ROLE OF SULFIREDOXIN INTERACTING PROTEINS IN LUNG CANCER DEVELOPMENT

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ROLE OF SULFIREDOXIN INTERACTING PROTEINS IN LUNG CANCER DEVELOPMENT

DISSERTATION

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the College of Medicine at the University of Kentucky

By

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ABSTRACT OF DISSERTATION

ROLE OF SULFIREDOXIN INTERACTING PROTEINS IN LUNG CANCER DEVELOPMENT

Sulfiredoxin (Srx) is an antioxidant enzyme that can be induced by oxidative stress. It promotes oncogenic phenotypes of cell proliferation, colony formation, migration, and metastasis in lung, skin, and colon cancers. Srx reduces the overoxidation of 2-cysteine peroxiredoxins in cells, in addition to its role of removing glutathione modification from several proteins. In this study, I explored additional physiological functions of Srx in lung cancer through studying its interacting proteins. Protein disulfide isomerase (PDI) family members, thioredoxin domain containing protein 5 (TXNDC5) and protein disulfide isomerase family A member 6 (PDIA6), were detected to interact with Srx. Therefore, I proposed that TXNDC5 and PDIA6 are important for the oncogenic phenotypes of Srx in lung cancer.

In chapter one, I presented background information about the role of Srx as an antioxidant enzyme in cancer. I also explained the functional significance of PDIs as oxidoreductase and chaperones in cells. In chapter two, I verified the Srx-TXNDC5/PDIA6 interaction in HEK293T and A549 cells by co-immunoprecipitation and other assays. In TXNDC5 and PDIA6, the N-terminal thioredoxin-like domain (D1) is determined to be the main platform for interaction with Srx. The Srx-TXNDC5 interaction was enhanced by H\textsubscript{2}O\textsubscript{2} treatment in A549 cells. Srx was determined to localize in the endoplasmic reticulum (ER) of A549 cells along with TXNDC5 and PDIA6. This localization was confirmed by both subcellular fractionation and immunofluorescence imaging experiments. In chapter three I focused on studying the physiological function of Srx interacting proteins in the ER. A549 subcellular fractionation results showed that TXNDC5 facilitates Srx retention in the ER. Moreover, TXNDC5 and Srx were found to participate in chaperone activities in lung cancer. Both proteins contributed in the refolding of heat-shock induced protein aggregates. In addition, TXNDC5 and PDIA6 were found to enhance the protein refolding in response to H\textsubscript{2}O\textsubscript{2} treatment. Conversely, Srx appeared to have an inhibitory effect on protein folding under same treatment conditions. Downregulation of Srx, TXNDC5, or PDIA6 significantly reduced cell viability in response to tunicamycin treatment. TXNDC5 knockdown decreased the time required for the splicing of X-box binding protein-1 (XBP-1). In either knockdown Srx or TXNDC5 cells, there was an observable decrease in the expression of GRP78 and the splicing of spliced XBP-1. These results suggest a possible role of Srx in unfolded protein response signaling. TXNDC5 and PDIA6, similar to Srx, contribute to the proliferation, anchorage independent colony formation and migration of lung cancer cells.
In this dissertation I concluded that Srx TXNDC5, and PDIA6 proteins participate in oxidative protein folding in lung cancer. Srx and TXNDC5 can modulate unfolded protein response (UPR) sensor activation and growth inhibition. Furthermore, TXNDC5 and PDIA6 can promote tumorigenesis of lung cancer cells. Therefore, the molecular interaction of Srx with TXNDC5/PDIA6 has the potential to be used as novel therapeutic targets for lung cancer treatment.

KEYWORDS: Sulfiredoxin, thioredoxin domain containing protein 5, protein disulfide isomerase A isoform 6, interaction, function
ROLE OF SULFIREDOXIN INTERACTING PROTEINS IN LUNG CANCER DEVELOPMENT

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April 26, 2016
I dedicate this dissertation to my family
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CHAPTER ONE
INTRODUCTION

1.1 Background

Lung cancer is the second most common cancer in men and women. Lung and bronchus cancer is the main cause of cancer death in the United States. It comprises approximately 27% of estimated cancer deaths (Siegel et al., 2016). The 5-year survival rate is only 16%. Among the major factors for lung cancer high fatality rate are poor early detection, difficulty of treatment, tumor recurrence and metastasis. The lifetime risk of lung cancer development is 1.3–1.4% in both male and female in non-smokers. The risk increases to 17% in male smokers and 13% in female smokers, respectively (Durham & Adcock, 2015; Zhang et al, 2016; Torre et al, 2016; de Groot et al, 2012). Cancer originates from the cells lining airways of bronchial lung tissues. Tumors arising from squamous cells and basal epithelial cells are termed non-small-cell carcinomas (NSCLC). Tumors consisting of undifferentiated smaller-than-normal cells are termed small-cell carcinomas (SCLC). NCSLCs are subdivided to adenocarcinoma, large-cell carcinoma, and squamous cell carcinoma, which account for 40%, 10%, and 30% of lung cancer cases, respectively. The remaining 20% of all cases are diagnosed as SCLC (Gadgeel et al, 2012; Durham & Adcock, 2015). For normal lung cells to become tumor cells, series of genetic mutations must occur, that lead to the formation of benign tumors. Additional genetic modifications cause the mutated cells to acquire metastatic and invasive behaviors, triggering them to take on the characteristic features of cancer cells (Cooper, 2005).

Among these verified changes are increases in cellular levels of reactive oxygen species (ROS). This feature is accompanied by enhanced competence of antioxidant systems in the cells (Kim et al, 2015; Watson et al, 2016). Cellular peroxidases,
including 2-cysteine containing peroxiredoxins (2-Cys Prxs), are among the antioxidant systems that contribute to the development of lung cancer (Kwon et al, 2015; Kim et al; 2011). For instance, Prxl and PrxIV have been found to enhance colony formation and migration in vivo metastasis of lung cancer cells (Jaing et al, 2014; Kwon et al, 2015). In general, Prxs strictly regulate H₂O₂ levels. 2-cys Prx reduce H₂O₂ to generate H₂O. At the same time, Prxs oxidize other protein substrates, such as protein disulfide isomerase family members, to generate disulfide bonds (Ramming & Appenzeller-Herzog, 2013; Kakihana et al, 2011). H₂O₂ oxidizes a highly nucleophilic active cysteine residue called peroxidative cysteine (Cᵰ) in 2-Cys Prx to sulfenic acid. This Cᵰ is then attacked by another cysteine residue, resolving cysteine (Cᵣ), which is present on the surface of a second 2-Cys Prx protein; to generate a disulfide linked Prx homodimer (Pace et al, 2013; Zhu et al, 2014). In the presence of high concentrations of H₂O₂, the sulfenic acid at the Cᵰ site can be overoxidized to generate sulfinic acid (and sulfonic acid) before the Cᵣ can form the disulfide bond. If unresolved, 2-Cys Prx loses its peroxidative activity and acquire additional features, such as the ability to form disulfide bonds independent stacks of decamers that possess chaperone activity (Pace et al, 2013; Schulte et al, 2011).

Hyperoxidation can stabilize these decameric forms of Prxl, II, and IV in cooperation with other proteins. The protein disulfide isomerase family members, predominantly, thioredoxin-domain-containing protein 5 (TXNDC5), protein disulfide isomerase family A member 6 (PDIA6), and protein disulfide isomerase (PDIA1), are reported to interact directly with overoxidized 2-Cys Prx, particularly PrxII and PrxIV. These PDI members act to stabilize the decameric structure of PrxIV (Pace et al, 2013; Sato et al, 2014; Zhu et al, 2014). TXNDC5 and PDIA6 interact with PrxIV in Hela cells to facilitate rapid formation of disulfide bonds in newly synthesized protein substrates in
a process of oxidative protein folding. PDIA1 functions at a slower pace to proofread and fold the nascent protein substrate. Thus high affinity of PDI members for overoxidized 2-Cys Prx shifts H2O2 signaling in cells (Pace et al, 2013; Sato et al, 2014).

As an adaptive mechanism against this prolonged overoxidation of Prxs, cells have adapted recycling machinery for reducing overoxidized Cp residue from sulfenic acid to sulfinic acid through differential expression of sulfiredoxin (Srx) enzyme, on the expense of ATP. Srx transfers ϒ-phosphate of ATP to Cp sulfinic acid modification on 2-Cys Prxs, to produce sulfinic phosphoryl ester. Glutathione and thioredoxin counterparts reduce sulfinic phosphoryl ester to sulfenic acid (Biteau et al, 2003; Woo et al, 2003; Chang et al, 2004). The presence of Srx appears to be necessary to maintain the oxidative stress balance in cancer. Srx inhibition results in oxidative stress-induced mitochondrial damage and caspase cascade activation that lead to apoptosis in lung adenocarcinoma cells (Kim et al, 2016; Baek et al, 2012). Beside H2O2 mediated signaling, Srx de-glutathionylates several proteins, including PrxI, protein tyrosine phosphatase 1B, and actin. Meanwhile, Srx itself is not glutathionylated since there is only one cysteine residue. In this term, Srx is known to be the first protein to function specifically in reductive de-glutathionylation (Findlay et al, 2006; Grek et al, 2013).

**Srx in cancer**

Srx is expressed under oxidative stress conditions. Srx expression is under the control of transcription factors nuclear erythroid 2-related factor 2(Nrf2) and activator protein 1 (AP-1) (Soriano et al, 2008; Ramesh et al, 2014). Nrf2 is required for Srx protein expression because cells that do not have Nrf2 do not express Srx. In normal lung cells, there is minimal expression of Srx. However, significantly higher expression level was observed in lung cancer, particularly in adenocarcinoma and squamous cell carcinoma (Merikallio et al, 2012; Wei et al, 2011). In fact, the Srx-PrxIV axis was found
to promote cancer progression through the modulation of phosphokinase signaling components. Srx is required for the activation of MEK1/2, and promotes the proliferation, colony formation, and metastasis of cancer cells. Srx and PrxIV are required in AP-1/matrix metallopeptidase 9 (MMP-9) axis activation, which can enhance tumorgenesis (Wei et al, 2011).

Additionally, Srx expression is upregulated in squamous cell carcinoma, basal cell carcinoma, sweat gland carcinoma, and melanoma in human skin relative to controls (Wu et al, 2014; Wei et al, 2008). As indicated by TUNEL assay, Srx-depleted tumor cells showed higher levels of apoptosis and there were fewer papillomas in mouse skin. Treating mouse skin with 12-O-tetradecanoylphorbol 13-acetate (TPA) prompted Srx expression via the activation of JNK and MAKP signaling pathways (Wu et al, 2014). Srx was also found to be upregulated in poorly differentiated colorectal cancers. And downregulation of Srx led to decreased cancer cell colony formation and cell migration in vitro. Srx depletion also reduced tumor formation and metastasis of colon cancer in mouse models. Srx limits the acetylation of tyrosine (K1037) on epidermal growth factor receptor (EGFR) thus increasing EGFR phosphorylation and the activity of the EGFR-MAPK signaling pathway (Jiang et al, 2015; Wei et al, 2013).

Srx interacts with S100 calcium-binding protein A4 (S100A4) protein to modulate its interaction with non-muscle myosin IIA protein (NMIIA) in lung cancer cell line. S100A4 and NMIIA form a complex that bind reversibly with actin filament. Srx regulate Ca^{2+} binding to S100A4 in a redox-mediated manner. Thus, Srx redox activities are believed to regulated motility, migration and adhesion of lung cancer cells (Bowers et al, 2012; Conti et al, 2008).

Out of six isoforms of Prxs (Prxl-VI), Srx can only reduce the sulfinic acid modification in four isoforms, 2-Cys Prxl–IV. The affinity of Srx is different for various 2-Cys Prxs. When GST fusion protein of Srx was expressed in Hela cells,
immunoprecipitation of H₂O₂ treated cell lysate indicated that Srx had more affinity toward PrxI and PrxII than toward PrxIII and PrxIV (Woo et al, 2005). In addition, the only cysteine residue of Srx, Cys99, which is responsible in the formation of thiosulfinate with protein substrates, did not affect Srx-2-Cys Prx interaction when mutated to serine (Woo et al, 2005). Immunoprecipitation and mass spectrometry results from Wei et al (2011) confirmed that Srx interacts with Prx I–IV when Flag-tagged Srx expressed in HEK293T and A549 cell lines. However, Srx was found to have more affinity toward PrxIV. The same study was also first to recognize the interaction between Srx with TXNDC5 and PDIA6 proteins in HEK293T and in A549 cells (Wei et al, 2011). TXNDC5 and PDIA6 belong to the same family of PDI enzymes. So far, the significance of Srx-TXNDC5/PDIA6 interactions has not yet been characterized.

The PDI family

The redox system in cells is generally categorized as reducing and hypoxic in the cytosol, oxidizing and normoxic in the extracellular milieu, and oxidizing in the ER (Jordan et al, 2005; Essex, 2009). Due to this variation in redox state, cells have developed many signaling pathways to regulate redox reactions. Redox reactions are implicated in many cellular processes, including stabilization of proteins through generation and rearrangement of disulfide bonds. PDIs are group of 21 enzymes that have a central role in thiol-disulfide reactions in cells. Structurally, most PDIs share one or more thioredoxin-like domains, cysteine-X-X-cysteine (CXXC), and ER retention signal (Figure 1.1). Hence, PDIs are believed to play a pivotal role in the biogenesis of approximately one third of proteins destined to enter the secretory pathway (Hatahet & Ruddock, et al 2009). PDIA1 was the first PDI to be discovered in 1963. It is a multi-functional oxidoreductase that participates in nascent protein folding in different cellular compartments, including the mitochondria, plasma membrane, and ER (Kozlov et al,
PDIA1 is involved in processes such as disulfide bond formation, reduction, isomerization, and disulfide exchange reactions (Figure 1.2). Disulfides generated in PDI originate from several sources, including endoplasmic reticulum oxidoreductin-1alpha (Ero1alpha), 2-Cys Prxs, vitamin K epoxide reductase (VKER), glutathione peroxidase (GPX) 7 and 8, and dehydroascorbate reduction to ascorbate pathways (Bulleid & Ellgaard, 2011; Lu & Holmgren, 2014; Bulleid, 2012; Shepherd et al, 2014). H$_2$O$_2$ can directly oxidize PDIs to generate disulfide bonds.

These pathways function as oxidants to transfer electrons to oxidize active-site cysteine residues on PDI. PDIs in turn oxidize newly synthesized protein substrates in the ER to ensure synthesis of physiologically functional native protein substrates (Figure 1.3). Oxidation of cysteine residue during disulfide bond formation is accompanied by oxidative protein folding. In this form, electrons are transferred from the reduced substrate to the PDIs active sites (Zito et al, 2010; Lu & Holmgren, 2014). Hence, PDIs are considered indispensable enzymes for providing maturation and structural stability for substrates (Sato et al, 2013; Lu & Holmgren, 2014). Several PDIs, particularly PDIA1 and PDIA6, have been found to take part in reduction and isomerization of receptor proteins. A related point to consider is that nascent protein substrates can also fold to their native form in pathways independent of PDI proteins. The quiescin sulfhydryl oxidase (QSOX) pathway can be considered one instance of this (Figure 1.4).

The enzymatic activity of PDI family members depends on four main structural features. The first feature is number and sequence of active site CXXC motifs. For instance, PDIA19 (ERdj5) has four thioredoxin-like domains, three of which have CXPC motifs function as thiol-disulfide reductants. ERdj5 reduces thioredoxin-like domain in PDIA6. The reduced PDIA6 interacts with unfolded protein response (UPR) sensor inositol-requiring enzyme 1 (IRE1) in the ER lumen (Oka et al, 2015). PDIs with CXHC thioredoxin like sequence are shown to act as efficient thiol-disulfide oxidants. For
instance, Ero1 selectively oxidizes PDIA1, which has two active site CGHC domains with two b domains, whereas Ero1 has less activity toward TXNDC5, which contains three CGHC folds and no b domains. PrxIV is shown to preferentially oxidize TXNDC5 and PDIA6 relative to other protein PDIs (Sato et al, 2013).

