A PRDX1-FOXO3 SIGNALING PATHWAY ACTS AS A REDOX SENSOR CONTROLLING FOXO3 SUBCELLULAR LOCALIZATION AND TARGET GENE TRANSACTIVATION

by

Barbara Lee Hopkins

BS, California Polytechnic State University, 2011

Submitted to the Graduate Faculty of

the Department of Human Genetics

Graduate School of Public Health in partial fulfillment

of the requirement for the degree of

Doctor of Philosophy

University of Pittsburgh

2017

UNIVERSITY OF PITTSBURGH

GRADUATE SCHOOL OF PUBLIC HEALTH

This dissertation was presented

by

Barbara Lee Hopkins

It was defended on

September 5, 2017

and approved by

Dissertation Advisor:
Carola Neumann, MD, Associate Professor
Department of Pharmacology and Chemical Biology, School of Medicine
University of Pittsburgh

Committee Members:

Ryan Minster, PhD, Associate Professor
Department of Human Genetics, Graduate School of Public Health
University of Pittsburgh

David Finegold, MD, Professor
Department of Human Genetics, Graduate School of Public Health
University of Pittsburgh

Adrian Lee, PhD, Professor
Department of Pharmacology and Chemical Biology, School of Medicine
University of Pittsburgh

Carola Neumann, MD

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ABSTRACT

Precision in redox signaling is attained through posttranslational protein modifications such as oxidation of protein thiols. The peroxidase peroxiredoxin 1 (PRDX1) regulates signal transduction through changes in thiol oxidation of its cysteines. We demonstrate here that PRDX1 is a binding partner for the tumor suppressive transcription factor FOXO3 that directly regulates the FOXO3 stress response. Heightened oxidative stress evokes formation of disulfide-bound heterotrimers linking dimeric PRDX1 to monomeric FOXO3. Absence of PRDX1 enhances FOXO3 nuclear localization and transcription that are dependent on the presence of Cys31 or Cys150 within FOXO3. Notably, FOXO3-T32 phosphorylation is constitutively enhanced in these mutants, but nuclear translocation of mutant FOXO3 is restored with PI3K inhibition. Here we show that on H_2O_2 exposure, transcription of tumor suppressive miRNAs let-7b and let-7c is regulated by FOXO3 or PRDX1 expression levels and that let-7c is a novel target for FOXO3. Conjointly, inhibition of let-7 microRNAs increases let-7-phenotypes in PRDX1-deficient breast cancer cells. Altogether, these data ascertain the existence of an H_2O_2-sensitive PRDX1-FOXO3 signaling axis that fine tunes FOXO3 activity toward the transcription of gene targets in response to oxidative stress.

The public health significance of this research lies in the fact that elevated levels of oxidative stress in a major cancer risk factor. In breast cancer it has been found that post-

menopausal women, who are generally at increased risk for breast cancer development, show an even higher level of oxidative stress markers. Unfortunately, antioxidant therapies have proven ineffectual. If we can gain a deeper understand of how these oxidative stress induced redox-signaling pathways work, it is possible to develop more effective therapies for those at-risk patients for whom therapies fail.

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PREFACE

Pursuing my PhD has been simultaneously the most challenging and rewarding experience of my life. The unending support, guidance, advice, and mentorship of quite a few people made my road through graduate school all the more manageable. Without these people, the work I present here would not have been possible.

First, I must acknowledge and profoundly thank my mentor, Dr. Carola Neumann. In the last five years, Dr. Neumann has introduced me to the world and wonders of redox chemistry, a subject I knew very little about, and never thought I would dedicate so much of my time to. In the last five years, I have grown, learned, and matured into a better scientist, and that is all because of the support and encouragement of Dr. Neumann. Whether it's waiting for a film to come out of the developer, or an image to finish loading on LiCor, Dr. Neumann is always front and center, excited to dive-in and start discussing what it all means, and where we could go next. Always pushing me to strive for more, to take the next step forward, I can't imagine a better mentor to help guide me and teach me. If I end up being even half the scientist she is, I will consider myself truly accomplished.

With a mentor like Dr. Neumann, it comes as no surprise that the members of her lab are people with whom I am truly honored to have worked alongside. Dr. Myriam Attar who helped to get me settled, and showed me the ropes (and only slightly teasing me while doing so). My fellow graduate students, Shireen Attaran and Alp Asan. you guys made lab an experience I

wouldn’t trade for the world. Whether it was help with an unfamiliar procedure, graciously lending just a couple plates worth of media, geeking out about Harry Potter or the newest Game of Thrones episode, talking about the latest news stories (there were a lot of them), sharing some truly frightful experiences in the hallways, or trading conspiracies as to where all our glassware really went and if we were in fact unknowing test subjects in a far bigger experiment, I could always count on you, and I can’t thank you enough.

I would be remised if I didn’t offer a most sincere thank you to Dr. John Skoko. I doubt there is any more accommodating, understanding, helpful, or knowledgeable post-doc out there, and I count myself unbelievably lucky to have had him to run any and every thought by. We may have been polar opposites when it came to certain issues, but when it came to science, hilarious and almost unbelievable stories, I can’t think of a better office mate.

I must thank my dissertation committee members, Dr Adrian Lee, Dr. David Finegold, and Dr. Ryan Minster for their sound and patient advice, suggestions, constructive criticism, and guidance.

A thank you is also owed to Dr. Kenneth Hillers who gave me a place in his lab at Cal Poly as an undergraduate, and how introduced me to just how fun, frustrating, and rewarding the lab environment is.

To the entire Department of Human Genetics, I can’t thank them enough for taking a chance, and giving me the opportunity to study, lean, and explore. To my fellow PhD students, I wouldn’t trade our monthly meet-ups for the world. And especially to Dr. Candace Kammerer who kept me and pretty much everyone on track, and genuinely cared about every student that came through her door.

I must thank the friends I've made. From cabining adventures, to cooking cake on a broom handle, becoming villains, and our countless game nights, Maura, Emily, and Sara kept me sane and made Pittsburgh feel a little like home (even if it is on the wrong coast).

I cannot put into words how much I owe my family. To Steve and Kelsey, I can't imagine a better brother and sister to look up to as I grew. Time or an ocean may be in our way, but there is no length of time, and no distance so great as to hinder my love for you, or weaken my connection to you.

Finally, to my mom (touch your face), to whom I dedicate this work. There is absolutely nothing I have done in life, or will ever do, that I don't owe completely to you. From the day I was born, you have been my number one fan, my loudest cheerleader, my most trusted advisor, and most importantly, my best friend. You have believed in me, encouraged me, and loved me, and for that, I can't thank you enough. Mommies love those babies, and no mommy has ever loved hers babies as much as you love yours. I love you, I love you, I love you!

ABBREVIATIONS

β-ME	Beta-mercaptoethanol
ASK1	Apoptosis signal-regulating kinase 1
CAT	Catalase
ChIP	Chromatin immunoprecipitation
Co-IP	Coimmunoprecipitation
Cys	Cysteine
EGFP	Enhanced green fluorescent protein
ERp46	Endoplasmic reticulum protein 46
FLAG	FLAG-epitope tag
FOXO	Forkhead box transcription factors of the O class
HA	Human influenza hemagglutinin
JNK	Jnk N-terminal kinase
MEF	Murine embryonic fibroblast
miRNA	MicroRNA
MKP	Map kinase phosphatase
MST	Mammalian sterile 20-like kinase 1
NEM	*N*-ethylmaleimide
p21	Cyclin-dependent kinase inhibitor 1
p27	Cyclin-dependent kinase inhibitor 1B
PI3K	Phosphatidylinositol-4,5-bisphosphate 3-kinase
PRDX	Peroxiredoxin
qPCR	Quantitative polymerase chain reaction
ROS	Reactive oxygen species
SGK	Serum and glucocorticoid-regulated kinase 1
sh	Small hairpin
SOD2	Superoxide dismutase 2
STAT3	Signal transducer and activator of transcription 3
UTR	Untranslated region
WT	Wild type

1.0 INTRODUCTION

1.1 BREAST CANCER

Breast cancer is the most common new cancer diagnosis, and the second leading cause of cancer related death in American women after lung cancer. In 2017, it is estimated that in the United Sates, 252,710 women will be diagnosed with breast cancer, accounting for roughly 30% of all cancer diagnoses, and 40,610 women will die as a result of the disease, accounting for roughly 14% of all cancer related deaths in women (1). Overall, an estimated one in every eight American women will develop an invasive breast cancer over the course of her lifetime (2). While being 100 times less prevalent in men than in women, men are still susceptible to breast cancer, with the lifetime risk of developing the disease at around one in one thousand. In addition, though it is rarer, breast cancer in men is usually considered more aggressive than breast cancer in women, as many men are not aware of their risk, and do not typically undergo screening. The American Cancer Society estimates that in 2017 there will be around 2,470 new invasive breast cancer diagnoses, and around 460 men dying from the disease (3).

Given all of these statistics, it is clear that breast cancer is a serious public health concern, and therefore it is important to understand the underlying mechanisms which lead to breast cancer development and progression. With a greater understanding of what processes drive and

promote breast cancer, possible treatments can be devised so that diagnosis and mortality rates can decrease.

1.1.1 Classification and stages

Commonly, breast cancer is classified based on its cell of origin. Ductal carcinoma *in situ* (DCIS) and invasive ductal carcinoma (IDC) both originate in the inner tissue lining the milk ducts. Lobular carcinoma *in situ* (LCIS) and invasive lobular carcinoma (ILC) both originate from the milk-producing glands, or lobules (4). As the names suggest, both IDC and ILC have spread from their points of origin and invaded into the surrounding breast tissue. DCIS and LCIS are still confined to points of origin had have the ability to transition into invasive forms. IDC accounts for roughly 80% of all invasive breast cancers, while ILC accounts for about 10%. IDC can be broken down into further subtypes, medullary, mucinous, papillary, or cribriform carcinomas, each with unique cell morphologies, growth patterns, prognoses, and differences in effected populations (5).

Breast cancer is categorized into stages 1-4, based on certain phenotypic criteria such as invasiveness, size, lymph nodes status, and metastases (6). DCIS is often termed stage “zero” as it is considered non-invasive, remaining limited to the duct and does not invade through the basal membrane. Once a tumor begins to increase in size and spread to the surrounding breast tissue and proximal lymph nodes, the cancer stage is upgraded. The extent of tumor size and invasiveness determines its categorization as stage 1, 2, or 3. A breast cancer is considered stage 4 once the primary tumor has metastasized to other organs including distant lymph nodes, the lungs, liver, bone, and brain (7).

A stage four metastatic breast cancer diagnosis most often accompanies a poor prognosis, with a mortality rate of about 90% following diagnosis (8). A cancer becomes metastatic when primary tumor cells acquire properties through genomic events that allow them not only to break away from the tissue barrier of primary breast tumor and invade into blood and lymph vessels and travel to distant sites (9), but also to survive assaults from the immune system and drug interventions as they travel to the secondary sites. The changes often result in drug insensitivity, meaning that therapies that may have worked on the primary tumor are ineffectual in the treatment of metastatic tumors (10). In general, changes between the primary and metastatic tumors have also been shown to lead to a resistance to treatments which makes metastatic breast cancer all the more difficult to treat (11).

1.1.2 Receptors

Breast cancer is also categorized by their receptor profile. The three significant receptors involved in breast cancer are the estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2 or ERRB2)) (12). ER and PR are hormone receptors and localized intracellularly; when their ligands bind (estrogen or progesterone), ER and PR translocate into the nucleus and bind to hormone-responsive element (HRE) sequences located in the promoters of target genes. When bound to the DNA, ER and PR then regulate the expression of transcription factors which in turn regulate the expression of numerous genes involved in processes such as cell cycle control, DNA replication and repair, and apoptosis. Dysfunctions in ER/PR activity can lead dysregulation of their gene targets, and result in tumorigenesis (13-15). HER2 is a tyrosine kinase receptor bound to the plasma membrane and functions by dimerizes with other members of the epidermal growth factor receptor (ErbB) family (EGFR, ErbB3,

ErbB4). The dimerization causes autophosphorylation of tyrosine residues on HER2, triggering conformational changes that lead to other proteins binding, and inducing downstream signaling cascades (16). Inhibition or degradation of these signaling cascades can promote cancer progression by increasing cell survival, proliferation, motility, invasion, and metastasis.

Based on a recently developed gene signature comprising 50 genes (pam50), breast cancer can be clinically divided into 4 subtypes (17). Accordingly, Lumina A breast cancers are positive for all three receptors, while Luminal B cancers are usually only positive for ER and PR. Hence, both groups are susceptible to estrogen based treatments, such as tamoxifen an estrogen antagonist, or aromatase inhibitors such as anastrozole, exemestane, and letrozole (18). Breast cancers only positive for HER2 show an over-expression of the HER2 receptor on the cell surface, resulting in increased growth factor binding and enhanced cell growth and proliferation beyond its normal limits. Drugs like Herceptin attach to the HER2 receptors, blocking them from receiving the excess growth signals, triggering the cell to arrest during the G1 phase of the cell cycle, and downregulate AKT activity (19). Breast cancers that do not express any of the aforementioned receptors are called triple negative, and belong according to the pam50 classification to the basal subtype. As this subtype does not express any of the druggable receptors (ER, PR or HER2), making them insensitive to endocrine or HER2 receptor targeting therapies, and thus very aggressive and difficult to treat (20).

Understanding the underlying pathways that drive breast cancer development and progression may aid in the discovery and development of more effective therapies for those patients for whom current therapies fail. Oxidative stress and redox signaling is considered one such driver.

1.2 REACTIVE OXYGEN SPECIES AND OXIDATIVE STRESS

Reactive oxygen species (ROS) are chemically reactive species containing oxygen and include superoxide anions (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radicals (·OH). Some ROS are also called free radicals and are characterized by unpaired valence electrons such as the hydroxyl radicals (·OH) (Figure 1.1) (21). O_2^- is a product of the naturally occurring one-electron reduction

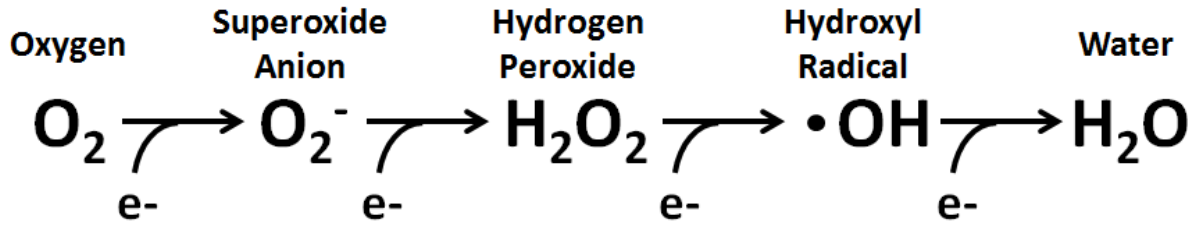

Figure 1.1: Reduction of molecular oxygen.
Molecular oxygen (O_2) requires the addition of four electrons (e^-) to be fully reduced to water (H_2O) [Not shown as a balance equation]

of molecular oxygen (dioxygen, O_2). While single oxygen has two unpaired electrons that are shared in dioxygen and is relatively stable, O_2^- contains an additional electron, making it very reactive. O_2^- is a product of the naturally occurring one-electron reduction of molecular oxygen and for example a byproduct of mitochondrial respiration and produced at complex I and complex III of the electron transport chain (22). As with O_2^-, H_2O_2 is very short lived and a byproduct of biochemical reactions in the cell. For example, the O_2^- scavenging dismutase produces H_2O_2 in the process of eliminating O_2^-. Also, during catabolism of very long chain fatty acids in the peroxisomes, H_2O_2 is made. H_2O_2 is under acidic conditions one of the most powerful oxidizers known and can through catalysis be converted into (·OH). For example,

during the Fenton reaction, ferrous (+2) iron is oxidized by H_2O_2 resulting in ferric (+3 ion), hydroxyl ion (OH^-) and reactive ·OH (22).

ROS can be produced both endogenously and exogenously. Endogenously, ROS are produced in cellular compartments in which oxygen consumption is high, such as the mitochondria, peroxisomes, and the endoplasmic reticulum and enzymatically through NADPH oxidases. ROS can also be produced by various exogenous sources that include ionizing radiation, pollutants, tobacco and xenobiotics (Table 1.1) (22). ROS are capable of inducing oxidative modifications on various cellular components, such as DNA, proteins, and lipids. These modifications can play an essential role in protein functioning, and when at physiologically relevant levels, the ROS included changes are reversible. The reversibility of these changes allows for signals to be transduced to various downstream targets, this phenomenon is known as reduction and oxidation (redox) signaling (23). As opposed to other signaling mechanisms which may require a series of different enzymatic reactions in order to occur, oxidation requires none of that. ROS need only to be present in an environment, and oxidation susceptible targets accessible, for

Table 1.1: Exogenous sources of ROS.

Water & Air Pollution	Heavy Metals (Fe, Cu, Co, Cr)
Alcohol	Transition Metals (Cd, Hg, Pb, As)
Tobacco Smoke	Drugs (Halothane, Doxorubicin, etc.)
Pesticides	Refrigerants
Ultraviolet Light	Industrial Cleaning Supplies

(Adapted from Phaniendra et. al., 2015) (22)

oxidation to occur. As a result of the relatively few requirements for ROS induced oxidation, oxidation occurs fairly non-discriminately. While certain levels of ROS are beneficial to the cell, if ROS continue to accumulate and reach elevated levels, the changes will continue to occur, and the modifications will become irreversible, ultimately leading to permanent damage. This damage is referred to as oxidative stress (24). A redox homeostasis must be established within cells in order to carefully maintain a balance between the two different sides of ROS, oxidative stress and their role as signaling molecules.

1.2.1 Oxidative stress induced damage

Oxidative stress is the result of sustained accumulation of ROS, which regardless of origin, leads to non-specific oxidation. Uncontrolled and unchecked ROS accumulation and oxidative stress can result in lipid peroxidation, DNA mutagenesis, and protein dysfunction and damage. (21,25). In lipids, peroxidation by ROS can result in irreversible alterations to the lipid membrane, including loss of membrane function, changes in fluidity, and dysfunction in the transmembrane proteins (26,27). In DNA, high levels of ROS induce damage such as single and double stranded breaks, a-basic site formation, irregular DNA-protein crosslinking, and incorrect base pairing. Any one of these changes can result in incorrect or failed transcription; any resulting protein may contain altered amino acid sequence, and alterations can lead to either gain of function or loss of function of the protein (28,29). RNA is even more susceptible to the damaging effects of ROS since it is single-stranded, and therefore lacks a template and the common repair pathways in DNA (30). Finally, the most common cellular targets of oxidative stress are proteins, accounting for an estimated 70% of all ROS induced modifications in the cell (31). When amino acid

residues are indiscriminately oxidized by ROS, this leads to a multitude of altered residues (Table 1.2) (32) which may in turn negatively alter protein function (33).

Table 1.2: ROS mediated oxidation of protein amino acid residue side chains.

Amino Acid	Product
Arginine	Glutamic-semialdehyde
Cysteine	Cysteine disulfides; Sulfenic acid
Histidine	2-oxo-histodine
Leucine	3-,4-,5-hydroxyleusine
Lysine	2-Amino-adipic-semialdyhyde
Proline	Glutamic-semialdehyde; 2-pyrrolidone; 4-,5-hydroxyproline
Threonine	1-amino-3-keto butyric acid

(Adapted from Stedtman et. al., 2003) (32).

Altogether, the oxidative stress induced damage to lipids, DNA/RNA, and proteins due to elevated ROS levels results in considerable alterations to protein function and inevitably lead to numerous severe physiological conditions due to disruptions to various pathways and processes involved in such conditions. Left uncontrolled, ROS accumulation and oxidative stress have been shown to contribute to and promote a wide array of human diseases, including aging, obesity, cardiovascular diseases, neurodegenerative diseases, and many forms of cancer (34-38). It is not an understatement to say that the negative effects of ROS are far reaching and impact every living creature.

1.2.2 Redox signaling

While the damaging effects of ROS accumulation are well documented and severe, organisms actually require ROS to survive, and H_2O_2 is considered a second messenger (39). Thus, the biological role ROS play in redox signaling has seen a significant growth in attention and importance in the past couple of decades (40,41).

For example, one well understood benefit of ROS is demonstrated in immune defense (42). NOX2, an NADPH oxidase comprised of six subunits, $gp91^{phox}$ and $p22^{phox}$ are membrane-bound components, whereas $p47^{phox}$, $p67^{phox}$, and $p40^{phox}$ are all cytosolic components that assemble with the membrane-bound portion upon activation of either Rac1 or Rac2 GTPases. NOX2 converts molecular oxygen to superoxide anions as a mechanism to eliminate bacteria (43,44). NOX2 activity is essential as patients who carry a mutation in the gp91 subunit develop an immunodeficiency called chronic granulomatous disease (CGD) which is characterized by frequent bacterial infections (45).

Another example of redox signaling is the regulation of the phosphatase and tensin homolog (PTEN) that is a widely expressed tumor suppressive protein which functions as a lipid and tyrosine phosphatase and inhibitor of the PI3K/AKT cellular proliferation and longevity signaling pathway. PTEN belongs to the group of protein tyrosine phosphatases which use the nucleophilic cysteine thiolate in their active site to remove phosphate groups from tyrosine by building a cysteinyl-phosphate enzyme intermediate. In the absences of PTEN, PI3K converts phosphatidylinositol (4,5)-triphosphate (PIP_2) to phosphatidylinositol (3,4,5)-triphosphate (PIP_3), and phosphorylates AKT, causing AKT to activate and localize to the cell membrane where it can initiate a number of downstream effects, many of which when constitutively active, are involved in tumorigenesis. When active, PTEN functions to dephosphorylate PIP_3 back to PIP_2,

thereby limiting AKT membrane binding, and decreasing its activity (46). However, upon oxidation of the active site cysteine (Cys124) in PTEN by hydrogen peroxide an intra-disulfide bond forms between Cys124 and the N-terminal Cys71 that inhibits PTEN's phosphatase activity, and allowing for the activation of AKT. Maintaining a careful balance between unoxidized and active PTEN and oxidized and inactive PTEN, is therefore important for proper PI3K/AKT signaling activity (47,48).

Through the reversible oxidation of thiol-containing amino acid residues of target proteins, ROS are capable of regulating a diverse range of biological processes as a result of their ability to transduce a signal (49,50). Protein targets of redox signaling include transcription factors, receptors, enzymes, and proteases. Some of the cellular processes modified by redox signaling include aging, cell growth and proliferation, and apoptosis (51).

1.2.3 Cysteine oxidation

As previously mentioned, ROS oxidize thiol-containing amino acid residues in order to regulate target protein activity. Cysteine is the primary amino acid susceptible to oxidation within a protein, contributing most to the protein's overall redox sensitivity (52). Briefly, in the presence of ROS such as H_2O_2, the thiol (-SH) gets oxidized to a thiolate ($-S^-$), which upon further ROS stimulation, is oxidized further into sulfenic acid (-SOH), the precursor to disulfide bonds. The oxidation of thiol to thiolate to sulfenic acid is reversible, and the disulfide bonds formed can be reduced upon reaction with a reducing agent such as thioredoxin (TRX). Cysteine can be further oxidized from sulfenic acid into sulfinic acid (SO_2H), and then from sulfinic acid into sulfonic acid (SO_3H). These oxidations, however, are irreversible and result in permanent protein damage (Figure 1.2) (50).

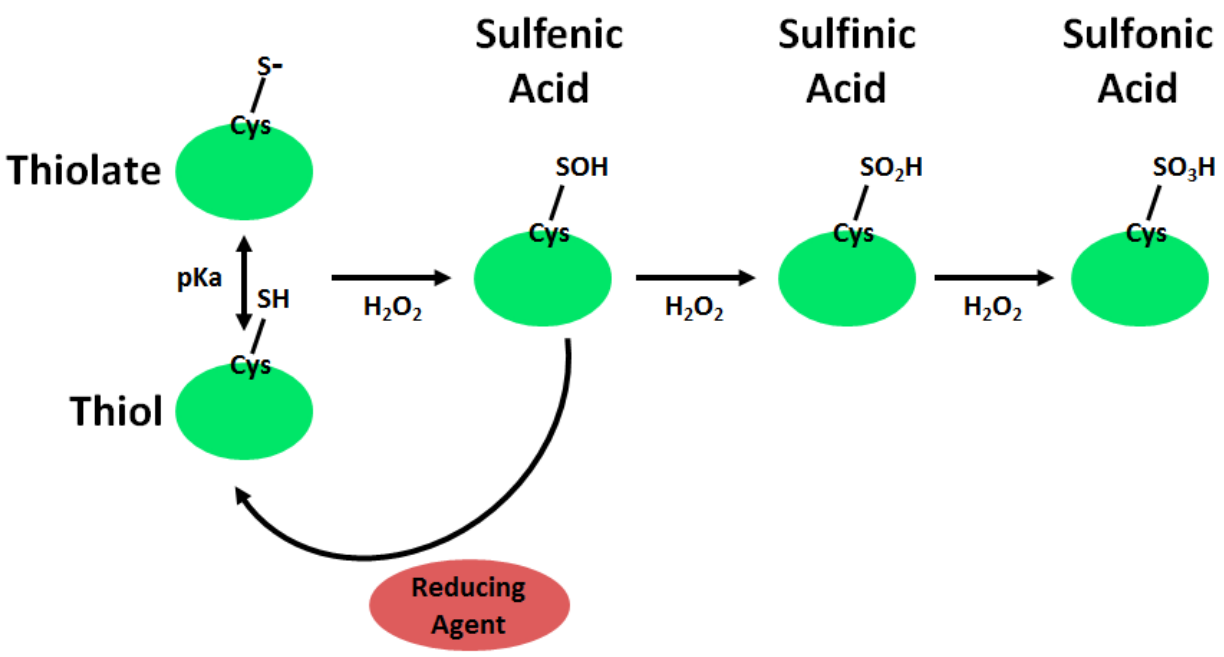

Figure 1.2: Stepwise oxidation of cysteine residues.
Oxidation of the sulfur atom within a cysteine residue can result in the stepwise formation of a reactive cysteine thiolate, sulfenic acid, sulfinic acid, and finally sulfonic acid. With the aid of a reducing agent, the oxidation to thiolate and sulfenic acid is reversible. Oxidation to sulfinic or sulfonic acid is irreversible.

