage and subsequent ferroptosis. [8] B. Antioxidant pathways. Cysteine is imported into the cell by (two members of the solute carrier family 7 member 11) SLC7A11/SLC3A2 complex for the synthesis of GSH. [8] GPX4 uses GSH as a substrate and reduces the membrane phospholipid hydroperoxide to harmless lipid alcohols, thereby preventing the accumulation of lethal lipid ROS and suppressing ferroptosis. Alternatively, cells utilize the FSP1 (ferroptosis suppressor protein 1) axis to suppress lipid peroxidation and prevent ferroptosis. [8]

Thioredoxin Reductase

Mammalian thioredoxin reductases (TrxR) are homodimers, homologous to glutathione reductase and have a selenocysteine residue in the conserved C-terminal sequence -Gly-Cys-SeCys-Gly. It is involved in the regulation of cellular redox reactions, growth and differentiation. It contains a selenocysteine residue at the C-terminal active site that is essential for catalysis. [8] Also has reductase activity on hydrogen

peroxide. [8] Superfamily of Thioredoxin Reductase represents the C-terminal domain in FAD- and NAD-linked reductases, FAD is a prosthetic group and catalyzes NADPH-dependent reduction, reduces disulfide protein thioredoxin (Trx) to its dithiol - containing form. The selenoprotein showed two isoelectric points at pH 5.2 and pH 5.3 [8]. TRXR activates the p53 tumor suppressor, manifests other tumor suppressor activities, and is specifically targeted by carcinogenic electrophilic compounds. p53 being a tumor suppressor, suggests that TRXR plays a major role in cancer prevention. Examination of a number of human and mouse cancer cell lines showed that they had elevated levels of thioredoxin reductase, but not other selenoproteins, and knockdown of TRXR in these cells disrupted characteristics of malignancy as well. [9] On the other hand, TRXR has a role in promoting cancer as well. Its over-expression in many cancer cell lines has made its exposure to many anti-cancer drugs and potent inhibitors that alter cancer-related properties of malignant cells. [9] This dual nature of the protein has made it an interesting target for further studies. However, the cancer promoting or cancer preventing properties of TRXR1 have greater influence on the fate of a cell may be difficult to predict. It possibly seems that lack of knowledge in many areas of selenium function has made it essential for additional genetic, mechanistic and animal studies before undertaking large, very expensive human trials. [9]

- Mechanism Followed By Thioredoxin Reductase Isoforms

The detailed mechanism of mammalian TrxR has not been known. The mammalian Se-containing TrxR reduces lipid hydroper-oxides and H₂O₂ and this activity is lost in the Cys mutant enzyme. The catalytic intermediates consist of a selenol, a putative selenenic acid, and the sulfenyl sulfide enzyme forms. [10] The initial reaction is that the selenenyl sulfide receives electrons from NADPH via the FAD and redox-active dithiol of the first subunit to produce a sulf-hydryl and a selenol (-SeH) in the second subunit. Because of the low pK_a value of the selenol, selenolate should be a predominant form under physiologic conditions. [10] Because it is a strong nucleophilic, selenolate is more susceptible to oxidation by H₂O₂ than thiols, yielding selenenic acid (-SeOH). [10] One cysteine thiol (most likely Cys497) reacts with the selenenic acid to produce water and to reform the sulfenyl sulfide. A second thiol (most likely Cys59 From the other subunit) would attack the bridge to regenerate the selenol. Therefore, the sulfenyl sulfide serves as either a catalytically essential redox center or transient intermediate during peroxide reduction. [10]

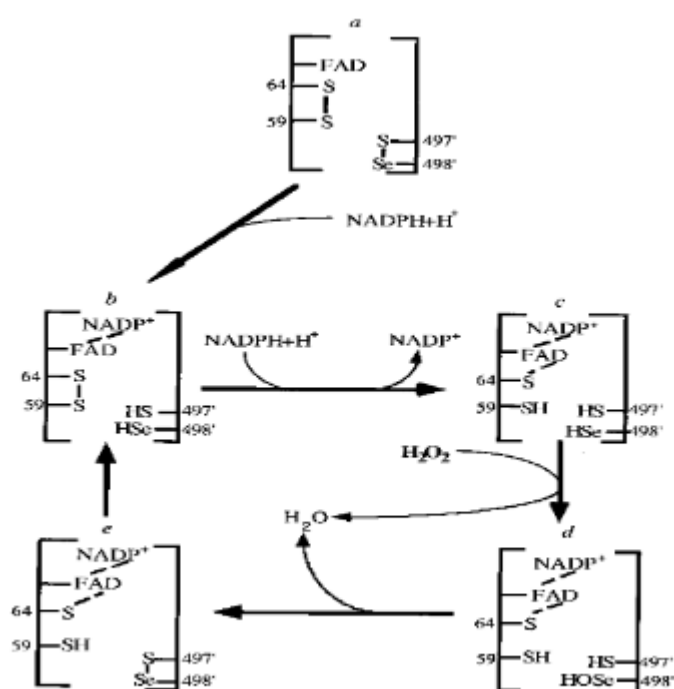


Fig.3 Simplified postulated mechanism for hydrogen peroxide reduction by mammalian TRXR.