The second factor in determination of PDI enzymatic activity is the presence of certain amino acid residues in PDI structure to modulate pKa of CXXC motif. Local movement of arginine residue is found to lower the pKa of active CXXC domain (Lappi et al, 2004). Arginine residues determine the time required for the client protein to bind to the active site in oxidation and isomerization reactions. The importance of this residue has been validated in PDIA1 PDIA3, PDIA4, and PDIA6 proteins (Lappi et al, 2004; Ellgaard & Ruddock, 2005). Another important factor in regulating CXXC activity is the presence of glutamic acid-lysine pairs in proximity to the thioredoxin like domain. Glutamic acid acts as an electron acceptor, which facilitates exchange of protons between PDIs and client protein substrates. A PDIA1 and PDIA3 oxidative activity is regulated by this residue (Ellgaard & Ruddock, 2005).

The fourth factor is the presence of high-affinity substrate binding pocket, b domain. The presence of b domain has been shown to be required for isomerization activity in PDIs. For instance, the thioredoxin like domain and b domain containing PDIA1 and PDIA3, are established to have oxidase and isomerase activities, whereas TXNDC5, and TMX3 (Thioredoxin-Related Transmembrane Protein 3), that lack the b domain, are proposed to be proficient in oxidation but not isomerization. PDI enzymes lacking thioredoxin-like domain, such as PDIA8, PDIA9, and TMX2, are proposed to be indirectly involved in disulfide bond exchange reactions (Bastos-Aristizabal, et al., 2014; Ellgaard & Ruddock, 2005). The variation in thiol-disulfide reactions and the selectivity of different PDIs toward different substrates suggests the presence of a hierarchy among PDIs toward these reactions. In fact, it has been shown that PDI members can exchange
disulfide with each other. In addition, certain PDIs facilitate thiol-exchange reactions with other PDIs. For instance, PDIA1 and TXNDC5 were found to be conduits for oxidation of other PDIs via the Ero1 oxidation pathway. Meanwhile, ERdj5 is the conduit for reduction reactions (Oka et al, 2015).

Figure 1.1. Domain architecture of the protein disulfide isomerase (PDI) family. Thioredoxin like domain is depicted as a red colored box. Substrate binding domain b is illustrated as a blue box. Transmembrane domain is labeled with TMD in a purple box (Hatahet et al, 2009; Kozlov et al, 2010).
Figure 1.2. Disulfide exchange reactions carried out by PDIs. Red: Reduces substrate. 
A) Reduction of disulfide bond from a disulfide bond to two sulfhydryl groups. B) Oxidation of the substrate from the reduced form to more oxidized disulfide bond. C. and D) Exchange of disulfide linkages without changing the overall oxidative nature of the substrate. C. Isomerization involves changing the overall folding of the substrate, D. disulfide bond alteration with limited changes on the protein conformation (Essex, 2009).
Figure 1.3. Mechanism of substrate oxidation and isomerization by PDIs. A) Oxidation of the client protein (C) by passage of disulfide bond from PDI. PDI exert a nucleophile attack on one of the substrate’ cysteine residue, followed by the formation of mixed disulfide bond with the substrate. Then the second sulfur atom of the PDI initiates a second attack on the mixed disulfide sulfur of PDI releasing the substrate active thiol ion from the PDI. The active thiol of the substrate then attacks the second Cys of on the same protein to form a disulfide bond. B) PDIs break the existing disulfide bonds within a protein substrate to allow for the formation of new bonds between two new Cys residues (Hatahet et al, 2009).
Figure 1.4. Possible sources of disulfide bonds in PDIs. A) Vitamin K epoxide reductase (VKOR) thioredoxin domain gets oxidized during reduction process of vitamin K epoxide to vitamin K; the disulfide bond is passed to PDIs. B) Glutathione peroxidase 7 and 8 (GPx7, 8) used oxidoreductin (Ero1)-produced H$_2$O$_2$ to oxidized PDIs. C) Ero1 used molecular oxygen to generate disulfide bond and releasing H$_2$O$_2$ then passes the disulfide bond to PDIs. D) Reduction of dehydroascorbate to ascorbate by PDIs. E) PrxIV consumes H$_2$O$_2$ to generate interchain disulfide bond (depicted as one unit), before passing it to PDIs. F) Quiescin-sulfhydryl oxidase (QSOX) couples generation of disulfide bonds to molecular oxygen reduction reaction to produce H$_2$O$_2$, and then the disulfide is passed directly to the substrates. Disulfide bond generated via coupling TXNDC5 or PDIA6 with Ero1 and PrxIV have been experimentally validated (depicted in the dashed box), the other pathways are hypothetical suggestions based on that documented for PDIA1 protein (Bulleid & Ellgaard, 2011; Lu & Holmgern, 2014; Bulleid, 2012; Shepherd et al, 2014).
PDI is shown to couple ER stress with oxidative stress through three ROS-generating pathways activated during UPR. Those pathways are ER flavor oxidase Ero1, Nox4 in Nox family NADPH oxidase isoform, and physical linkage with mitochondria. While H$_2$O$_2$ can promote protein folding, increased ER load of un/misfolded protein triggers UPR. Furthermore, UPR activation is generally accompanied by increases in ROS and ER stress (Laurindo et al, 2012). The un/misfolded proteins sequestered by 78 kDa glucose-regulated protein (GRP78), 94 kDa glucose-regulated protein (GPR94) and Calreticulin regulators. These modulators trigger UPR sensors in the cytosolic side of ER membrane. There are UPR tributaries to three response pathways: PRKR-like ER kinase (PERK)–eukaryotic translation initiation factor 2α (eIF2α), inositol-requiring protein 1α (IRE1α)–X-box binding protein 1 (XBP-1), and activating transcription factor 6α (ATF6α) (Wang et al, 2014). The initial results of UPR activation are adaptation and correction of this ER stress through halting of overall protein synthesis, increasing expression of chaperone proteins to correct the misfolded proteins, and direction of the misfolded proteins to the ERAD (ER associated degradation) degradation pathway. Furthermore, UPR activation can arrest the cell cycle at the G1 phase through the PERK pathway. PERK activation causes decrease in the expression of cyclin D1 via the phosphorylation of translation elongation initiation factor eIF2α (Brewer & Diehl, 2000). However, if the cells are incapable of recovering ER homeostasis, death-signaling pathways are triggered. Extended IRE activation can initiate apoptotic ASK/JNK and Bcl2 pathways. CCAAT/enhancer-binding protein homologous protein (CHOP) transcription factors are activated via PERK/activating transcription factor 4 (ATF4) or ATF6 pathways resulting in apoptosis (Laurindo et al, 2012). It is important to note that the ROS generated UPR in some cells do not show increases in oxidative stress due to antioxidant system upregulation (Laurindo et al, 2012; Watson, 2013). PDIs, particularly PDIA1, are recognized to be key enzymes in
oxidative protein folding in the ER to prevent protein misfolding and aggregation. Moreover, the redox regulation of PDIA1 chaperone activity has been shown to play a protective role in preventing activation of the UPR pro-apoptotic pathway (Wang et al, 2014; Xu et al, 2013). It is well established that tumor cells activate UPR during oncogenic transformation as survival strategy in stressful microenvironments and that UPR activation is associated to cancer growth and chemoresistance (Wang & Kaufman, 2014). ATF6α activation upregulates the expression of GRP78. GRP78 overexpression has been associated with the malignancy of colon, skin, kidney, and ovary cancer. Increased XBP-1 splicing has been associated with chemoresistance and short survival in lymphoma B cells (Wang & Kaufman, 2014). Furthermore, the deletion of PERK interrupts mammary tumor development and reduces metastasis of lung cancer cells (Bobrovnikova-Marjón et al, 2010).

Even though the roles of PDI family members in cancer development have not been established, many studies have indicated PDIs overexpression in different types of cancer. PDIA1 is associated with clinical outcomes and tumor survival and progression. PDIA1 is overexpressed in lymphoma, brain, kidney, lung, prostate, and ovary cancers. PDIA1 inhibition by bacitracin was found to sensitize aplidin-resistant Hela cells to aplidin (Xu et al, 2014). PDIA3 is upregulated in cancers of prostate (Pressinotti et al, 2009), laryngeal (Choe et al, 2015), breast (Ramos et al, 2015), colon (Caorsi et al, 2015; Ren et al, 2006). Similar to PDIA1, PDIA3 protected Hela cells from aplidin-induced cytotoxicity (Laurindo et al, 2012). PDIA4 has been found to be upregulated in leukemia (Voss et al, 2001), and liver cancer (Chen et al, 2008; Laurindo et al, 2012). However, the association between the levels of the majority of PDI members and cancer progression requires further investigation. The following paragraphs focus on expression of the Srx-interacting PDIs TXNDC5 and PDIA6 in cancer.
TXNDC5 and PDIA6 in cancer

The TXNDC5 gene is located at Chr6p24.3 and encodes a 432 amino acid protein. It has N-terminal signal peptide of 32 amino acids, and C-terminal KDEL ER retention signal and three CGHC domains. TXNDC5 was discovered in 2003 in two parallel studies by Sullivan and Knoblach to protect normal and tumor endothelial cells from undergoing apoptosis. Under hypoxic conditions, TXNDC5 becomes overexpressed and modulates anti-apoptotic proteins. However, the biochemical mechanisms of TXNDC5 have not been studied, particularly its role in disulfide bond exchange, chaperon activity, and UPR activation in lung cancer. TXNDC5 expression is upregulated in lung (Vincent et al, 2011), prostate (Wang et al, 2014), colon (Wang et al, 2007), gastric (Wu et al, 2015), breast, cervical, esophageal, liver (Nissom et al, 2006), and ovarian cancers (Chang et al, 2013). Because of its role in enhancing cancer cells proliferation, colony formation and cell migration; TXNDC5 is believed to act as an oncogene (Chang et al, 2013; Wu et al, 2015).

Cell fractionation and microscopic studies show that most of TXNDC5 proteins are located in the ER and Golgi organelles. Thus, they are believed to be involved in protein folding and anterograde transport. Meanwhile, nearly one fifth of the total TXNDC5 proteins are present on the surface of the plasma membrane, and they are presumably involved in events on the cell surface (Charlton et al, 2010). For instance, knockdown of TXNDC5 affects the presence of AdipoR1 (Adiponectin Receptor 1) presence on surface of plasma membrane through affecting its trafficking and endocytosis process (Charlton et al, 2010). TXNDC5 interacts directly with Androgen receptor (AR), which is enhanced by dihydroxytestosterone. TXNDC5 binds and stabilizes AR structure, and consequently AR transcription activity and responsiveness to different ligands changes. An example of these ligands is estrogen, which enhances signaling of AR to promote growth of prostate cancer cells. As a result, TXNDC5 acts as
an alternative pathway for AR activation when androgen hormone is limited in castration-resistant prostate cancer (CRPC) (Wang et al, 2014). TXNDC5-AR interaction protects AR from degradation and assists its translocation to the nucleus, which indicates that TXNDC5 acts as a chaperone for AR.

TXNDC5 is upregulated in androgen naïve prostate cancer and in CRPC. There was more protein expression in androgen-independent cancer than in androgen-dependent cancer. Also, TXNDC5 promotes LNCaP adenocarcinoma cell proliferation in the presence of androgen. It has been shown that downregulation of TXNDC5 in androgen-independent LNCaP-Al, PC3, and VCap cells decreases their invasiveness. In vivo studies have shown larger tumors when TXNDC5-derived LNCaP cells are injected into xenograft models of male Balb/c athymic nude mice (Wang et al, 2014). TXNDC5 overexpression in prostate cancer, which occurs in response to low levels of androgen hormone, sensitizes the cells to activated protein kinase B (AKT), extracellular-signal-regulated kinases (ERKs)1/2, and human epidermal growth factor receptor 2 (HER2) (Wang et al, 2014). TXNDC5 knockdown in Hela cells increased phosphorylation of AMP-activated protein kinase (AMPK). However, the phosphorylation of P38 mitogen-activated protein kinases (p38MAPK) was reduced (Duivenvoorden et al, 2014, Charlton, 2010). In renal cell carcinoma (RCC), AdipoR1 has the opposite effect, where the reduced AdipoR1 and adiponectin levels are associated with increased aggressiveness in RCC. Overexpression of TXNDC5 aggravates metastasis of clear-cell RCC in vitro and increases RCC tumor growth after subcutaneous injection into nude mouse models (Duivenvoorden et al, 2014).

TXNDC5 expression is elevated in pre-cancerous lesions in gastric cancer. In gastric adenocarcinoma, MKN45, and normal gastric epithelial cell line, HFE145, demonstrated that TXNDC5 could affect cell characteristics in normal and cancer cells. TXNDC5 overexpression enhances tumor formation by affecting the proliferation rate of
the cell. Zhang et al. reported that TXNDC5 upregulation increases number of cells in the G2/M phase of cell cycle, while TXNDC5 downregulation increases number of cells in G0/G1 phase (2010). They also reported more cell migration and colony formation and a lower rate of apoptosis in gastric cells with overexpressed TXNDC5 (Zhang et al., 2010). This anti-apoptotic action is further supported in pancreatic cancer, where the pro-oncogene NR4A1 orphan nuclear receptor and TXNDC5 are overexpressed. NR4A1 downregulation leads to ER defragmentation and concurrent alteration of expression of many of ER stress proteins, such as ATF-4, GRP78, TXNDC5, and CHOP (Lee et al., 2014). NR4A1 is involved in sustaining endoplasmic reticulum stress and directly regulates TXNDC5 transcription in pancreatic and renal cancers. Thus, NR4A1 enhances the overexpression of TXNDC5 protein to reduce ER stress, consequently leading to decreases in pro-apoptotic CHOP activities (Lee et al., 2014; Hedrick et al., 2015).

TNFα (tumor necrosis factor-alpha) -treated human umbilical vein endothelial (HUVE) cells showed less phosphorylation of ERK1/2 when TXNDC5 was knockdown by siRNA, and this decrease was attributable to inactivation of Ras and Raf. However, the phosphorylation of p38 MAP and JNK kinase was found to be unaffected by TXNDC5 downregulation. NF-κB (nuclear factor of kappa light polypeptide gene enhancer in b-cells 1) signaling represented by IκBα degradation and ICAM expression. ERK inactivation has been found to reduce activity of AP-1, which results in decreased expression of angiogenesis inducing proteases, matrix MMP9 and cathepsin B. For this reason, TXNDC5 is believed to enhance angiogenesis of endothelial cells (Camargo et al., 2013). Moreover, studies on rheumatoid arthritis (RA) show that TXNDC5 may lead to an increase in MMP1 and MMP13 proteases. MMP1 enzyme is overexpressed in cancers and is suggested to be involved in cancer cell invasion and metastasis (Sauter et al., 2008). MMP13 has been associated with promoting tumor angiogenesis through
focal adhesion kinase and ERK signaling and secretion of vascular endothelial growth factor A from endothelial and fibroblast cells (Kudo et al, 2012). It is likely that TXNDC5 modulates the activity metalloprotease to enhance metastasis, invasion, and angiogenesis of cancer cells.

TXNDC5 protein is highly expressed in endothelial cells of tumor tissues and atherosclerotic plaques, particularly under hypoxic conditions. Knockdown of TXNDC5 reduces endothelin-1, adrenomedullin, and CD105 levels. These protein molecules are known to protect endothelial cells from hypoxia. It has been proposed that TXNDC5 has a protective role against hypoxia-induced cell death in endothelial cells (Sullivan et al, 2003). In prostate cancer, androgen-deprivation treatment (ADT) leads to increases in TXNDC5 protein expression under hypoxic conditions through the HIF-1α and miR-200b signaling pathway. In addition to TXNDC5, expression of AR also increases. Moreover, the hypoxic condition fortifies the interaction between TXNDC5 and AR and their downstream signaling pathways (Wang et al, 2014). The hypoxic conditions caused by CoCl2 in TXNDC5-overexpressing PC cells also sensitize the cells to ERK1/2, AKT pathways. Hypoxia induces TXNDC5 expression in RA. Hypoxia’s effect on TXNDC5 expression can be explained by activity of miR-200b, which targets 3' UTR TXNDC5 mRNA (5'-CAGUAUUU-3'), which reduces TXNDC5 expression. HIF-1α and miR-200b are involved in prostate cancer progression, and it is possible that HIF-1α indirectly regulates TXNDC5 expression under hypoxic conditions through miR-200b pathway, where HIF-1α stabilization leads to miR-200b downregulation (Wang et al, 2014). miR-200b downregulation is associated with increased angiogenesis and cell migration (Chan et al, 2011).