While cysteine is considered the most physiologically relevant amino acid reside due to its thiol group, its position within a folded protein, and the surrounding residues do play a part in cysteines reactivity. Cysteine has a high acidic dissociation constant (pKa) of approximately 8.2, so at physiological pH, it is more likely to be found in its protonated and therefore less reactive thiol state. However, if a cysteine is surrounded by positively charged amino acid residues, the pKa, can be lowered to such a level that the cysteine can exist as a deprotonated nucleophilic thiolate, greatly increased its and the protein's redox sensitivity (31,50).

Arguably, one of the most significant outcomes resulting from the oxidation of cysteine's thiol in terms of redox signaling, is its ability to form reversible disulfide bonds with other thiol functional groups. In the presence of ROS, a thiolate group can be oxidized into a sulfenic acid intermediate, which then can react with another thiol (both intra- or inter-molecularly), creating a

reversible disulfide bond (53). Disulfide bond formation can have a great impact on protein structure, stability, and function. Often, a disulfide is required for proper protein folding, and the absence of the bond would result in a dysfunctional or inactive protein (54). Oxidation of a thiol and disulfide bonds have another important function in redox signaling, and that is acting to regulate the activity of other proteins. One of the better understood methods of protein regulation is through a mechanism known as the thiol-disulfide exchange, and is also thought to have a role in intra- and intermolecular redox homeostasis (55). In short, due to their negative charge, thiolate anions (S^-) are highly reactive because of their availability of electrons, and therefore will readily react with other reactive species in order to become more stable and neutralize their negative charge. During the thiol-disulfide exchange, a reactive thiolate anion 'attacks' one of the sulfur atoms of a disulfide bond. The original disulfide bond then begins to break as a new one forms, creating a trisulfide-like transition state, a "tri-thiol" ion (56). Eventually the original disulfide is entirely broken, and a new disulfide bond containing the 'attacking' thiolate is formed, along with the release of a new thiolate anion (Figure 1.3) (57). This reaction is highly transient, with the newly formed thiolate free to go on and react, possibly forming or reforming other bonds and triggering further downstream reactions. In summary, disulfide bond formation's role in protein folding, stabilization, activation, and regulation, and the transient nature of the thiol-exchange serves to highlight the importance of oxidation as a redox signaling mechanism.

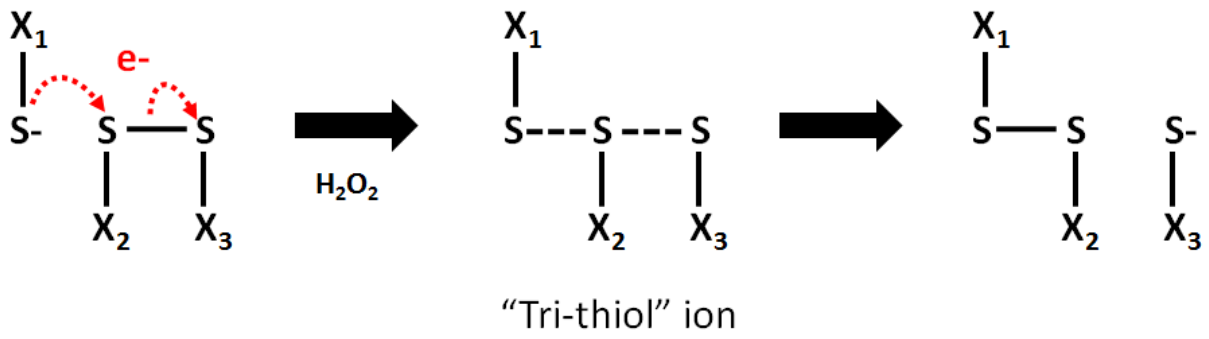

Figure 1.3: Thiol-disulfide exchange.
The thiolate group on protein X_1 attacks a sulfur atom of protein X_2 of the disulfide bond, creating a temporary "tri-thiol" ion, and ultimately displacing the other sulfur atom in the disulfide (protein X_3) and forming a new disulfide bond between proteins X_1 and X_2, and a new thiolate ion on protein X_3.

1.2.4 Antioxidants

As discussed above, accumulation of ROS can cause permanent damage and dysfunction on both the cellular and organismal levels. Fortunately, cells have a way to combat detrimental effects in the form of antioxidants that inhibit the oxidation of other molecules. Antioxidants include vitamins, minerals, flavonoids and antioxidant enzymes. When there is a buildup of ROS in a cell or in the cellular environment, antioxidants restore redox homeostasis by neutralizing the ROS into non-reactive products and repairing any erroneously oxidized molecules before permanent damage can occur. The importance of antioxidants is highlighted by the fact that loss of antioxidant activity has been shown to be a contributing factor in numerous diseases (58,59).

Enzymatic antioxidants such as superoxide dismutase (SOD), catalase, thioredoxins, glutathione peroxidase, and peroxiredoxins, all function to detoxify any excess ROS, often working in combination and in a stepwise fashion (Figure 1.4) (60). As an example, SOD can convert two superoxide anions into hydrogen peroxide and molecular oxygen (60). The hydrogen peroxide can then immediately be converted by catalase into water and another molecular

oxygen. Glutathione peroxidase, with glutathione as a co-factor, and peroxiredoxin, using thioredoxin as an electron donor, can also reduce hydrogen peroxide to water and molecular oxygen (61,62). This redundancy in antioxidant activity, with multiple different antioxidants able to reduce the same ROS, highlights their importance in the cell, and the importance of neutralizing any ROS accumulation.

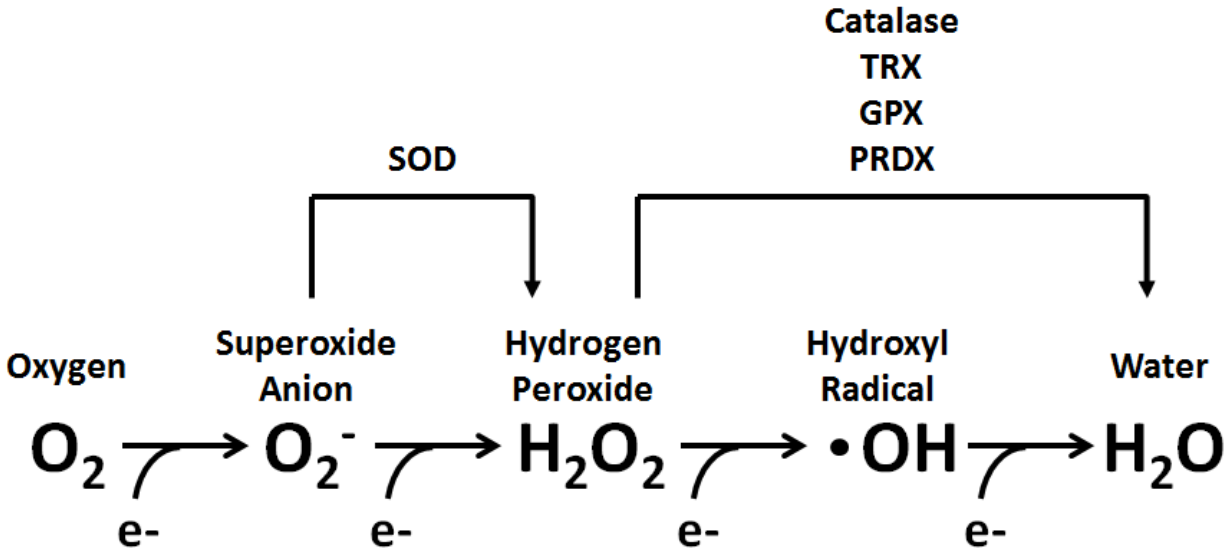

Figure 1.4: Neutralization of ROS by antioxidants.
SOD: Superoxide dismutase; TRX: Thioredoxin; GPX: Glutathione peroxidase; PRDX: Peroxiredoxin. [Not shown as a balance equation]

Besides their function as ROS detoxifying agents, antioxidants also play a role in redox signaling, reducing signaling targets and redox regulators in order to inactivate or restore signaling capacity.

1.3 PEROXIREDOXIN-1

Peroxiredoxins (PRDXs) are a family of six ubiquitous hydrogen peroxide scavengers (PRDX1-6), antioxidant enzymes that reduce hydrogen peroxide (H_2O_2) via the oxidation of a peroxidatic (or catalytic) cysteine to sulfenic acid (63). The peroxiredoxins can be subdivided into three main subclasses based on their cysteine complement: typical 2-Cys PRDXs (PRDX1-4), atypical 2-Cys PRDX (PRDX5), and 1-Cys PRDX (PRDX6) (64,65). The names 1-Cys and 2-Cys do not refer to the numbers of cysteines actually present in the protein, but rather the number of cysteines active in the catalysis of hydrogen peroxide. 1-Cys PRDX has only one cysteine involved, while 2-Cys PRDXs have two. The difference between typical and atypical 2-Cys PRDXs is that the typical 2-Cys PRDXs form a disulfide bond between two different PRDX proteins, forming a homodimer, while the atypical 2-Cys PRDX forms the disulfide between two cysteines within the same protein (66). In contrast to the 2-Cys PRDXs, in the catalytic cycle of PRDX6, the only 1-Cys PRDX, the single C-SOH does not form a disulfide bond, as another C-SH is unavailable. PRDX6 oxidized at the peroxidatic cysteine is reduced by glutathione (67). 2-Cys PRDXs contain a thioredoxin binding domain, which is necessary to disulfide bond reduction and maintenance of their catalytic activity (Figure 1.5).

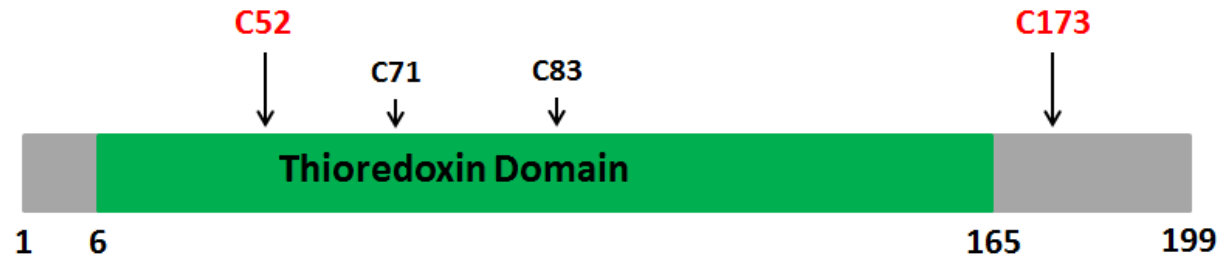

Figure 1.5: PRDX1 as example of typical 2-Cys PRDX domain structure.
Peroxidatic cysteine (C52) and resolving cysteine (C173) are shown in red, along with other cysteines present in the protein.

1.3.1 PRDX1 oxidation

As mentioned above, all PRDX proteins contain a cysteine known as the peroxidatic cysteine (C_P) which can be found in the N-terminal domain. It is this cysteine that reacts with hydrogen peroxide during catalysis. In PRDX1, this is C52. In the majority of PRDXs, there is a second evolutionarily conserved resolving cysteine (C_R) found in the C-terminal domain, that allows for an intermolecular disulfide bond to form between two PRDX molecules. In PRDX1, this is C173 (68).

In the presence of hydrogen peroxide, PRDX1 C52 is oxidized to a sulfenic acid (C52-SOH). The sulfenic acid can then react with the thiol of C173 (C173-SH) in a second PRDX1 molecule, forming an intermolecular disulfide bond and a head to tail PRDX1 dimer (66). Unlike some other antioxidant enzymes that can be reduced by various other reducing agents, PRDX1, like other 2-Cys PRDXs, can only be reduced via a thiol-disulfide exchange by TRX, which was mentioned before as another antioxidant involved in redox control. Thus, the reducing equivalents responsible for restoring PRDX1's catalytic activity come from NADPH via thioredoxin reductase (TRXR) and TRX (Figure 1.6) (69).

Compared to other antioxidants like catalase, PRDXs are very sensitive to ROS, and have a high affinity for H_2O_2, functioning at relatively low to moderate concentrations. Catalase, which reduces H_2O_2 following an exponential decay, the reduction rate depends linearly on hydrogen peroxide concentration (70), functions fast and efficiently when H_2O_2 concentrations are high. However, once concentrations drop to a certain level, PRDXs can scavenge more efficiently due to their higher affinity. Because of the high affinity to H_2O_2, PRDX1 can be very easily over-oxidized from sulfenic acid to sulfinic or sulfonic acid at its peroxidatic cysteine,

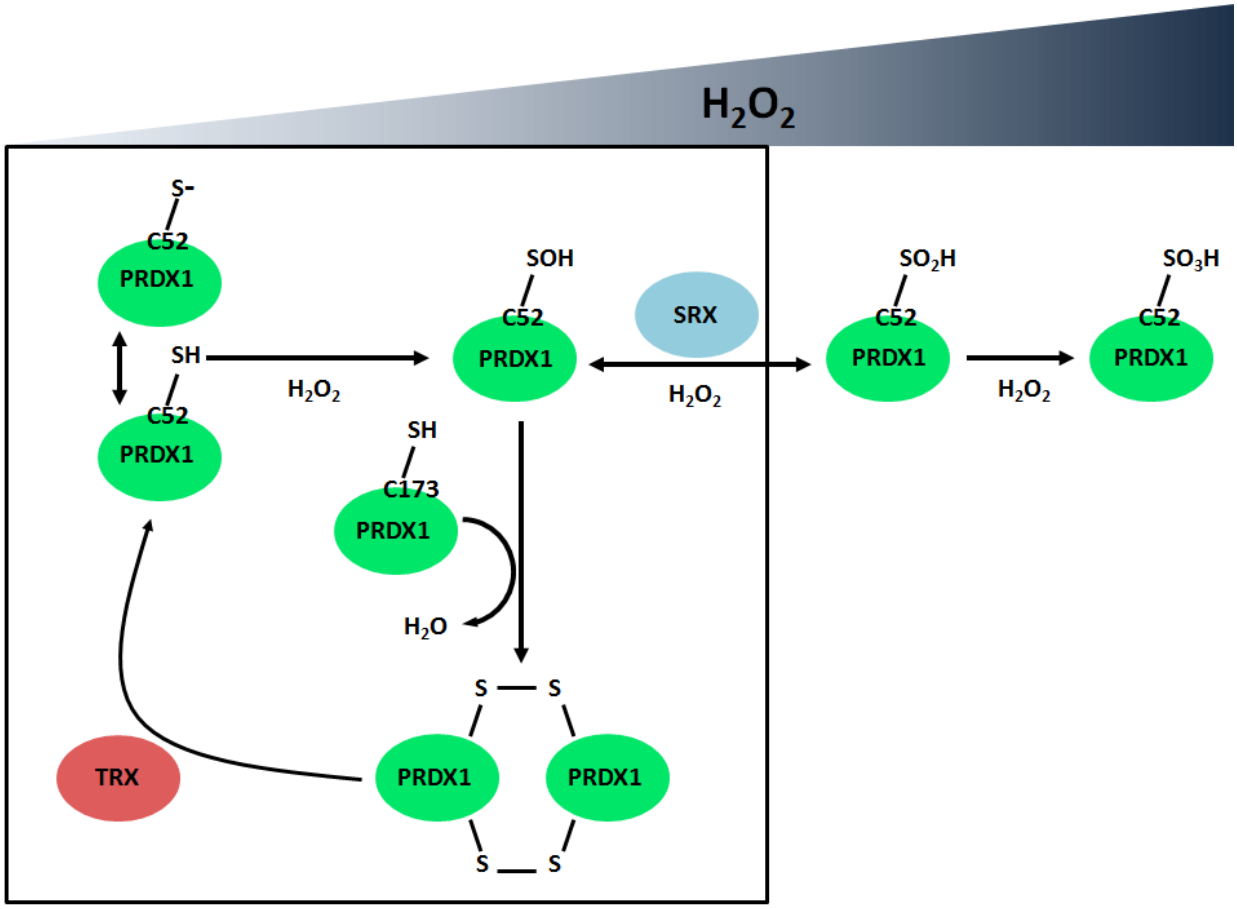

Figure 1.6: The PRDX1 oxidation cycle.
In the presence of H_2O_2, the peroxidatic cysteine of PRDX1 (C53), is first deprotonated to a thiolate anion and then further oxidized to sulfenic acid. In the sulfenic acid, C52 can form a disulfide bond with the resolving cysteine (C173) of a second PRDX1 molecule, creating a PRDX1 homodimer and releasing a water molecule. The dimer can be reduced by thioredoxin (TRX) to restore PRDX1's peroxidase activity [cycle shown in box]. Further oxidation by H_2O_2 leads to the formation of sulfinic acid and sulfonic acid, both reactions are irreversible.

blocking its antioxidant activity (71). One possible explanation that has been suggested for the easy over-oxidation of C52, is that during catalysis C52 exists as a thiolate ($C52\text{-}S^-$) whilst the three other cysteines (C71, C83, and C173) all remain protonated as thiols. As discussed above, thiolates are nucleophilic and highly unstable, and will react with any available thiol to form either a disulfide bond, or to irreversibly oxidize further into sulfinic or sulfonic acid (72).

Interestingly, examinations of the crystal structures of both PRDX1 and PRDX2 showed that their peroxidatic cysteines can be stabilized and protected in its thiolate state within a folded N-terminal active site pocket, by forming salt bridges with other amino acid residues nearby due to protein folding (notably arginine and threonine) (73,74). This, of course, begs the question, if a thiolate can be stabilized and protected, what exactly causes the over-oxidation observed during catalysis? It was found that over-oxidation is correlated with an increased presence of TRX (75). Investigators speculate that when TRX binds to PRDX1 to reduce the disulfide, the reduced C52-SH becomes susceptible to over-oxidation due to TRX blocking the sequestration of the reduced C52 into the active-site, thus exposing it to hydrogen peroxide present (68).

While over-oxidation of PRDX1 does trigger a loss of antioxidant activity, it does not render PRDX1 useless or non-functional. Rather, over-oxidation of PRDX1 functions as an effective switch over to molecular chaperone function in the form of a decamer composed of five homodimers (76,77).

1.3.2 Cell signaling through PRDX1

PRDX1 plays an active role in redox signaling, regulating the activity protein binding partners involved in a number of cellular processes and biological pathways. Under increased H_2O_2

stress, PRDX1 shows a decreased affinity for certain signaling molecules, and can increase the dissociation of PRDX1 from said molecules. The loss of complex formation can then lead to the activation (e.g. c-Abl) or inactivation of PRDX1's binding partners (e.g. PTEN, MKP-1). Other binding partners have been described to enhance activity once bound to PRDX1 (e.g. MKP-5 and MST1) or inactivity (e.g. JNK) (78-80).

As discussed previously, the nucleophilic cysteine in the active site of the PTP-PTEN is required for dephosphorylation of it substrate, but because of this it is very sensitive to oxidation. Interestingly, it's been shown that PRDX1 binds to PTEN under mild H_2O_2 stress, promoting PTEN membrane binding, and fully protecting PTEN lipid phosphatase activity from any oxidation induced inhibition (78). However, as H_2O_2 concentrations increase and PRDX1 C52 becomes over-oxidized and the PRDX1-PTEN interactions breaks, leaving PTEN susceptible to inactivating oxidation, inhibition of its phosphatase activity, and subsequent tumorigenesis by way of overexpressed PI3K/AKT downstream targets.

Mammalian Ste20-like kinase-1 (MST1), like PTEN, is another binding partner activated through an interaction with PRDX1. MST1 is a Ser/Thr protein kinase. In response to apoptosis inducing stimuli such as H_2O_2 accumulation, MST1 autophosphorylates, which allows it to phosphorylate target proteins, and ultimately trigger cell death mechanisms (81). The phosphorylation of transcription factors like FOXO family members in mammalian cells, and histone subunits in yeast, are just some examples of the cell death pathways activated by MST1 and its homologs (82,83). It is an association with PRDX1 by which MST1 mediates these apoptotic pathways. It is assumed that in response to H_2O_2, PRDX1 decamers form an oligomeric complex with MST1, thus interfering with the MST1 inhibitory domain and protecting the autophosphorylation domain, thereby enhancing MST1 activation. As expected, in

the absence of PRDX1 or decreased oligomerization, MST1 shows decreased autophosphorylation levels and decreased activity (79).

PRDX1 binding does not just activate its partners, but can also lead to their inactivation or inhibition. JNK, or c-Jun N-terminal kinases, are a group of kinases originally identified as phosphorylating the c-Jun subunit of the transcription factor AP-1 but have been shown to phosphorylate a number of nuclear substrates, mostly transcription factors. Interestingly, in a study examining PI3K-driven tumorigenesis in PTEN loss of function mutants, it was found that the JNK signaling pathway was enriched as a downstream target of PI3K activity. However, while it had been previously established that constitutively active PI3K was sufficient to trigger JNK activation (84), upregulation of JNK activity was found to be independent of AKT activation, suggesting that the JNK and AKT pathways, while both regulated PI3K and PTEN, work in parallel to one another instead of linearly to promote tumorigenesis (85). One common inhibitory mechanism of JNK is the formation of a complex between the C-terminal domains of phosphorylated glutathione S-transferase P1 (GSP1) and JNK, preventing JNK from phosphorylating its downstream targets. The dissociation of this GSTP1-JNK complex via stress stimuli (gamma irradiation, heat shock, oxidative stress, etc.) restores JNK activity (86). It's been shown, however, that PRDX1 is able to bind to the GSTP1-JNK complex, preventing the release of JNK, insuring JNKs further inhibition, and suppressing JNKs activity, even following a stress stimulus like irradiation. Interestingly, when the peroxidatic cysteine was overoxidized or mutated into a serine, and therefore catalytically inactive, PRDX1 was still able to bind to the GSTP1-JNK complex. This finding suggests that PRDX1's JNK inhibitory function is independent of its antioxidant activity (80).

As mentioned above, PRDX1 displays two functions, one as an antioxidant, neutralizing hydrogen peroxide to prevent the damaging effects of ROS build-up and the other one as a key signaling molecule that is capable of using its oxidation by hydrogen peroxide as a secondary messenger. With both functions, PRDX1 regulates the activity of many cellular processes, activating and inhibiting numerous biological pathways, and even playing a role in several diseases and dysfunctions.

1.3.3 PRDX1 and cancer

As discussed in the previous section, PRDX1 is involved in the regulation of many cell signaling pathways and biological processes, and as a result of this involvement, plays a role in the development and progression of many diseases, the most paramount of which is cancer. In a study in which PRDX1 was knocked out in mice, it was found that the mice lacking PRDX1 (*Prdx1*$^{-/-}$) had significantly decreased lifespans as compared to mice containing one or both copies of PRDX1 (WT and *Prdx1*$^{+/-}$). In addition, the mice lacking at least one copy of PRDX1 developed, and eventually succumbed to severe hemolytic anemia and several types of malignancies, including pancreatic cancer, lung cancer, and breast cancer (87). Similar findings were found in a *Caenorhabditis elegans* model, in which *C. elegans* lacking cytosolic 2-Cys PRDXs were short-lived and showed signs of accelerated aging (88). Taken together, these findings suggest that PRDX1 possesses some tumor preventive functions.

Many have investigated PRDX1's tumor preventative function, and found that it is conferred through the regulation of signaling molecules involved in pathways responsible for cell death and apoptosis, cell cycle arrest, and cell growth and cell proliferation. PRDX1 protects the tumor suppressor PTEN from oxidative stress induced degradation. As mentioned before, by

binding to and protecting PTEN, PRDX1 thereby enables PTEN to regulate AKT levels, and maintain a proper balance of cell death and proliferation, and inhibiting tumorigenesis (78). Acting as an intermediary for the critical tumor suppressor p53, PRDX1 oligomers bind to MST1, activating the kinase which then goes on to regulate apoptotic pathways (79). Shown in lung cancer, PRDX1's binding to the GSTP1-JNK complex, preventing the release of the kinase JNK, inhibits JNK activity (80).

While for the most part PRDX1 is considered to be a tumor preventer, aiding in the regulation of many tumorigenesis inhibitory pathways, the antioxidant has been shown to play a tumor supportive role as well. When looking at its relationship with the mitogen-activated protein kinase (MAPK) p38α, PRDX1 acts as a redox specific sensor in cellular senescence. As demonstrated in breast cancer, PRDX1 differentially associates with and regulates the MAPK phosphatases MKP-1 and MKP-5, depending on its oxidation state. Both MKP-1 and MKP-5 dephosphorylate and deactivate the senescence inducing p38α. These MKPs have a low pKa catalytic cysteine residue within a conserved active site, and as a result, at physiologically normal pH levels this cysteine is susceptible to oxidation. Oxidation at the catalytic cysteine leads to loss of the protein's phosphatase activity, and the formation of a disulfide-based oligomeric structure (89,90). However, PRDX1 binding does depend on concentration of H_2O_2, and oxidation state of PRDX1 C52. Under H_2O_2-indused stress, PRDX1 dissociates from MKP-1, which then allows for the oligomerization of MKP-1, and the loss of its phosphatase activity towards p38α. In contrast to MKP-1, under high concentrations of H_2O_2, PRDX1 binding to MPK-5 was promoted, protecting MKP-5 from oxidation-induced inactivation, and actually promoting MKP-5's phosphatase activity towards p38a. Over oxidation of PRDX1 C52 was also found to be enhanced in the PRDX1 – MKP-5 complex. Taken together, these findings suggest

that PRDX1 fine tunes MPK/p38a signaling in a dose-dependent manner by way of C52's oxidation status (91). Interestingly, in pancreatic cancer, PRDX1 was found to co-localize with phosphorylated (active) p38α, and form complexes at the leading edge of migrating pancreatic cancer cells, suggesting PRDX1 plays a role promoting tumor cell invasion though an interaction with p38α (92).