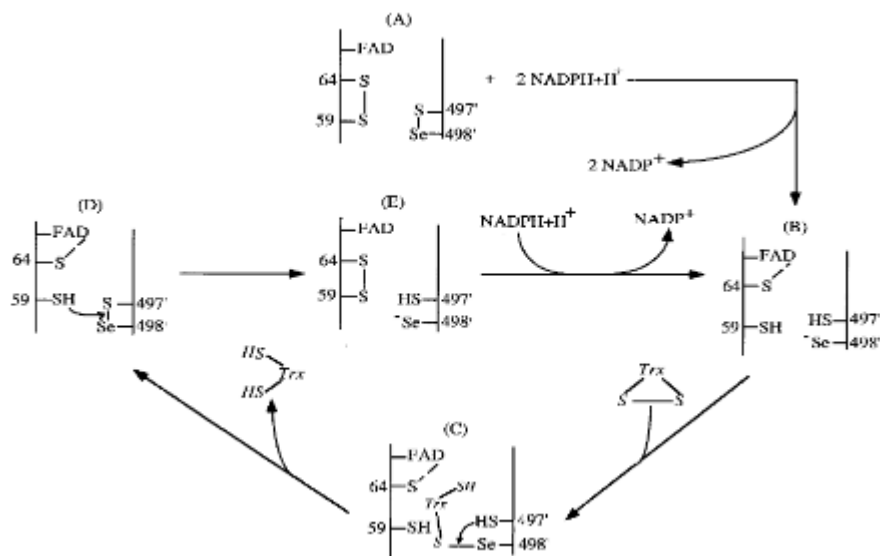


Fig.4 Postulated mechanism for Trx reduction by mammalian TrxR.

The catalytic reaction starts by reduction of the sulfenyl sulfide to the selenolate anion as for H₂O₂ reduction. The selenolate anion (-Se-) attacks the disulfide of Trx. The resulting enzyme-Trx-mixed sulfenyl sulfide (C) is attacked by Cys497 to regenerate the sulfenyl sulfide, which will be reduced by the active-site thiolate from the other subunit again (D). During the reaction, the active-site dithiol maintains the selenol in the reduced state.[10]

StateOfArt

Isoforms	Active Site Residue	Biological Unit	Catalytic Site	Chromosome Location	PDB Structure
GPX1	U	Homotetramer	Gln82, Trp160	Chr. 3 p21.3	U-G (2F8A)
GPX2	U	Homotetramer	Gln74, Trp152	Chr. 14 q24.1	U-C (2HE3)
GPX3	U	Homodimer	Gln107, Trp181	Chr. 5 q23	U-G (2R37)
GPX4	U			Chr. 19 p13.3	6ELW
GPX5	C	Homotetramer	Gln107, Trp181	Chr. 6 p21.32	213Y
GPX6	U in humans, C in rodents	Monomer	-	Chr. 6 p21	-
GPX7	C	Monomer	Gln92, Trp142	Chr. 1 p32	2P31
GPX8	C	Monomer	Gln92, Trp142	Chr. 5 q11.2	3CYN
TRXR1	U	Homodimer	Gly-Cys-SeCys-Gly Conserved	Ch37 / hg19	3QFA (Crystal structure of the human thioredoxin reductase-thioredoxin complex)
TRXR2	U	Homodimer	Gly-Cys-SeCys-Gly Conserved	Ch37 / hg19	1W1E (Crystal structure of the human thioredoxin reductase-thioredoxin complex)
TRXR3	U	Homodimer	Gly-Cys-SeCys-Gly Conserved	Ch37 / hg19	3H8Q (glutaredoxin domain of human thioredoxin reductase 3)

Table. 2 Structural information of glutathione peroxidase and thioredoxin reductase

Glutathione peroxidase was discovered in 1957 by Gordon C. Mills. (GPX) (EC 1.11.1.9) is the general name of an enzyme family with peroxidase activity whose main biological role is to protect the organism from oxidative damage.[12] The class of this enzyme is Oxidoreductases. The biochemical function of glutathione peroxidase is to reduce lipid hydroperoxides to their corresponding alcohols and to reduce free hydrogen peroxide to water. [11]

Several isozymes are encoded by different genes, which vary in cellular location and substrate specificity. Glutathione peroxidase 1 (GPX1) is the most abundant version, found in the cytoplasm of nearly all mammalian tissues, whose preferred substrate is hydrogen peroxide. Glutathione peroxidase 4 (GPX4) has a high preference for lipid hydroperoxides; it is expressed in nearly every mammalian cell, though at much lower levels. Glutathione peroxidase 2 is an intestinal and extracellular enzyme, while glutathione peroxidase 3 is extracellular, especially abundant in plasma.[13] Eight different isoforms of glutathione peroxidase (GPx1-8) have been identified in humans. Mammalian GPx1, GPx2, GPx3, and GPx4 have shown to be selenium-containing enzymes, whereas GPx6 is a selenoprotein in humans with cysteine-containing homologues in rodents. GPx1, GPx2, and GPx3 are homotetrameric proteins, whereas GPx4 has a monomeric structure, its antioxidative protective system depends heavily on the presence of selenium. The catalytic triad consisting of C462, Q81, and W136 is localized at a flat impression of the protein surface in GPX4.[14] GPx4 is unique among GPx-isoforms. The lack of structural elements in GPX4 when compared to

Fig.6 Multiple Sequence Alignment of different isoforms of glutathione peroxidase found in humans.
Conserved Resides - L,G,E,K - shown in *
Catalytic Triad - Cysteine/Selenocysteine, Tryptophan, Asparagine - shown in \$
GSH domain residues - shown in :
N-terminal ER retention sequence of the ER homologs in GPX7 and GPX8 - shown in .
Cysteine Block - shown in ^

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Theoretically such a replacement is not expected to have a major impact on catalysis, because either the Gln nitrogen or the Ser oxygen could function as a proton acceptor in the active site for the interaction with hydroperoxide. [20] GPx7 is a luminal protein and GPx8 is a transmembrane protein. Both the GPx7 and the GPx8 sequences include an N-terminal signal peptide, which is cleaved off in the mature protein with an ER retention sequence at the C-terminus. Since, both enzymes are ER-resident proteins with different topologies. [20]