The PDIA6 gene is located at Chr2p25.1 and encodes a protein of 440 amino acids. It has an N-terminal signal sequence of 19 amino acids and C-terminal KDEL sequence. PDIA6 contains two CGHC domains and one b domain. It has been reported
that PDIA6 has both chaperone and isomerase activity. Isomerase activity has been credited mainly to the N-terminal cysteine residues at both active CGHC sites. The promoter region of PDIA6 contains ERSE elements to which spliced ER XBP-1 binds after activation of UPR. Thus, PDIA6 expression can be induced during ER stress (Eletto et al, 2014; Groenendyk et al, 2014). Furthermore, PDIA6 can bind to ER stress marker, GRP78, suggesting a possible role of PDIA6 in assisting GRP78 in protein folding. PDIA6 has been verified to assist in platelet aggregation and activation through modulating receptors on the surface of plasma membranes (Galligan & Petersen, 2012).

However, few studies available regarding the role of PDIA6 protein in the development of lung cancer. Nonetheless, PDIA6 has been found to be upregulated in head and neck squamous cell carcinoma. It has also been found to protect Hela cells from aplidin-induced cytotoxicity (Laurindo et al, 2012). PDIA6 exerts a chemopreventive effect in cisplatin-resistant non-small-cell lung cancer cells (Tufo et al, 2014). It was found to translocate to the nuclei of treated tumor cells but not in normal cells. PDIA6 knockdown prompted increases in caspase 4 activation causing receptor-interacting serine/threonine-protein kinase 1 (RIPK1) upregulation, thus activating programmed necrotic death pathways (Tufo et al, 2014). PDIA6 overexpression is associated with neoplastic transformation in invasive ductal carcinomas of breast cancer. More PDIA6 was observed in metastatic lymph nodes than in controls (Ramos et al, 2015).
**Research objectives**

Srx connects several main redox-regulating systems in cells. Therefore, additional elucidation of its function could make it a creditable target for lung cancer treatment. In this study we aimed to find additional function of Srx in cancer through its interacting proteins. Moreover, we attempted to understand the consequences of Srx-protein interaction on the physiology of the cells and in lung cancer development. We hypothesis that Srx-interacting protein are essential for enhancing oncogenic characteristics of Srx in lung cancer cells. TXNDC5 and PDIA6 oncogenic phenotype recapitulate that of Srx enzyme.

**Specific aims:**

A. In chapter two, I aimed to substantiate Srx interaction with each of TXNDC5 and PDIA6 in cells and determine the protein domains responsible for these interactions. Moreover, through subcellular fractionation Srx was determined to localize in the ER. The co-localization of Srx with TXNDC5 and PDIA6 were characterized by immunofluorescence and immunoprecipitation methods.

B. Experiments of chapter three were aimed at clarifying physiological functions of Srx, TXNDC5, and PDIA6 in lung cancer cells, particularly in the ER compartment. Role of each protein in chaperone activity, in response to different ER stressor, is examined, in addition to their role in UPR sensors activation. Luciferase refolding assay, XTT assay, RT-PCR, and Western blot methods were employed to elucidate these functions. The roles of TXNDC5 and PDIA6 in oncogenic phenotypes in lung cancer cells were characterized. Cell proliferation, anchorage independent colony formation, and cell migration were examined. In addition, role of TXNDC5 and PDIA6 in phosphokinase signaling in response to epidermal growth factor was demonstrated.
1.2. Materials and methods

Cell lines and chemicals

Human embryonic kidney cells (HEK293T) and human lung adenocarcinoma cell lines (A549, H226 and H2030) were purchased from ATCC. Dulbecco's Modified Eagle Medium (DMEM) (Thermo scientific; Lonza Bio Whittater) supplemented with 10% fetal bovine serum (FBS) (Atlanta Biologicals) was used to culture HEK293T cells. Roswell Park Memorial Institute-1640 (RPMI-1640) medium (Hyclone) supplemented with 10% FBS was used to culture the cancer cell lines. Penicillin- streptomycin solution (Hyclone, Thermo scientific) [penicillin is 100 U/ml and streptomycin 100 µg/ml] and 5 µg/ml gentamycin (gibco, Life Technologies) were added to the media before use. Puromycin (gibco, Life Technologies), 1 µg/ml, was added to maintain the lentiviral generated stable cell lines. The cells were incubated in humidified atmosphere with 5% (v/v) CO₂ at 37°C. Hydrogen peroxide and tunicamycin were purchased from Sigma-Aldrich.

Plasmid constructs and lentiviral production

A. pcDNA 3.1/ TXNDC5- Myc-His, pcDNA 3.1/ PDIA6- Myc-His constructs, and pcDNA 3.1/ Srx- Myc-His construct:

1. Total RNA was extracted from HEK293T cells following RNeasy Mini Kit (Qiagen).

2. First Strand cDNA of TXNDC5 and PDIA6 were synthesized by ProtoScript II Reverse Transcriptase kit (New England Biolab).

3. The PCR was set to Initial denaturation temperature of 94°C for 5min, denaturation 94°C 30sec, annealing 68 °C, extension 68°C and final extension 72°C for 10min, in a total of 30 cycles.
4. The products were digested with either Xho I and Xba I restriction enzymes for TXNDC5, HindIII and Xbali restriction enzymes for PDIA6, or BamHI and EcoRI restriction enzymes for Srx.

5. Empty pcDNA 3.1/ - Myc-His constructs were digested with the corresponding restriction enzymes for each of the cDNAs.

6. After DNA ligation, the constructs were amplified by Top 10 bacteria (Invitrogen), followed by Maxi prep (Qiagen)

7. The correct sequence for each of TXNC5, PDIA6, and Srx were verified by sequencing (Appendix).

B. **Constructs for stable overexpressed cells:**

1. To tag cDNA of TXNDC5 and PDIA6 with Flag sequence, the cDNA of these genes were subcloned from pcDNA3.1 myc-His A vector to p3xFLAG-CMV-14 vector.

2. For TXNDC5-Flag NotI and XbaI restriction sites were used while for PDIA6 HindIII and Xbali enzymes were used.

3. Then the cDNA of the genes tagged with Flag were sub-cloned from p3XFLAG-CMVTM-14 vector to PLVX - IRES-PURO expression vectors.

4. Primers with restriction BstB1 and Sall enzymes were generated for both TXNDC5 and PDIA6. Forward primer with Sall restriction site was: 5’ CATCGTCGACGTGCTAAGCTTGC 3’. The reverse primer with BstB1 restriction site was: 5’ CGGTTCGAAGGATCACTACTTTGCG 3’.

5. After DNA ligation, the constructs were amplified by Top 10 bacteria (Invitrogen), followed by Maxi prep (Qiagen)

6. The correct sequence for each of TXNC5 and PDIA6, were verified by sequencing.
C. Constructs for stable knockdown cells:

1. Short hairpin constructs were purchased from Sigma-Aldrich to knockdown TXNDC5 and PDIA6 expressions.

2. All shRNA constructs including MISSION pLKO.1-puro control vector (vector control), MISSION Non-Target shRNA (ShNT) and shRNAs specifically targeting either TXNDC5 (ShTXNDC5), or PDIA6 (ShPDIA6) were commercially obtained (Sigma-Aldrich).

D. Generation of lentiviral particles

1. The lentiviral particles were generated following instructions provided in MISSION Lentiviral Packaging Mix manual (Sigma-Aldrich).

2. Lentiviral particles were produced in HEK293T cells using the provider's plasmid packaging system and PolyJet transfection reagent (3:1). And the viral soup stored in -80C.

3. To establish stable cell lines, A549 and H2030 cells were cultured in 35mm dish till 90% cell confluence.

4. The cells were infected with 1ml of the viral soup.

5. The remaining steps was followed as instructed by the manual.

6. Cells were maintained in puromycin containing medium to establish stable cells.

7. A549 Flag Srx, HEK293T Flag Srx, and H226 knockdown cells were provided by Dr. Qiong Wei lab, University of Kentucky.
Site directed mutagenesis

A. Mutation of Srx cysteine 99 residue (C99A)

1. Site directed mutagenesis was used to mutate cysteine99 residue (TGC) to alanine (GCC).

2. Protocol provided by QuikChange II site directed mutagenesis kit (Agilent Technologies) was followed as instructed.

3. Forward 5’ TACTCCTTTGGGGCGCCCAACCCTACGCGGCC 3’ and reverse 5’ GGCGCGTAGCGGTGGGCGCCCCCAAAGGAGTA 3’ primers were used.

4. The generated constructs were amplified by Top 10 bacteria (Invitrogen), followed by Maxi prep (Qiagen).

5. The correct sequence for each of TXNC5 and PDIA6, were verified by sequencing (Integrated DNA Technologies).

B. Thioredoxin like fold deletion in TXNDC5:

1. Site directed mutagenesis was also used for thioredoxin like domain deletion, pcDNA 3.1/ TXNDC5- Myc-His plasmid. One deletion per construct was prepared.

2. Protocol provided by QuikChange II site directed mutagenesis kit (Agilent Technologies) was followed as instructed.

3. The primers used are listed below:

   The primers for N-terminus thioredoxin like domain (D1) deletion (Del1):

   Forward primer: 5’ATCCAGAGCGCGCACAATGACCTGGGAGAC 3’

   Reverse primer:

   5’ GTCTCCAGGTCATGGCGCGCGCTCTGGAGAC 3’.
The primers for second thioredoxin like domain (D2) deletion (Del2):
Forward primer:
5’ GTTGCACAAGGCGACCACGACGAGCTGGCTCTGGGCCTTG 3’
Reverse primer:
5’ CAAGGCCCAGAGCCAGCTGCTCGTGGTGCGCTTTGTGCAAC 3’
The primers for the C-terminus thioredoxin like domain (D3) deletion (Del3):
Forward primer
5’ CGATGACACCATTGCAGAAGGAATAACCGAGGAACTCTCTAAAAAGG
3’
Reverse primer
5’ CTTTTTTAGAGAGTTCTCGTATTCTCTCTGCAATGGTGTCATC3’
4. Presence of the desired deletion was verified by sequencing (Integrated DNA Technologies).

C. Thioredoxin like fold deletion in PDIA6:
1. Site directed mutagenesis was also used for thioredoxin like domain deletion, pcDNA 3.1/ PDIA6- Myc-His plasmid. One deletion per construct was prepared.
2. Protocol provided by QuikChange II site directed mutagenesis kit (Agilent Technologies) was followed as instructed.
3. The primers used are listed below:
The primers for N-terminus thioredoxin like domain (D1) deletion (Del1):
Forward primer:
5’ GTTATTCAGAGTGATAGTTTGTGGAAGAAAGCAGCAACTGC 3’
Reverse primer:
5’ GCAGTTGCTGCTTTCTTCCACAAACTATCACTCTGAATAAC 3’

The primers for the C-terminus thioredoxin like domain (D2) deletion (Del2):
Forward primer:
5’GGACAGTGAAGATGTTTGGGCTGCCGCAGCTTCAG 3’
Reverse primer:
5’TAGGCTCTAGAGTCAGCCACAACACAGGCTGGTGCTCCTC3’

The primers for Truncation of C-terminal amino acids in PDIA6 are:
Forward primer:
5’ GTTAAGCTTATGGCTCTCCTGGTGCTCGGTCTGGTG3’
Reverse primer:
5’ TAGGCTCTAGAGTCAGCCACAACACAGGCTGGTGCTCCTC3’

4. Presence of desired deletion was verified by DNA sequencing (Integrated DNA Technologies).

Protein purification

Prokaryotic system was used to purify Srx and TXNDC5 recombinant proteins.

1. Srx and TXNDC5 cDNA were subcloned from pcDNA 3.1/ Myc-His plasmids to pRSET B constructs.
2. BL21 bacteria were used to amplify translation of the genes.
3. 1M IPTG was used to induce translation of the proteins in the bacterial culture. The cells were pelleted and lysed.
4. Then Ni-Affinity Chromatography was used to purify the proteins. Ni was employed to bind Histidine tagged TXNDC5 or Srx proteins.
5. The proteins eluded by 250mM imidazole buffer.

6. The imidazole was exchanged with 1X PBS by dialysis using Pur-A-Lyzer Midi Dialysis Kit (Sigma) Samples stored in -20°C before the co-immunoprecipitation experiments.

Immunoprecipitation and immunohistochemistry

A. Immunoprecipitation

1. The cells lysed using either NP-40 lysis buffer (rpi CORP) or immunoprecipitation buffer (50mM HEPES pH7.5, 150mM NaCl, 1mM EDTA, 10% Glycerol, 0.1 % Tween-20, and 0.5% Triton X100) mixed with protease inhibitors.

2. A volume of 250-400µl of lysis buffer was added to each 100mm cell culture plate. The plates were incubated at 4°C for 1-2hr.

3. The lysate centrifuged at 15000rpm for 10min at 4°C.

4. A volume of 100µl supernatant was used as input control. The rest of the lysate was mixed with 20µl of magnetic beads for co-immunoprecipitation part.

5. Dynabeads Protein A (life technologies), Protein G magnetic beads (New England BioLab) or ANTI-FLAG M2 Magnetic Beads (Sigma-Aldrich,) were used.

6. The samples incubated overnight at 4°C. After using magnetic separator to collect the beads, the lysate was discarded at the end of the incubation period.

7. The beads washed in cold immunoprecipitation buffer (without Triton x100) and re-suspended in 1X Laemmli sample buffer with β-mercaptoethanol before boiling at 90°C for 10min.
8. Magnetic separated was used to separate the denatured samples from the beads before loading on SDS-PAGE gel for Western blot.

9. When antibody-free magnetic beads were used, 10µl of the primary antibody was incubated with the supernatant overnight, and then the beads were added. The samples incubated for additional 2hr at 4°C before proceeding to Western blot.

10. For purified recombinant Srx and TXNDC5 proteins co-immunoprecipitation experiment, 2µg of Srx protein was mixed with 10µg of TXNDC5 protein in 500µl immunoprecipitation buffer. The mixture was incubated for 2hr at 4°C. Then 10µl of anti-Srx antibody was added to the mix and incubated at 4°C overnight. A Dynabeads Protein magnetic bead, 20 µl per sample, was mixed with the samples before incubation for 2hr at 4°C. Then beads were washed and processed for Western blot.

11. The samples loaded on commercial SDS-PAGE gels, 4-12% gel (Invitrogen), and transferred using nitrocellulose or PVDF membranes

**B. UPR experiment**

1. The cells were seeded in 35 mm dishes and treated for either 4,6,8,12, and 24hr with 2µg/ml of Tunicamycin diluted in RPMI medium, 10% FBS.

2. The cells were scarped to a microcentrifuge tube and pelleted at 1500rpm for 5min.

3. The pellet were suspended in 100µl NP-40 buffer and lysed for 5min at 4C. The lysate centrifuged at 15000rpm for 10min at 4C.

4. The supernatant was saved as a cytosolic fraction.

5. The pellets re-suspended in another 100µl NP-40 and sonicated for 30 sec and used as a nuclear fraction.
6. Both fractions were mixed with Laemmli sample buffer and β-mercaptoethanol before being boiled at 95C.

7. The samples were loaded on 12% SDS-PAGE gel and transferred to PVDF membranes. The membranes were blotted for GRP78, XBP-1 (Santa Cruz Biotechnology), phosphorylated eIF2α (Cell Signaling), and β-Actin (Sigma) antibodies. The band detected in nuclear fraction near 50 kDa was considered as Spliced XBP-1 protein.

C. Phosphokinase signaling experiment

1. A549 cells cultured in 35 mm dish, serum free RPMI medium with antibiotics was used to starve the cells for 16hr.

2. Epidermal growth factor (EGF), 100ng/ml, prepared in 10% FBS RPMI medium was added to the cells for different durations: 0, 7,15,30,60 or 120min.