PRDX1's role in tumorigenesis, both inhibitory and protective, is complex. It is only by investigating its functions beyond that of simply mitigating ROS induced stress, by studying the different processes in which PRDX1 is involved, identifying its binding partners, and determining the various mechanism by which it acts, that we may gain a deeper understanding of full extent of PRDX1's biological role. Recently, the transcription factor known to have a role in cancer, FOXO3 was identify through mass spectroscopy as a possible PRDX1 binding partner (93).

1.4 FOXO3: AN ESSENTIAL TUMOR SUPPRESSOR

First identified in *C. elegans*, the Forkhead box (FOX) transcription factors are superfamily of transcription factors which contain an evolutionarily conserved DNA binding domain (DBD) known as the forkhead box or winged helix domain (WHD). Variations in sequence outside of the DNA-binding domain between the smaller FOX subfamilies (FOXA-R), lead to significant differences in regulation, protein function, and distribution within a body (94).

The Forkhead Box, Class O (FOXO) subfamily consists of four proteins, FOXO1, FOXO3, FOXO4, and FOXO6, and contains a conserved GDSNS amino acid sequence inserted within the DNA-binding domain, which is missing in the other FOX proteins (95). In addition to

the unique DNA-binding domain, FOXO proteins also contain three other conserved domains; a nuclear localization signal (NLS), a nuclear export sequence (NES), and the transactivation domain (TA) (Figure 1.7) (96). While all FOXOs are expressed at basal levels in most tissues, the different isoforms can be tissue specific. FOXO1 is more highly expressed in the ovaries and fat tissue, FOXO3 is found at high levels in skeletal muscle, and FOXO6 can be found in the hippocampus and amygdala, suggesting it may play an important role in cognitive function (97,98).

1.4.1 DNA-binding domain structure

The evolutionarily conserved DNA-binding domain contains three α–helices (H1-3), three β-sheets (S1-3), and two loops known as 'wings' (W1-2) which protrude from and connect β-sheets. The N-terminal domain is formed by H1-S1-H2-H3, while the C-terminal domain is formed by S2-W1-S3-W2 (99). Conserved amino acid residues (N208, R221, H212, and S215) within H3 of the N-terminal domain, recognize the FOXO consensus binding domain (GTAAA(C/T)A) in target genes, and interact with the major groove of the DNA through hydrogen bonds and van der Waals forces (100). The two β-sheets (S2 and S3) of the C-terminal domain interact with the minor groove, while the two wings interact with the phosphate backbone of the target DNA, increasing binding stability (101).

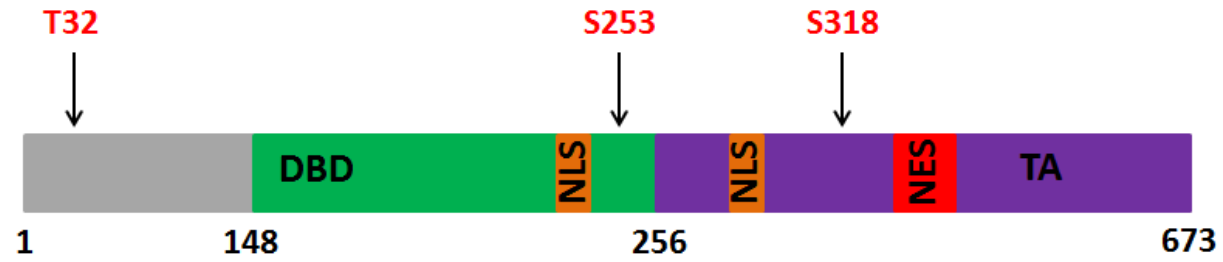

Figure 1.7: FOXO3 as example of FOXO domain structure.
DBD: DNA binding domain; TA: Transactivation Domain; NLS: Nuclear Localization Signal; NES: Nuclear Export Signal. AKT phosphorylation sites (T32, S253, and S318) shown in red.

1.4.2 Post-translational modification

FOXO activity is highly regulated through post-translational modifications (PTM) such as phosphorylation, acetylation, and ubiquitination (102).

Phosphorylation by the kinase AKT is a key negative regulator of FOXOs. AKT phosphorylates FOXO at specific serine or threonine residues (T32, S253, and S315 on FOXO3), which generates a binding site for the chaperone protein 14-3-3 (103). 14-3-3 binding to a FOXO protein phosphorylated at any one of the three AKT sites obscures the nuclear localization signal, thereby preventing nuclear entry or re-entry. 14-3-3 binding also results in a conformational change that exposes the nuclear export sequence, so that any FOXO present in the nucleus is subsequently expelled (Figure 1.8). As an end result, AKT phosphorylation of FOXO proteins, and the subsequent binding of 14-3-3, sequesters the transcription factor in the cytoplasm, inhibiting transcriptional activity (104). Phosphorylation by AKT can also trigger other subsequent PTMs discussed later.

Phosphorylation can also lead to nuclear import and accumulation of FOXO proteins, some examples of which are by the kinases MST1, JNK, and p38α in FOXO3 (82,105,106). Phosphorylation by these kinases results in a disruption of 14-3-3 binding to FOXO3 in the cytoplasm, allowing it to accumulate in the nucleus and bind to its protein targets (99). MST1 phosphorylates at S209, JNK at S574, and p38α at S7. Interestingly, as was discussed previously, PRDX1 has been shown to regulate the activities of all three of these kinases, suggesting there are multiple layers to PRDX1's control of FOXO3.

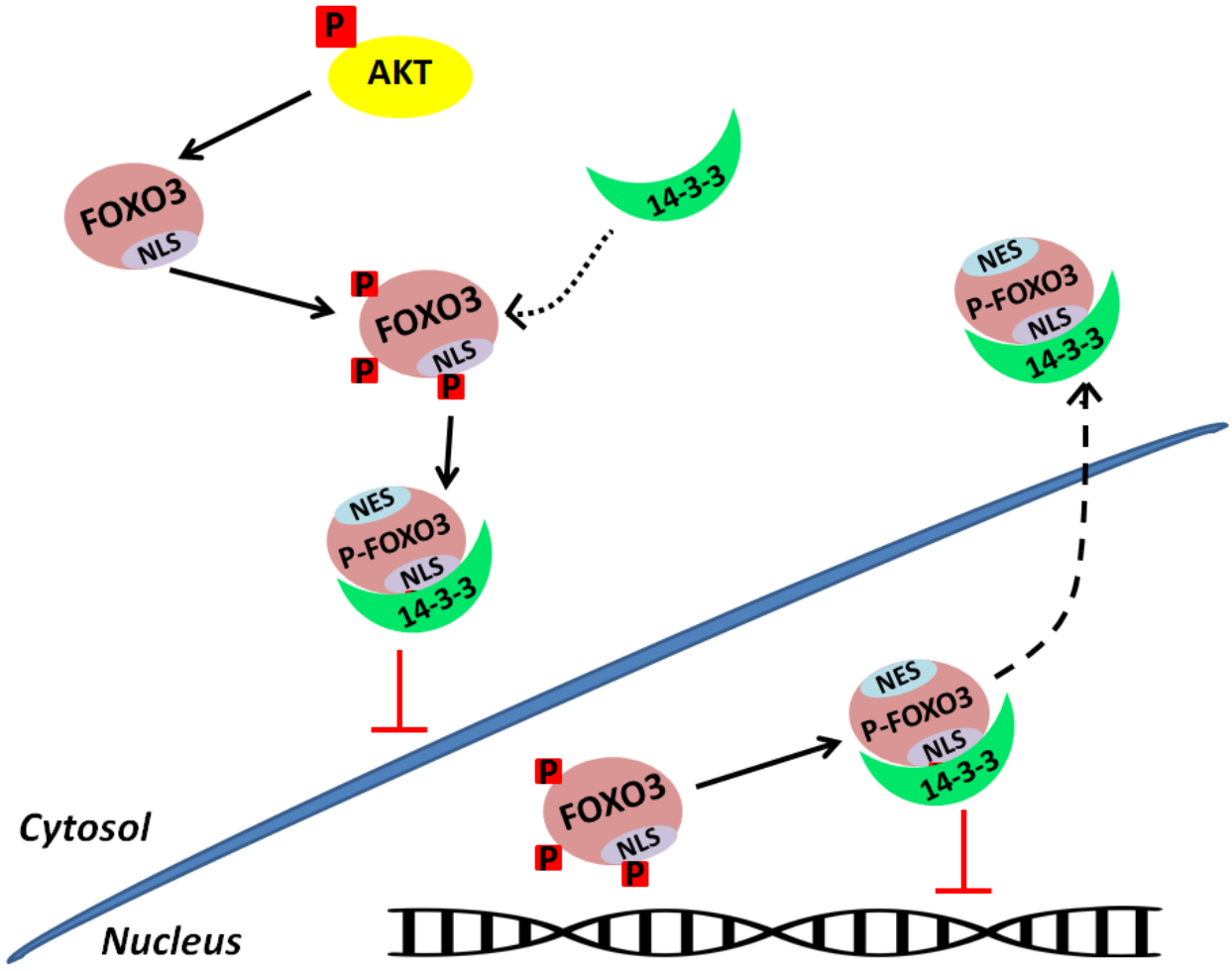

Figure 1.8: 14-3-3 binding regulates FOXO3 localization.
AKT phosphorylates FOXO3 on three sites, including one proximal to the nuclear localization sequence (NLS). 14-3-3 can bind to phosphorylated FOXO3, covering the NLS, thus preventing nuclear translocation. 14-3-3 binding triggers a conformational change, exposing the nuclear export signal (NES) so that if FOXO3 is phosphorylated within the nucleus, binding triggers nuclear expulsion. Binding of 14-3-3 also prevents the DNA binding domain from interacting with target DNA thereby blocking transcriptional activity.

Other PTMs exist which also influence FOXO activity. One example of a modification is the susceptibility of lysine residues in FOXO proteins (K242 and K245 in FOXO3) to acetylation by cAMP-response element binding (CREB)-binding protein (CBP) (96). The acetylation of these residues decreases the affinity of FOXO for the consensus sequence found in target DNA, thereby, leading to the FOXO-DNA complex to become less stable. It has also been proposed that acetylation at these lysines leads to an increase in phosphorylation at the AKT phosphorylation site, S253, found nearby (101). Interestingly, it has been shown in FOXO4 that acetylation of the lysine residues by CBP is mediated through a redox sensitive disulfide bridge between cysteines on FOXO4 and CBP. Under H_2O_2 induced stress, the bridge between FOXO4 and CBP forms, allowing CBP to acetylate FOXO4's lysines. TRX can then come in, reduce the cystine disulfide through an intra-disulfide-thiol exchange, thereby releasing the acetylated FOXO4 molecule (107). This finding suggests redox and cysteine oxidation could play a role in other forms of FOXO regulation.

Finally, FOXO proteins can be regulated by mono- and polyubiquitination, which can both increase and decrease transcriptional activity (96). In the case of FOXO4, monoubiquitination increases activity. Certain lysine residues found in the nuclear localization signal (K199 and K211) can be monoubiquitinated under oxidative stress conditions. This monoubiquitination interferes with and disrupts the acetylation of other nearby lysines, which would otherwise lead to a decrease in nuclear localization and decreased activity (108). In FOXO1 and FOXO3, polyubiquitination at key lysine residues such as K63, in conjunction with phosphorylation, can promote proteasomal degradation. Phosphorylation by AKT triggers the binding of the FOXO1 or FOXO3 to MDM2, an E3 ubiquitin ligase, which then promotes the polyubiquitination and eventual degradation of the FOXO protein (109). Independent of AKT, I

kappa B kinase b (IKKb) has been shown to phosphorylate FOXO3 at S644 which leads to the polyubiquitination of K63 by an E2-E3 ubiquitin ligase complex composed of Rad6 (E2) and Bre1 (E3) (110,111).

1.4.3 FOXO3 function

Like the other members of the FOXO family, FOXO3 acts as a tumor suppressor, regulating the transcription of gene targets which play integral roles in a number of cellular pathways such as apoptosis, cell cycle arrest, and resistance to oxidative stress (Table 1.3). (96).

FOXO3 activates the expression of a number of pro-apoptotic proteins, such as FasL and BIM. FOXO3 binding to the FOXO consensus sequence in the Fas receptor ligand (FasL) promoter leads to the upregulation of FasL, a transmembrane protein which when bound to the Fas receptor, activates the Fas-dependent cell death pathway through the activation of caspase 8 (112-114). FOXO3 has also been shown to upregulate the expression of Bcl-2-like protein 11 (BIM), a member of the pro-apoptotic Bcl-2 family. Once transcribed, BIM forms either homo- or heterodimers with other Bcl-2 proteins. The dimer then releases cytochrome c into the cytoplasm, and apoptotic protease activating factor-1 (Apaf-1) is in turn activated, forming an oligomeric apoptosome, leading to apoptosis (112).

FOXO3 is also known to regulate the cell cycle, specifically to induce the expression of cell cycle inhibitors, an example of which is the cyclin-dependent kinase (CDK) inhibitor, p27kip1 (p27) (115). p27 functions by binding to either the cyclinE-CDK2 or the cyclinD-CDK4 complex, preventing the complex's function and leading to its ultimate inactivation. When either complex is inactivated, cell cycle arrest is induced at the G0 to G1 phase (116). It's also been

Table 1.3: FOXO3 target genes.

Gene (Protein)	Function	Reference
BCL2L11 (BIM)	Apoptosis	Gilley *et al.* 2003 (117)
FASLG (FasL)	Apoptosis	Brunet *et al.* 1999 (118)
TNFSF10 (TRAIL)	Apoptosis	Modur *et al.* 2002 (119)
BBC3 (PUMA)	Apoptosis	Ekoff *et al.* 2007 (120)
PMAIP1 (NOXA)	Apoptosis	Obexer *et al.* 2007 (121)
BCL6 (BCL6)	Apoptosis	Fernández de Mattos *et al.* 2004 (122)
NOLC1 (P130)	Cell Cycle Control	Kops *et al.* 2002 (123)
CDKN1A (p21)	Cell Cycle Control	Seoane *et al.* 2004 (124)
CDKN1B (p27)	Cell Cycle Control	Medema *et al.* 2000 (125)
CCND1 (Cyclin D1)	Cell Cycle Control	Ramaswamy *et al.* 2002 (126)
CCND2 (Cyclin D2)	Cell Cycle Control	Ramaswamy *et al.* 2002 (126)
CCNG2 (Cyclin G2)	Cell Cycle Control	Martínez-Gac *et al.* 2004 (127)
GADD45 (GADD45)	DNA Repair	Furukawa-Hibi *et al.* 2002 (128)
DDB1 (DDB1)	DNA Repair	Ramaswamy *et al.* 2002 (126)
SOD2 (MnSOD)	Oxidative Stress	Kops *et al.* 2002 (129)
CAT (Catalase)	Oxidative Stress	Nemoto and Finkel. 2002 (130)
TXNIP (TXNIP)	Oxidative Stress	Papadia *et al.* 2008 (131)
SESN3 (Sestrin 3)	Oxidative Stress	Nogueira *et al.* 2008 (132)
IGFBP1 (IBP-1)	Cell Growth	Yang *et al.* 2016 (133)
GOLPH3 (Golph3)	Cytoskeletal Adhesion	Eijkelenboom *et al.* 2013 (134)
CIDEA (CIDEA)	Milk Production	Pelosi *et al.* 2013 (135)
FOXC2 (FOXC2)	Transcription Factor	Pelosi *et al.* 2013 (135)
GALT (GALT)	Metabolism	Halperin *et al.* 2007 (136)

found that when FOXO3 is sequestered in the cytoplasm, rendering it transcriptionally inactive, there is a corresponding decrease in the expression of cyclin D, which too leads to an arrest of cell cycle progression, adding another layer of regulation by FOXO3 (137).

Lastly, FOXO3 has also been shown to upregulate targets associated with oxidative stress resistance, including manganese superoxide dismutase 2 (MnSOD2) and catalase (129,130). As a reminder, MnSOD2 like SOD, catalyzes the reaction that converts superoxide ions (O_2^-) into hydrogen peroxide (H_2O_2) and oxygen (O_2). Catalase then catalyzes the further reaction of hydrogen peroxide into water and molecular oxygen. This upregulation of ROS detoxifying gene targets by FOXO3 serves to protect the cell from oxidative stress induced damage.

1.4.4 FOXO3 and cancer

As stated before, FOXO3 is considered to be a tumor suppressor with the ability to regulate a number of cellular functions involved in tumorigenesis, including cell cycle progression and apoptosis. When sequestered in the cytoplasm, FOXO3 is rendered inactive, inhibiting the transcription of FOXO3's cell cycle inhibitor and pro-apoptotic gene targets. As a result of the inhibition of these targets, cells can turn malignant, giving rise to a number of different cancer types, including breast cancer (110).

Numerous studies have been published showing the protective role FOXO3 plays in decreasing and inhibiting breast cancer tumorigenesis. FOXO3 is known to inhibit estrogen-dependent cancer cell proliferation. Through interactions with ER-α and ER-β proteins, FOXO3 inhibits 17β-estradiol(E2)-dependent and ER-regulated transcriptional activities. When expressing FOXO3, ER-positive cells show decreased levels of expression of several ER-

regulated genes, including those involved in cell growth and proliferation. Over-expression of FOXO3 *in vivo* has also been shown to suppress E2-induced tumorigenesis (138). Investigations into patient survival have found that elevated levels of FOXO3 is significantly correlated with long-term patient survival, and that FOXO3 expression can be utilized as a favorable prognostic indicator of overall breast cancer patient survival (139).

When FOXO3 is inhibited, an opposite effect is observed, with tumorigenesis being promoted. As mentioned previously, S644 of FOXO3 can be phosphorylated by IKKb, leading to its polyubiquitination, and subsequent degradation. This decrease in FOXO3 nuclear activity, is shown to correlate with poor survival in breast cancer (110).

While there is very strong support for FOXO's role as a tumor suppressor, a converse relationship in which FOXO3 actually acts to promote tumor progression, has been observed. In one study, cancer cells undergoing serum starvation showed increased localization of FOXO3 to the nucleus, and a corresponding upregulation of the matrix metallopeptides (MMP) MMP-9 and MMP-13. When active, MMPs degrade the extracellular matrix (ECM), resulting in an increase in cell invasion. Increased cancer cell invasion was observed in the serum starved breast cancer cells (140). Building on this, it has been found that constitutively nuclear, and thereby constitutively active, FOXO3 can lead to poorer prognosis in breast cancer patients, and an enhance hyperactivation of the PI3K/AKT signaling pathway (141). More specifically, this sustained FOXO3 activity has been linked to increased lymph nodal metastasis in invasive ductal carcinoma (IDC) (142).

FOXO3 must be active within a certain range to be most effective in its role as a tumor suppressor, too little or too much activity and FOXO3 leads to cancer progression and poor prognosis. These contradictory roles FOXO3 plays in cancer development, both suppressing and

promoting tumorigenesis, highlight the need for a greater understanding of the mechanisms through which FOXO3 activity is regulated.

1.5 SIGNIFICANCE AND PUBLIC HEALTH RELEVANCE

Breast cancer is the most commonly occurring cancer in US women, with 1 in 8 women developing an invasive breast cancer in her lifetime. While there have been extraordinary steps made in the development and of targeted therapies, breast cancer is still the second leading cause of cancer related death in US woman (1). There are still thousands for whom current therapies do not work, or lose their efficiencies (143). A greater understanding of cancer development and progression is essential to finding new ways to identify and target the drivers of cancer development and progression. One such avenue down which to explore, is oxidative stress. Elevated levels of oxidative stress in a major risk factor in breast cancer, and it's been found that post-menopausal women, who are generally at increased risk for breast cancer development, also show increased markers for oxidative stress (144). Unfortunately, antioxidant therapies have proven ineffectual. If we can gain a deeper understand of how these oxidative stress induced redox-signaling pathways work, it is possible to develop more effective therapies for those at-risk patients for whom therapies fail.

In this dissertation, we define the mechanism by which oxidative stress and the antioxidant enzyme PRDX1 regulates the activity of the tumor suppressor FOXO3, and the effect such regulation has on FOXO3 downstream transcriptional targets. Such a mechanism is novel,

giving us a deeper understanding of transcriptional regulation through oxidative stress and redox signaling.

2.0 MATERIALS AND METHODS

*This chapter has been partially published in Antioxidants & Redox Signaling (145) and permission has been obtained from the journal regarding the copy-right. ARS correspondence: Karen Ballen (KBallen@liebertpub.com)

2.1 CELL CULTURE

HEK 293T (293T) and HeLa cells were obtained from ATCC. These cells were grown in DMEM (Mediatech) supplied with 10% heat-inactivated FBS (HyClone), 100 units/ml penicillin, 100 mg/ml streptomycin (Mediatech) and 2 mM l-glutamine (Mediatech) (complete DMEM) in a 37° C incubator supplied with 5% CO_2. *Prdx1*$^{+/+}$ and *Prdx1*$^{-/-}$ MEFs were generated as described in (78) from *Prdx1* knockout and parental mice (82) and grown in the same conditions as the 293T. Except when otherwise stated, chemicals used were obtained from Sigma.

2.2 PLASMIDS

pcDNA3-FLAG, pcDNA3-FLAG-HA, pcDNA3-FLAG-FOXO3 and pcDNA3-FLAG-HA-FOXO3 have previously been described (146). Site-directed mutagenesis of FOXO3 plasmids

were performed using Stratagene's QuickChange II XL kit (Agilent Technologies) following the manufacturer's guidelines. The oligonucleotides used were designed using the online QuickChange Primer design application (Agilent Technologies) and were synthesized from Integrated DNA Technologies. The pcDNA3-FLAG-HA-FOXO3 plasmid was used as a template to generate all five single C-to-S mutants. Each clone obtained was sequenced with four different sequencing primers to span, with overlaps, the entire coding sequence. To generate the double, quadruple and quintuple C-to-S mutants, newly synthesized single Cys-to-Ser mutants were used as templates for successive mutagenesis rounds with full-length sequencing after each round. To generate the Cys-to-Ser mutants of pcDNA3-FLAG-FOXO3, the inserts of the several C-to-S mutants of pcDNA3-FLAG-HA-FOXO3 were excised with a BamH1-Xho1 (New England Biolabs) restriction enzyme digestion and cloned into the pcDNA3-FLAG-FOXO3 plasmid, also digested with BamH1 and Xho1 to remove the un-mutated FOXO3 insert. shRNA for FOXO3 (NM_001455.x-2766s1c1 clone) was purchased from Sigma. shPRDX1 expression constructs were used as previously described (91).

2.3 FOXO3 – H_2O_2 DOSAGE

HEK 293T cells (5×10^5) were transiently transfected with 2 µg pcDNA3-FLAG-HA (EV) or pcDNA3-FLAG-FOXO3 plasmids, using the Fugene 6 system for 48 hours. Cells were serum starved for 30 min, then treated with 0, 25, 100, 250, or 500 µM H_2O_2 for 30 min. Samples were lysed using a tris lysis buffer (50mM Tris; 2% Triton X-100; 0.5 mM EDTA; 0.5 mM EGTA; 150 mM NaCl; 10% glycerol; 50 mM NaF; 1 mM $NaVO_4$; 40 mM β-glycerophosphate), supplemented with 30 µg/ml catalase from bovine liver (Sigma), and proteinase inhibitors.

Protein concentrations were quantified using the Pierce BCA Protein Assay kit, according to the manufacturer's instructions (Thermo). 1 mg of cell lysate was incubated with 20 µL of acid treated Anti-FLAG M2 Affinity Gel (Sigma) and 400 µL lysis buffer, at 25˚ C for 3 h, with rotation. Precipitated samples were collected and washed four times with lysis buffer, and once with 1x TBS. Beads were boiled in Laemmli sample buffer (BioRad) in the presence or absence of β-mercaptoethanol (Sigma) for 10 min. 20 µg of whole cell lysate input was prepared in Laemmli sample buffer as above for 5 min.

2.4 FOXO3 C-TO-S MUTANTS TRANSFECTION

HEK 293T cells (5 x 10^5) were transiently transfected with 2 µg EV, pcDNA3-FLAG-FOXO3, or pcDNA-FLAG-HA-FOXO3 constructs containing various C-to-S mutations, using the Fugene 6 system for 48 h. Cells were serum starved for 30 min, then treated with 0 or 25 µM H_2O_2. Samples were lysed using the tris lysis buffer detailed above, supplemented with 30 µg/ml catalase from bovine liver (Sigma), and proteinase inhibitors. Protein concentrations were quantified using the BCA protein assay (Thermo). IP of 1000 µg (or 1500 µg for nuclear localization) of cell lysate was processed as detailed above.