Human Glutathione Peroxidases:

>gi|41406084|ref|NP_000572.2| glutathione peroxidase 1 [Homo sapiens]
MCAARLAAAAAQSVAFSARPLAGGEPVSLGSLRGKVLLENVSLTGGTTVRDYTMNELQRRLGPRGLVVLGFPCNQFGHQENAKNEEILNSLKYPVPGGGFEPN
FMLFEKCEVNGAGAHPLFAFLREALPAPSDATALMTDPKLIWSPVCRNDVAWNFEKFLVGPDPVPLRRYSRRFQTIDIEPDIEALLSQGPSCA

>gi|54038169|gb|AAH05277.1| Gastrointestinal glutathione peroxidase 2 [Homo sapiens]
MAFIKASFYDLAISLDGEKVDFNTFRGRAVL IENVASLTGGTTTRDFTQLNELQCRFPRLVVLGFPCNQFGHQENCNEEILNSLKYPVPGGGYQPTFTLVQKCEVN
GQNEHPVFAYLKDKLPYPYDDPFLMTDPKLIWSPVRRSDVAWNFEKFLIGPEGEFRRYSRTFTINIEPDIKRLKVAI

>gi|6006001|ref|NP_002075.2| Plasma glutathione peroxidase 3 [Homo sapiens]
MARLLQASCLLSLLAGFVSQSRGQEKSKMDCHGGISGTIYEGALTIDGEEYIPFKQYAGKYLVFNVASYTGLTGQYIELNALQEELAPFGLVILGFPCNQFGKQE
PGENSEILPTLKYPVPGGGFVPNFQLFEKGDVNGEKEQKFYTLKNSCPPTSELLGTSDRLFWPEMKVHDIRWNFEKFLVGPDPGIPMRWHHTTVSNVKMDILSYMR
RQAALGVKRRK

>gi|90903238|ref|NP_001034936.1| Glutathione peroxidase 4 [Homo sapiens]
MSLGRLCRLKLPALLCGALAAPLAGTMCASRDDWRCARSMHEFSAKDIDGHMVNLDKYRGFVCIVTNVASQTGKTEVNYTQLVDLHARYAECGLRILAFPCNQFGKQ
EPGSNEEIKEFAAGYNVKFDMFSKICVNGDDAHLWKWMIQPKGKILGNAIKWNFTKFGHRLSTVPHRQERLRGEALRTHGGAPGDPREGPAPLFLAPQVCGPARAP
AHALGAFHRHS

>gi|3288455|emb|CAA06463.1| Glutathione peroxidase type 5 [Homo sapiens]
MTTQLRVVHLLPLLLACFVQTSPKQEKMKMDCHKDEKGTIYDYEAIALNKNEYVSFKQYVGKHLFVNVAITYGLTAQYPELNALQEELKPYGLVVLGFPCNQFGKQE
PGDNKEILPGLKYVPGGGFVPSFQLFEKGDVNGEKEQKVFSFLKHSCPHSEILGTFSISWDPVKVHDIRWNFEKFLVGPDPGIPMRWSHRATVSSVKTDILAYLK
QFKTK

>gi|33186887|ref|NP_874360.1| Glutathione peroxidase 6 [Homo sapiens]
MFQQFQASCLVLFVLGFAQQTLPQNRKVDNCNKGVTGTIYEGALTNGEEYIQFKQFAGKHVLFVNVAAYTGLAAQYPELNALQEELKNFGVIVLAFPCNQFGKQE
PGTNSEILLGLKYVCPGSGFVPSFQLFEKGDVNGEKEQKVFTFLKNSCPPTSDLLGSSSQFLWPEMKVHDIRWNFEKFLVGPDPGVPMWHFQAPVSTVKSDILEYLK
QFNTH

>gi|119627182|gb|EAX06777.1| Glutathione peroxidase 7 [Homo sapiens]
MVAATVAAAWLLWAAACAQQEQDFYDFKAVNIRGKLVLSLEKYRGSVSLVNVVASEGFTDQHYRALQQLQRLDGPHEFNVLAFPCNQFGQEPDSNKEIESFARTY
SVSFPFMFSKIAVTGTGAHPAFKYLQATSGKEPTWNFWKYLVPADGKVVGAWDPTVSVVEVRPQITALVRKLILLKREDL

>gi|192455698|ref|NP_001008398.2| Glutathione peroxidase 8 [Homo sapiens]
MEPLAAYPLKCSGPRAKVFAVLLSIVLCTVTLFLLQLKFLKPKINSFYAFEVKDAKGRTVSLEKYKGKVSLLVNVASDQLTDRNYLGLKELHKEFGPSHFSVLAFC
NQFGESEPRPSKEVESFARKNYGVTFPIFHKIKILGSEGEPAFRFLVDSSKKEPRWNFWKYLVPNEGQVVKFWKPEEPIEVIRPDIAALVRQVIKKKEDL

Fig.7 Human Glutathione Peroxidases Sequences

Thioredoxins are small disulfide-containing redox proteins that have been found in living organisms. Thioredoxin serves as a general protein disulfide oxidoreductase. It interacts with a broad range of proteins by a redox mechanism based on reversible oxidation of 2 cysteine thiol groups to a disulfide, accompanied by the transfer of 2 electrons and 2 protons. The net result is interconversion of a disulfide and a dithiol. [21], [22] The thioredoxins are maintained in their reduced state by the flavoenzyme thioredoxin reductase, in a NADPH-dependent reaction [23]