3. The cells lysed using 200µl RIPA buffer with protease inhibitors.

4. The lysate centrifuged at 15000 at 4C for 10min.

5. The supernatant was mixed with Laemmli buffer and β-mercaptoethanol, and boiled for 10min at 90C.

6. The samples loaded on 4-12% SDS-PAGE commercial gel (Invitrogen) for Western blot.

D. Immunohistochemistry staining

Normal human and lung cancer tissue microarray slides were purchased from Biomax.US. Slide of multiple organ normal tissue array, 47 cases/99 cores (Cat# FDA 999 J256) and lung disease spectrum tissue microarray with 99 cases /100 cores (Cat# BC04002 057) were used. The lung cancer slide contained five cases of carcinoid, five cases of inflammatory pseudotumor, and ten cases of metastatic carcinoma, ten of each of small cell undifferentiated carcinoma and alveolar cell carcinoma, twenty cases of each squamous cell carcinoma and
adenocarcinoma, in addition to tuberculosis, adjacent tissue, adjacent normal tissue and normal tissue.

1. The slides rehydrated in xylene and ethanol 100, 95, 70% respectively.

2. The slides immersed in antigen retrieval solution made of 10% target retrieval solution (10x) (Dako, Ref S1699), 90% glycerol, and 1mM EDTA (Fisher Scientific).

3. Primary anti TXNDC5 antibody (Santa Cruz biotechnology, Cat# sc-271465) prepared in mouse in a dilution of 1:50 (Antibody Diluent, DakoCytomation, Ref # S0809) and it is added to the slides for 2hr.

4. Then biotin-streptavidin conjugated horseradish peroxidase (HRP) secondary antibody added Chromatogen DAB (3, 3-diaminobenzidine) was used as a HRP substrate that produced a dark brown reaction product as instructed by Dako LSAB2 System-HRP kit (Ref K0673).

5. The slides stained for 1 min with Hematoxylin, followed by dehydration in 70, 95, 100% and clearing with xylene.

6. Images and quantification of the slide staining in each core is analyzed by measuring the intensity of the brown stain by using Image Scope software, version 11.2.0, (Aperio Technologies).

7. Percent of positive pixel is plotted against the tissue organs of each slide. The data is presented as Mean±SEM.

E. Antibodies used

7. Anti-TXNDC5 antibody (Santa Cruz biotechnology, Cat# sc-271465) was diluted 1:1000 in 5% BSA. PDIA6 (Santa Cruz biotechnology, Cat# sc-271465), PrxIV (Abcam, ab59542), PrxIII (Santa Cruz biotechnology, Cat#, sc-33574), β-actin (Sigma, A2228), flag (Sigma, F3165) antibodies were incubated in 5% dry milk prepared in TBS. Antibodies for phosphorylated c-Jun, Total c-Jun,
phosphorylated ERK\(_{1/2}\), Total ERK\(_{1/2}\), total AKT and phosphorylated AKT (Cell Signaling), in 1:1000 dilutions in 5% Bovine serum albumin (BSA) were used. Secondary anti-mouse and anti-rabbit antibodies (R&D systems; cell signaling) used in concentrations of 1:5000 for 1hr at room temperature. SuperSignal West Dura Chemiluminescent Substrate (Thermoscientific) was used to enhance HRP signals from the secondary antibodies.

**Prediction of tertiary structures and docking sites**

1. Protein sequenced for Srx, TXNDC5, or PDIA6 protein (Appendix) was entered to I-TASSER server for protein structure and function prediction (http://zhanglab.ccmb.med.umich.edu/I-TASSER/).
2. The predicted structure with the highest score was used in the experiments.
3. Molecular visualization of the results was prepared by Pymol software (Delano Scientific LLC).
4. Srx and TXNDC5, or Srx and PDIA6 structures in step 2 were inserted into ZDOC server, to predict Srx docking sites with either TXNDC5 or PDIA6 (http://zdock.umassmed.edu).
5. The model with the highest score was used in the experiments to hypothesize for Srx-TXNDC5/PDIA6 complex formation.

Methods references: (Roy et al., 2010; Yang et al., 2015; Pierce et al., 2014)
**Subcellular fractionation**

1. A549 cells were cultured in 100mm dishes until 90% confluence. Three dishes prepared per group.
2. The cells lysed in chilled homogenization buffer (0.25M sucrose, 25mM KCL, 10mM HPES). Protease inhibitor cocktail was added to the lyses buffer before use.
3. The lysate were moved to 2ml microcentrifuge tubes filled to 1/4th with 1.0 mm glass beads (BioSpec).
4. MiniBeadBeater-16 (BioSpec, Model 607) was used to homogenize the cells for 3min.
5. A fraction of cell lysate was saved as a total cell fraction and stored at -20°C.
6. The rest of the sample centrifuged at 15000rpm at 4°C for 15min to remove mitochondrial, nuclear, and cytoskeletal fractions.
7. After centrifugation a part of the supernatant was saved as cytosolic fraction. The cytosolic fraction was centrifuged for additional 30min, at 20 000 rpm to remove microsomal fractions.
8. The rest of ER containing supernatant was centrifuged at 35 000 rpm for 70min at 4°C using Beckman SW41 Ti rotor.
9. The supernatant was discarded and the microsomal fractions washed with 1X PBS by centrifugation at 35 000 rpm for 10min.
10. Then the sediments were lysed using 1X Laemmli sample buffer with β-mercaptoethanol prepared in ddH₂O.
11. Equal amount of total and cytosolic fractions lysed using Laemmli sample buffer with β-mercaptoethanol and loaded on 4-12% gel for Western blot.
12. Anti-Calnexin (Santa Cruz) was used as an ER marker. Anti-GAPDH (Santa Cruz) was used as a cytosolic marker and Anti-PrxIII (Santa Cruz) antibody was used as a mitochondrial marker.

**Immunofluorescence imaging**

1. 3000 cells per chamber were cultured in either DMEM or RPMI medium for HEK293T and A549 cells respectively.
2. The media were supplemented with 10% FBS for 24hr.
3. After 24hr, the cells were fixed with chilled methanol for 10min. followed by wash step with 1X PBS, pH 7.5.
4. The cells were incubated in 5% goat serum blocking buffer (goat serum diluted in 1% BSA prepared in 1X PBS), for 45-60min at room temperature. Followed by a 5min wash step with TBST (0.01% Tween 20).
5. The primary and secondary antibodies prepared in dilution of 1:100, and 1:1000 respectively, prepared in 1% BSA diluted in 1X PBS. The 150µl of the primary antibodies added to each chamber and incubated for 2hr at room temperature.
6. The excess primary antibody was removed by TBST wash.
7. The fluorescently labeled secondary antibody was added for 1hr, in a dark humid container.
8. Same steps starting from blocking buffer were repeated to blot for the second primary antibody.
9. The slides slightly air-dried. A drop of DAPI containing prolog-anti fade was added to the slide and covered with cover slip.
10. The slides stored a dark container in 4°C until analysis by confocal microscope.
Intracellular refolding assay

1. A549, H2030, or H226 cells were cultured in 12 well plate (n=4) till 85% confluence.
2. PolyJet reagent was used to transfect the cells with 1µg pGL3 luciferase reporter vector for 24hr.
3. To control for luciferase translation during refolding process, Cyclohexamide (10µg/ml) was added to the growth medium 30min prior to treatment.
4. The cells either treated with either non-lethal doses of heat-shock, 42°C for 30min in water bath, or 1mm H$_2$O$_2$ at 37°C for 10min.
5. The medium in the well changed to fresh RPMI medium (10% FBS) and the cells returned back to 37°C incubator.
6. The cells were scraped in 1ml RPMI growth medium at different time points and centrifuged at 15000 for 1min.
7. The pellets lysed by adding 100µl passive lyses buffer (Promega) per sample.
8. The samples were mixed with luciferase substrate and read by Luminometer of GloMax-Multi Detection System (Promega).
9. The readings are represented as Mean±SEM of the samples. A detailed procedure of intracellular refolding assay can be found in Walther et al publication (2012).

Clonogenic cell survival assay

Moderate hyperthermia has been demonstrated to sensitize cells to radiation and chemotherapy-induced responses, in vitro as well as in vivo. This hyperthermia-induced response inhibits tumor growth (Urano, 1986; Carper, 1987). To test if TXNDC5 or
PDIA6 affects lung cancer cells tolerance to heat treatment, clonogenic cell survival assay was employed. The procedure used is as follows:

1. A549 cells were cultured in RPMI medium in 35mm dish till 90% confluence.
2. Then the cells subjected to heat at 42°C in a water bath for different durations: 30min, 60min, or 120min.
3. After treatment, equal number of cells were cultured in 35mm dish, under standard growth condition of 37°C for 8 days.
4. The cells were fixed with 100% methanol and stained with 0.1% crystal violet.
5. After removing excess stain by 1x PBS, image of the plates were taken.
6. Then the colonies were scraped and re-suspended in water.
7. Absorbance of the stain was measured at 560nm. The readings of the treated groups were compared to that of the control group.
8. The data are presented as Mean±SEM of the samples (n=3).

Reverse transcription polymerase chain reaction (RT-PCR)

1. A549 or H226 (4X10^5) cells were seeded in 35 mm dishes for 24hr.
2. Tunicamycin (1-2 µg/ml) was used to treat the cells for different durations in their growth medium.
3. Total RNA extracted by RNeasy Mini Kit (Qiagen). First Strand cDNA Synthesis Kit using Protoscript II Reverse Transcriptase kit (New England Biolab) was used to amplify the cDNA of interest.
4. PCR reaction contained primers for either XBP-1 or GAPDH in a total reaction mixture of 50µl:
   Forward XBP1: 5’ TTACGAGAGAAAACCTCATGGCC 3’
   Reverse XBP1: 5’ GGGTCCAAGTTGTCCAGAATGC 3’
   Forward GAPDH: ‘CAACGAATTTGGCTACAGCA 3’
Reverse GAPDH: 5’ AGGGGTCTACATGGCAACTG 3’

5. The PCR setting included 30 sec of 94C of initial denaturation, 94C for 45sec denaturation, 52C for 45sec annealing, and 72C for 30 sec of extension in a total of 34 cycles, and 72C for 8min of final extension.

6. MetaPhor Agarose (Lonza) was used to prepare electrophoresis gel in concentration of 3%. 1X TBE buffer (54g Tris-Base, 27.5g Boric acid, 20ml of 0.5M EDTA in 1L water to make 5X buffer) was prepared to run the gel.

7. 10-12 μl of PCR products mixed with cyber green (10X) and run on the agarose gel at 80V for 4-5 hr.

8. Image of the amplified gene of interest was captured.

9. The percent of spliced XBP-1 (sXBP-1) band intensity was compared to the total XBP-1 band intensities (sXBP-1+ unspliced XBP-1 (uXBP-1) at each time point, GAPDH was used as a control for the procedure.

**XTT Assay**

1. In 96 well plates, 2X10³ cell were seeded per well in 100μl RPMI, phenol red free mediums.

2. XTT reagents (Roche Cell Proliferation Kit II [XTT], or Trevigen’s TACS XTT cell proliferation assay kit) were added starting from the day of seeding, referenced as day 0, up to 5 days.

3. After adding XTT reagents, cells incubated for 3-4hr at 37°C. Reading of the cell media were measured at 490nm and 600nm by GloMax-Multi Detection System (Pormega), and the values subtracted.

4. For cell proliferation assay the values are plotted as fold increase in growth in each day compared to day 0 (Mean±SEM).
5. In cell viability assay, the cells treated with different concentrations of tunicamycin (μg/ml) for 4 days. Then the cell viability was measured. Microsoft excel was used to calculate IC50.

**Anchorage independent colony formation assay**

1. Six well plates (35mm dishes) were used to layer a 1.5ml of agar (0.5%) (Sigma). The agar prepared in RPMI medium, supplemented with 10% FBS and left to solidify.
2. A top agar layer (0.35%) prepared in RPMI was mixed with 7X10³ – 10X10⁴ cell/well. 1.5ml of this mixture added to each well and left to solidify.
3. The colonies nourished with 1.5 ml of RPMI medium, 10% FBS.
4. To avoid fungal contamination 25ng/ml of amphotericin A (Fisher Scientific) was added to medium.
5. The plate incubated for 30 days at 37˚C, with occasional change of the medium.
6. The wells fixated for 10min with absolute methanol, and stained for 20min with 0.25% Crystal Violet. Excess stain was removed with 1XPBS.
7. The colonies were examined under 4X to calculate colonies larger than50 µm used for statistical analysis.

**Wound healing assay**

1. A549 cells (5x10⁵) were cultured in 35 mm dishes (n=4) and supplied with RPMI medium containing 10% FBS.
2. The following day a scratch (wound) was made in the cells monolayer by using 200-microlitter tips, perpendicular to the surface of the cells.
3. The floated cells were washed twice with 1XPBS.
4. RPMI, supplied with 10%FBS, added to the cells. The cells incubated at 37˚C.
5. Image of the cells were taken each 24hr, starting from day 0 up to day 3.
6. The remaining wound gap was measured in each day and compared to image of the same scratch of day 0.
7. The data represented as Mean±SEM of remaining gap.

**Subcutaneous or tail-vein injection Of A549 cells into SCID mice**

1. Severe combined immunodeficiency (SCID) female mice, 6-week of age, were injected in tail vein with nearly $5 \times 10^5$ cells/mouse (in 100 μl of PBS).
2. After 8 weeks the animals were sacrificed.
3. Lung tissues were collected and fixed with 4% paraformaldehyde and saved in 70% ethanol.
4. The tissues proceeded with standard paraffin embedding, sectioning, H&E staining and histopathological examination.
5. Anti-TXNDC5 antibody (Santa Cruz) was used to immunoblotted for TXNDC5 proteins.
6. The intensity of the TXNDC5 staining in the tumor site is compared to the normal surrounded lung tissue.

**Statistical analysis**

Data were analyzed by two-tailed student t-test. Numbers presented as mean ± SD (or SEM). 95% was used as confidence interval. The difference was defined to be statistically significant at p-values ≤ 0.05.
CHAPTER TWO
VERIFICATION OF THE SRX-TXNDC5/PDIA6 INTERACTION

2.1. Results

2.1.1 Co-immunoprecipitation of Flag tagged Srx with TXNDC5 and PDIA6

TXNDC5 and PDIA6 proteins were co-immunoprecipitated with Flag-Srx in HEK293T cells (Wei et al., 2011). The identities of the PDI proteins were confirmed by mass spectrometry in HEK293T and A549 cells (Figure 2.1).

![Figure 2.1. Co-immunoprecipitation of Flag tagged Srx with TXNDC5 and PDIA6. A) Silver staining showing pull-down of approximately 48 kDa band of TXNDC5 and PDIA6 with Srx tagged with Flag in HEK293T cells, In addition to immunoprecipitation of 2-Cys Prxs (I, II and IV). B) Mass Spectrometry was used to verify identity of TXNDC5 and PDIA6 proteins (Wei et al., 2011).](image-url)
2.1.2 Srx interacts directly with TXNDC5 in vitro

In order to verify Srx-TXNDC5 interaction, purified recombinant Srx and TXNDC5 proteins were co-immunoprecipitated. The Western blot result confirms pull down of TXNDC5 with Srx indicating their direct interaction in vitro (Figure 2.2).

Figure 2.2. Srx interacts directly with TXNDC5. Co-immunoprecipitation of purified recombinant Srx and TXNDC5 proteins. Srx and TXNDC5 form a protein complex in vitro.
2.1.3 The active site cysteine residue of Srx is dispensable in the Srx-TXNDC5/PDIA6 interaction

Since cys99 is considered the key of catalytic center in Srx, it was expected Srx-TXNDC5/PDIA6 interaction to be cys99 residue dependent (Figure 2.3A). To test this prediction HEK293T cells were transfected with either wild type or mutant Srx constructs (C99A), tagged with Flag. Co-immunoprecipitation results do not display significant differences in the detected TXNDC5 or PDIA6 protein levels pulled down with Srx. Thus, cys99 of Srx is unessential for Srx-TXNDC5/PDIA6 interaction (Figure 2.3B).