2.5 PRDX1 C-TO-S MUTANTS TRANSFECTIONS

HEK 293T cells (5 x 10^5) were co-transfected with pcDNA-FLAG-FOXO3 and pcDNA3-HA-PRDX1 or pcDNA3-HA-PRDX1 constructs containing various C-to-S mutations using the

Fugene 6 system for 48 h. Cells were serum starved for 30 min, then treated with 0 or 25 µM H_2O_2. IP samples were prepared as above with 1.5 mg of cell lysate was for relative protein quantification.

2.6 FOXO3-PRDX1 INTERACTION IN MEFS

Confluent *Prdx1*$^{+/+}$ and *Prdx1*$^{-/-}$ MEFs were serum starved for 30 min then treated with 0, 25, or 250 µM H_2O_2 for 30 min. Cells were lysed in the aforementioned tris lysis buffer and 80 µg of cell lysate was used for relative protein quantification.

2.7 WESTERN BLOTTING

Prepared IP samples and corresponding whole cell lysates were fractionated by SDS-PAGE and transferred to a nitrocellulose membrane according to manufacturer (BioRad). Membranes were blocked with 5% BSA in TBS for 30 min, and incubated with antibodies against FOXO3 (1:1000) (Abcam), P-FOXO3 T32 (1:1000) (Cell Signaling), PRDX1 (1:4000) (Abcam), PRDX-SO3 (1:500) (Abcam), 14-3-3 (1:1000) (Cell Signaling), or actin (1:1000) (Oncogene), overnight at 4 °C. Membranes were washed four times for 5 min in TBST (0.05% Tween-20), and visualized by IR or chemiluminescent detection. For IR processing, membranes were incubated with a 1:15000 dilution of anti-goat, anti-rabbit, or anti-mouse IRDye (LI-COR), for 30 min at 25˚ C. Blots were washed with TBST 3 times and with TBS once, and imaged on an Odyssey (LI-COR) imager. Membranes processed by chemiluminescence were incubated in a 1:10000

dilution of HRP-conjugated anti-mouse or anti-rabbit antibodies for 1 h at 25˚ C. Blots were washed four times with TBST for 5 min, and exposed to ECL for 1 min.

2.8 FOXO3 NUCLEAR LOCALIZATION IN 293T CELLS

Nuclear localization of 5.0 x 10^4 293T cells transiently transfected with Fugene 6 for 24 h with 50 ng EGFP-FOXO3 or FOXO3-EGFP C31S or C150S mutants, transferred to complete media for 24 h, serum starved for 1 h, then treated with 100 µM H_2O_2 or vehicle and 20 µM LY294002 or vehicle for 30 min, and fixed with 2% paraformaldehyde for 15 min was quantified by blinded scoring of triplicate images from three separate experiments using a IX83 microscope (Olympus).

2.9 FOXO LUCIFERASE ASSAY

FOXO signaling activity was quantified in mouse embryonic fibroblasts or HEK 293T cells utilizing the dual-luciferase Cignal FOXO Luciferase Reporter assay (Qiagen). 2.5 x10^5 MEFs were transiently co-transfected with 350 ng Cignal reporter plasmids, 0.5 µg of PRDX1 and FOXO3 or FOXO3 cysteine mutant plasmids and compared to FOXO3 plus vector control samples. Transfections were performed with Fugene 6 for 24 h. Luciferase activity was normalized to the internal Renilla control. The effect of oxidative stress on FOXO3 activity was quantified in 293T cells by transfection with 350 ng Cignal reporter plasmid, 50 ng FOXO3 or the FOXO cysteine mutants. 500 ng PRDX1 was transfected into the cells 8 h later and incubated

for an additional 14 h Luciferase activity was measured 1.5 h following 30 min 0 to 250 µM H_2O_2 treatment. Dual luciferase activity was measured in 1x passive lysis buffer using the manufacturer's protocol (Promega).

2.10 ENDOGENOUS TRANSCRIPT QUANTIFICATION IN 293T CELLS

HEK 293T cells (2.5×10^5) infected with pLKO.1 control or shPRDX1 plasmids were serum starved for 1 h then treated with 0 or 250 µM H_2O_2 for 16 h. RNA was isolated utilizing the GeneJET RNA Purification Kit (Thermo Scientific) and converted to cDNA with the qScript cDNA synthesis kit (Quanta Biosciences). SESN3, P27, BIM, SOD2, CAT and P21 transcripts were quantified by SYBR green real-time PCR (BioRad) relative to YWHAZ control using custom primers (Table 2.1) and accounting for PCR efficiency.

Table 2.1: qPCR primers for endogenous transcript quantification.

Primer Name	Sequence
SESN3Fw	AGAGAAGGAAGTTGTCCAAGCA
SESN3Rv	GTAAGAACACTGATGTCTAGCTGC
P21Fw	AGACCCCAGAAATAAAGGATGACA
P21Rv	ATACTCCCCACATAGCCCGT
BIMFw	GTGCAATGGCTTCCATGAGG
BIMRv	TCCAATACGCCGCAACTCTT
SOD2Fw	GAACCCAAAGGGGAGTTGCT
SOD2Rv	GAAACCAAGCCAACCCCAAC
CATFw	CTCCGGAACAACAGCCTTCT
CATRv	ATAGAATGCCCGCACCTGAG
P27Fw	GCAGCTTGCCCGAGTTCT
P27Rv	AGAAGAATCGTCGGTTGCAGG
YWHAZFw	ACTTTTGGTACATTGTGGCTTCAA
YWHAZRv	CCGCCAGGACAAACCAGTAT

2.11 QUANTITATIVE RT-QPCR

Cell lysates were generated with the Cells-to-CTkit (Life Technologies). RT enzyme, individual Taqman assays and all PCR enzymes, dyes and buffers were purchased from Life Technologies and used according to the manufacturer's suggestions. All qPCRs were run on an ABI 7900HT instrument.

2.12 CELL VIABILITY ASSAY

HEK 293T cells (5×10^5) were transiently transfected with 2 µg pcDNA3-FLAG-FOXO3 plasmids, using the Fugene 6 system for 24 h. Cells were then trypsinized, and then $2x10^4$ cells were plated into the wells of a 96-well assay plate. The next day, the cells were treated with 0, 250, or 500 µM H_2O_2 for 3 h. Viability, cytotoxicity, and apoptosis were then measured using the ApoTox-Glo Triplex Assay (Promega), following the manufacturer's protocol.

2.13 WOUND HEALING ASSAY

One million MDA-MB-453 cells were infected with a pLKO.1 shPRDX1 or control construct then transfected in a 12-well plate with 25 nM let-7 miRIDIAN microRNA hairpin inhibitor (GE Dharmacon) or miRIDIAN microRNA hairpin inhibitor negative control #1 (GE Dharmacon) and 25 nM siGLO Green Transfection Indicator (GE Dharmacon) utilizing 5 µl DharmaFECT 2

transfection reagent (GE Dharmacon) per well. Confluent cultures were scratched with a 10 µl pipette tip in the presence of mitomycin C and photographed after 48 h to measure wound healing. Wound area was quantified with the Image J plugin MRI Wound Healing Tool (Volker Bäcker). let-7b expression was used as an indicator of let-7 inhibition. The relative wound area was compared in PRDX1-deficient cells and control cells treated with the let-7 miRIDIAN inhibitor normalized to control microRNA hairpin inhibitor. Average values were compared from 4 separate experiments + SEM.

2.14 CHIP ASSAY

293T cells were seeded (4 x 10^6 cells/dish 150mm) and 24 h later transfected with EGFP-FOXO3 plasmid (14 µg/dish) by Calcium Phosphate method. 48 h after transfection complete medium was replaced with serum-free medium for 30 min, followed by H_2O_2 (100 µM) treatment for 30 min. Cells were cross-linked with 1 % formaldehyde for 10 min at 25˚ C, then the reaction was stopped by addition of glycine to a final concentration of 0.125 M. Cross-linked chromatin was immunoprecipitated with 1 µg of FOXO3 antibody (ab12162 Abcam) as previously described (147). The genomic regions in the host gene and intronic promoter, close to FOXO3 binding sites, were amplified with primers designed by the Primer-Blast NCBI software (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). The following primer sequences were used: MIR99HG oligo#1 FW: 5'-CTATGCGCCACTCTGTGCAA-3'; MIR99HG oligo#1 RV: 5'-CTAATTACCGCGCACAAGCTG-3'; let-7c intronic prom oligo #2 FW: 5'GGCATAAACCCGTAGATCCG-3'; let-7c intronic prom oligo #2 RV: 5'-GAGCTTGTGCGGTCCACTT-3. Quantification of cDNA was performed in triplicate on an

Applied Biosystems 7500 Real-Time PCR System SDS v1,2, using the SYBR green dye detection method. ChIP assay results were evaluated by the double delta C_T method.

3.0 A PEROXIDASE PEROXIREDOXIN 1-SPECIFIC REDOX REGULATION OF THE NOVEL FOXO3 MIRORNA TARGET LET-7

*This chapter has been partially published in Antioxidants & Redox Signaling (145) and permission has been obtained from the journal regarding the copy-right. ARS correspondence: Karen Ballen (KBallen@liebertpub.com)

3.1 INTRODUCTION

Appropriate cell responses to stress are necessary to decide cell fate. Recent evidence establishes protein sulfhydryl groups as modulators of signaling events through oxidation state changes, which impact protein interactions and activity (148). This post-translational calibration requires a dynamic reversibility of the protein modification. Interestingly, in contrast to (de)/phosphorylation events induced by the interplay of protein kinases and phosphatases, protein sulfhydryl group oxidation to highly oxidized forms such as sulfonic acid can be irreversible, resulting in protein degradation or in the case of peroxiredoxins, a change of function. Peroxiredoxin (PRDX) family members (typical 2-Cys: PRDX1-4, atypical 2-Cys: PRDX5 and 1-Cys: PRDX6) are antioxidant enzymes that reduce peroxides via catalytic cysteine oxidation to sulfenic acid. Evidence is accumulating that demonstrates 2-Cys PRDXs as important redox sensors in signaling (68) through two unique features: a) a highly reactive

catalytic cysteine that converts to a protein sulfenic acid moiety that produces a disulfide bond with a resolving cysteine, which can be reduced via thioredoxin to reset catalytic function (66); and b) during recycling the catalytic cysteine of PRDX can be further oxidized, which promotes formation of PRDX decamers that display chaperone functionality, but lack peroxidase activity (68). These features equip PRDX1 to sense and react to changes in redox signaling accordingly by controlling protein-binding partners. For example, we have recently shown heightened oxidative stress can over-oxidize PRDX1, which causes PRDX1 to dissociate from MKP1 as well as increase association and activity of MKP5 thereby regulating senescence (91). A similar mechanism has been described for PRDX1 and PTEN (78) or MST1 (79) and for PRDX2 and ERp46 (149). Recently, PRDXs have gained attention to act as redox-relays involving the catalytic and resolving cysteine to form disulfides with the partnering protein to transfer oxidative equivalents. This has been suggested for PRDX1 and ASK1 (150,151) and most recently, a transient PRDX2-STAT3 redox-relay has been described that resulted in STAT3 oligomerization and inactivation (152).

Our studies shown here demonstrate that PRDX1 interacts with the transcription factor FOXO3 through disulfide bonds. The mammalian forkhead box transcription factors of the O class (FOXOs) comprises of four family members (FOXO1, 3, 4 and 6) that are highly related tumor suppressors that provide resistance to oxidative stress, halt cell cycle progression, and control the induction of cellular apoptosis. Although FOXOs serve as major cellular ROS arbitrators (153), the mechanisms by which FOXOs sense and integrate ROS signals to define transcriptional outcomes are still poorly understood (154). A recent mass spectrometry analysis indicated FOXO cysteines to be involved in protein binding underscoring its role for redox-sensing (93). In response to oxidative stress, FOXO proteins translocate to the nucleus due to

phosphorylation by mammalian Sterile 20-like kinase 1 (MST1) and Jun N-terminal kinase (JNK) as well monoubiquitination (96). FOXO is negatively regulated through the phosphoinositide 3-kinase (PI3K)/AKT signaling pathway, which promotes cytoplasmic sequestration of FOXO via AKT-induced phosphorylation and therefore causing FOXO-inactivation in many cancers (96,155).

As cancer cells carry a higher pro-oxidant burden compared to normal cells (156), we examined the role of PRDX1 in FOXO3 function and binding under pro-oxidant conditions. We establish that PRDX1 binds FOXO3 under H_2O_2 stress and regulates FOXO3 nuclear localization and activity through disulfide bridges involving the PRDX1 peroxidatic C52 and resolving C173 and surprisingly C71, which has not been previously seen. Within FOXO3, PRDX1-binding engages C31 and 150, the latter of which is not conserved among FOXO family members. Mutation of these FOXO3 cysteines cause changes to the phosphorylation levels of AKT substrate sites and heightened cytoplasmic localization that is responsive to PI3K inhibition in comparison to FOXO3WT. We also demonstrate for the first time that let-7c, a member of the let-7 family of microRNAs (miRNA) first identified in the nematode *C. elegans* as regulators of development, and widely regarded as a tumor suppressor miRNA, is a novel FOXO3 target. let-7c and let-7b miRNAs are significantly increased by H_2O_2 exposure in a FOXO3 and PRDX1-dependent manner, and inhibit breast cancer cell migration. Notably, several functional and mechanistic parallels exist for FOXO proteins and let-7. Both mediate tumor suppression and glucose homeostasis (157) and expression of the let-7 miRNA family has similarly been shown to be governed by various ROS-inducing stressors (158,159). Importantly, loss of let-7c expression in cancer cells promotes migration and invasion (160,161). Taken together, these data

provide compelling evidence for an existence of a redox-specific signaling axis comprised of PRDX1, FOXO3 and let-7c miRNAs in regulating oxidative stress signaling in breast cancer.

3.2 RESULTS

3.2.1 PRDX1 interacts with FOXO3 and regulates its nuclear function

We confirmed a PRDX1 FOXO3 interaction by precipitating endogenous FOXO3 bound to PRDX1 by immunoblot (Figure 3.1). Precipitation of FLAG-FOXO3 from transfected 293T cells indicated PRDX1 binding was elevated following treatment with 25 to 500 μM H_2O_2 in the presence of lysis buffer containing N-ethylmaleimide (NEM), a chemical agent that alkylates the thiol group on cysteines, therefore reducing post-lysis oxidation events that might otherwise occur (Figure 3.2). Interestingly, the PRDX1-FOXO3 complex displayed time-dependent binding dynamics when transfected 293T cells were treated with 250 and 500 μM H_2O_2 (Figure 3.3). Maximal PRDX1-FOXO3 oligomerization was seen at 30 min, which returned to baseline after 3 hrs. An assay to measure apoptosis, cell viability, and cytotoxicity found no significant changes in any of the three measurements, between treatment and no treatment over a period of 3 h, suggesting that the decrease in PRDX1-FOXO3 binding was not a result of cell death (Figure 3.4). Further experiments in the absence of NEM found that binding dynamics of FLAG-FOXO3 and PRDX1 followed a U-shaped H_2O_2 dose curve with maximal binding at 100 μM H_2O_2 and complex dissociation at higher concentrations (Figure 3.5). In addition, we could not detect any over-oxidized PRDX1 binding to FOXO3, suggesting that PRDX1 over-oxidation on its catalytic cysteine may induce FOXO3 release (Figure 3.5).

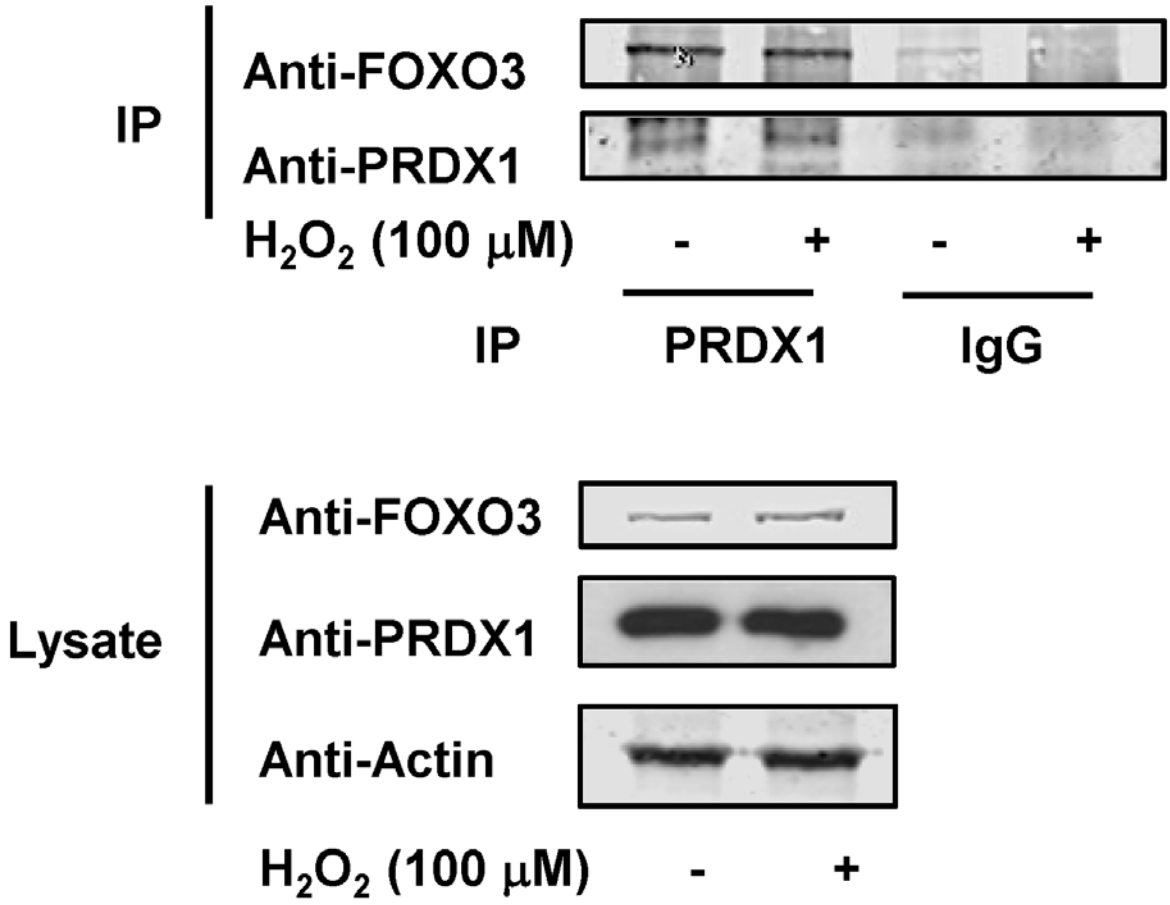

Figure 3.1: PRDX1 endogenously binds to FOXO3.
Immunoprecipitation of pre-cleared lysate with PRDX1 or IgG antibodies found PRDX1 bound-FOXO3. 293T cells underwent serum starvation for 30 min and were then treated with the 0 or 100 μM H_2O_2 for an additional 30 min.

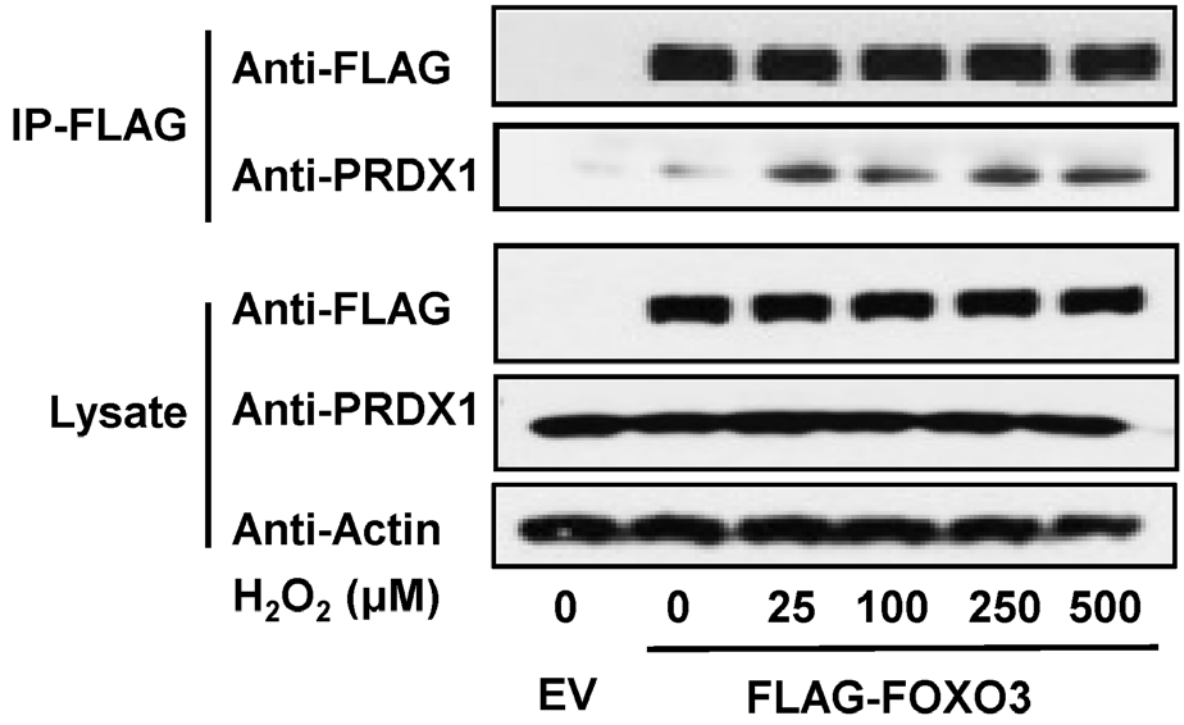

Figure 3.2: PRDX1 binds to FOXO3 in presence of H_2O_2.
293T cells were transfected with pcDNA3-FLAG-FOXO3A or EV and treated with increasing concentrations of H_2O_2 for the indicated times. Prior to lysis, cells were washed with 20 μM N-ethylmaleimide (NEM) in PBS to block lysis-induced disulfide bond formation. FLAG-labelled proteins were immunoprecipitated and detected by immunoblot with FLAG and PRDX1 antibodies.

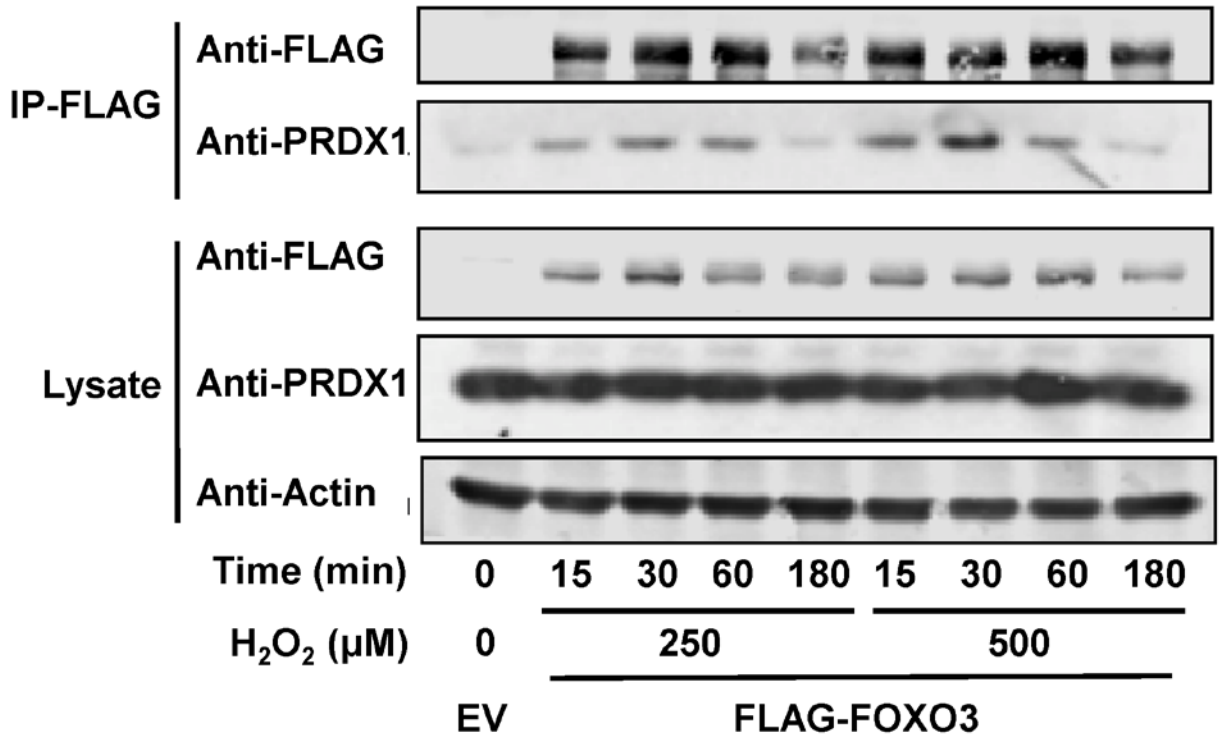

Figure 3.3: PRDX1 binds to FOXO3 in H_2O_2 time dependent manner.
293T cells were transfected with pcDNA3-FLAG-FOXO3A or EV and treated with the indicated concentrations of H_2O_2 for increasing periods of time. Prior to lysis, cells were washed with 20 μM N-ethylmaleimide (NEM) in PBS to block lysis-induced disulfide bond formation. FLAG-labelled proteins were immunoprecipitated and detected by immunoblot with FLAG and PRDX1 antibodies.