Thioredoxin is a 12-kD oxidoreductase protein. Thioredoxin proteins also have a characteristic tertiary structure termed the thioredoxin fold (The thioredoxin fold is a protein fold common to enzymes that catalyze disulfide bond formation and isomerization.) [24] The active site contains dithiols in a CXXC motif. These two cysteines are the key to the ability of thioredoxin to reduce other proteins. [25] For Trx1, this process begins by attack of Cys32. [25] Almost immediately after this event Cys35, the other conserved Cys residue in Trx1, forms a disulfide bond with Cys32, thereby transferring 2 electrons to the substrate which is now in its reduced form. Oxidized Trx1 is then reduced by thioredoxin reductase, which in turn is reduced by NADPH as described above. [25]

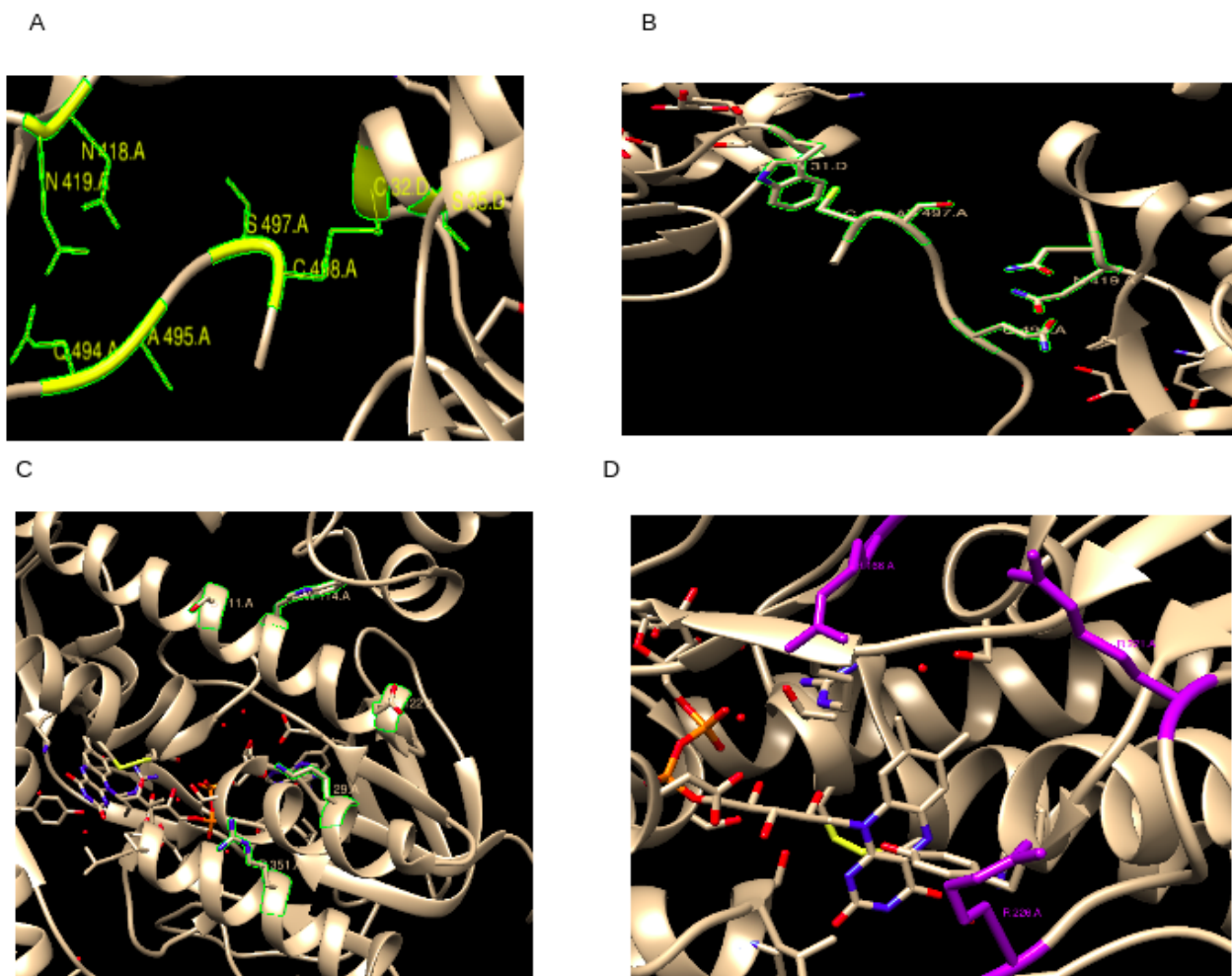


Fig. 8 K29 would interact with the carboxyl group of the C terminus and R351 with Q494. Sulfur atoms of C497 and C498 still point into the inner side of the enzyme. In such a conformation electron transport to the substrate Trx would not be possible. The TrxR wild-type possessing U498 Instead of C498, would have a negative charge at physiological pH due to the lower pKa of the selenol compared to a thiol. The structure implies that this negative charge inside the TrxR molecule is stabilized by K29. [26] The side-chain of E122 is close to the carboxyl end of the C terminus. The C497 and C498 sulfur atoms are solvent-exposed and therefore able to interact with substrates. The included tryptophan (W31) can interact with W114 in a similar way as W114 interacts with W114 of another dimer in the TrxR structure. The swapping W114 side-chain might represent a signal for Trx binding and then S111 could already be in a favorable position to facilitate deprotonation of C497 during catalysis. For this reason, it is plausible that W114 might be involved in Trx binding. Residues W114, S111 and R117 are conserved in rat TrxR1 and mouse TrxR2, suggesting a similar Trx binding in these enzymes. [26]

Human Thioredoxin Reductases (large or "animal" type of TRs):