![Diagram of Srx architecture and co-immunoprecipitation results](image)

Figure 2.3. The interaction of Srx with TXNDC5/ PDIA6 is independent of Srx catalytic function. A) The architecture of human Srx. The only catalytically active cysteine residue (C99) was mutated to alanine by site directed mutagenesis. B) Co-immunoprecipitation of Srx tagged with Flag with TXNDC5 and PDIA6 in HEK293T cells transfected with either wild type Flag-Srx (WT) or cysteine 99 mutant Srx (C99A) constructs. PrxIV is used as a control.
2.1.4 The interaction of Srx with TXNDC5/PDIA6 is thioredoxin-like domain dependent

To identify protein domain in TXNDC5 responsible for its interaction with Srx, I-TASSER prediction software was used to obtain the tertiary structure of both proteins (Figure 2.4. A and B). The model with the highest score in Z-doc software was selected to predict the domain of Srx-TXNDC5 interaction. Based on this prediction, it was hypothesized that TXNDC5 N-terminal (D1), and C-terminal (D3) thioredoxin like domains to be responsible for its interaction with Srx (Figure 2.4.D). To test this assumption HEK293T flag tagged Srx cells were transfected with c-Myc tagged TXNDC5 constructs, with deleted thioredoxin like domains (Figure 2.5. A). The lysates were immunoprecipitated for Flag, then the Western blot membrane immunoblotted for TXNDC5 using c-Myc antibodies. The results indicate the N-terminal thioredoxin like fold (D1) to be required for the Srx-TXNDC5 interaction, whereas the third thioredoxin like fold (D3) to be only partially involved in this interaction (Figure 2.6).

I-TASSER prediction software was used to obtain the tertiary structure of PDIA6 (Figure2.4. C). In Z-doc predicted model with the highest score, PDIA6 was expected to interact with Srx via PDIA6 C-terminal domain (Figure 2.4.E). However, our immunoprecipitation result showed no effect of this domain on Srx-PDIA6 interaction (data not shown). Therefore, similar to TXNDC5, It was hypothesized thioredoxin like fold to be responsible for this interaction. To test this hypothesis, HEK293T flag tagged Srx cells were transfected with constructs of PDIA6 tagged with c-Myc, with a deleted thioredoxin like domain (Figure 2.5 B). The cell lysates were immunoprecipitated for Flag, and then the Western blot membranes were immunoblotted for PDIA6 using c-Myc antibodies. The result shows the N-terminal thioredoxin like fold (D1) to be required for
the Srx-PDIA6 interaction, whereas the second thioredoxin like fold (D2) to be only partially involved in this interaction (Figure 2.7).

Figure 2.4. Prediction of domains responsible for the Srx-TXNDC5/PDIA6 interaction. A, B, and C) Predicted full-length Srx, TXNDC5 and PDIA6 proteins, respectively, using I-TASSER server for protein structure and function prediction, Blue: N-terminal first methionine amino acid residue, red: CGHC motif (labeled as D), and yellow: C-terminal KDEL. D) D-doc prediction result of Srx (Blue) interaction with TXNDC5 (Green) through D1 and D3 of TXNDC5. E) D) D-doc prediction result of Srx (Blue) interaction with PDIA6 (Green) C-terminal domain.
Figure 2.5 Constructs generated for examining the Srx-TXNDC5/PDIA6 interactions by site directed mutagenesis. A) The architecture of human TXNDC5. Each thioredoxin like domain consists of 18 amino acids, domain 1 (D1) starts from 81 to 99, domain 2 (D2) starts from 209 to 227, and domain 3 (D3) starts from 342 to 360 amino acids. B) The architecture of human PDIA6. Each thioredoxin like domain consists of 18 amino acids, domain 1 (D1) starts from 45 to 63, domain 2 (D2) starts from 389 to 407. Thioredoxin like motif consists of cysteine-glycine-histidine-cysteine (CGHC) sequence located within thioredoxin like domains. Both TXNDC5 and PDIA6 have N-terminal signal peptide (SP) and C-terminal Lys-Asp-Glu-Leu (KDEL) ER sequence retention signal.
Figure 2.6. Srx interacts with thioredoxin like fold of TXNDC5. Immunprecipitation of TXNDC5 with Srx is mainly dependent on N-terminal thioredoxin-like domain (D1) of TXNDC5 in HEK293T-Flag-Srx cells. Only one thioredoxin like domain deleted in the transfected c-Myc-tagged TXNDC5 construct. Del1 indicates deletion of D1, amino acids from 81-99. Del2 indicates deletion of D2, amino acids from 209-227. Del3 indicates deletion of D3, amino acids from 342-360 by site directed mutagenesis.

Figure 2.7. Srx interacts with thioredoxin like fold of PDIA6. Immunprecipitation of PDIA6 with Srx is mainly dependent on N-terminal thioredoxin-like domain (D1) of PDIA6 in HEK293T-Flag-Srx cells. One thioredoxin like domain deleted in transfected c-Myc-tagged PDIA6 construct. Del1 indicates deletion of D1, amino acids from 45-63. Del2 indicates deletion of D2, amino acids from 389-407 by site directed mutagenesis.
2.1.5. Srx co-localizes with TXNDC5/PDIA6 in the ER

Both TXNDC5 and PDIA6 are known to be ER resident proteins. Therefore, I sought to find if Srx could enter the ER. I treated A549 with increasing concentration of H₂O₂ for 10min. The Western blot result verified presence of Srx in the ER fraction, even in the absence of H₂O₂. And that TXNDC5 increases in the ER in correlation with increased doses of H₂O₂ (Figure 2.8). However, there are no obvious changes in PDIA6 and PrxlV levels.

The immunofluorescent staining of Srx with ER maker Calnexin verified co-localization of Srx with Calnexin in lung adenocarcinoma cells (Figure 2.9. A). I further verified co-localization of Srx with each of TXNDC5 and PDIA6 in HEK293T and A549 cells (Figure 2.9. B, and C, respectively). HEK293T has low endogenous Srx expression therefore I used HEK293T overexpressed Srx tagged with Flag and used anti-Flag primary antibody to blot for Srx. Srx is labeled with red fluorescent color in all panels of Figure 2.9, whereas Calnexin, TXNDC5, and PDIA6 labeled with green fluorescent color secondary antibodies. The overlap of the two colors produced a yellow staining indicating their co-localization (Figure 2.9).
Figure 2.8. Srx co-localizes with TXNDC5 and PDIA6 in the ER. Western blot for A549-Flag-Srx cells treated for 10min with increasing concentration of H$_2$O$_2$ (mM). Srx can be detected in the ER fraction along with TXNDC5 and PDIA6. ER resident PrxIV is blotted as a control.
Figure 2.9. Srx co-localizes with TXNDC5 and PDIA6 in the ER. A) Immunofluorescent staining for Srx (red) and ER marker Calnexin (green) showing their localization (yellow) in A549 cell. B) Immunofluorescence staining for Srx (red) and TXNDC5 (green) showing their localization (yellow) in HEK293T and A549 cell lines. C) Immunofluorescence staining for Srx (red) and PDIA6 (green) showing their localization (yellow) in HEK293T and A549 cell lines.
2.1.6. Srx-TXNDC5 interaction is enhanced under acute oxidative stress conditions

As Srx interaction with TXNDC5 and PDIA6 is redox sensitive thioredoxin like domain dependent, Srx-PDIs interaction was hypothesized to enhance under oxidative stress conditions. Immunprecipitation of A549 overexpressed TXNDC5 cells, tagged with Flag, was employed. The cells were treated with increasing concentration of H$_2$O$_2$ for 10 min and immunoblotted with Srx antibodies. The results show discernible correlation between increased Srx affinities to TXNDC5 with increased concentration of H$_2$O$_2$. These results suggest that Srx-TXNDC5 interaction is boosted under oxidative stress conditions (Figure 2.10).

To investigate role of acute exposure of H$_2$O$_2$ on Srx affinity to PDIA6 in lung cancer cells, A549 overexpressed Srx cells, tagged with Flag, were treated with H$_2$O$_2$. The co-immunoprecipitation experiments show a slight increase in PDIA6 binding to Srx at the highest treated dose of H$_2$O$_2$ of 1mM, whereas, no change in PrxIV affinity to Srx was observed in response to acute H$_2$O$_2$ exposure (Figure 2.11).
Figure 2.10. Increased binding of Srx to TXNDC5 under oxidative stress conditions. A) Verification of TXNDC5 knockdown (ShTXNDC5) and overexpression (Flag-TX) in A549 cells. B) Co-immunoprecipitation of Flag tagged TXNDC5 with Srx in A549 cells after treatment with H₂O₂ (mM) for 10min. The result shows a gradual increase in Srx binding to TXNDC5 in response to increase in H₂O₂ concentration.
Figure 2.11. Co-immunoprecipitation of Srx with PDIA6 in A549 cells. Srx co-immunoprecipitation with PDIA6 in A549 cells after treatment with \( \text{H}_2\text{O}_2 \) (mM) for 10min. The result shows slight increase in Srx binding to PDIA6 only the highest concentration of in \( \text{H}_2\text{O}_2 \) treatment, whereas no change in PrxIV affinity to Srx is observed.
2.2 Discussion

Srx provides a critical defense mechanism against oxidative stress through reactivation of overoxidized 2-Cys Prx in cells. Reactivation of catalytic activities of 2-Cys Prx, hence, facilitates \( \text{H}_2\text{O}_2 \) scavenging during oxidative stress (Pace et al., 2013). Among four members of 2-Cys Prxs (Prx I-IV), Srx preferentially binds with PrxIV. In fact, Srx-PrxIV axis is considered critical for enhancing oncogenic characteristics in lung cancer. Depletion of PrxIV recapitulates the oncogenic phenotypes similar to that observed in Srx knockdown cells (Wei et al., 2011). In this dissertation, I sought to examine additional functions of Srx in lung cancer, through studying Srx interacting proteins.

Besides PrxIV, ER resident proteins, TXNDC5 and PDIA6 I are identified to interact with Srx. Both proteins belong to PDI family of enzymes. PDIs are a group of more than twenty enzymes; which structurally share one or more thioredoxin-like domains. This redox sensitive domain contains CXXC fold, which is implicated in oxidoreductase activity of PDIs. The specific function of the majority of PDIs is not identified. However, many members are known to take part in oxidation (formation), reduction (breakdown), and isomerization (rearrangement) of disulfide bonds in nascent proteins to facilitate their maturation to physiologically functional proteins in the ER (Bulleid, 2012). PDIs become oxidized before interacting with their reduced protein substrates. There are several well-established pathways involved in the oxidation of PDI CXXC fold in mammalian cells. Ero1 and 2-cys Prxs are among these pathways (Bulleid, 2012; Bulleid et al., 2011; Lu et al., 2014). TXNDC5 holds three CGHC thioredoxin-like fold. The C-terminal thioredoxin-like domain has extended long loop, offering TXNDC5 extended V-shape configuration compared to the U-shaped feature of other PDI family members, such as protein PDIA1, PDIA3, and PDIA4. Additionally, the TXNDC5 CGHC folds
function independently from one another, providing the enzyme the capacity for rapid involvement in oxidoreductase activities. In contrast to TXNDC5, the two CGHC domains of PDIA1 function cooperatively and in a slower rate during substrate oxidative folding. The dimeric PrxIV is reported to form a mixed disulfide bond with the second thioredoxin-like domain of TXNDC5 during oxidative folding (Kojima et al., 2014). In this study, a novel interaction between TXNDC5 and Srx is identified. The N-terminal thioredoxin-like fold of TXNDC5 is determined to be primary interaction site while the C-terminal fold is only partially involved. This Srx-TXNDC5 interaction, which is Srx Cys99 independent, is enhanced by oxidative stress. Previous cell fractionations experiments reported that the majority of TXNDC5 proteins are present in the ER and Golgi compartments. Therefore, it was proposed TXNDC5 to function in protein folding in the ER and retrograde transport of substrates from Golgi to ER (Charlton et al., 2010). This may explain the TXNDC5 concentrations increase in the ER fraction in correlation to increased H$_2$O$_2$ concentration. Even though our results detected Srx in the ER, no changes in Srx levels were observed with increased H$_2$O$_2$ concentration in A549 cells. TXNDC5 is reported to interact with specificity with hyperoxidized forms of PrxII and PrxIV (Pace et al., 2013). It is well established that Prx hyperoxidation stabilizes the Prxs decameric structures. In contrary, reduced forms of Prxs causes disruption of decamer interactions, including PrxII-TXNDC5 interaction (Pace et al., 2013); therefore, it is likely that Srx interacts with TXNDC5 to prevent TXNDC5 from stabilizing the decameric structures of PrxIV during acute oxidative stress. Hence, leaving more PrxIV available in the ER to participate in H$_2$O$_2$ scavenging. This interpretation can be supported by enhanced Srx-TXNDC5 interaction in correlation to increased H$_2$O$_2$ concentration observed in the results.

PDIA6 has two thioredoxin-like folds and one b domain. Thus, in addition to CGHC related oxidoreductase activity, PDIA6 is expected to have isomerase activities
as well. The results demonstrate that PDIA6 interacts with Srx through PDIA6 N-terminal thioredoxin-like domain. This interaction insinuates redox-sensitive oxidoreductase activity between PDIA6 with Srx. However, the results do not display change in the level of Srx-PDIA6 interaction with increased H₂O₂ concentration. Therefore, I propose Srx to have a higher affinity toward TXNDC5 compared to its affinity to PDIA6 under acute oxidative stress conditions.

In summary, in this chapter, a novel interaction between antioxidant protein Srx with each of TXNDC5 and PDIA6 were corroborated. PDIs are the main players in ER quality control system that permits for only correctly folded secretory proteins to enter the secretory pathway. PDIs play an important role in the processes of disulfide bond oxidation, reduction, and isomerization in the client proteins. Consequently, the substrates will be retained in the ER until they are correctly folded. Otherwise, PDI proteins will direct the terminally misfolded substrates to the ER degradation pathway. Furthermore, PDIs can pass electrons to members of antioxidant systems in the ER, such as Ero1 and 2-CysPrx pathways; the resulted disulfide bonds in PDIs are used to oxidize substrates in the process of oxidative protein folding. Therefore, Srx interaction with the redox sensitive thioredoxin like folds in TXNDC5 and PDIA6 suggests a novel function of Srx in modulating members of the ER quality control system in response to oxidative stress in lung cancer.

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3.1. Results

3.1.1. TXNDC5 facilitates the retention of Srx in the ER

Even though there are no reports regarding its biochemical functions in lung cancer, TXNDC5 is involved in androgen receptor (AR) stabilization in prostate cancer to facilitate AR translocation to the nucleus. Therefore, TXNDC5 or PDIA6 were proposed to interact with Srx to stabilize Srx presence in the ER. The Western blot results for A549 subcellular fractionation supported this prediction for TXNDC5. As the ER level of Srx is significantly reduced in TXNDC5 knockdown cells compared to the control cells. The results also demonstrate less PrxIV in the cytosolic fraction of ShTXNDC5 cells. Therefore it is suggested that TXNDC5 is required for Srx retention in the ER in lung adenocarcinoma cells. However, PDIA6 deficient cells did not show any obvious change in the level of Srx and PrxIV compared to the control cells (Figure 3.2).
Figure 3.2. TXNDC5 facilitates Srx retention in the ER of A549 cells. A) Western blot for A549 TXNDC5, and PDIA6 knowdown cells showing decrease in Srx presence in the ER in ShTXNDC5 but not in that ShPDIA6 cells after normalization with ER marker Calnexin. B) and C) Quatitative analysis of relative band intensity for Srx protein in TXNDC5, and PDIA6 knowdown cells, respectvily. D) and E) Quatitative analysis of relative band intensity for PrxIV protein in TXNDC5, and PDIA6 knowdown cells, respectvily. GAPDH is used as a cytosolic marker. PrxIII is used as mitochondrial marker.
3.1.2. TXNDC5 and Srx protects against heat-shock induced protein aggregates

Cell exposure to nontoxic doses of stressors such as metals, ethanol, low heat-shock temperatures (40-45°C) and oxidants acquire cells the capacity to develop a temporary endurance (thermotolerance) to otherwise lethal doses of that stressor after succeeding exposure. These stressors cause protein aggregation and misfolding. Molecular chaperones are proteins with various cellular functions including disassembly of protein aggregates, protein refolding, and substrate transport (Freeman et al, 2000; Hoffmann et al, 2010; Hartl et al, 2011). Several members of PDI family, such as PDIA1 and PDIA3, are known to have chaperone activities (Vinaik et al., 2013).