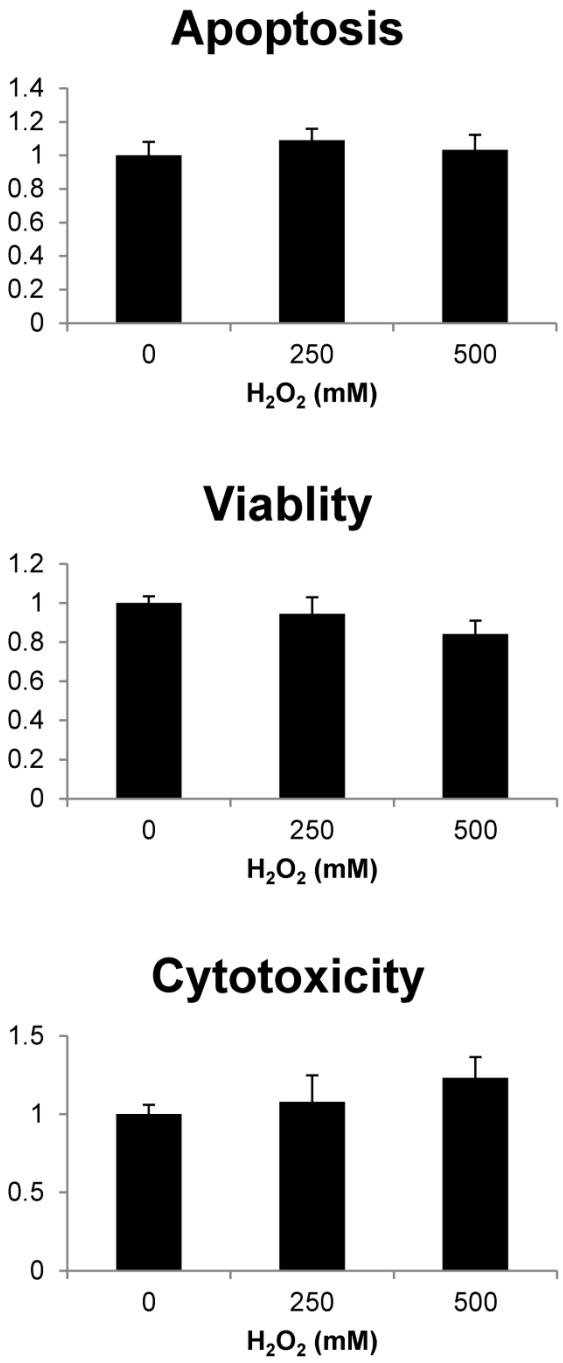

Figure 3.4: Changes in PRDX1-FOXO3 binding are not a result of cell death.
293T cells were transfected with FLAG-FOXO3 DNA. Cells were treated with H_2O_2 as indicated for 3 h. Apoptosis, cell viability, and cell cytotoxicity were measured using the Promega ApoTox-Glo Triplex Assay. Values normalized to the 0 μM H_2O_2 samples. (N=3).

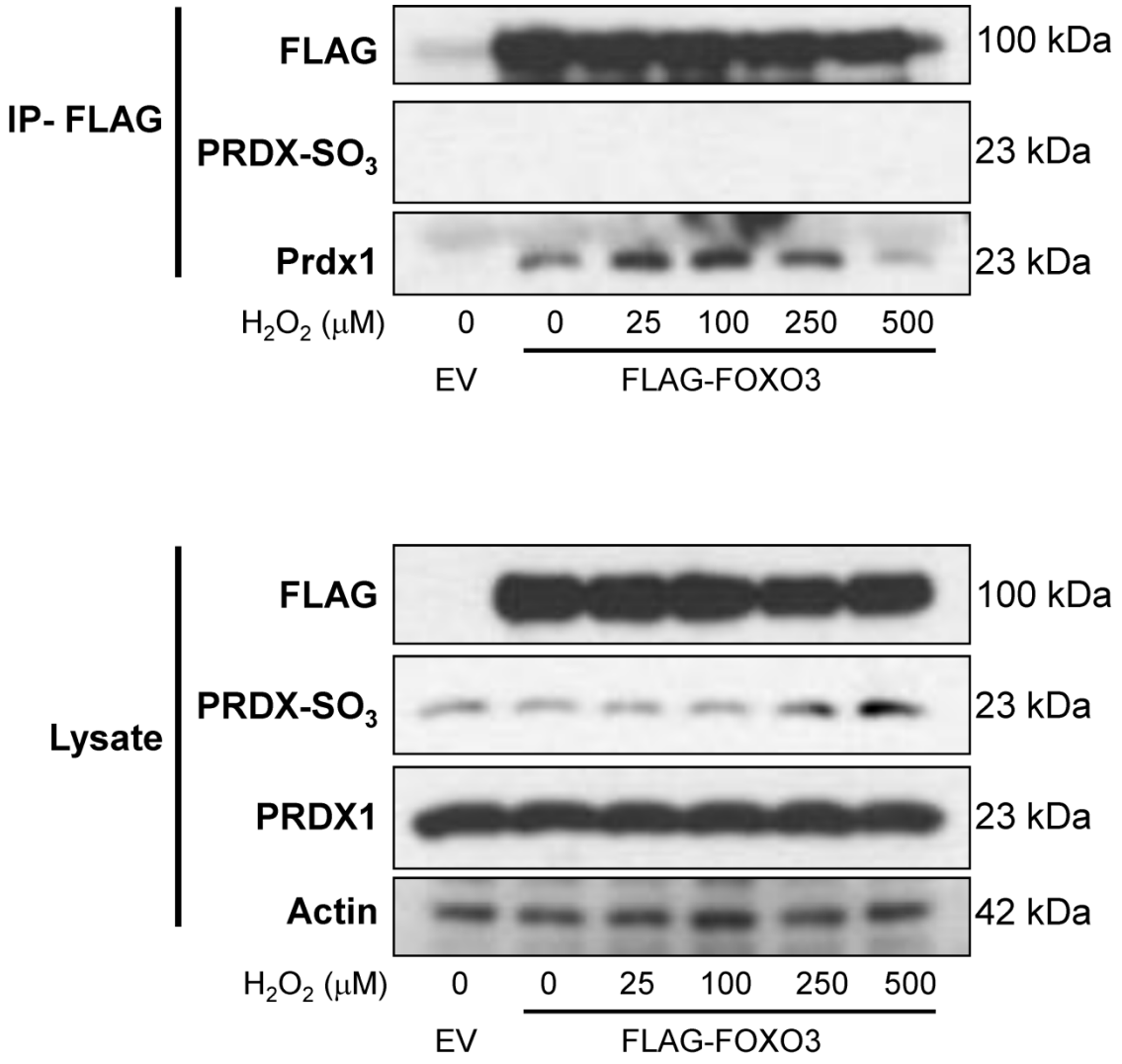

Figure 3.5: Over-oxidation of PRDX1 leads to FOXO3 release.
293T cells were co-transfected with FLAG-FOXO3. Before lysis, cells were treated with H_2O_2 as indicated for 30min. Cells were lysed with lysis buffer NOT containing NEM. Co-IPs were performed using anti-FLAG coated beads and Co-IPs were resolved under reducing conditions, followed by immunoblot with FOXO3, PRDX1, and PRDX1-4 SO3 antibodies. **(**EV=control vector only).

To investigate if PRDX1 binding to FOXO3 has a functional impact, we assessed FOXO3 nuclear translocation in *Prdx1*$^{-/-}$ and *Prdx1*$^{+/+}$ MEFs. Interestingly, MEFs lacking PRDX1 displayed primarily nuclear FOXO3 localization in contrast to wild-type MEFs expressing PRDX1 (Figure 3.6). Given the low transfection efficiency of MEFs, we next utilized 293T cells with reduced PRDX1 expression transfected with a FOXO3-EGFP reporter construct (Figure 3.7). As Figure 3.8 shows, FOXO3 nuclear localization was significantly increased in 293T cells harboring 90% less PRDX1, suggesting PRDX1 deterred FOXO3 nuclear translocation. Investigating the role of PRDX1 on FOXO3 function further, we next evaluated FOXO3 activity specifically using a dual-luciferase reporter assay in *Prdx1*$^{-/-}$ MEFs transiently transfected with FOXO3 and/or PRDX1. As expected, PRDX1 decreased luciferase signals by 50% (Figure 3.9). Investigating FOXO3 target gene expression by qPCR showed that PRDX1 knockdown increased expression of several FOXO3 targets in H_2O_2 treated 293T cells compared to control, where SESN3 and P27 differences were significantly enhanced (Figure 3.10).

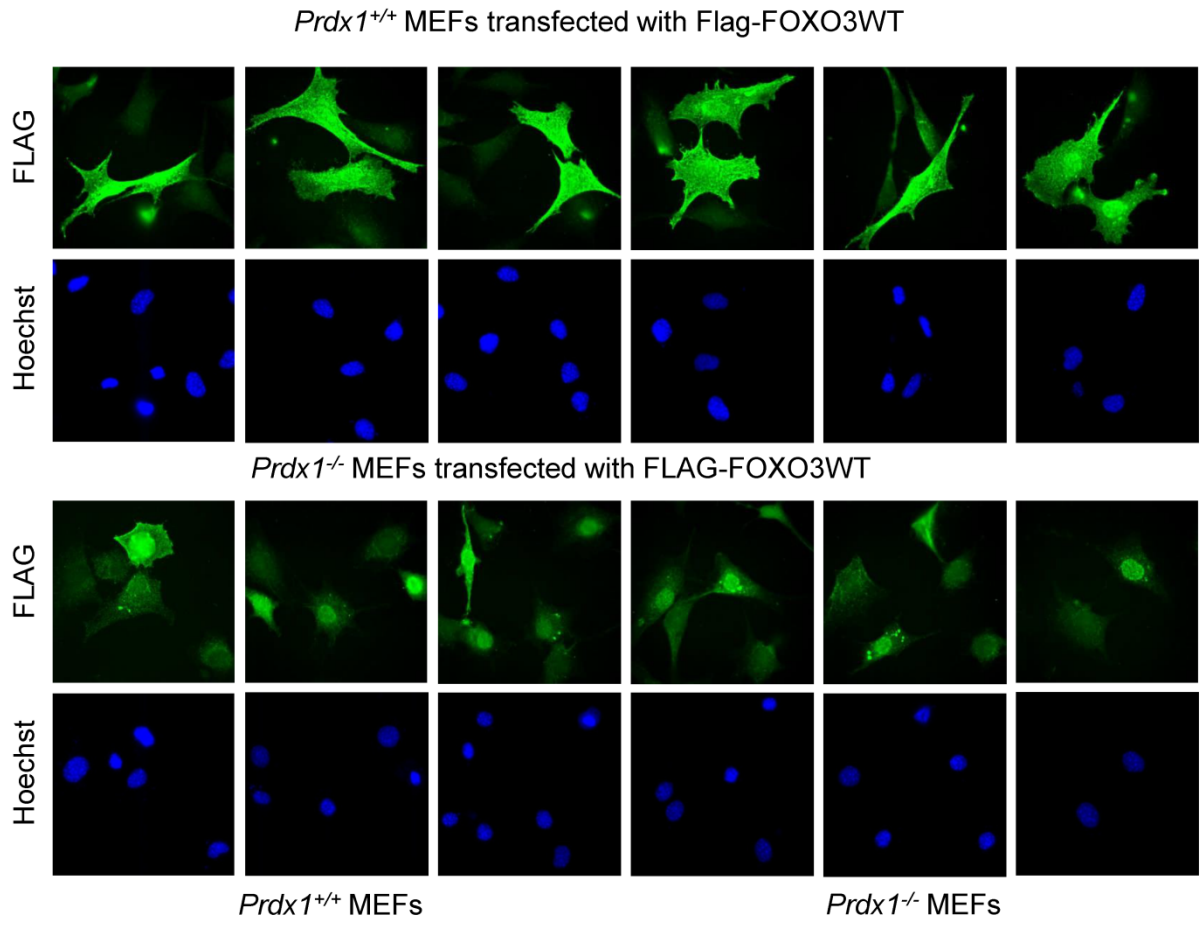

Figure 3.6: Absence of PRDX1 results in FOXO3 nuclear localizationin MEFs.
Representative pictures of *Prdx1*$^{+/+}$ MEFs and *Prdx1*$^{-/-}$ MEFs nucleofected with pcDNA3-FLAG-FOXO3 WT for 48 hours followed by fixation and analysis by IF with anti-FLAG antibodies. Co-staining with Hoechst indicates nuclear staining.

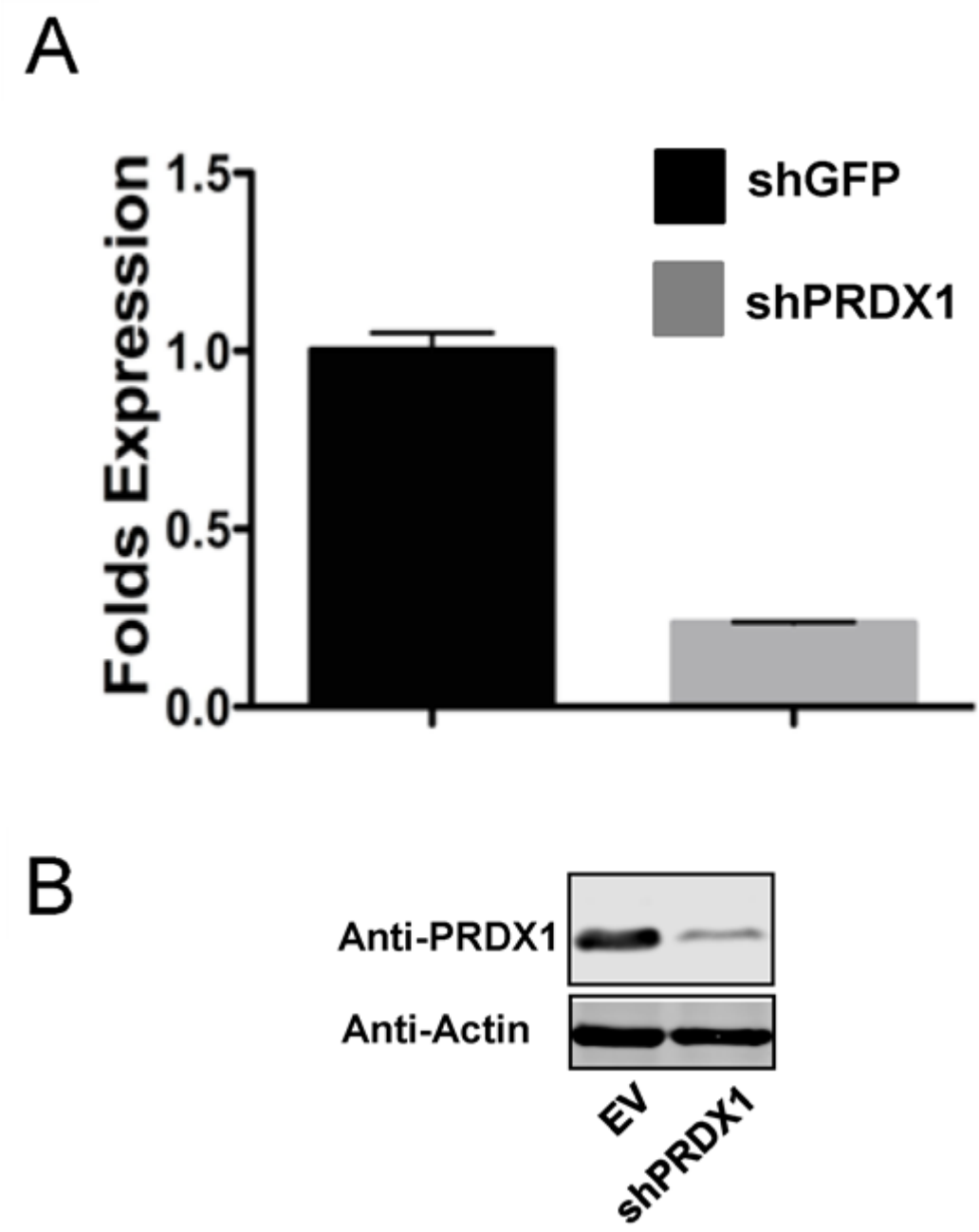

Figure 3.7: PRDX1 knockdown in 293T cells.
(A) PRDX1 protein expression following lentiviral infection with shPRDX1 compared to EV in 293T cells. **(B)** Representative western blot.

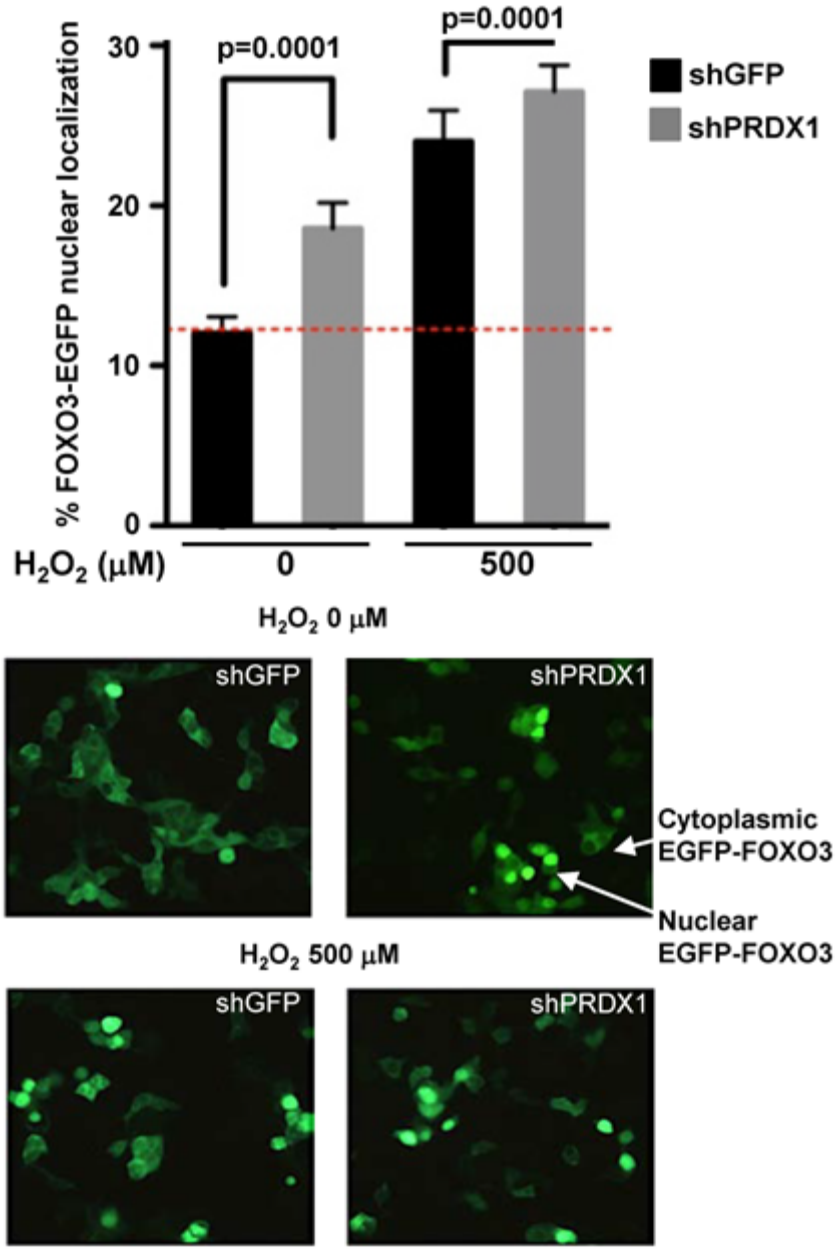

Figure 3.8: Enhanced FOXO3 nuclear localization in PRDX1 knockdown cells.
The percentage of 293T cells displaying nuclear FOXO3-EGFP localization was enhanced with reduction of PRDX1; 150 or more cells analyzed per sample, $p < 0.0001$ (t-test). Cells infected with shPRDX1A or control lentivirus for 48 h, followed by transfection with FOXO3-EGFP for 24 h. H_2O_2 was added during the last 30 min (Contributions by M. Nadler).

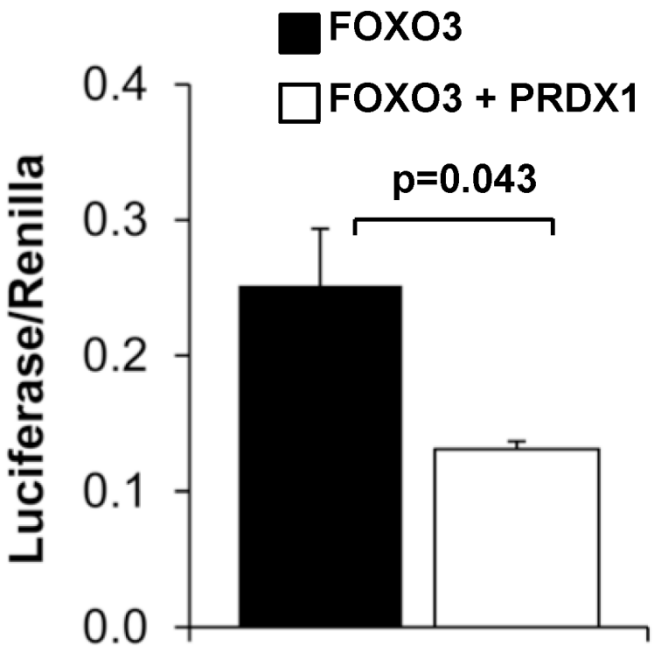

Figure 3.9: PRDX1 decreases FOXO3 activity.
HA-PRDX reduced FLAG-FOXO3 activity when transiently co-transfected into MEF and analyzed utilizing a dual luciferase reporter assay. Values (mean + SE) were normalized to vehicle treatment. *p < 0.05, t-test (N= 3).

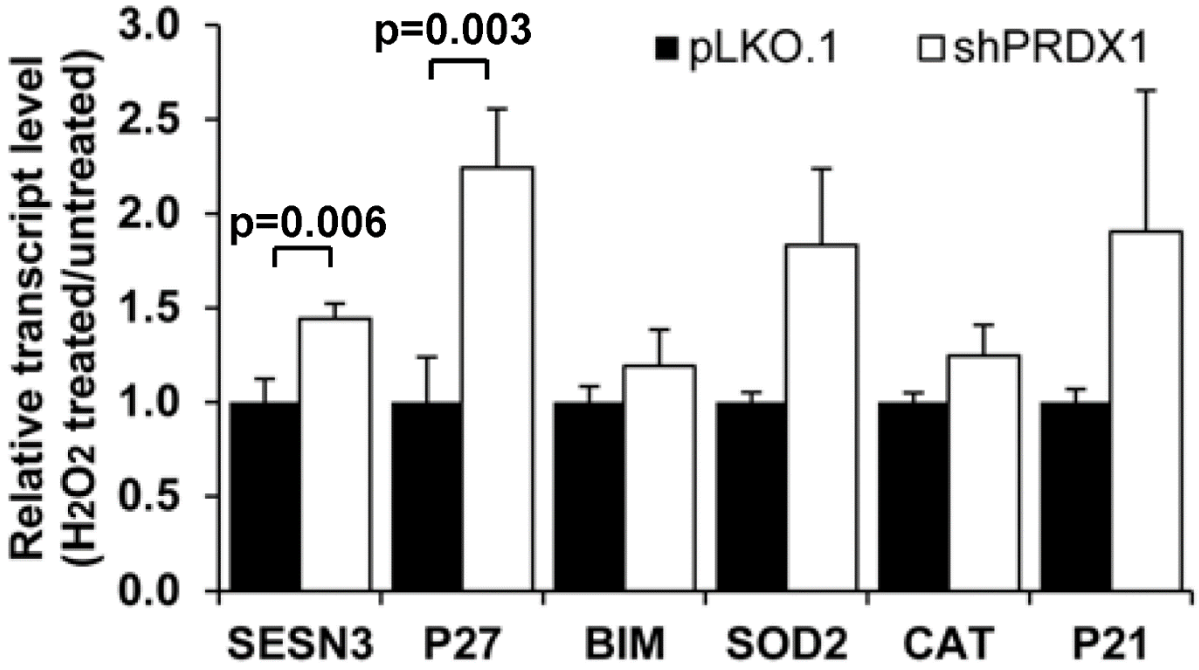

Figure 3.10: FOXO3 target expression increases following PRDX1 knockdown.
qPCR gene transcription of SESN3 and P27 was increased in PRDX1-deficient 293T cells (white bars) treated with 250 μM H_2O_2 compared to pLKO.1 control cells (black bars) after 16 h. Values (mean + SE) were normalized to vehicle treatment. *$p < 0.05$, t-test (N= 3). (Contributions by J. Skoko)

3.3 PRDX1 AND FOXO3 ASSOCIATE THROUGH DISULFIDE BONDS

Typical 2-Cys peroxiredoxins and FOXO proteins have been suggested to form disulfide bonds with target proteins (93). We explored whether covalent cysteine disulfide bridges exist between PRDX1 and FOXO3. Five cysteines are present in FOXO3 at positions 31, 150, 190, 362 and 622, while PRDX1 has 4 cysteines at positions 52, 71, 83 and 173. To investigate possible disulfide bridge interactions between PRDX1 and FOXO3, we analyzed *Prdx1*$^{+/+}$ and *Prdx1*$^{-/-}$ MEFs using two-color infrared (IR) antibody detection and identified one band staining positive for both PRDX1 and FOXO3 around 140 kDa after H_2O_2 treatment (Figure 3.11), which was reduced when lysates were treated with β-ME. This suggests an oligomeric PRDX1-FOXO3 protein complex composed of monomeric FOXO3 (90 kDa) disulfide bound to dimeric PRDX1 (2x 23 kDa). To gain further insight into the specific cysteines responsible for the PRDX1-FOXO3 interaction, co-immunoprecipitations (co-IPs) of endogenous PRDX1 with FLAG-FOXO3 single cysteine mutants revealed that loss of FOXO3 C31 or C150 decreased binding to PRDX1 (Figure 3.12). Conversely, co-precipitating endogenous FOXO3 with HA-PRDX1 single cysteine mutants showed that PRDX1 C52, C71 or C173 mutants decreased binding of endogenous FOXO3 as well (Figure 3.13). PRDX1 C71 has yet to be described to play a role in PRDX1 protein associations. Additionally, disulfide-dependent complex formation was further analyzed and demonstrated that, as shown in Figure 3.12, while H_2O_2 treatment induced endogenous PRDX1 binding to wild-type FLAG-FOXO3, and decreased PRDX1 binding to C31 and C150 single mutants, the FLAG-FOXO3 double mutant (C31,150S) and the quintuple cysteine to serine mutant FLAG-FOXO3

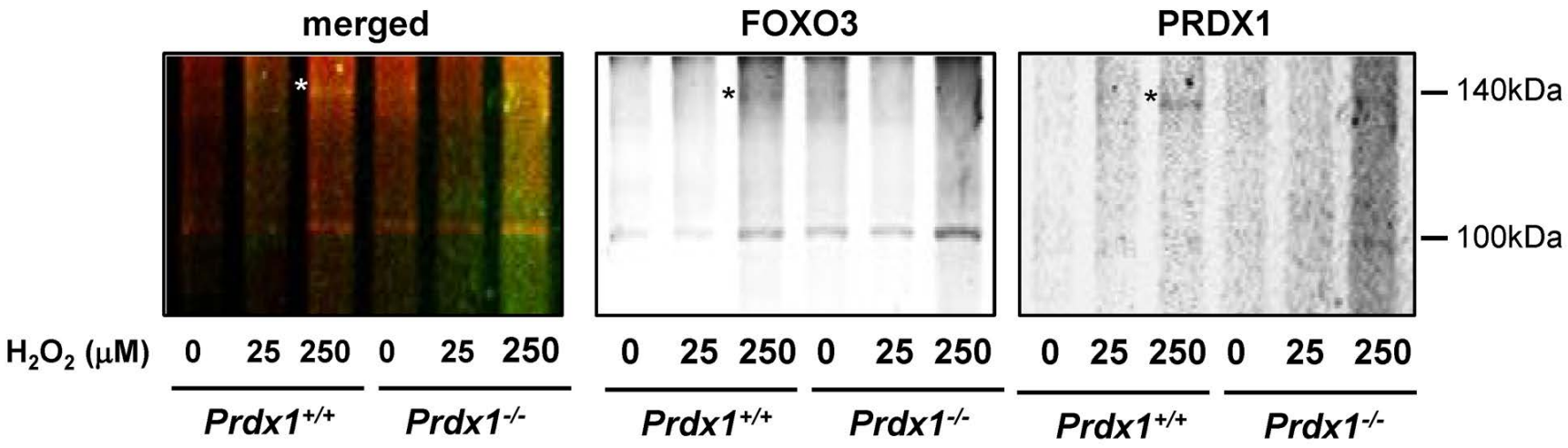

Figure 3.11: PRDX1 and FOXO3 form an oligomer via disulfide bonds.