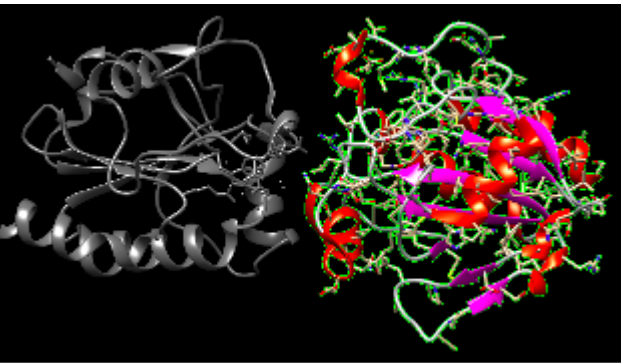
>gi|148277071|ref|NP_001087240.1| thioredoxin reductase 1 (TR1), cytoplasmic isoform 3 [Homo sapiens]
MGCAEGKAVAAAPTLEQTKGKNGDGRRRSAKDHHPGKTLPENPAGFTSTATADSRALLQAYIDGHSVVIFSRSTCTRCTEVKKLFKSLCVPYFVLELDQTEDGRALE
GTLSELAETDLPPVVFVKQRKIGGHGPTLKAYQEGRLQKLLKMNGPEDLPKSYDYDLIIIGGGSGGLAAAKEAAQYGKKVMVLDVFTPTPLGTRWGLGGT**CVNVGCI**P
KKLMHQAALLGQALQDSRNYGWKVEETVKHWDWMIEAVQNHIGSLNWGYRVALREKKVVYENAYGQFIGPHRIKATNNKGKEIYSAERFLIATGERPRYLGIIPGDK
EYCISDDLFSLPYCPGKTLVVGASYVALECAAGFLAGIGLDVTVMVRSILLRGFDQDMANKIGEHEHGIKFIQFVPIKVEQIEAGTPGRLRVVAQSTNSEEIEG
EYNTVMLAIGRDACTRKIGLETVGKINEKTGKIPVTDEEQTNVPIYIYAGDILEDKVELTPVAIQAGRLAQRLYAGSTVKCDYENVPTTVFTPLEYGACGLSEEKA
VEKFGEENIEVYHSYFWPLEWTIPSRDNNKCYAKIICNTKDNERNVVGHHVLPNAGEVTQGFAAALCKGLTKKQLDSTIGIHPVCAEVFTTSLVTKRSGASILQAGC**U**
G

>gi|291045266|ref|NP_443115.1| thioredoxin reductase 2 (TGR or TR2) isoform 1 [Homo sapiens]
MERSPPQSPGPGKAGDAPNRRSGHVRGARVLSPGRRARLSSPGPSRSEAREELRRHLVGLIERSRVVIFSKEY**Q**PHSTRVKELFSSLGVECNVLELDQVDDGARVQ
EVLSEITNQKTVPNIFVNKVHVGCDQTFQAYQSGLLQKLLQEDLAYDYDLIIIGGGSGGLSCAKEAAAILGKKVMVLDVFPVSPQGTWGLGGT**CVNVGCI**PKKLMHQ
AALLGQALCDSRKFGWEYNQQRHNMWETMTKAIQNHISLWGYRLSLREKAVAYVNSYGEFVEHHKIKATNNKGQETYYTAAQFVIATGERPRYLGIQGDKEYCITS
DDLFSLPYCPGKTLVVGASYVALECAAGFLAGIGLDVTVMVRSILLRGFDQEMAQVGSYMEQHGKFLRKFIQFVPIKVEQIEAGTPGRLRVVAQSTNSEEIEG
LAIGRDSCTRKIGLEKIGKINEKSGKIPVNDVEQTNVPIYIYAGDILEDKVELTPVAIQSGKLLAQRLLFGASLEKCDYINVPTTVFTPLEYGCCGLSEEKAIEVYKK
ENLEIYHTLFWPLEWTVAGRENNTCYAKIICNKFDHDRVIGFHILGPNAGEVTQGFAAAMCKGLTKQLLDDTIGIHPCTCEVFTTLEITKSSGLDITQKGC**U**G

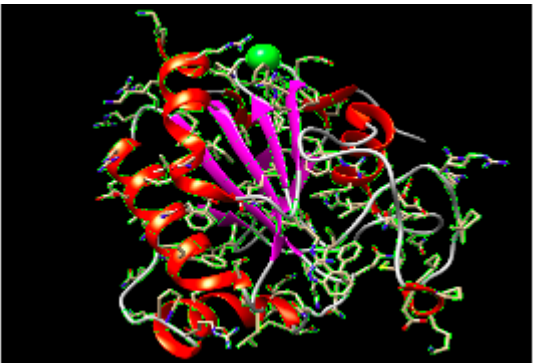
>gi|22035672|ref|NP_006431.2| thioredoxin reductase 3 (TR3), mitochondrial isoform 1 [Homo sapiens]
MAAMAVLRGLGGRFRWRTQAVAGGVGAARGAAAGQRDYDLLVVGGSGLACAKEAAQLGRKVAVVDYVEPSPOGTRWGLGGT**CVNVGCI**PKKLMHQAALLGGLIQ
DAPNYGWEVAQPVPHDWRKMAEAVQNHVKSLSNWGHVQLQDRKVYFNIAKSFVDEHTVCGVAKGKGEILLADHIIIIATGGRPRYPHTIEGALYGITSDDIIFWLKE
SPGKTLVVGASYVALECAAGFLAGIGLDVTIMMRSIPLRGFDQMSMVEHMASHTGTRFLRGCAPSRVRRLLPDGQLQVTWEDSTTGKEDTGTFTDLWAIQGRVPDTRS
LNLEKAGVDTSPDTQKILVDSREATSPHIIYAGDVVEGRPELTPIAIMAGRLLVQRLFGGSSDLMDYDNVPTTVFTPLEYGCVGLSEEEAVARHGQEHVEVYHAHYK
PLEFTVAGRDAQCYVVMVCLREPPQLVLGLHFLGPNAGEVTQGFALGKCGASYAQMVRTVGIHPTCSEEVVKLRISKRSGLDPTVTGC**U**G