To investigate the chaperone activities of Srx, TXNDC5, and PDIA6 intracellular-refolding assay was employed. The cells were transfected with luciferase coding construct for 24hr. The cells treated with heat at 42°C for 30min to generate protein aggregates. Then the cells capacity to recover the aggregates represented by luciferase activity over times was measured. The results show decrease in luciferase activity in ShTXNDC5 A549 cells compared to the control cells; meanwhile Flag-TXNDC5 cells appear to protect luciferase from losing its activity (Figure 3.4 A). Decrease in luciferase activity was also observed in H2030 shTXNDC5 cells (Figure 3.4 B). Heat shock treatment of A549 cells did not demonstrate significant differences in aggregate recovery in PDIA6 and Srx downregulated and overexpressed cells. (Figures 3.5 and 3.6 A, respectively). However, significant decrease in luciferase activity was observed in H226 Srx knockdown cells compared to the control cells under same treatment conditions (Figure 3.6 B).
Figure 3.3 Western blot showing no increase in translation of luciferase proteins after heat-shock (HS) treatment. A549 cells are treated with Cyclohexamide 30min in advance of heat-shock treatment to inhibit translation of luciferase gene.
Figure 3.4. TXNDC5 participates in the recovery of heat-shock induced protein aggregates. A) intracellular refolding assay in A549 cells. B) intracellular refolding assay in H2030 cells. Both cell lines were treated with heat at 42°C for 30min. Recovery of luciferase activity was followed over a period of 4hr after treatment. The data were analyzed by student t-test. Numbers presented as mean ± SEM, using 95% as confidence interval. The difference was defined to be statistically significant at p-values ≤ 0.05.
Figure 3.5. PDIA6 does not affect recovery of heat-shock induced protein aggregates. A549 cells were treated with heat at 42°C for 30min. Recovery of luciferase activity was followed over a period of 2hr after treatment. Data were analyzed by using student t-test. Numbers presented as mean ± SEM, using 95% as confidence interval. The difference was defined to be statistically significant at p-values ≤ 0.05.
Figure 3.6. Srx participates in the recovery of heat-shock induced protein aggregates in H226 cells. A) intracellular refolding assay in A549 cells. B) intracellular refolding assay in H226 cells. H226 cells demonstrates a protective role of Srx in preventing aggregate after heat shock treatment at 42°C for 30min. Data were analyzed by using student t-test. Numbers presented as mean ± SD (or SEM), using 95% as confidence interval. The difference was defined to be statistically significant at p-values ≤ 0.05.
3.1.3. TXNDC5 and PDIA6 enhance cellular capacity for oxidative protein folding

Wang et al (2011) demonstrated that the chaperone activity of PDIA1 is redox regulated. And that oxidation of PDIA1 activates its chaperone activity through PDIA1 conformation change. Moreover, Sato et al (2013) revealed that oxidized TXNDC5 to interact with PrxIV decameric structure that participates in oxidative substrate folding. This interaction was verified to abolish under reduced conditions. Therefore, it was anticipated that TXNDC5 and PDIA6 to show similar activities to PDIA1 in lung cancer cell lines. The cells were subjected acutely to H2O2 for 10min. The luciferase activity was followed at different time points. The results indicate significant decrease in the recovery of luciferase activity in ShTXNDC5 cells in both A549 as well as H2030 adenocarcinoma cell lines compared to the control cells (Figure 3.7 A and B, respectively). Luciferase activity was also reduced in PDIA6 deficient cells subjected to H2O2 treatment compared to the control A549 cells (Figure 3.8). The results demonstrate TXNDC5 and PDIA6 promote the process of protein folding under oxidative stress conditions.
Figure 3.7. TXNDC5 promotes protein folding under acute oxidative stress conditions. A. A549 cells. B) H2030 cells. In both H$_2$O$_2$ (mM) treated cell lines, luciferase activity is reduced in TXNDC5 downregulated cells compared to the control cells. While TXNDC5 overexpression enhances luciferase activity in A549 cells. Data were analyzed by student t-test. The data represented as Mean ± SD, using 95% confidence interval. The difference was defined to be statistically significant at p-values ≤0.05.
Figure 3.8. PDIA6 enhances protein folding under acute oxidative stress conditions in A549 cells. H$_2$O$_2$ (mM) induced luciferase activity is decreased in PDIA6 knockdown cells compared to control cells. PDIA6 overexpression enhances luciferase activity. Data were analyzed by student t-test. The data are presented as Mean ± SD, using 95% confidence interval. The difference was defined to be statistically significant at p-values ≤0.05.
3.1.4. Srx suppresses oxidative protein folding

ShSrx A549 and ShSrx H226 cell lines showed significant increase in luciferase activity after acute exposure to H$_2$O$_2$, for 10min(Figure 3.9 A and B). Srx overexpression reduced luciferase activity in A549 cells. Hence, the results indicate Srx to oppose folding process under oxidative stress conditions. The results also display PrxIV to enhance oxidative protein folding in lung cancer (Figure 3.9 C); supporting previously published chaperone function of PrxIV under oxidative stress conditions.
Figure 3.9. Srx suppresses protein folding under acute oxidative stress conditions. H$_2$O$_2$ (mM) induced luciferase activity is promoted in Srx knockdown A549 cells (A), and in Srx knockdown H226 (B) cells. While PrxlIV overexpression enhances luciferase folding (C). Data were analyzed by student t-test. The data presented as Mean ± SD, using 95% as confidence interval. The difference was defined to be statistically significant at p-values ≤ 0.05.
3.1.5. TXNDC5/PDIA6 are dispensable in heat-shock induced thermotolerance

Our result shows no observable change in lung cancer cell ability to survive different duration of heat shock treatment when TXNDC5 protein expression was downregulated or overexpressed in A549 cells (Figure 3.10). Therefore, TXNDC5 does not protect lung cancer cells against hyperthermia induced tumor growth inhibition. Our result shows no observable change in lung cancer cell ability to survive different duration of heat-shock treatment when PDIA6 protein expression was manipulated in A549 cells (Figure 3.11). Thus, similar to TXNDC5, PDIA6 does not provide thermotolerance against hyperthermia induced tumor growth inhibition.

A.
Figure 3.10. TXNDC5 does not provide thermotolerance in clonogenic cell survival assay. TXNDC5 does not affect the ability of cells to form colonies in response to different durations of heat shock treatment. A) Crystal Violet stained TXNDC5 downregulated and overexpressed cells. B) Quantification of fold change in the intensity of Crystal Violet stain measured at 560nm, showing no significant differences compared to control cells.
Figure 3.11. PDIA6 does not provide thermotolerance in clonogenic cell survival assay. PDIA6 does not affect the ability of cells to form colonies in response to different durations of heat shock treatment. A) Crystal Violet stained PDIA6 downregulated and overexpressed cells. B) Quantification of fold change in the intensity of Crystal Violet stain measured at 560nm, showing no significant differences compared to control cells.
3.1.6. The Srx-interacting proteins protect against tunicamycin induced growth inhibition in lung cancer

Tunicamycin induces ER stress through inhibition of protein glycosylation. An overall accumulation of under-glycosylated proteins in the ER of malignant tumors has been reported to inhibit cell growth and promote apoptosis. For instance, tunicamycin induces apoptosis in melanoma cells via depletion of functional insulin-like growth factor 1 receptor at the cell surface (Dricu et al, 1997). The results of cell viability assay demonstrate TXNDC5 to have protective effect on sensitizing A549 (Figure 3.12 A) and H2030 (Figure 3.12 C) to tunicamycin induced growth inhibition. Lung cancer PDIA6 deficient cells showed similar response to tunicamycin treatment in both A549 as well as H2030 cells (Figure 3.13 A and C, respectively). Half maximal inhibitory concentration (IC50) of tunicamycin was reduced from 1.49 μg/ml to 0.51 μg/ml in A549 cells (Table 3), and from 1.49 μg/ml to 0.93 μg/ml in H2030 cells (Table 5). The results also indicate more prominent tunicamycin induced cell proliferation inhibition in ShSrx A549 cells compared to the control cells measured by XTT (Figure 3.15). IC50 was reduced from 1.49 μg/ml to 0.2 μg/ml (Table 5).
A.

A549 Viability

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<th>Mean IC50 (µg/ml)</th>
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</tr>
<tr>
<td>ShTXNDC5</td>
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B.
Figure 3.12. TXNDC5 protects against tunicamycin induced growth inhibition. TXNDC5 depletion sensitizes cancer cells to tunicamycin in A549 (A) and H2030 (C) cells. Mean values of replicates are shown. B and D) Calculated mean of IC_{50} of tunicamycin in ShNT and ShTXNDC5 cells for A and C, respectively.
A.

B.

<table>
<thead>
<tr>
<th>A549 Viability</th>
<th>Mean IC50 (µg/ml)</th>
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</tr>
<tr>
<td>ShPDIA6</td>
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Figure 3.13. PDIA6 protects against tunicamycin induced growth inhibition. PDIA6 depletion sensitized cancer cells to tunicamycin in A549 (A) and H2030 (C) cells. Mean values of replicates are shown. B & D) Calculated mean of IC₅₀ of tunicamycin in ShNT and ShPDIA6 cells for A and C, respectively.
Figure 3.14. Srx protects against tunicamycin induced growth inhibition in A549 cells. A) Srx depletion sensitizes cancer cells to tunicamycin. Mean values of replicates are shown. B) IC$_{50}$ of tunicamycin in ShNT and ShSrx cells.
3.1.7. TXNDC5 downregulation accelerates XBP-1 splicing

Reverse transcription PCR (RT-PCR) was applied to study role of Srx, TXNDC5, and PDIA6 in UPR induction lung cancer cells. ShTXNDC5 cells were treated with tunicamycin (1µg/ml) for 0, 1, 2, 4, and 6 hr. Whereas ShPDIA6, and ShSrx cells were treated for 30min, 1, 2, and 4 hr. After extraction of mRNA, PCR was made for reverse transcribed XBP-1 (spliced and un-spliced). Our results indicate that TXNDC5 knockdown cells are more prone to have XBP-1 spliced (sXBP-1) than the control. As it is shown in figure 3.15, majority of the XBP-1 is in spliced after 1hr of chemical treatment, while in the control group was spliced after 2hr tunicamycin treatment. ShPDIA6 and ShSrx cells subjected to tunicamycin treatment did not display observable differences in the ratio of spliced to total XBP-1 compared to the control cells.
Figure 3.15. TXNDC5 depletion accelerates XBP-1 splicing in response to tunicamycin treatment in A549 cells. A) RT-PCR result of XBP-1 cDNA run on 3% agarose gel. B) Quantification of percent of spliced to total XBP-1 in A. sXBP-1: spliced XBP-1, uXBP-1: unspliced XBP-1, bands are compared at each time point. C) Representation of percent relative XBP-1 band intensity at each time point.
Figure 3.16. PDIA6 depletion does not affect splicing of XBP-1 in response to tunicamycin treatment in A549 cells.  A) RT-PCR result of XBP-1 cDNA run on 3% agarose gel. B) Quantification of percent of spliced to total XBP-1 in A. sXBP-1: spliced XBP-1, uXBP-1: unspliced XBP-1, bands are compared at each time point. C) Representation of percent relative XBP-1 band intensity at each time point.
Figure 3.17. Srx depletion does not affect splicing of XBP-1 in response to tunicamycin treatment in H226 cells. A) RT-PCR result of XBP-1 cDNA run on 3% agarose gel. B) Quantification of percent of spliced to total XBP-1 in A. sXBP-1: spliced XBP-1, uXBP-1: unspliced XBP-1, bands are compared at each time point. C) Representation of percent relative XBP-1 band intensity at each time point.
3.1.8. TXNDC5 depletion accelerates XBP-1 splicing

Western blot experiment for ShTXNDC5 H2030 cells, treated for 0, 4, 6, 8, 12, or 24 hr with 2 μg/ml tunicamycin, showed faster XBP-1 splicing compared to the control cells. More sXBP-1 protein was detected 4 hr after tunicamycin treatment in comparison to the control cells (Figure 3.18). The overall expression of sXBP-1 and GRP78 are decreased in ShTXNDC5 cells compared to the control cells. Thus, the results suggest a protective role of TXNDC5 against premature splicing of XBP-1, besides regulating ER chaperone GRP78 expression. Western blot experiment showed decrease in UPR sensor activation, sXBP-1, in response to 2 μg/ml tunicamycin, when ShSrx H226 cells treated for different durations, 4, 6, 8, 12, 24 hr (Figure 3.19). Besides, less GPR78 was expressed 12 hr after the treatment compared to the control cells. Therefore, it is concluded here that Srx to play a vital role in XBP-1 and GRP78 signaling during tunicamycin induced UPR.

A.
Figure 3.18. TXNDC5 depletion accelerates XBP-1 splicing. A) Western blot for H2030 ShTXNDC5 cells treated for different durations (hr) with tunicamycin (2μg/ml) showing spliced XBP-1, and GRP78 protein expression change. C: control with no treatment. B and C) Quantification of intensity of Western blot band of sXBP-1, and GRP78 proteins in A, respectively.
Figure 3.19. Srx depletion decreases XBP-1 and GRP78 signaling. A) Western blot for H226 ShSrx cells treated for different durations (hr) with tunicamycin (2μg/ml) showing spliced XBP-1, and GRP78 protein expression change. C: control with no treatment. B and C) Quantification of intensity of Western blot band of sXBP-1, and GRP78 proteins in A, respectively.
3.1.9. TXNDC5 is upregulated in lung cancer

To screen for TXNDC5 protein expression in normal tissues, human tissue microarray slides were immunobotted for TXNDC5. In contrary to Srx protein that is only expressed under oxidative stress conditions, TXNDC5 expression varies in different tissues (Figure 3.20). The immunohistochemistry results of lung tissues shows TXNDC5 protein expression to be significantly lower in normal tissues than cancer tissues in adenocarcinoma, bronchioloalveolar carcinoma, squamous cell carcinoma and small cells carcinoma represented by the intensity of brown staining (Figure 3.21).
Figure 3.20. Variation in TXNDC5 protein expression in normal tissues A) TXNDC5 protein expression in panel of normal tissue B) Quantitative analysis of positive staining in A.

A.

B.

Figure 3.21. TXNDC5 is upregulated in lung cancer. A) Immunohistochemistry for TXNDC5 protein expression in different lung cancer tissues. B) Quantitative analysis of positive staining in A. The data presented as Mean ± SD, using 95% as confidence interval. Lung cancer tissue compared to the control and the difference was defined to be statistically significant at p-values ≤ 0.05.
3.1.10. TXNDC5 promotes tumorigenesis in lung cancer

To investigate effect of TXNDC5 in sustaining proliferative rate of A549 cells, XTT cell proliferation assay was used. Growth of TXNDC5 knockdown cells was followed over a period of 5 days. The results show an observable decrease in the growth of downregulated TXNDC5 cells compared to the control cells (Figure 3.22). To characterize the role of TXNDC5 in malignant transformation of lung cancer cells, anchorage independent colony formation assay was used. In which the ability of TXNDC5 to promote cell growth independent of a solid surface was tested. The results show that depletion of TXNDC5 caused inhibition of colony formation. Besides, counterpart overexpression of TXNDC5 promoted colony formation. These results show expression of TXNDC5 is vital to stimulate tumor growth in A549 lung adenocarcinoma cells in vitro (Figure 3.23). To study role of TXNDC5 in migratory characteristics of lung cancer, wound healing Assay was employed. The wound generated in a monolayer of A549 cells was followed over a period of 3 days. The migratory characteristics of cells to heal the wound imitate in vivo cell migration. The images of the cells were taken each day. The wound remaining in ShTXNDC5 cells was nearly 20% more than that of the control ShNT, in which the gap was completely filled by 3 days. These results show TXNDC5 to stimulate migratory characteristics of lung cancer cell (Figure 3.24).