A PRDX1-FOXO3 complex was detected under nonreducing conditions in *Prdx1*$^{+/+}$, but not *Prdx1*$^{-/-}$ MEFs, when treated with the indicated concentrations of H_2O_2 for 30 min by immunoblot with two-color IR antibody detection.

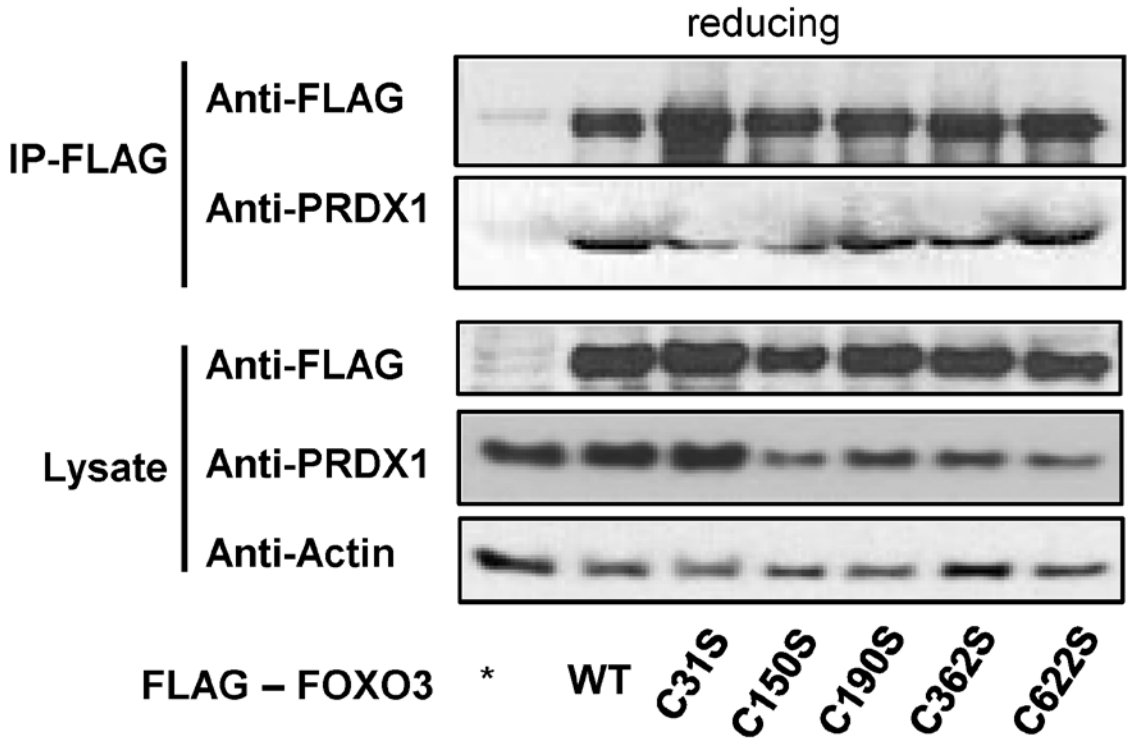

Figure 3.12: FOXO3 C31 and C150 are involved in binding to PRDX1.
Anti-FLAG IP of 293T cells transfected with EV or FLAG-FOXO3 (WT or single C-to-S mutants) displayed reduced PRDX1 binding to FOXO3 C31S or C150S when treated with H_2O_2 for 30 min by immunoblot.

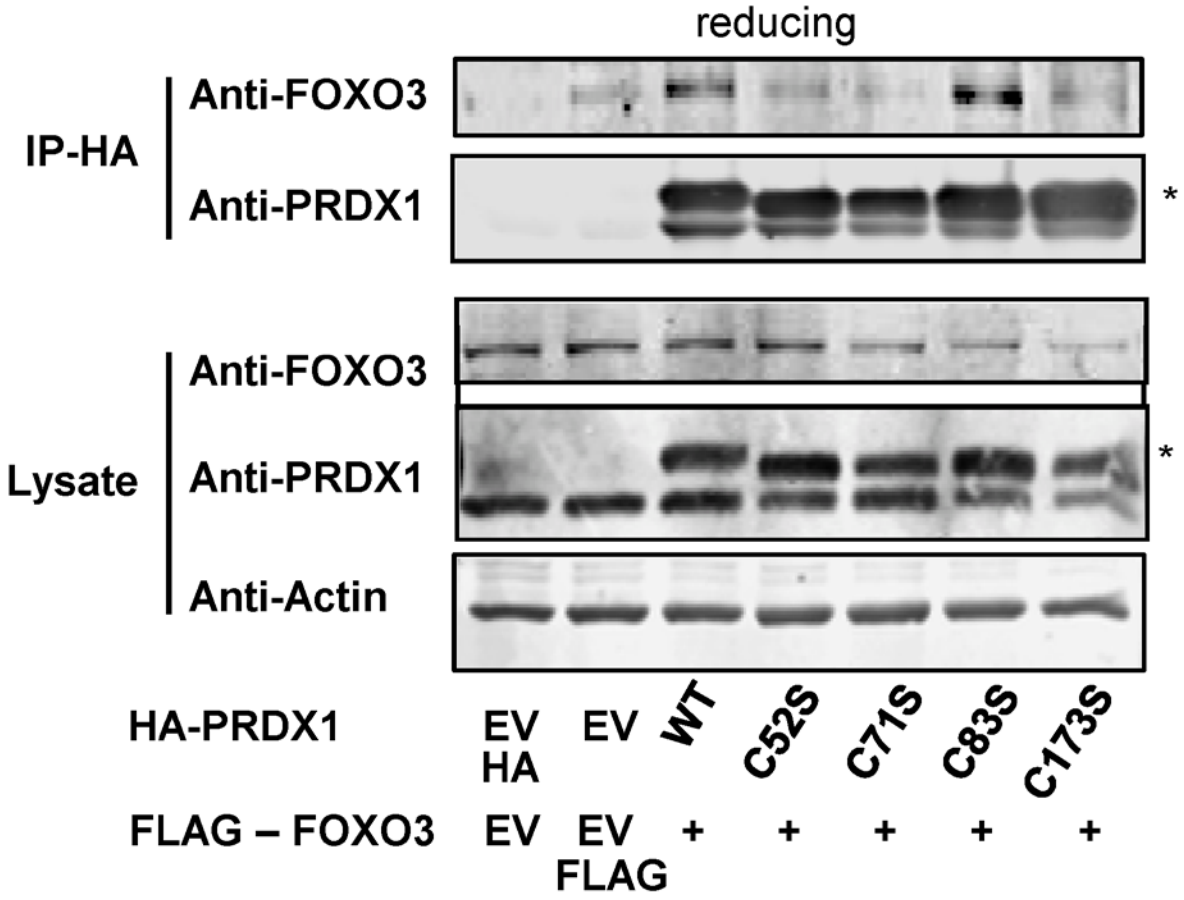

Figure 3.13: PRDX1 C52, C71 and C173 are involved in binding to FOXO3.
Anti-HA IP of 293T cells transfected with EV or HA-PRDX1 (WT or C-to-S mutants) showed reduced FOXO3 binding with PRDX1 C52S, C71S, or C173S mutants when treated with H_2O_2 for 30 min (*HA-PRDX1).

(CΔS), showed no PRDX1 binding (Figure 3.14). To further examine the importance of FOXO3 C31 and C150 in oligomeric formation, we expressed FLAG-FOXO3WT, C31S and C150S in 293T cells. Figure 3.15 shows the FOXO3-PRDX1 oligomer can be detected with wild-type FLAG-FOXO3, but not with single cysteine FOXO3 mutants. As FOXO3C31 and FOXOC150 are important for PRDX1 binding, we next examined how cysteine mutant FOXO3 activities were responding to PRDX1 mediated decrease under H_2O_2-induced stress. Using a dual-luciferase reporter assay we observed that under lower H_2O_2 treatments PRDX1 is able to decrease FOXO3 activity significantly, and under higher H_2O_2 doses (250 μM), PRDX1-induced decrease of FOXO3 was abrogated (Figure 3.16A). Additionally, PRDX1 did not further decrease FOXO3 C31 or C150 activity, nor did higher H_2O_2 doses increase mutant activity (Figures 3.16B and 3.16C). In contrast to wild-type FOXO3, PRDX1 was unable to lower FOXO3 (ΔC) luciferase activity (Figure 3.17).

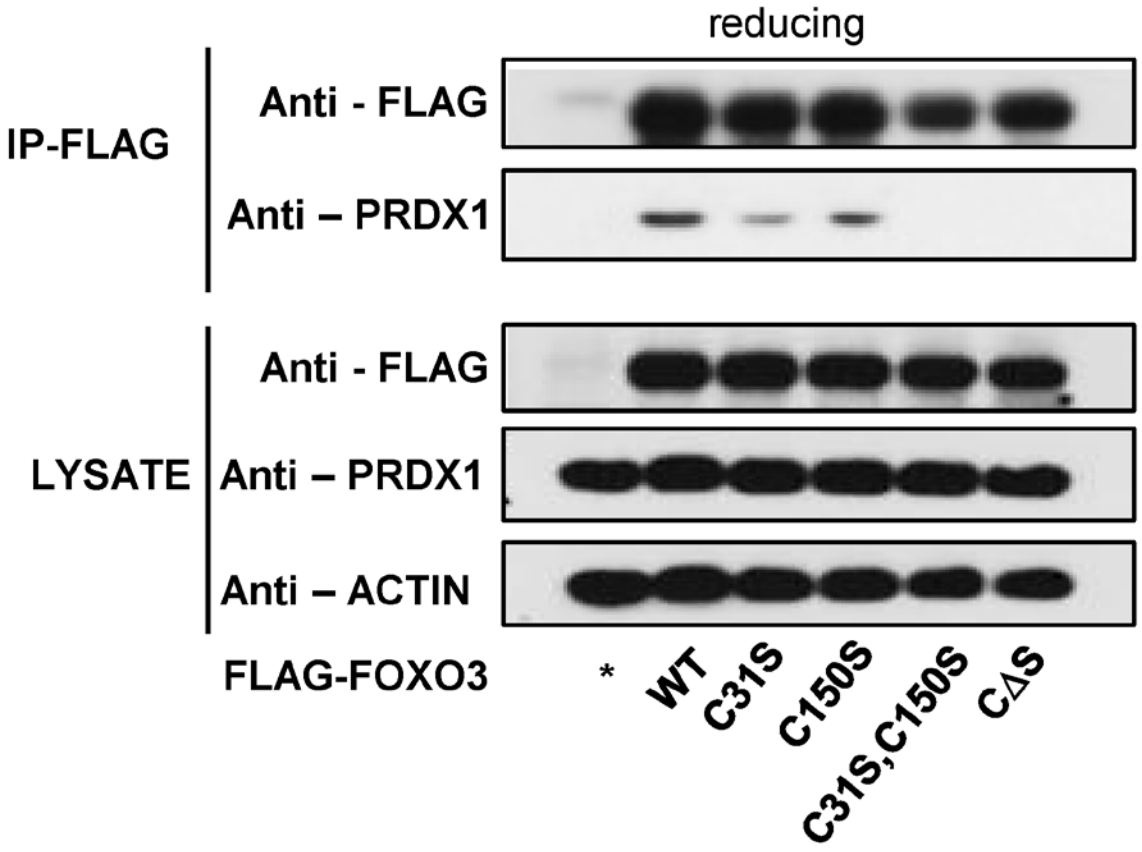

Figure 3.14: FOXO3 C31, 150S double mutant cannot bind to PRDX1.
Mutation of FOXO3 cysteines reduced PRDX1 binding when treated with 25 μM H_2O_2 for 30 min. Anti-FLAG IP of 293T cells transfected with EV or FLAG-FOXO3 (WT, C31S, C150S, C31,150S double mutant, or ΔCys mutants) was detected by immunoblot.

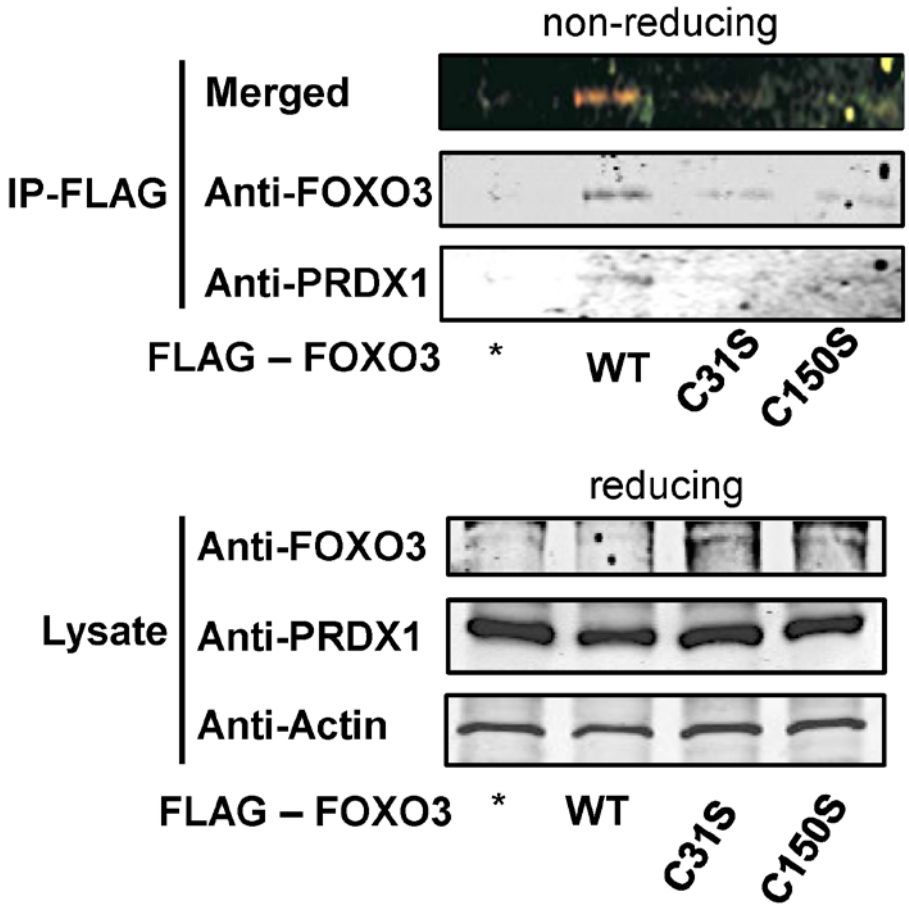

Figure 3.15: FOXO3 C31 and C150 mutants do not form the PDX1-FOXO3 oligomer.
A PRDX1-FOXO3 complex was detected in FLAG-FOXO3 WT, but not C31S or C150S mutant Anti-FLAG samples under nonreducing conditions in 293T cells co-transfected with FLAG-FOXO3 constructs and HA-PRDX1 treated with 25 μM H_2O_2 for 30 min.

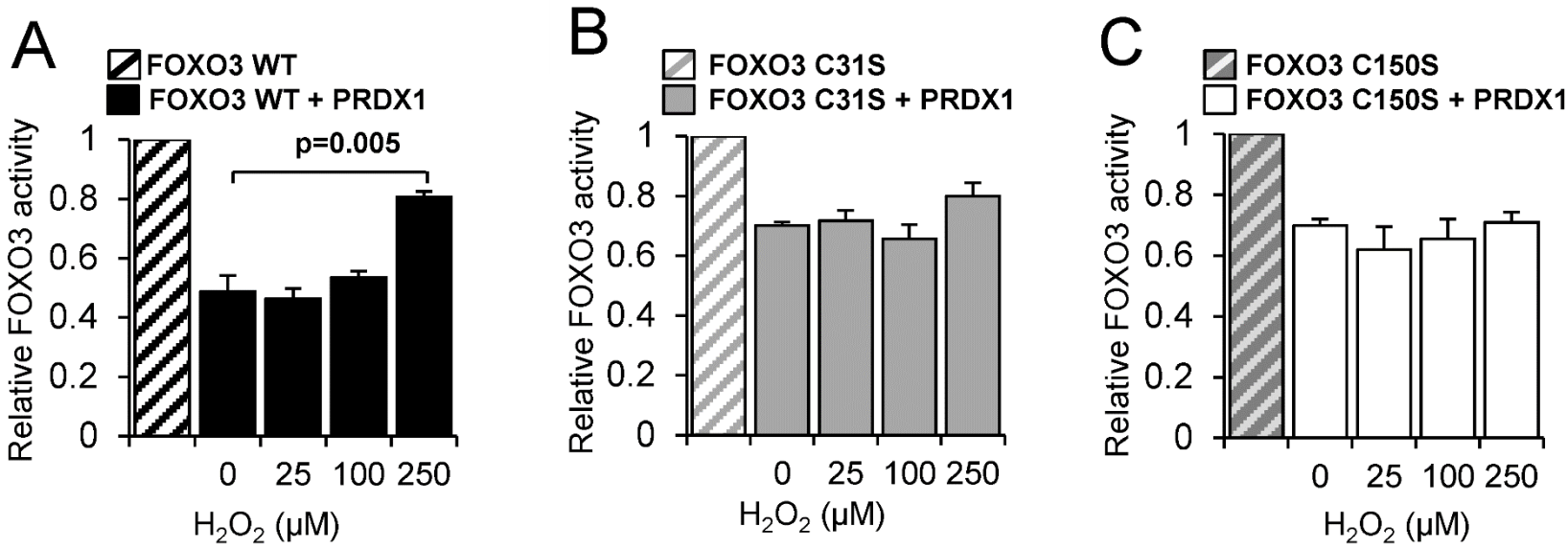

Figure 3.16: Effect of PRDX1 on FOXO3 C31S and C150S mutant activity.
The ability of PRDX1 to reduce FOXO3 activity was inhibited with H_2O_2 treatment in 293T cells in **(A)** FOXO3 WT, but not **(B)** C31S or **(C)** C150S mutants. Cells were transiently transfected with FOXO3 and PRDX1 constructs in a dual-luciferase assay treated with 0–250 μM H_2O_2. Values (mean + SE) were normalized to FOXO3 vehicle treatment. *$p < 0.05$, t-test (N = 3). (Contributions by J. Skoko)

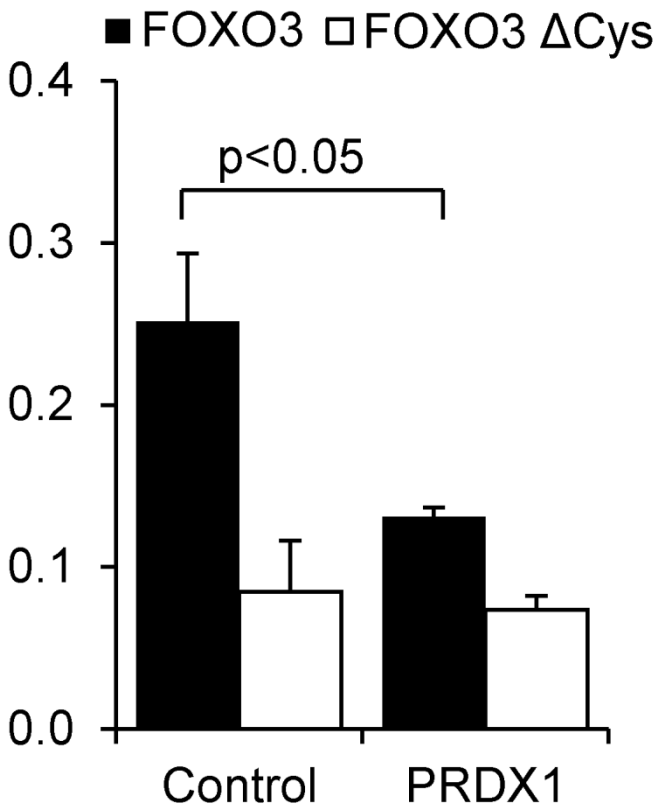

Figure 3.17: PRDX1 decreases FOXO3 activity via cysteine interactions.
HA-PRDX reduced FLAG-FOXO3 activity, but not FLAG-FOXO3 ΔCys, when transiently co-transfected into MEF and analyzed utilizing a dual-luciferase reporter assay. Values (mean + SE) were normalized to vehicle treatment. *p< 0.05, t-test (N = 3).

3.4 FOXO3 DISULFIDE BONDING WITH PRDX1 DETERMINES SUBCELLULAR LOCALIZATION UNDER H2O2 STRESS

Several lines of evidence demonstrate that FOXO proteins translocate to the nucleus under increased levels of ROS, reviewed in (162). Importantly, nuclear localization of FOXO proteins is a pre-requisite for their transcriptional activity, as such FOXOs are highly regulated through mechanisms that alter nucleo-cytoplasmic shuttling (154). To address if the PRDX1-FOXO3 disulfide bound complex influences FOXO3 protein nuclear localization, we expressed WT-, C31S- or C150S-FOXO3-EGFP reporter constructs in 293T cells and quantified nuclear localization following H_2O_2 exposure for one hour. Figure 3.18 shows that unlike WT FOXO3, C31S- and C150S-mutant FOXO3s fail to translocate to the nucleus, even during high dose H_2O_2 treatment. These data indicate that FOXO3 C31 and C150 regulate FOXO3 nuclear translocation. A well-known mechanism regulating FOXO3 nuclear localization is phosphorylation on T32 by AKT, which sequesters FOXO3 in the cytosol (118). We therefore examined the AKT phosphorylation status of C31S and C150S FOXO3 mutants. Figure 3.19A shows FOXO3 WT T32 phosphorylation levels were increasing following treatment with H_2O_2, which corresponded with binding of 14-3-3 to the PRDX1-FOXO3 complex (Figure 3.20). Interestingly, both mutants showed increased basal levels of T32 phosphorylation compared to FOXO3 WT, with phosphorylation further increasing with additional H_2O_2 treatment. We also examined phosphorylation at S318, another AKT phosphorylation site known to enhance FOXO3 nuclear export (163), and found no change in phosphorylation levels between FOXO3

WT, and the C31S, and C150S single FOXO3 mutants (Figure 3.19B), suggesting the binding of PRDX1 to FOXO3 has a site-specific effect on FOXO3 phosphorylation. Given that FOXO3 WT, and the C31S, and C150S single FOXO3 mutants (Figure 3.19B), suggesting the

binding of PRDX1 to FOXO3 has a site-specific effect on FOXO3 phosphorylation. Given that FOXO3 C31S and C150S mutants have increased T32 phosphorylation and dysfunctional translocation in response to high doses of H_2O_2 (Figure 3.18 and 3.19A), we questioned if PI3K inhibition is capable of promoting FOXO3 C31S and C150S mutant nuclear translocation. To do so, we applied experimental conditions that would decrease the activation of kinases such as STK4/MST1, JNK and p38 (82,106) known to induce FOXO3 nuclear localization by phosphorylating FOXO3. For example, MST1 phosphorylation on FOXO3 is known to overcome its AKT-induced cytoplasmic retention by decreasing 14-3-3 binding to FOXO3 (82). EGFP-FOXO3 localization was compared in 293T cells treated with 100 μM H_2O_2 for 30 min. Under this treatment 14-3-3 binding to FOXO3 was not decreased (Figure 3.20). In addition, as FOXO3 nuclear localization is dependent on DNA concentration (Table 3.1), we assessed EGFP-FOXO3 localization of 50 ng of EGFP-FOXO3 in a sub-confluent population cells to ensure AKT activity towards FOXO3 (164). We found that 293T cells were transfected with indicated concentration of EGFP–FOXO3 WT DNA for 24 hours, then transferred to complete media for an additional 24 hours. FOXO3 localization was then scored as either nuclear, cytoplasmic, or intermediate. Percentage of cells displaying nuclear FOXO3 is indicated.