Fig.9 Human Thioredoxin Reductases Sequences

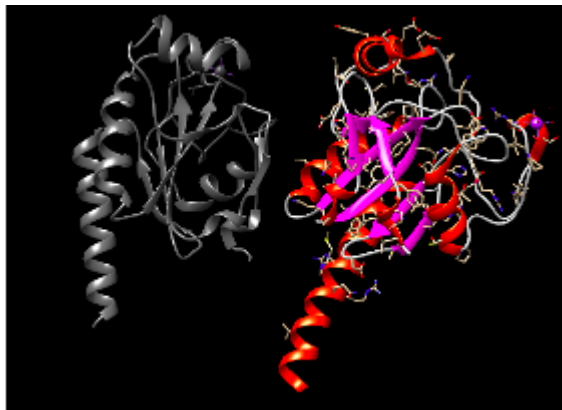
- The location of variants on the structures of GPX and TRXR. A- GPX1, B- GPX2, C- GPX3, D- GPX4, E- GPX5, F- GPX7, G- GPX8, H- TRXR1, I- TRXR2, J- TRXR3



GPX1 (A)



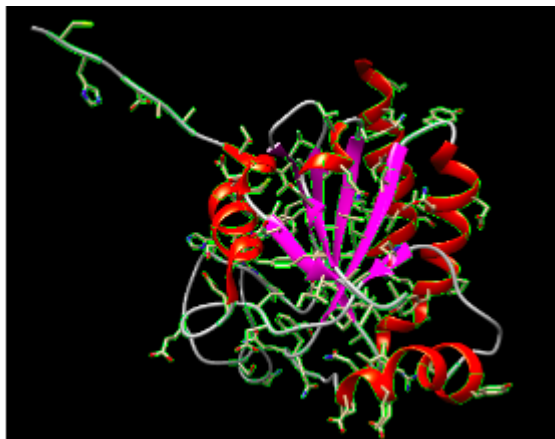
GPX2 (B)



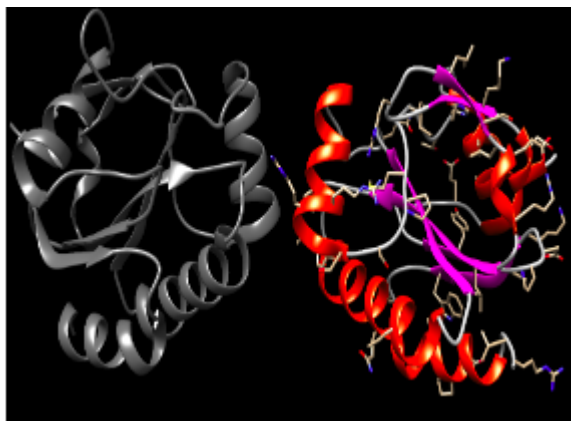
GPX3 (C)



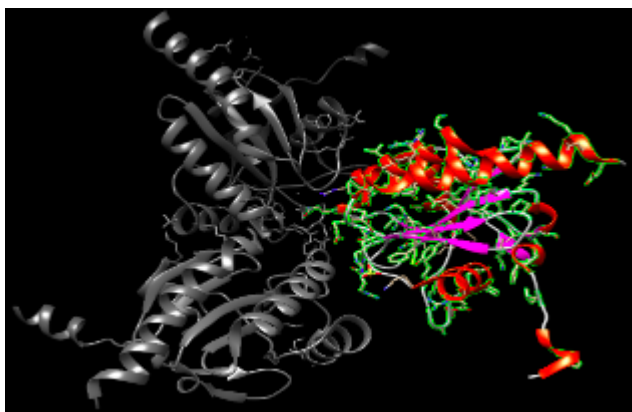
GPX4 (D)



GPX5 (E)



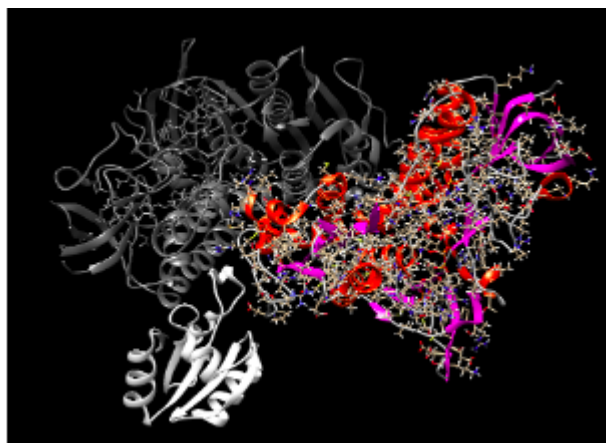
GPX7 (F)



GPX8 (G)



TRXR1 (H)



TRXR2 (I)



TRXR3 (J)