To examine role of TXNDC5 in A549 cells invasiveness, three dimensional tumor spheroid invasion assays was used. The area covered by outgrowth extended form a spheroid of ShTXNDC5 and Flag-TXNDC5 cells were measured. The area was measured after 6 days in a medium supplemented with EGF. The results show no significant different in the distance traveled by cells when TXNDC5 protein expression was manipulated (Figure 3.25). To study role of TXNDC5 in tumor formation in vivo, A549 cells were injected in the tail of mice. Immunohistochemistry staining for TXNDC5
shows formation of multiple tumor nodules in the lung. The result shows presence of cell aggregates with high TXNDC5 protein expression away from the nodules, possibly detached and migrated from the nearby nodules (Figure 3.26).

To investigate role of TXNDC5 in MAPK signaling, ShTXNDC5 and Flag-TXNDC5 cells were treated with EGF (100ng/ml) for different time points after serum starvation. Then phosphorylation of each c-jun, AKT, ERK1/2 proteins were measured, that are activated by Srx-PrxIV signaling. The Western blot results show TXNDC5 knockdown to induce phosphorylation of AKT and ERK1/2. This result was further supported by opposing effects observed in TXNDC5 overexpressed A549 cells (Figure 3.27). Suggesting possible role of TXNDC5 in modulating protein phosphorylation in lung cancer cells.

Figure 3.22. A549 proliferative rate decreases with TXNDC5 depletion. Cell proliferation assay in TXNDC5 knockdown A549 cells measured by XTT assay over a period 5 days (Mean ± SEM).
Figure 3.23. TXNDC5 increases anchorage independent colony formation. A) A549 ShTXNDC5 and Flag-TXNDC5 cells followed for a period of 30 days in soft agar medium. B) Quantification of number of colonies larger than 50μm in diameter.
Figure 3.24 TXNDC5 enhances the migration of cancer cells A) One directional cells migration in TXNDC5 knockdown A549 cells. B) Quantification of gap remaining between the wounded cells in A (Mean±SEM).
Figure 3.25. TXNDC5 does not demonstrate invasive behavior in A549 cells in three dimensional tumor spheroid assay. A) A549 cells spheroids treated with epidermal growth factor for 6 days (200ng/ml) K) Quantitative analysis of area covered by spheroid outgrowths in A.
Figure 3.26. TXNDC5 enhances tumor migration and metastatic in vivo. A549 cells injected in the tail of mice, immunohistochemistry staining for TXNDC5 shows formation of multiple of tumor nodules in the lung. Zoom in view of the same slide, shows presence of aggregates of the cells in other locations, possibly detached and migrated from the nearby nodules.
<table>
<thead>
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<th>EGF (100ng/ml)</th>
<th>ShNT</th>
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<td>7 15 30 60 120</td>
<td>0 7 15 30 60 120</td>
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- **p-c-jun (S63)**
- **c-jun**
- **p-ERK1/2**
- **ERK1/2**
- **p-Akt (S473)**
- **Akt**
- **β-Actin**
Figure 3.27. TXNDC5 depletion increase AKT and ERK$_{1/2}$ phosphorylation in A549 cells. 

A) Western blot of TXNDC5 depleted (ShTXNDC5) and TXNDC5 overexpressed (Flag-TXNDC5) cells treated for different durations with 100ng/ml epidermal growth factor (EGF). B and C) Quantification of the intensity of phosphorylated ERK$_{1/2}$ (p-ERK$_{1/2}$) band after normalization for total ERK$_{1/2}$ in ShTXNDC5 and Flag-TXNDC5 cells, respectively. D and E) Quantification of the intensity of phosphorylated AKT (p-AKT) band after normalization with total AKT in ShTXNDC5 and Flag-TXNDC5 cells, respectively.
3.1.11. PDIA6 promotes tumorigenesis in lung cancer

For investigate the role of PDIA6 in promoting the growth of lung cancer cells, growth of PDIA6 knockdown cells were followed over a period of 5 days. The result shows a noticeable decrease in the growth rate of A549 cells when PDIA6 was downregulated (Figure 3.28). To study the role of PDIA6 in anchorage independent colony formation in vitro, the cells ability to form colonies away from a solid surface over a period of 30 days was measured. The results show a significant decrease in the number of colonies formed in PDIA6 deficient cells. In contrast, a substantial increase in colony formation was observed in Flag-PDIA6 cells, signifying PDIA6 role in stimulating tumor growth (Figure 3.29). To study the role of PDIA6 in migratory characteristics of lung cancer, wound healing Assay was employed. The wound generated in a monolayer of A549 cells was followed over a period of 3 days. The images of the cells were taken each day. The wound remaining in ShPDIA6 cells was nearly 30% more than that of the control ShNT, in which the gap was completely filled by 3 days (Figure 3.30). These results show PDIA6 to stimulate migratory characteristics in lung cancer cell.

To examine PDIA6 role in cancer invasiveness, three dimensional tumor spheroid invasion assays was employed. In which the area covered by spheroid outgrowths of ShPDIA6 and Flag- PDIA6 cells were measured. The spheroids were treated with a medium supplemented with EGF for 6 days. The results show no significant differences in the distance traveled by the cells when PDIA6 protein expression was manipulated (Figure 3.31). The phosphokinase signaling experiment shows that knockdown of PDIA6 improved phosphorylation of each c-jun, AKT, ERK1/2 proteins. This result was further supported by opposing effect observed in PDIA6 overexpressed A549 cells measured under same treatment conditions (Figure 3.32).
Presenting a possible role of PDIA6 in regulating phosphorylation pathways in lung cancer cells.

Figure 3.28 PDIA6 depletion reduces A549 proliferation rate. Cell proliferation assay in PDIA6 knockdown A549 cells, measured by XTT assay over a period of 5 days (Mean ± SEM).
Figure 3.29  PDIA6 increases anchorage independent colony formation. A) A549 ShPDIA6 and Flag-PDIA6 cells followed for a period of 30 days in soft agar medium. B) Quantification of number of colonies larger than 50μm in diameter.
Figure 3.30 PDIA6 depletion lowers cancer cells migration. A) One directional cells migration in PDIA6 knockdown A549 cells. B) Quantification of gap remaining between the wounded cells in A (Mean±SEM).
Figure 3.31 PDIA6 does not demonstrate invasive behavior in A549 cells in three dimensional tumor spheroid assay. A) A549 spheroids treated with epidermal growth factor for 6 days (200ng/ml) K) Quantitative analysis of area covered by spheroid outgrowths in A.
Figure 3.32. PDIA6 depletion increases c-jun, AKT, and ERK_{1/2} phosphorylation in A549 cells. A) Western blot of PDIA6 depleted (ShPDIA6) and PDIA6 overexpressed (Flag-PDIA6) cells treated for different durations with 100ng/ml epidermal growth factor (EGF). B and C) Quantification of the intensity of phosphorylated c-jun (p-c-jun) band after normalization for total c-jun in ShPDIA6 and Flag PDIA6 cells, respectively. D and E) Quantification of the intensity of phosphorylated ERK_{1/2} (p-ERK_{1/2}) band after normalization for total ERK_{1/2} in ShPDIA6 and Flag PDIA6 cells, respectively. F and G) Quantification of the intensity of phosphorylated AKT (p-AKT) band after normalization for total AKT in ShPDIA6 and Flag PDIA6 cells, respectively.
3.2 Discussion

Under oxidative stress conditions, Srx acts to subdue protein folding in the ER of lung cancer cells. Srx modulates UPR sensor activities in response to accumulation of misfolded proteins, in particular activity of XBP-1 transcription factor and GRP78. TXNDC5 and PDIA6 participate in oxidative protein folding. TXNDC5 delays activation of spliced XBP-1 transcription factor and it is needed to promote GRP78 proteins expression during ER stress. In addition both TXNDC5 and PDIA6 promote oncogenic phenotypes in lung cancer in signaling mechanisms different than that of Srx.

As it was previously reported, TXNDC5 and PDIA6 are involved in rapid oxidative folding of protein substrates (Sato et al., 2013; Kojima et al., 2013). This rapid folding can introduce abnormal disulfide bonds in the client proteins (Marciniak et al., 2004; Jessop et al., 2009). Therefore, other PDI family members, for instance, PDIA1 proofreads and amends the incorrect disulfide bonds generated by TXNDC5 and PDIA6 (Sato et. al, 2013). Hence, it is possible that Srx interacts with the PDI members to decelerate their contribution to rapid oxidative folding; consequently Srx prevents accumulation of misfolded proteins. This inference is substantiated by the results of intracellular refolding assay, in which rapid increases in the luciferase activity was detected in A549 and H226 Srx knockdown cells after acute H$_2$O$_2$ exposure. Furthermore, PrxIV-TXNDC5/PDIA6 complexes are previously described to participate in oxidative substrate folding in Hela cells (Sato et al., 2013; Jessop et al., 2009; Meunier et al., 2002). In this study, the results support involvement of TXNDC5, PDIA6 in promoting oxidative protein folding in lung cancer. Significant decreases in luciferase activities detected in each of TXNDC5, PDIA6 or PrxIV knockdown A549 and H2030 cells after acute exposure to H$_2$O$_2$ treatment. Furthermore, TXNDC5 contributes to chaperone activities, represented by its role in preventing heat stress-induced protein
aggregates. In contrast, PDIA6 does not contribute to resolving heat-induced protein aggregates.

In general, protein folding is enhanced under oxidative stress conditions (Bhandary et al., 2013). The chaperone activities of PDIs can be regulated by the redox status. Under stress conditions, the second thioredoxin-like fold is oxidized triggering a conformational change in the structure of PDIA1 protein. Consequently, the shielded hydrophobic amino acids, that are involved chaperone activity of PDIA1, are exposed (Wang et al., 2012). Thus, the oxidoreductase activity of PDIA1 can be coupled with substrate folding (Wang et al., 2012). It has been reported previously that the reduced forms of TXNDC5 and PDIA6 enhance the fidelity of PrxIV in oxidative protein folding. PrxIV has preferences to bind to and oxidize TXNDC5 and PDIA6 than other PDI members. TXNDC5 and PDIA6 function as a catalyst for Ero1a and PrxIV substrate folding pathways (Sato et al., 2013). However, overoxidation can introduce abnormal disulfide bonds in the protein substrates (Marciniak et al., 2004; Jessop et al., 2009). Therefore, we propose that Srx modulates protein folding in lung cancer cells, by orchestrating the PDIs function during oxidative stress. The D1 domain of TXNDC5 and PDIA6, to which Srx binds, might be mechanistically responsible for the PDIs involvement the chaperone activity. However, further studies are needed to test this assumption.

Srx is implicated in attenuating TXNDC5 oxidative folding activity; in turn, TXNDC5 facilitates presence of Srx in the ER. The results of this study demonstrate a significant decrease in Srx protein levels in the ER following knockdown of TXNDC5 in A549 cells. In previous studies, the role of TXNDC5 as stabilizing chaperone has been observed in prostate cancer as well. TXNDC5 stabilizes dimeric form of the androgen receptor (AR), to protect AR from degradation. TXNDC5 facilitates AR binding to its ligands and plays a role in AR translocation to the nucleus (Wang et al., 2015). Also,
knockdown of TXNDC5 in Hela cells increased the availability of AdipoR1 receptor on the plasma membrane; Therefore, TXNDC5 is believed to participate in AdipoR1 retention in the ER. And it may function as an adaptor in AdipoR1 signaling (Charlton et al., 2010).

To date, no studies are available regarding TXNDC5 transcriptional regulation in lung cancer cells, and its role ER stress. However, orphan nuclear receptor NR4A1 is reported to participate in sustaining ER stress in pancreatic cancer through upregulation of TXNDC5 at transcriptional levels. TXNDC5 upregulation causes decrease in pro-apoptotic CHOP activities (Lee et al., 2014). In this study, TXNDC5, PDIA6, or Srx deficient lung cancer cells were more sensitized to proliferation inhibition induced by tunicamycin than their control cells. TXNDC5 knockdown cells were more prone to XBP-1 splicing after tunicamycin treatment compared to the control cells. Decrease in Srx levels reduced spliced XBP-1 levels in the nuclear fraction. Also, TXNDC5 and Srx knockdown cells expressed less GRP78 proteins compared to the control cells. Spliced XBP-1 is one of the major components in UPR adaptive phase. Spliced XBP-1 codes for upregulation of chaperones that correct unfolded/misfolded proteins in the ER. Spliced XBP-1 also codes for transcribing ERAD pathway to degrade the terminally misfolded proteins. Moreover, spliced XBP-1 codes for transcription of GRP78 that sequesters and corrects the folding of protein aggregates in the ER (Fu et al. 2008; Yan et al. 2015; Clarke et al., 2015). XBP-1 splicing also activates cyclin A1 related activities that is one of the major components in promoting cell cycle (Yan et al., 2015). Taken together, we concluded that each of TXNDC5 and Srx contribute to cell response to ER stress in lung cancer through modulation of XBP-1 signaling pathway. However, more studies are required to recognize possible Srx/ TXNDC5 interaction with XBP-1 in lung cancer.
TXNDC5, similar to Srx, is involved in enhancing oncogenic phenotypes of cell proliferation, anchorage independent colony formation, and one directional cell migration in cell lines and metastasis in mouse models. Nevertheless, the signaling mechanism does not recapitulate that of Srx. Srx interaction with PrxIV forms an axis that contributes to promoting cancer through modulating MAPK/AP-1/MMP9 signaling at MEK activation level. Knockdown of Srx results in insufficient activation of CREB, AP-1, and MAPK signaling (Wei et al., 2011). Whereas the data in this study indicate TXNDC5 deficient cells to have more activation of MAPK through ERK\textsubscript{1/2} and AKT activation. Meanwhile, the activation level of c-Jun does not change. This finding was further supported by opposite effect observed in TXNDC5 overexpressed A549 cells. The increase in Adiponectin-stimulated AMPK phosphorylation with TXNDC5 knockdown was reported previously in Hela cells. In which knockdown of TXNDC5 increases AdipoR1 availability for signaling on the cell surface (Charlton et al., 2010). Hence, TXNDC5 depletion might cause a release of signaling molecules that activates phosphorylation of ERK\textsubscript{1/2} and AKT.

PDIA6 is one of the least studied PDI members in lung cancer. The results show PDIA6 promotes oncogenic phenotypes of cell proliferation, anchorage independent colony formation, and one directional cell migration. But similar to TXNDC5, the signaling in PDIA6 knockdown A549 cells does not repeat that of signaling in Srx depleted cells. Significant increase in phosphorylation of c-Jun, ERK\textsubscript{1/2} and AKT was observed in PDIA6 deficient cells. While in overexpressed PDIA6 cells, phosphorylation of these proteins was significantly reduced. Further study is required to identify proteins that modulate TXNDC5 and PDIA6 role in MAPK signaling in lung cancer.

In breast cancer, ErbB2 was activated with overexpression of PDIA6. PDIA6 was associated with increased cells migration, invasion, and in vivo metastasis. At the
same time, ErbB2 was determined to be required for the tumorigenic properties of PDIA6 in MCF7 and MDA-MB-436 cells (Gumireddy et al., 2007). It is possible that PDIA6 modulates the release of these proteins in the secretory pathway. In fact, most of the studies on PDIA6 show this protein to function outside the ER. PDIA6 has been associated with reduction and isomerization reactions on the cell surface. Fractionation of platelet plasma membrane revealed PDIA6 recruitment to the cell surface in response to platelet activation. PDIA6 interacts with integrin αIIbβ3. It has been associated with platelet aggregation, fibronectin binding, and P-selectin exposure (Jordan et al., 2005). PDIA6 is believed to exert a thiol isomerase activity when it binds to the β3 subunit of integrin. This isomerase activity changes the conformation of αIIbβ3 causing its activation (Jordan et al., 2005). Also, PDIA6 is reported to form a complex with PDIA1, PDIA4, and GRP78 at the cytosolic face of ER thereby blocking translocation of prion form ER to the cytosol via the Sec61p site (Stockton et al., 2003). Furthermore, PDIA6 has been associated with ERAD pathway in the NIT-1 pancreatic beta cell line. Where PDIA6 was believed to reduce disulfide bonds in misfolded pro-insulin proteins in the preparation of its translocation from the ER to the cytosol for degradation (Gorasia et al., 2016). ERjd5 and PDIA1 are associated with reducing protein substrates in the ER before directing the substrate to ERAD pathway. For instance, Cholera toxin chain A and α1-antitrypsin are unfolded by of PDIA1 reductase activities (Tsae et al., 2001) and ERdj5 (Ushioda et al., 2008), respectively. Because Srx is well documented to reduce overoxidized 2-Prxs. Therefore, it is possible for Srx to modulate reduction of PDIA6 in compartments not limited to the ER.