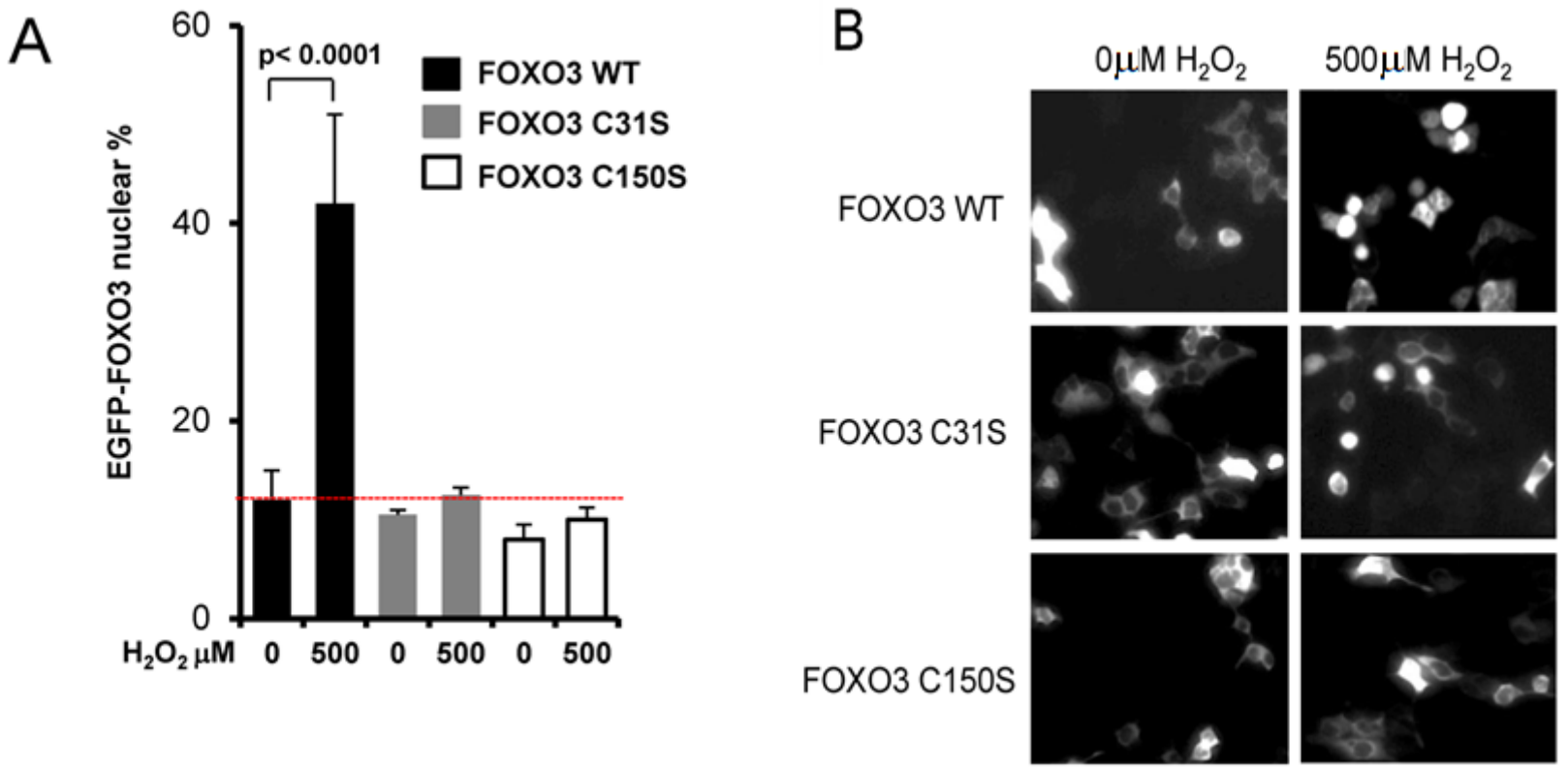

Figure 3.18: Disulfide bonds between PRDX1 and FOXO3 modulate FOXO3 subcellular localization.
(A) 30 min of H_2O_2 treatment enhanced nuclear EGFP-FOXO3 content in WT, but not C31 or C150 mutant constructs, 48 h following transfection in 293T cells. Percentage of cells displaying nuclear FOXO3-EGFP localization is indicated. **(B)** Representative pictures of **(A)**.

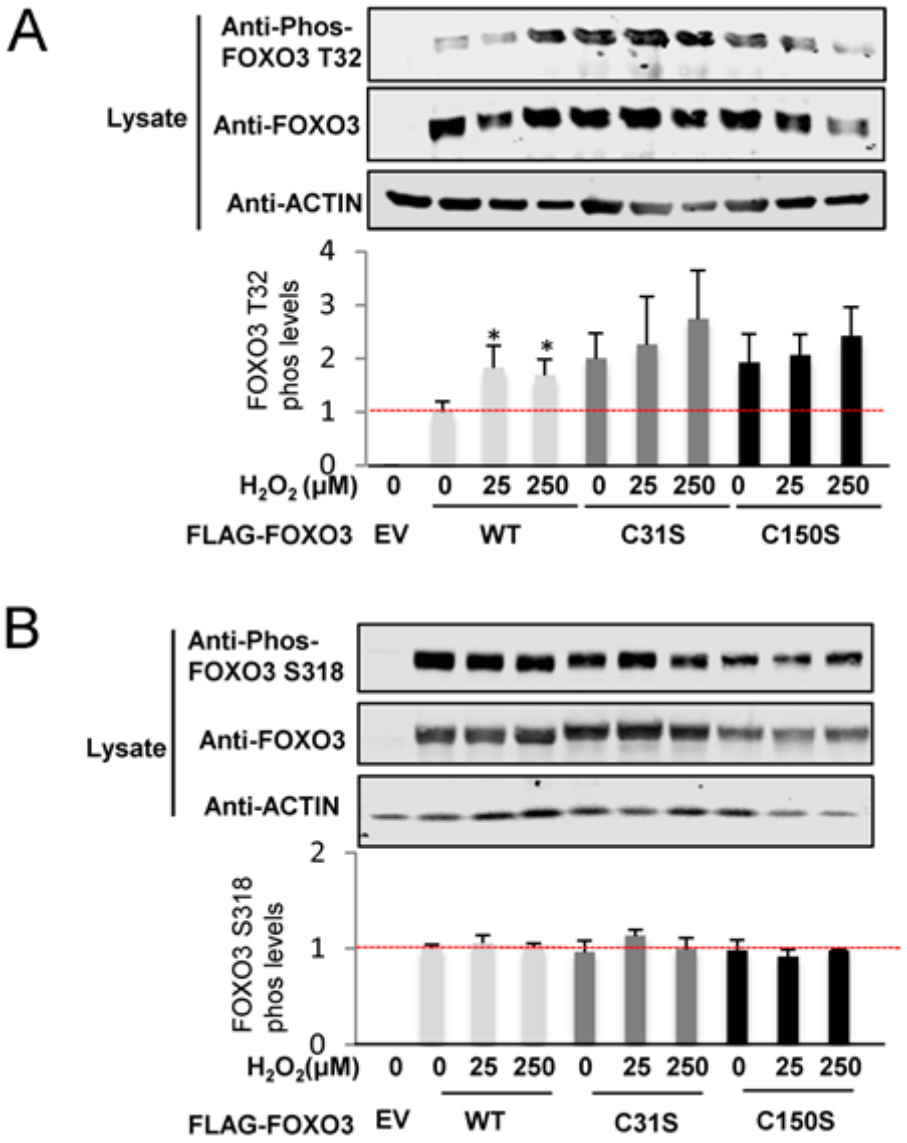

Figure 3.19: PRDX1 binding differentially modulates FOXO3 phosphorylation.
293T cells transfected with FLAG-FOXO3 (WT, C31, or C150 mutants) were treated with H_2O_2 and immunoblotted for FOXO3 Phospho-T32, FOXO3 Phospho-S318, FOXO3 and Actin. **(A)** T32 phosphorylation of FOXO3 C31S or C150S was heightened under basal conditions compared to FOXO3 WT. **(B)** S318 phosphorylation of FOXO3 WT, C31S and C150S was unaffected by H_2O_2 treatment. Phospho-T32 and Phospho-318 signals were normalized to total FOXO3. Values represent mean + SD normalized to WT 0 μM H_2O_2. *$p < 0.05$ (N = 3).

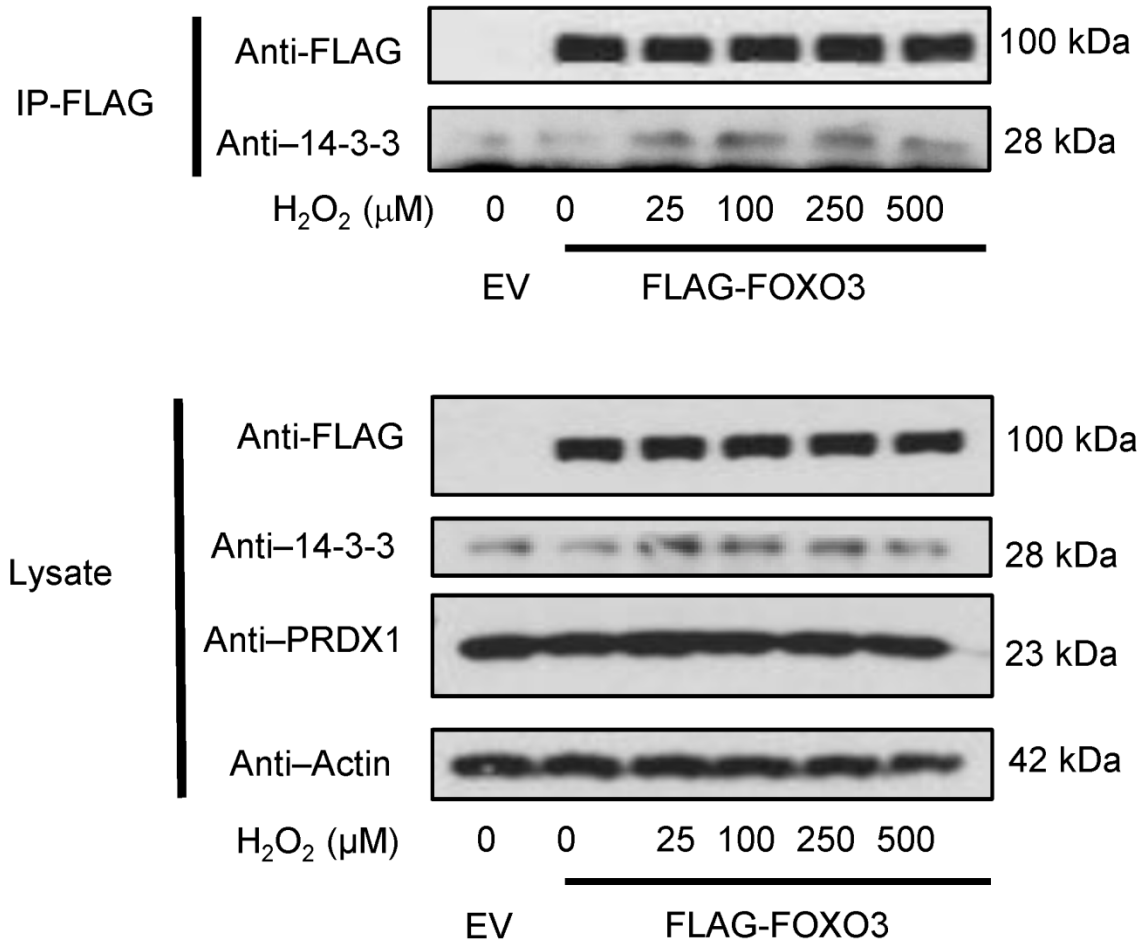

Figure 3.20: 14-3-3 binding increases with H_2O_2 concentration.
293T cells were co-transfected with FLAG-FOXO3. Prior to lysis, cells were treated with H_2O_2 as indicated for 30 min. Co-IPs were performed using anti-FLAG coated beads and Co-IPs were resolved under reducing conditions, followed by immunoblot with 14-3-3, Flag, PRDX1, and Actin antibodies.

Table 3.1: FOXO3 nuclear localization.

EGFP-FOXO3 Concentration (ng)	Nuclear (#)	Total (#)	% Nuclear
400	337	406	83%
200	198	301	66%
100	52	144	36%
50	21	131	16%

LY294002 (PI3K inhibitor) treatment significantly increased nuclear localization by 5.7%, 11.7% and 25% in FOXO3WT, FOXOC31S and FOXOC150C, respectively, compared to untreated cells (Figure 3.21). These results suggest again that AKT-induced phosphorylation of FOXO3 sequesters FOXO3 cysteine mutants in the cytosol.

3.4.1 Loss of PRDX1 enhances FOXO3 dependent H_2O_2-induced let-7 miRNA up-regulation

As prior studies have reported, increased let-7 family member expression is induced by ionizing radiation, genotoxic stress and peroxide (158,159). In C. elegans, let-7 family members have overlapping functions with FOXO proteins to regulate development, aging, glucose metabolism, stress responses, and act as tumor suppressors (165). We were interested to examine if let-7 miRNAs are regulated by FOXO3 under oxidative stress. Using the UCSC Genome Browser we analyzed the 5' UTR of let-7b and let-7c in several species for putative FOXO3 binding

sequences (YTXXACA) (166). As shown in Figure 3.22, FOXO3 binding sites are preserved in the 5' UTR of *MIRLET7B* and *MIRLET7C*.

To confirm let-7 miRNAs as a potential novel FOXO3 target, we established that H_2O_2 treatment of HeLa cells enhanced the expression of both let-7b and let-7c miRNAs (Figure 3.23). To determine if FOXO3 is involved in H_2O_2-mediated enhanced expression of let-7b and let-7c in HeLa cells, we performed either an over-expression or a shRNA-mediated knockdown of FOXO3 prior to treatment with H_2O_2. When FOXO3 expression increased, levels of let-7b and let-7c were 3-fold and 2-fold higher, respectively (Figure 3.24). As FOXO3 expression was significantly diminished, the ability of H_2O_2 to induce either let-7b or let-7c miRNA expression was lost (Figure 3.25). As final confirmation of FOXO3's role in the H_2O_2-mediated enhancement of let-7 expression, a chromatin IP (ChIP) assay was performed showing that FOXO3 binds directly to both of the let-7c promoter regions, the distal host gene and the proximal intronic promoter, both considered functional in let-7c transcription (147) (Figure 3.26). FOXO3 promoter binding was further enhanced following 100μM H_2O_2 treatment compared to no treatment. These data suggested that FOXO3 very likely regulates the expression of *MIRLET7B* and *MIRLET7C*. Next, we examined if loss of PRDX1 magnifies let-7 miRNA transcription in the context of H_2O_2 addition. Figures 3.27A and 3.27B show when PRDX1 expression was more than 90% reduced, let-7b and let-7c expression levels were augmented. This effect was further enhanced with 100 μM H_2O_2 treatment. Moreover, SOD2, an established target of FOXO3, was also increased upon H_2O_2 treatment in the context of PRDX1 knockdown (Figure 3.27C). In conclusion, these data suggest that a PRDX1/FOXO3 signaling axis exists that directly regulates let-7c expression under H_2O_2-induced stress.

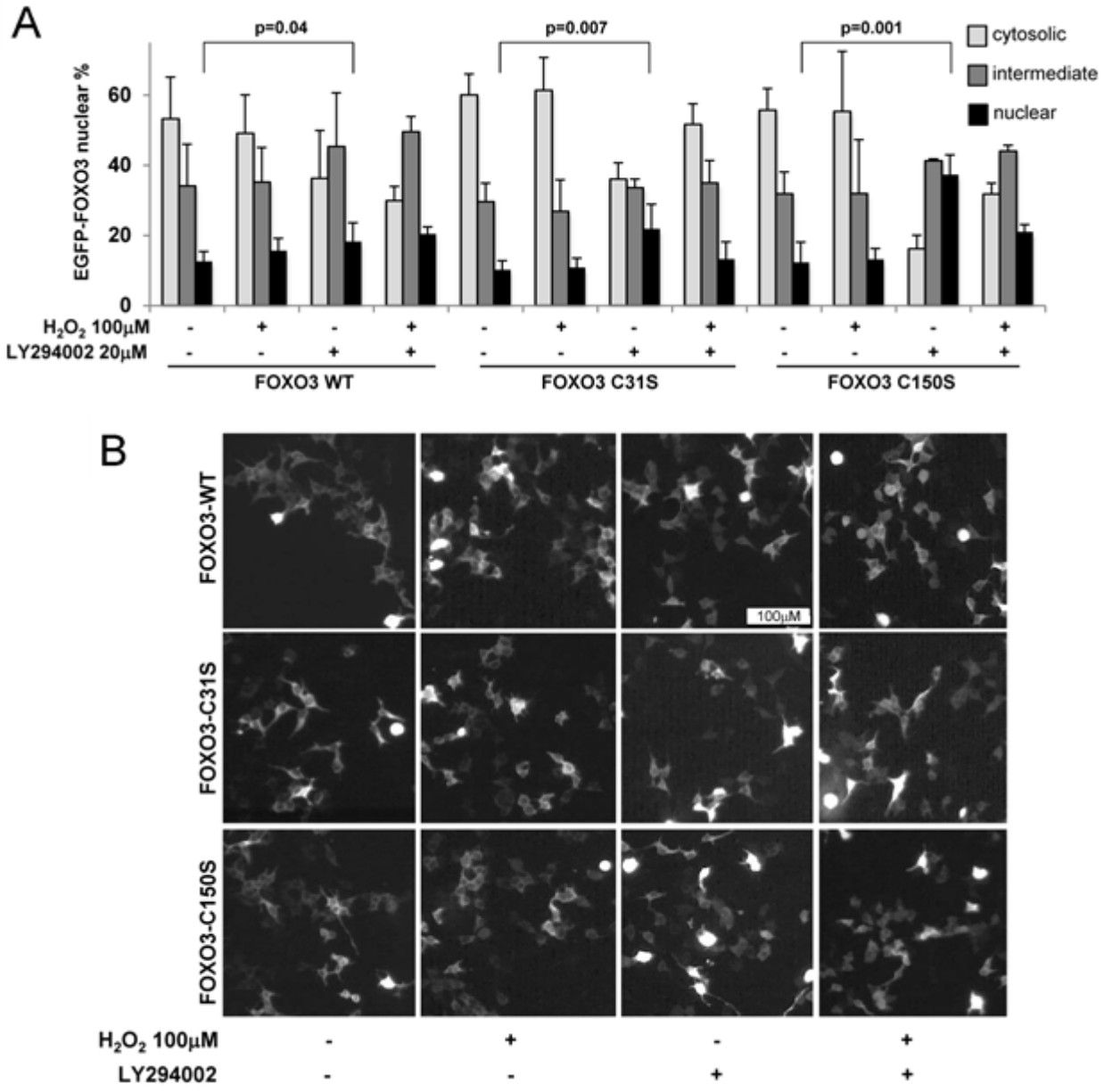

Figure 3.21: AKT-induced phosphorylation of FOXO3 sequesters FOXO3 cysteine mutants in the cytosol.

PI3K inhibition with 20 µM LY294002 enhanced nuclear FOXO3-EGFP WT, C1S, or C2S mutants in transiently transfected 293T cells after 24 h. Values represent mean + SE (N= 3) with 150–400 cells counted per sample by fluorescence microscopy. Experiment was repeated twice. **(B)** Representative pictures of **(A).**

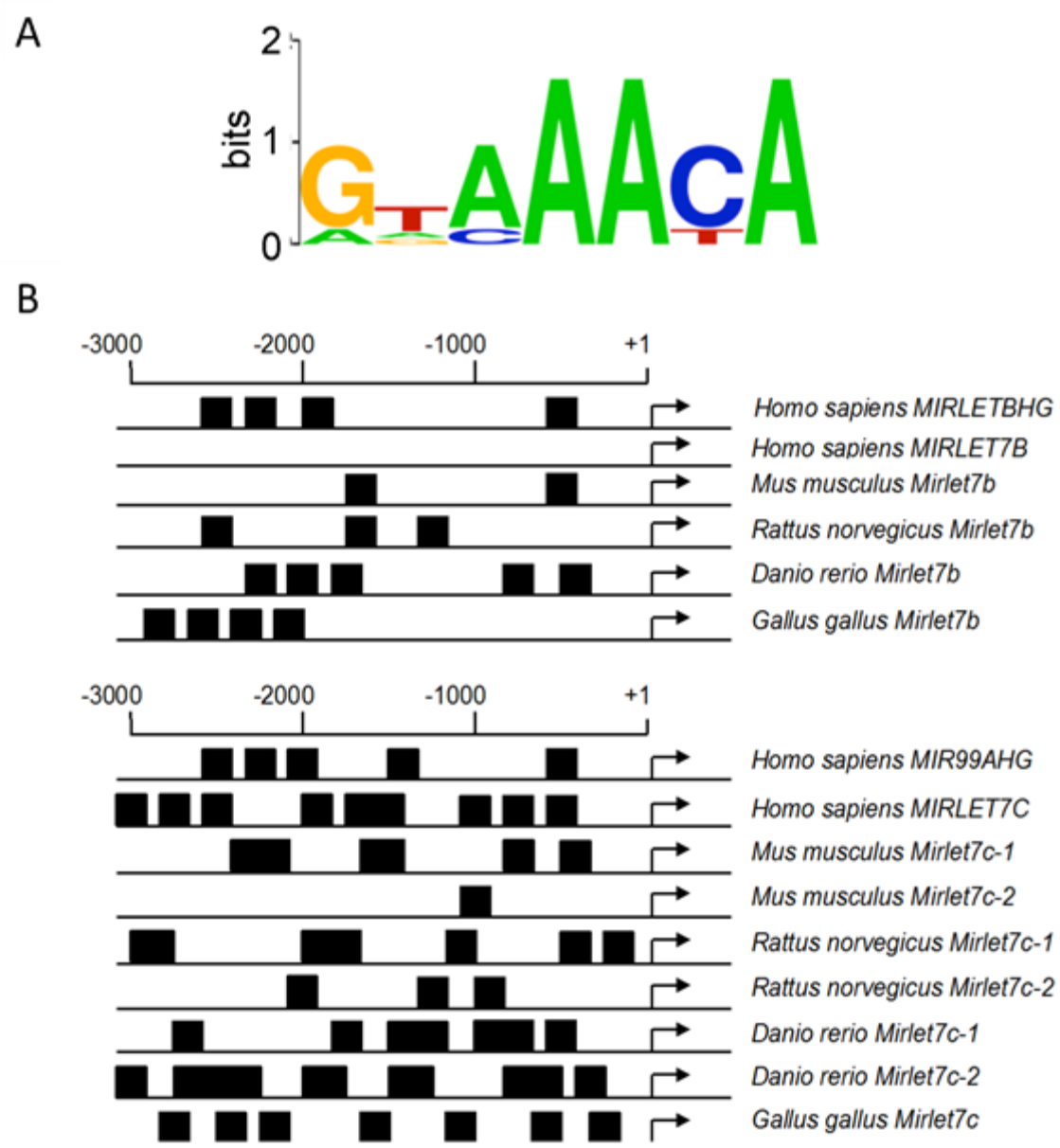

Figure 3.22: FOXO3 consensus binding sequence found in let-7b and let-7c promoter region.
(A) Putative FOXO3 binding sequence. **(B)** Putative FOXO binding motifs in forward and reverse are located in the 5` UTR of let-7b and -7c in several species. The consensus FOXO binding sequences in the WebLogo (black boxes) are indicated. Numbering is relative to the transcription start site obtained through the University of California Santa Cruz Genome Browser. UTR = untranslated region.