- Interacting Proteins

Most HCC cell lines have a minimal SBP1 expression, with the exception of SMMC7721. SBP1 only exhibited its impact on cancer cell proliferation and apoptosis following treatment with hydrogen peroxide; these results indicated that SBP1 might exert its tumor suppressive power through modulation of the tumor redox microenvironment. According to [27] SBP1 could greatly inhibit the activity, but not expression of GPX1 in cancer cells both in vitro and in vivo. The poor responses of patients with HCC to chemotherapy might be due to low SBP1 expression and high GPX1 activity, thus increasing SBP1 expression and decreasing GPX1 activity could be a novel strategy for cancer treatment. [27] GPx7 interacts with numerous proteins including ADF, GRP75, HSP7C, ERp72, eEF1A-1, U-Tmod, ErJ3, and Histone H1b, in addition to the GRP78, PDI, and XRN2 [28]. The biological significance of these interactions between GPx7 and binding proteins remains to be explored. For example, the ADF protein, which regulates cargo transportation together with Cofilin in the trans-Golgi network [28], U-Tmod (TMOD3), which binds to actin monomer to determine cell polarity [28], could be regulated by GPx7 via thiol-disulfide shuttling. On the other hand, GPx7 may cooperate with GRP75, which contains mitochondrial targeting sequences [28] and binds to transcription factors such as p53 [28], to shuttle mitochondrial proteins into the nucleus to regulate transcription [28]. It is currently unclear why non-ER resident proteins such as the elongation factor EF1A-1 (eEF1A-1), Histone H1b, or HSP7C interact with GPx7 in response to oxidative stress. FOXC1 was proved to be a transcription factor of GPX8 and mediated GPX8 expression to regulate cell development processes. [29] A study found that GPX8 overexpression increased the expression of wnt1, wnt3a and β -catenin and decreased the expression of p-GSK3 β , thereby activating the Wnt signaling pathway. [29]

Oxidized Trx is reduced by TrxR to maintain a pool of reduced Trx. Trx reduces peroxiredoxin (Prx), ribonucleotide reductase (RNR), and methionine sulfoxide reductase (Msr). [30] These reductive enzymes catalyze the reduction of peroxides, ribonucleotides, and methionine sulfoxides, respectively. Trx also directly interacts with redox-sensitive molecules, such as apoptosis signal-regulated kinase 1 (ASK1), thioredoxin interacting protein (Txnip), and phosphatase and tensin homolog (PTEN). Apart from reductive enzymes, Trx also interacts with redox-sensitive signaling molecules. [30] There are a number of transcription factors that are redox regulated and contain redox-sensitive cysteines in their DNA binding domain. [30] These include activator protein 1 (AP-1), NF- κ B, protein 21 (p21), protein 53 (p53), hypoxia-inducible transcription factor-1 α -ph (HIF-1), the glucocorticoid receptor, the estrogen receptor, PEBP2, EPF, Nrf2, Oct-4, and TFIIC (172). [30] Txnip reacts with reduced Trx and forms an intermolecular disulfide cysteine 247. (B) No disulfide exchange reaction is possible between Txnip and oxidized Trx. [30]

- Variants and their effect on PPI

Polymorphisms in selenoproteins have been studied for over years. It is seen that the variant associated at different locations is an important study for many diseases. Epidemiological studies have implicated several selenoprotein genes in cancer risk or outcome based on associations between allelic variations and disease risk or mortality. In addition to GPX1 polymorphisms, other selenoprotein genes have also been linked to cancer risk when assessed in conjunction with selenium status. [31] Homozygous mutation in any component of the thioredoxin antioxidant system leading to inherited disease in humans.[32]

Polymorphisms in the genes for two members of the thioredoxin reductase family of selenoproteins (TRXR1 and TRXR2) and Selenoprotein K (SELENOK) associated with prostate cancer risk only when selenium status was also considered, but they did not reside in the coding sequence or 3'-UTR, leaving the mechanism behind these associations subject to future studies.[31] The best known polymorphism of GPX1 is Pro198Leu, with the Leu allele being less responsive to enzyme activity during selenium supplementation.[33] Leu carriers for rs1050450 (Pro198Leu) in GPX1 have shown an increased risk of developing non-ductal breast cancer. However, this study of Pro198Leu polymorphism did not show susceptibility towards disease, but CAT which protects the cells against severe oxidative stress, also linked to GPX1 activity, led to the risk of increase in spontaneous abortion.[34]

A higher frequency of the Leu variant of rs1050450 (GPX1) was found in breast tumor DNA compared to normal tissue, probably due to loss of heterozygosity in the tumor cells. Risk was increased in individuals carrying both the Leu variant in GPX1 and the Ala variant. Since enzymatic assays have previously shown that the Leu protein variant is less active than the Pro counterpart,[34] proposed that high GPx1 activity is required to counterbalance the levels of ROS and related damage occurring during initiation or progression of the disease and hypothesize that the capacity of an individual to deliver Se to the breast tissue, generating high or low GPx1 activity determines the susceptibility of their breast tissue to oxidative damage and carcinogenesis. [34]

235 SNPs determined across the 6 genes, gave a result of 5 non-synonymous variants, 6 synonymous variants and 224 non-coding SNPs. The coding region SNPs identified were located in the GPX1 (P75R,L91L, A192T and P198L), GPX3 (L13L), GPX4 (L193L),TXNRD1 (L55L, L80L and C383C) and SEPP1 (K19E,A234T) (5). SNPs of potential functional importance include the GPX1 P75R and P198L variants, a high frequency GPX4 SECIS region SNP. There is strong LD (Linkage disequilibrium (LD) is the correlation between nearby variants such that the alleles at neighboring polymorphisms (observed on the same chromosome) are associated within a population more often than if they were unlinked.) between the GPX1 P198L, and nearby gene RHOA which is a ras onco-gene family gene, thus there is a possibility of the variant to be associated with cancer risk.[35] It has been found for the first time that the C718T polymorphism of the glutathione peroxidase-4 gene can be considered as a risk factor for cerebral stroke in hypertensive patients. The allele C718, the 718TC and 718CC genotypes of the GPX4 gene were found to be associated with it.[36] The OMIM database has another variant of GPX which causes Sedaghatian-type spondylometaphyseal dysplasia. [37] The first variant, c.587+5G;A, which was also identified in the mother, changes a highly conserved nucleotide, while the second, c.588-8_588-4del, is 5-bp deletion upstream of the 3' splice acceptor site. [37]