In this chapter, we elucidated the functional significance of Srx, TXNDC5, and PDIA6 in the tumorigenesis of lung cancer cells. TXNDC5 contribute to chaperone activities and it is required for Srx retention in the ER. It participates, along with Srx, in the process of substrate refolding in response to the presence of misfolded protein.
aggregates. Both PDI members, TXNDC5, and PDIA6, increase protein folding under acute oxidative stress conditions, whereas Srx suppresses protein folding under same treatment conditions. TXNDC5 modulates UPR sensor activation in response to ER stress. It delays XBP-1 splicing. Possibly to prevent arrest of protein synthesis and activation of pro-apoptotic pathways in cancer cells. Whereas Srx may contribute to modulating splicing of XBP-1, and GRP78 protein expressions in response to ER stress. Maintaining ER homeostasis can be among many pathways by which Srx, TXNDC5, and PDIA6 promote cancer development. Since Srx and its interacting proteins are found to participate in tumorigenesis of lung cancer cells.
DISCUSSION AND FUTURE DIRECTION

This dissertation explains the role of Srx interacting proteins in lung cancer development. Srx interacts with redox sensitive thioredoxin-like folds in each of TXNDC5 and PDIA6 in the ER of lung cancer cells. TXNDC5 and PDIA6 engage in nascent protein folding in the ER. The PDIs involvement in chaperone activity is enhanced under oxidative stress conditions, whereas Srx has an inhibitory effect on oxidative protein folding. TXNDC5 maintains Srx localization to the ER. Meanwhile, Srx can bind to TXNDC5 and PDIA6, perhaps, to regulate their participation in the mechanism of protein folding, to prevent the formation of oxidative stress induced protein aggregates. Both TXNDC5 and Srx play important roles in XBP-1 activation regulation.

Srx knockdown initiates ER stress and UPR activation, as indicated by the change in GRP78 protein expression and XBP-1 splicing. The spliced mRNA of XBP-1 codes for a transcription factor that in turn codes for ER chaperone GRP78 expression and PDIA6. GRP78 is implicated in several ER functions, including folding, assembly and degradation of proteins. It is also an important component in UPR sensor activation and ER Ca^{2+} binding (Li & Lee, 2006). In cancer, GRP78 has been associated with tumor growth and drug resistance (Yan et al., 2015). In lung cancer, GRP78 is upregulated, and it has been related to promoting migration and invasion of tumor cells (Ma et al., 2015; Yu et al., 2016). Treatment of downregulated TXNDC5 lung cancer cells with tunicamycin triggered a significant increase in XBP-1 splicing at early doses of treatment compared to control cells. Event though, the overall sXBP-1 levels reduced in the nuclear fraction. Meanwhile TXNDC5 knockdown decreased expression of GRP78.

PDIA6 gene exhibits self-regulation during ER stress. It regulates the duration of IRE1α phosphorylation in mouse fibroblast cells as well as C. elegans. PDIA6 reduces disulfide bonds in the activated IRE1α oligomer resulting in IRE1α dephosphorylation.
Prolonged IRE1α activation causes an increase in XBP-1 splicing and activation of apoptotic pathways (Eletto et al., 2014). Meanwhile, XBP-1 splicing regulates PDIA6 expression. Spliced XBP-1 can bind to ERSE elements in the promoter region of PDIA6 to induce PDIA6 expression during UPR activation (Lee et al., 2003; Galligan & Petersen, 2012). Moreover, cleaved ATF6 was found to bind to ERSE element in ischemic cardiac myocytes of mouse providing protection against ER stress (Vekich et al., 2013). Therefore, PDIA6 functions to prevent apoptosis through termination of prolonged UPR activation (Eletto et al., 2014). PDIA6 is shown to prevent RIPK1 activation, which is involved in necrotic signaling pathways in lung cancer. PDIA6 downregulated cells show decreased GRP78 protein expression (Tufo et al., 2014). In fact, PDIA6 is known to cooperate with specificity toward GRP78 to form a non-covalent interaction (Jessop et al., 2009). Therefore, it is plausible to propose that TXNDC5, similar to PDIA6, is involved in the termination of XBP-1 to prevent their over-activation, which triggers UPR induced cell death pathways. As it is shown in our results, TXNDC5 deficient cells are more sensitive to early XBP-1 splicing compared to the control cells.

Splicing of XBP-1 is also associated with cyclin D1 activation that promotes cell cycle progression (Yan et al., 2015; He et al., 2011; Zucal et al., 2015; Xie et al., 2015). This may explain inhibition of growth in Srx and TXNDC5 deficient cells in response to tunicamycin treatment. Based on these foundations we propose TXNDC5 to negatively regulate XBP-1 splicing, possibly through termination of transiently activated IRE1 signaling. And that Srx regulates IRE1 signaling pathway activation. Our data suggests that Srx contributes to UPR through regulation of XBP-1 splicing, and GRP78 expression in response to ER stress.

Moreover, TXNDC5 and PDIA6 are oncogenes that participate in promoting tumorigenesis in lung cancer cells. The results show that both proteins promote cell proliferation, anchorage independent colony formation, and cell migration. It is possible
that these two proteins participate in the oncogenesis of cancer cells through regulating ER quality control system in response to stress conditions.

In summary, in this study, we examined the function of Srx in lung cancer cells, through its interacting TXNDC5, and PDIA6 proteins. TXNDC5 and PDIA6 are oncogenes that contribute to promoting cells division, colony formation and cell migration. TXNDC5 contributes to modulating ERK\textsubscript{1/2} and AKT signaling, while PDIA6 modulates phosphorylation of c-Jun, ERK\textsubscript{1/2}, and AKT proteins. Srx interacts with the redox-sensitive thioredoxin-like fold of TXNDC5 and PDIA6 in response to oxidative stress. The N-terminal (D1) of TXNDC5 or PDIA6 acts as the main platforms for this interaction. Srx is found to co-localize with TXNDC5 and PDIA6 in the ER, to modulate their role in oxidative protein folding. Srx-PDIs interaction is vital in preventing protein misfolding and UPR activation. Perhaps to avoid activation of UPR mediated cells death. Therefore, we suggest Srx-TXNDC5/PDIA6 axis to be a valuable target for future development of therapeutic methods in lung cancer.

A better understanding of function of Srx, TXNDC5 or PDIA6 in UPR of lung cancer can present a unique opportunity for development of therapeutic targets. Therefore, in the future it would of interest to study molecular function of Srx and its interacting proteins in activation of the other UPR sensors in lung cancer, ATF6 and PERK sensors. Because Srx interaction with the PDIs mainly occurs through the N-terminal thioredoxin like fold, it would be of interest to study the role of this redox sensitive domain in the PDIs chaperone activities, and its implication to UPR sensors activation. Then This Srx-PDIs axis can be utilized for development of therapeutic drugs to sensitize cancer cells to UPR induced growth inhibition and apoptosis. Beside, PDIs
role in Nrf2 and MAPK signaling can be further investigated in the future to understand their potential role in transcriptional regulation of Srx in lung cancer.
Figure 3.33. Proposed model for Srx-TXNDC5/PDIA6 interaction under oxidative stress conditions. Srx interacts with the N-terminal thioredoxin like fold in TXNDC5 and PDIA6. TXNDC5 and PDIA6, along with Ero1 and PrxIV participate in oxidative protein folding. In contrary, Srx has an inhibitory effect on oxidative protein folding. Srx-TXNDC5 interaction is enhanced under oxidative stress conditions. TXNDC5 facilitates Srx retention in the ER compartment, to sustain homeostasis of oxidative protein folding in lung cancer cells.
Figure 3.34. Proposed model for role of Srx and TXNDC5 in UPR activation. Srx contributes to the activation of UPR adaptive response pathway via activation of spliced XBP-1 (sXBP-1) transcription factor in the nucleus fraction, under ER stress conditions. TXNDC5 participates in the process of correctly folding unfolded/misfolded proteins in the ER. Thus TXNDC5 delays activation of sXBP-1 transcription factor. Both Srx and TXNDC5 activities contribute to boosting GRP78 protein expression during tunicamycin induced ER stress.
APPENDIX
LIST OF ABBREVIATIONS

Adipor1: Adiponectin Receptor 1
AP-1: Activator Protein-1
AR: Androgen Receptor
ATF6: Activating Transcription Factor 6
CHOP: C/EBP Homologous Protein
D: Thioredoxin like domain (CGHC)
ERAD: Endoplasmic Reticulum-Associated Protein Degradation
ERK1/2 Extracellular Signal-Regulated Kinase 1/2
ERO1: Endoplasmic Reticulum oxidoreductin-1
GRP78: Glucose-Regulated Protein, 78kDa
IRE1 Inositol-Requires Enzyme 1
MAPK: Mitogen-Activated Protein Kinase
Nrf2: Nuclear Factor (Erythroid-Derived 2)-Like 2
PDI: Protein Disulfide Isomerase
PDIA1: Protein Disulfide Isomerase Family A, Member 1
PDIA6: Protein Disulfide Isomerase Family A, Member 6
PERK: PRKR-Like Endoplasmic Reticulum Kinase
Prx: Peroxiredoxin
SRX: Sulfiredoxin
TXNDC5: Thioredoxin Domain Containing Protein 5
UPR: Unfolded Protein Response
XBP-1: X-Box Binding Protein 1
TXNDC5 cDNA was inserted to pcDNA3.1 myc-His A Plasmid using XhoI and XbaI restriction sites. PDIA6 gene was inserted using Hind III and XhoI restriction sites. And Srx gene was inserted using BamHI and EcoRI restriction sites. (Plasmid image: SnapGene viewer).
To tag cDNA of each of TXNDC5 and PDIA6 with Flag sequence, the cDNA of these gene were subcloned from pcDNA3.1 myc-His A to p3xFLAG-CMV-14 vector. For TXNDC5-Flag NotI and Xbal restriction sites were used while for PDIA6 HindIII and Xbal enzymes were used. (Plasmid image: SnapGene viewer)
The cDNA of each of TXNDC5 and PDAI6 tagged at the C-terminal with Flag sequence were subcloned from p3xFLAG-CMV-14 vector to pLVX-IRES-Puro vector. SalI and BstBI restrictions sites were used to cut the genes from p3xFLAG-CMV-14 vector and they were inserted to XhoI and BstBI sites in pLVX-IRES-Puro vectors for both genes. (Plasmid image: SnapGene viewer)
Based on the homology modeling and results of structure prediction from I-TASSAR and Z-doc interaction prediction software, the following constructs were used for investigating Srx TXNDC5/PDIA6 interactions:

C-terminal Flag tagged TXNDC5 cDNA showing thioredoxin like domains (underlined), thioredoxin like folds (yellow), and Flag tag sequence (lower case nucleotides at the C-terminus):

ATGCCGCAGCCAGGACGCCTCCTCCCCGCTGCTGGCCCAGGGAGGGCGGCGCCCTG
ACTGCCTGCTGCTGCTGCTGCTGCGCCATGGCGGCGGCGGGGGCGCTGGGGCGC
GGGCCGGCAGGAGCGGCGGCGCAGGCGGCGGAGGGGCCGCCGCCAGACG
GGCGAGGAGCAGAGCAGGCGGAGGAGCGAGCAGACGACTGACCAAGACGAGGACGCC
AGAGTGGCAGGATGAGCCACTGACGGTTGCTGCAGCGTACTTACCCACCA

Translation of TXNDC5 sequence:

MPARPGRLPLLARPAATLALLLLLLGHHGGGGRWGARAQEEAAAAADGPPAAADGEDG
QDPHSHKLHTADMFTGHIQSAHHFVMFAPWCGHCRQLQPTWDLGDKYNSMEDAK
VYVAKVDCTAHSVDVCSAGGVRGYPFTKLKFGQGEAVKYQGPRDFQTLWMLQLVNE
EPVTPEPEPSAPELQKLYELSAENFELHVAOQDHFIKKFAPWCGHCKDALPTE
QLALGHESETVPSGVGJDQTVYHELSONCNVRGYPFTLWFRDGKKDVISKGRDLESL
REYVESQLQRQETGATETVPSEAPVLAEEPKGTYLALTTENNFDDTIAEGTIFK
APWCGHCKLAPTWEESSLKKEFPGLAGVKEVIAEVDCTAERNICSKYVRGYPFTLLFRG
GKVKVEHSGGDRDLSLHRFVSQAKDEL
C-terminal Flag tagged PDIA6 cDNA showing thioredoxin like domains (underlined), thioredoxin like folds (yellow), and Flag tag sequence (lower case nucleotides at the C-terminus):

ATG GCT CCT CTG GTG CCT CGT GCT GGT GCT GCT GAT TAG ATG
TCT GAT TTT CTG TGG CTC CTC TCT TCT TCT CTC

ATG GCT CCT CTG GTG CCT CGT GCT GCT GAT TAG ATG
TCT GAT TTT CTG TGG CTC CTC TCT TCT TCT CTC

ATG GCT CCT CTG GTG CCT CGT GCT GCT GAT TAG ATG
TCT GAT TTT CTG TGG CTC CTC TCT TCT TCT CTC

ATG GCT CCT CTG GTG CCT CGT GCT GCT GAT TAG ATG
TCT GAT TTT CTG TGG CTC CTC TCT TCT TCT CTC

ATG GCT CCT CTG GTG CCT CGT GCT GCT GAT TAG ATG
TCT GAT TTT CTG TGG CTC CTC TCT TCT TCT CTC

Translation of PDIA6 sequence:

MALLVLGLVSCTFFLAVNGLVSDDVIDELTSNFRVIQSDSLWLFETYAPWCGHCQ
RLTPWEKKAALKTVKGVAVADHKHSLGGQYVGQFGPTIKIFGSNIKNRPEDYQG
GRTGEAIVDAALSRLQKVRDLGRSGGYSSGKQRSDSSSKDVIELTDFKDVNL
LDSEDEVMVPFAPWCGHCNLEPEWAAAASEVEKTQSKVLAADVATVNVLAVS
RYGIRGFTIKIFGKESPVYDGRTSNDVRSALFDSNAPPELLEINEDIARKTC
EEHQLCVDVAVPHILDTGAAGRNSYLEVLKLADKYKKMWMGWLTWQAQSELETAL
GGGFYPMMAAIAKNMKFALLKGSFSEQGINELRSLFGTGASTPVGGAFTIVE
REPWDGRDGELPVEDDIDLSVELOLDLGKDEL
SrX sequence in pcDNA3.1 myc-His A plasmid (Cys99 is shaded with yellow color):

ATGGGGCTGCTGCTGAGGAGAACGCTGGGCAAGGCGGCGGGCTGGGGGGCGCCCGAGGGGCCCGGGCCGAGCGGCGGCGCGCAGGGCGGCAGCATCCACTCGGCGCCGCATCGGCAGGCCTGGTACACTACTATTCTCTTTGGGGGCCTGCCACCGCTACGCGGCCTACCAGCAACTGCAGCGAGAGACCATCCCCGCCAAGCTTGTCCAGTCCACTCTCTCAGACCTAAAGGGGTACCTGGGAGCATCCACACCAGACTTGCAGTAG

Translation of SrX sequence:

MGLRAGGTLGRAGAGRGAPEGPGPSGGAQGSGSIHSGRIGAVHNVPLSRLRSVLPSVLDSPAKVQSLVDTIREDPSVPPIDWIKGAQGGDIFYFSFGGCHRGAAYQQLQRETIPAKLVQSTLSDLRVLGASTPDLQ
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