The geno-micnucleotide sequence variations in theTXNRD3NT1 gene that may alter the concentration or activity of these proteins and ultimately contribute to the development of psoriasis. Part of this gene overlaps the previously described thioredoxin reductase 3(TXNRD3) gene, we have named it TXNRD3NT1(TXNRD3 new transcript). Two silent (SNPs) (A/G) were identified within the 5'-untranslated region (UTR) at the nucleotide positions 23 and 26. One polymorphic site (GGC/DEL) was found within intron 4 at nucleotide position 36889. These two SNP sites (T/C) in the 5' promoter region were identified at nucleotide positions -203 and -196.[38] The studies gathered through these literature emphasize on exploring the variants, to understand how they affect the activity of the enzyme, investigating their role in

functional changes and how they are associated with cancer.

SOD is involved in the dismutation of superoxide into oxygen and hydrogen peroxide, and it is represented in three forms: SOD1, which is located in the cyto-plasm, SOD2 (also called MnSOD because it has manganese ion in its catalytic cleft), which exists in the mitochondria, and SOD3, which functions in the extracellular matrix. [39] Of these isoforms, SOD2 was found to modify cancer susceptibility, largely due to several functional single nucleotide polymorphisms in the SOD2 gene. [39] The most well-studied SNP in the SOD2 gene is SOD2 47T/C (rs4880), or Val16Ala, as it causes an amino acid substitution of valine (val) to alanine (val) on the 16th residue. [39] in 2003, Hu and Diamond revealed a direct relationship between the presence of the variant T allele of rs1050450 polymorphism and a dramatic decrease in the selenium-dependent activation of GPX1 enzyme in breast cancer patients, suggesting its role in susceptibility to cancer and other diseases. The rs4880 polymorphism of SOD2 gene and the rs1050450 polymorphism of GPX1 gene are the most commonly studied polymorphisms. [39] The T allele of the rs713041 polymorphism of the GPX4 gene was found to be involved in colorectal carcinogenesis. [39]

Hypothesis

- Questions
 1. How do genomic variants give an insight of changes in the protein-protein interaction network of GPX and TRXR ?
 2. Is their role of both enzymes in ROS metabolism the only reason for action in both tumorigenesis and cancer protection ?
 3. How is the enzymatic activity of GPX and TRXR affected by genomic variations ?
 4. How do the presence of variants in these two proteins affect other organisms ?
 5. Why do some isoforms have selenocysteine while some cysteine on the active site ?
 - Hypothesis
 - 1. The active site and the overall structure along with reaction kinetics has a fundamental role in distinguished activities of proteins, which may be strongly affected by variants
 2. It has been also stated that mutations in GPX and TRXR might lead to alteration in protein-protein interaction
 3. Homeostasis, redundancy of the biochemical networks can be affected due to the variants
-

Workflow

- **STRUCTURAL STUDY :**
 - [Goals](#)
 - [Methodologies](#)
- **STRUCTURAL STUDY :**
 - [Goals](#)
 - [Methodologies](#)

- **REACTION KINETICS :**
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- **PROTEIN-PROTEIN INTERACTION :**
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The PhD thesis includes three interconnected areas of research, corresponding to structure, reactivity and biological function (in terms of protein-protein interactions -PPI- analysis). For each of the three blocks we have set some specific goals and a set of tools to develop and apply.

STRUCTURAL STUDY :

Goals

1. Understanding the structural features at the secondary, supersecondary, tertiary and quaternary level that characterize the two families of proteins.
2. Identifying the motifs that present variants in human genome and the possible effect in the structures of the two families.
3. Unravelling the effect of the variants in the dynamics of the protein structures.

Methodologies

1. Multiple sequence alignment of the isoforms of two proteins for checking the missing residues, catalytic residues, and conserved sequence patterns.
2. Phylogenetic analysis of different isoforms of GPX and TRXR.
3. Exploring the modelled, unmodelled regions in the proteins with respect to the sequence, variant and structure in parallel.
4. Identifying different Variants in the databases to check their location on the structure of proteins
5. Evaluating alterations in the active and catalytic site of the protein structures due to specific mutations.

REACTION KINETICS :

Goals

1. Characterizing the reaction mechanisms of the proteins in the two families under study.
2. Understand the effect of benign and pathogenic variants in the catalytic activity of the proteins.
3. Identify the effect of collective motions in the catalytic activity of both wild type and mutated proteins.

Methodologies

6. EVB (Empirical Valence Bond Simulations) on known mechanism
7. QM/MM calculations for mechanisms not known completely

8. Checking for the residues involved in catalysis of the reaction in presence of mutants through free energy calculations
9. Homeostasis and redundancy of the biochemical networks due to presence of mutations in mammals and other organisms - To understand if mammalian mutations affect other organisms biological network as well

PROTEIN-PROTEIN INTERACTION :

Goals

1. Review and identify the network of physical interactions of the two protein families, as well as their role in metabolism pathways.
2. Characterize the interfaces in the PPIs the proteins in the two families use for their interactions.
3. Quantify the effect of both benign and pathogenic variants on the PPIs.

Methodologies

10. Identifying hotspot residues at PPI interfaces in GPX, TRXR
11. Evaluating the effect of mutations on PPI network present in the two proteins

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