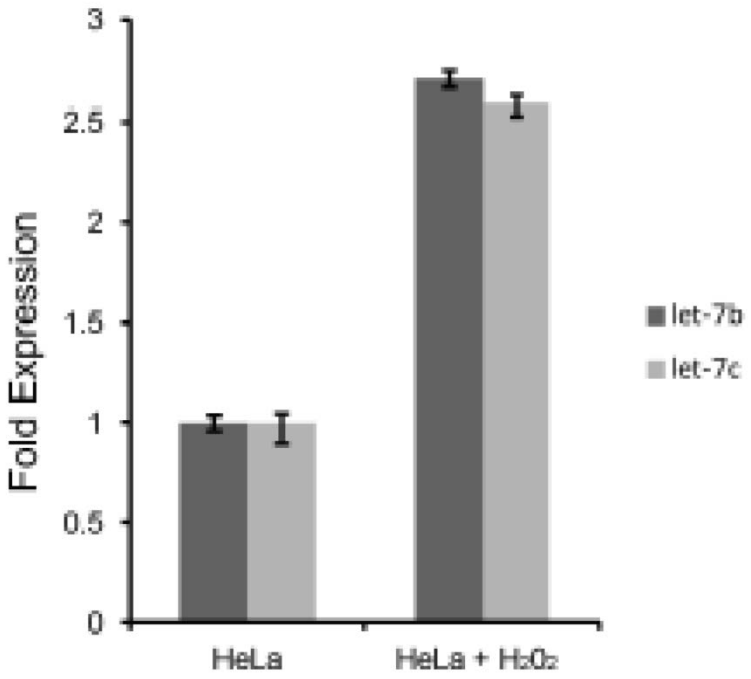

Figure 3.23: H_2O_2 enhances let-7b and let-7c miRNA expression.
HeLa cells treated with or without 500 μM H_2O_2 for 18 h were harvested, lysed and analyzed for let- 7b and let-7c expression by individual Taqman microRNA expression assays using the delta C_T method with U18 as the internal standard. (Contributions by M. Nadler)

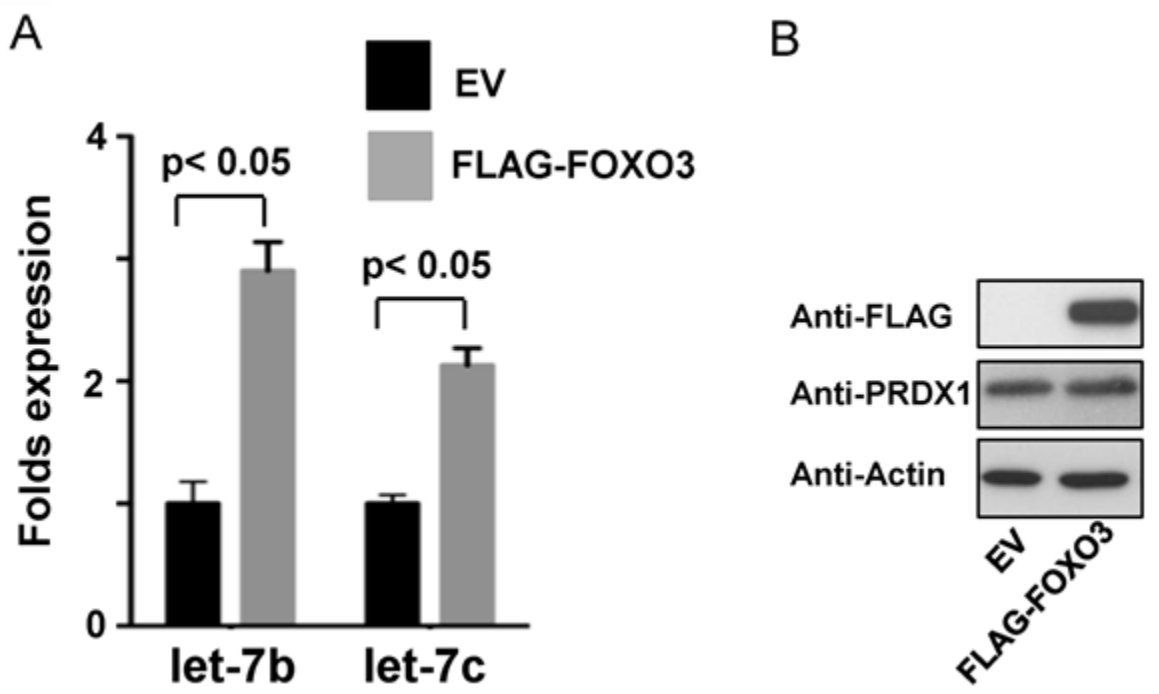

Figure 3.24: FOXO3 enhances let-7b and let-7c miRNA expression.
(A) Overexpression of FLAG-FOXO3 enhanced let-7b and c transcription in Hela cells 48 h after transfection. Cells were harvested, lysed, and analyzed for miRNA expression by TaqMan assays, using the delta C_T method with mir-30c as the internal standard. **(B)** Representative western blot of cell lysis and immunoblotting with FOXO3, PRDX1 and Actin antibodies. (Contributions by M. Nadler)

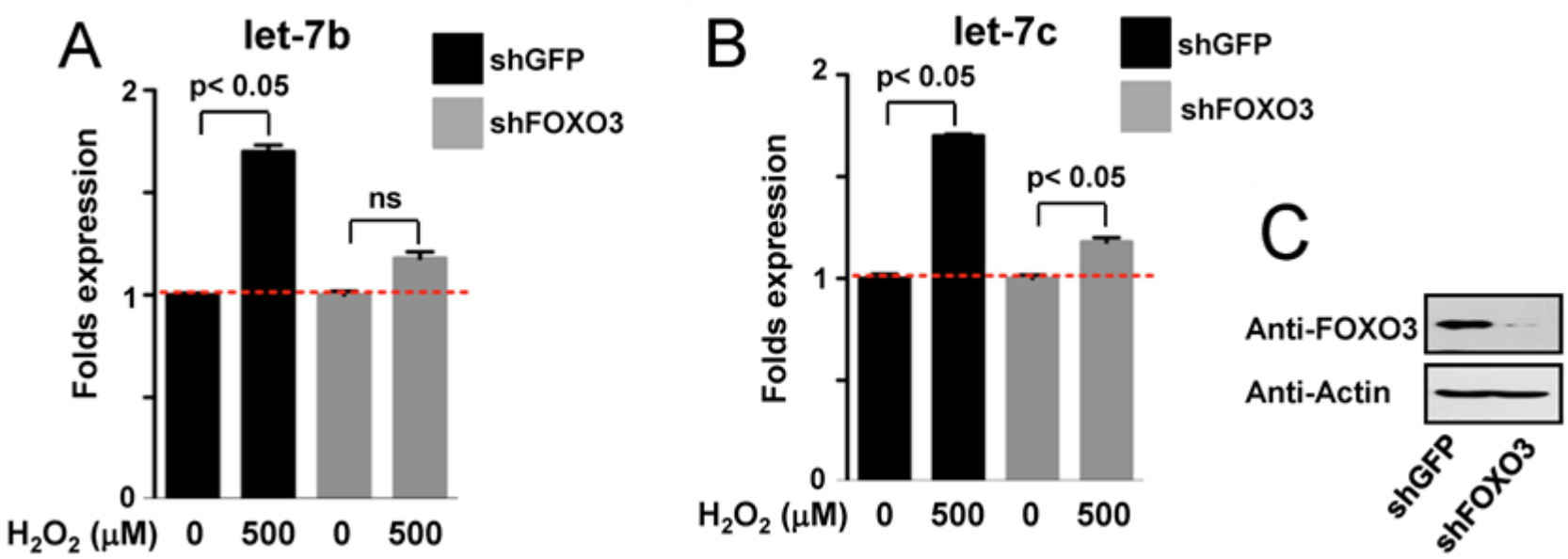

Figure 3.25: FOXO3 is required or H_2O_2 induced let-7b and let-7c miRNA expression.

(A-B) FOXO3-deficient HeLa cells were nonresponsive to H_2O_2 treatment. 30 h following transfection of shGFP or shFOXO3, cells were treated with or without H_2O_2 for 18h. Expression profiles of let-7b and let-7c were assessed by individual TaqMan assays with U18 as the internal standard (N= 3). (C) Western blot of cell lysis and immunoblotting with FOXO3 and Actin antibodies

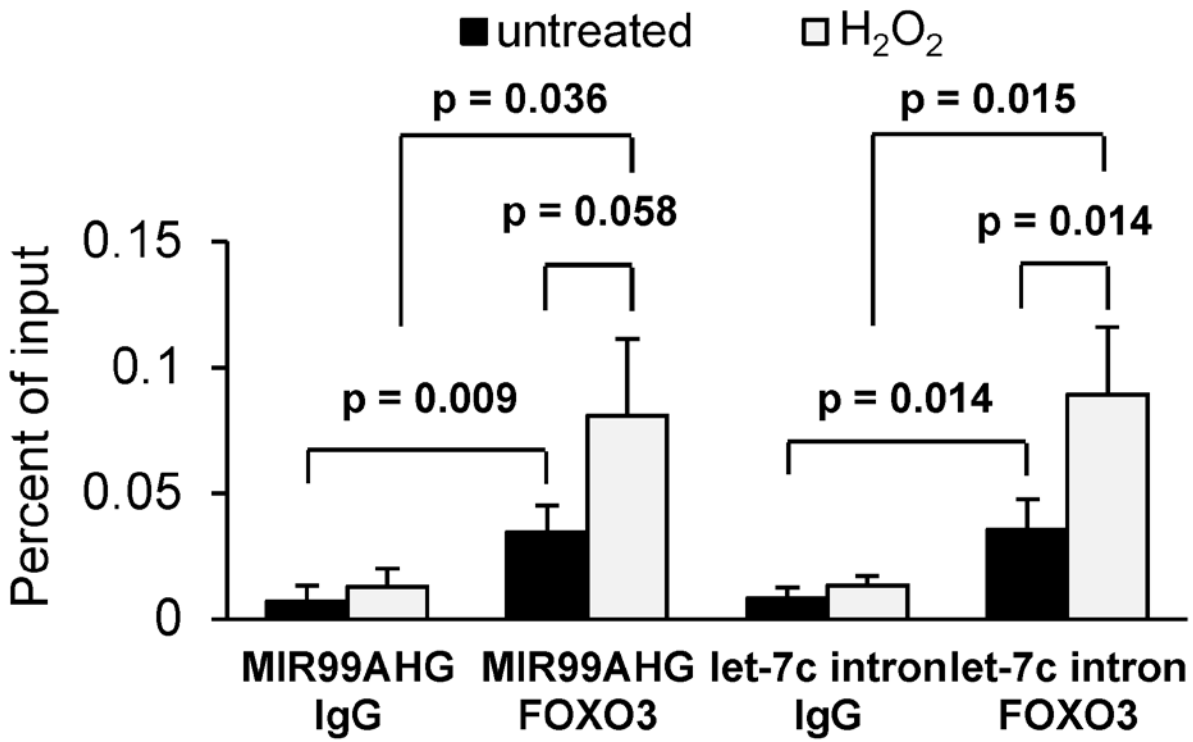

Figure 3.26: ChIP assays of FOXO3 binding to let-7c promoter region.
ChIP assays indicate transfected FLAG-FOXO3 binds to the MIR99AHG and intronic let-7c promoter regions and is enhanced following 100 μM H_2O_2 treatment for 30 min in 293T cells. Quantification of immunoprecipitated DNA was performed in triplicate by quantitative PCR and evaluated by the delta C_T method. Values of each immunoprecipitated sample are expressed as a percentage relative to their respective input (no antibody). (Courtesy of A.Pelosi)

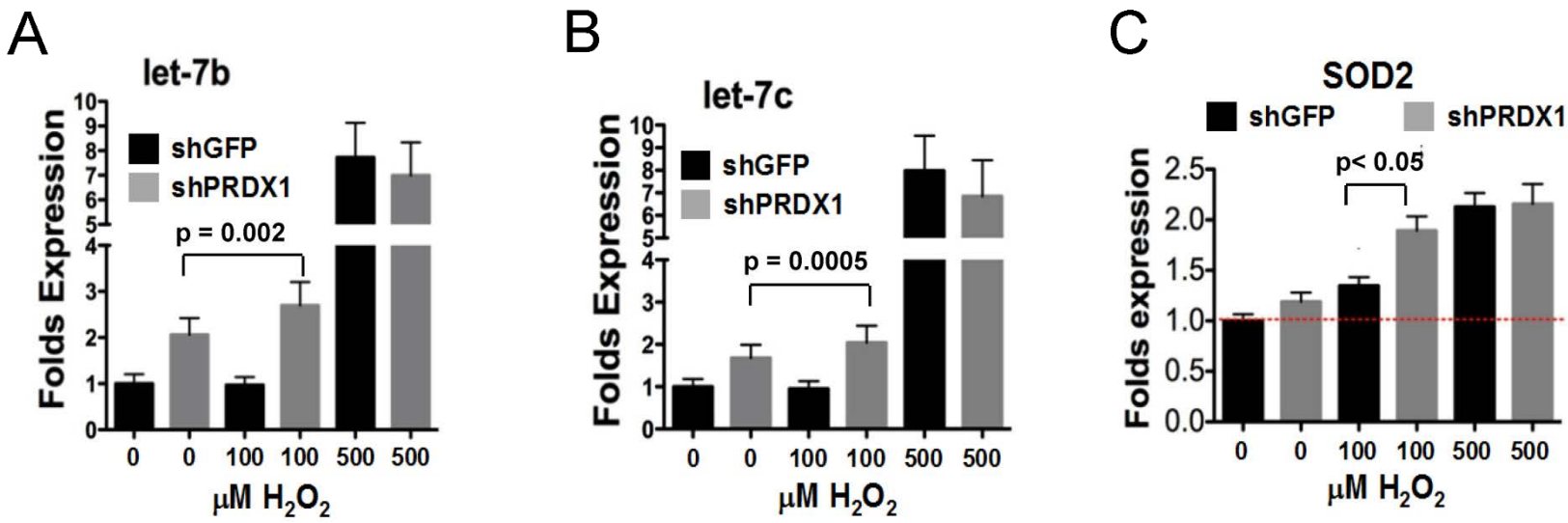

Figure 3.27: Loss of PRDX1 magnifies H_2O_2 induced let-7b and let-7c miRNA expression.

(A) let-7b, **(B)** let-7-c, and **(C)** SOD2 transcription was increased in PRDX1-deficient cells. HeLa cells were transfected with either a shGFP or shPRDX1 construct for 56 h, followed by treatment with or without H_2O_2 for 18 h. HeLa cells were analyzed for gene expression by TaqMan expression assays using the delta C_T method with U18 as the internal standard.

3.4.2 The PRDX1-FOXO3-let-7 axis regulates breast cancer cell migration

Lastly, we sought functional confirmation of the PRDX1-FOXO3-let-7 axis. As genetic alterations in cancer cells elevate the production of ROS, reviewed in (167) and let-7b and let-7c suppress cancer development (165) including breast cancer (168-170), we compared let-7c expression levels in normal and breast cancer cases (171). We found let-7c expression to be significantly lower in breast cancer tissues compared to normal breast tissue (Figure 3.28). FOXO3 showed a similar expression pattern to let-7c, while PRDX1 expression was increased in breast cancer compared to normal tissue (Figure 3.29). Comparing changes to PRDX1 and FOXO3 expression in normal and breast cancer tissues using Pearson correlation demonstrated

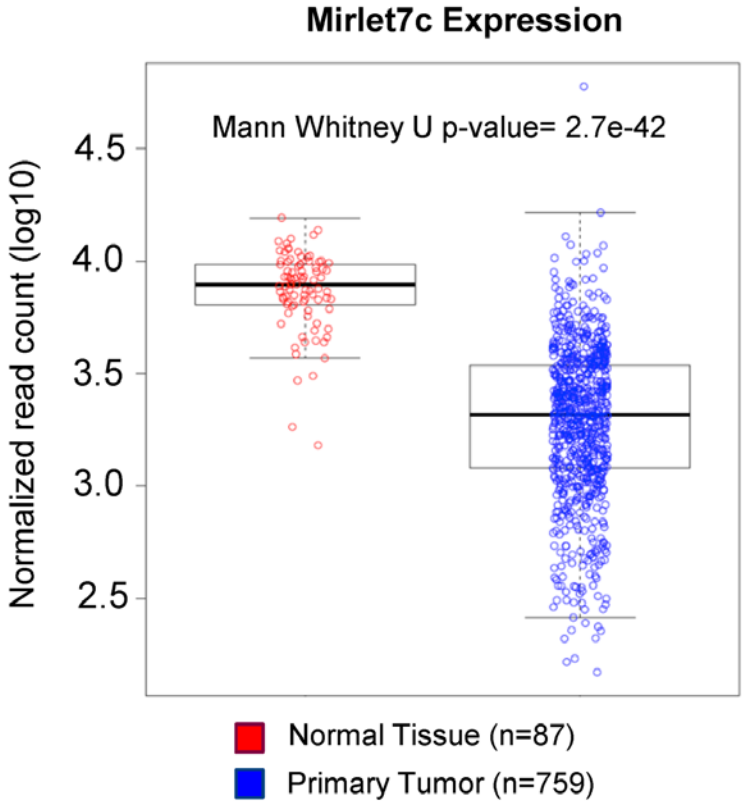

Figure 3.28: Mirlet7 expression in normal and breast cancer tissue.
Data from The Cancer Genome Atlas (TCGA). (Courtesy of K. Levine)

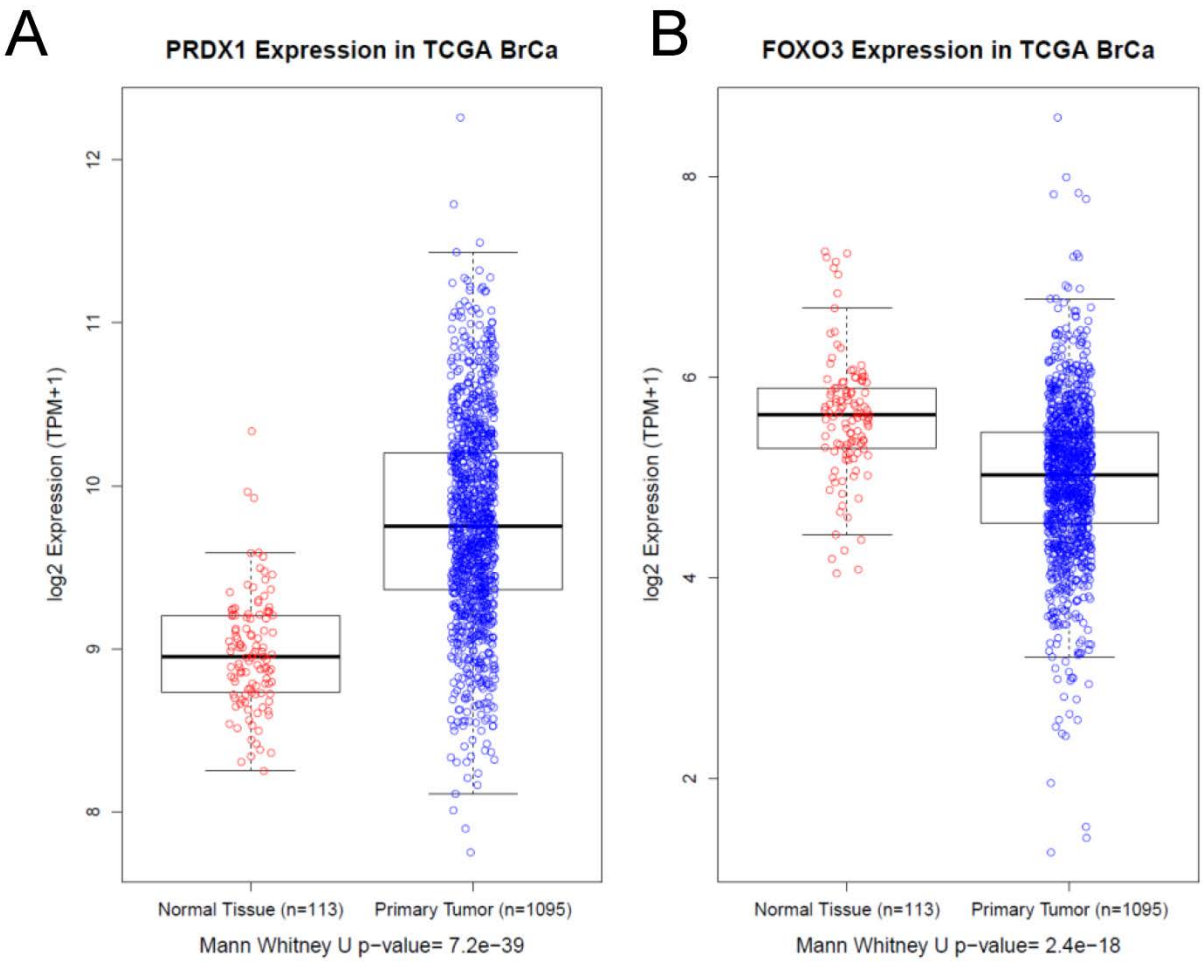

Figure 3.29: PRDX1 and FOXO3 expression in normal and breast cancer tissue.
(A) PRDX1 expression. **(B)** FOXO3 expression. Data from The Cancer Genome Atlas (TCGA). (Courtesy of K. Levine)

a significant negative correlation for PRDX1 and FOXO3 in this data set downloaded from GEO Accession: GSE62944 (172) (Figure 3.30), suggesting functional significance of our findings. This was further substantiated analyzing breast patient cancer survival in data sets from the Kaplan Meier Plotter (173) and the Gene Expression-based Outcome for Breast Cancer Online (GOBO) (174), which showed that lower expression of FOXO3 as well as higher expression of PRDX1 correlate with shortened patient survival (Figure 3.31).

FOXO3 suppresses cancer development in different ways including the inhibition of cell motility (175-180). In contrast, while PRDX1 prevents cancer initiation (87,181), its role in cancer is less understood. Interestingly, we have previously shown PRDX1-deficiency in MCF-10A, MCF-7 and MDA-MB-231 cells increased p38 activation (91), which induces FOXO3 nuclear localization in MCF-7 cells (106,182). We therefore hypothesized that inhibition of let-7b and let-7c would affect migration differently in breast cancer cells (MDA-MB-453 or MCF-7) with knockdown of PRDX1 (shPRDX1) (Figure 3.32) compared to control vector treatment. Intriguingly, cells with reduced PRDX1 showed a 30% increase in let-7 expression (Figure 3.33) and a 50% (MDA-MB-453) and a 30% (MCF-7) increased wound closure, respectively when transfected with single-stranded RNA oligonucleotide inhibitors that compete with let-7b and let-7c seed sequences (Figure 3.34). However, when compared with a negative control single-strand RNA oligonucleotide, we saw little to no wound closer between the control and the shPRDX1 cells. Altogether, these data demonstrate a PRDX1 regulated signaling axis exists in breast cancer and is required for FOXO3 and let-7 miRNA upregulation to suppress breast cancer cell migration.

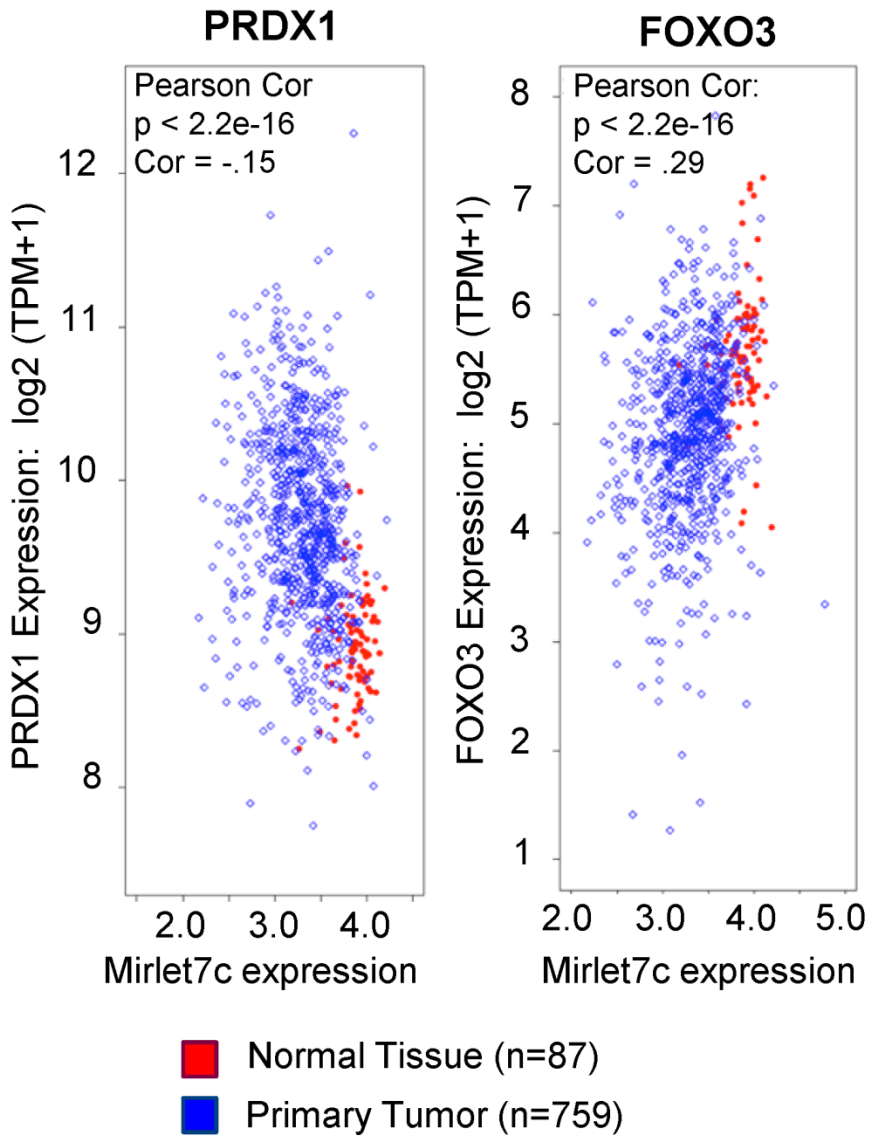

Figure 3.30: Comparing let-7c expression to PRDX1 and FOXO3 expression in normal versus breast cancerous tissues.
Expression of FOXO3 and PRDX1 from TCGA breast cancer cases and normal tissue was compared to Mirlet7c expression

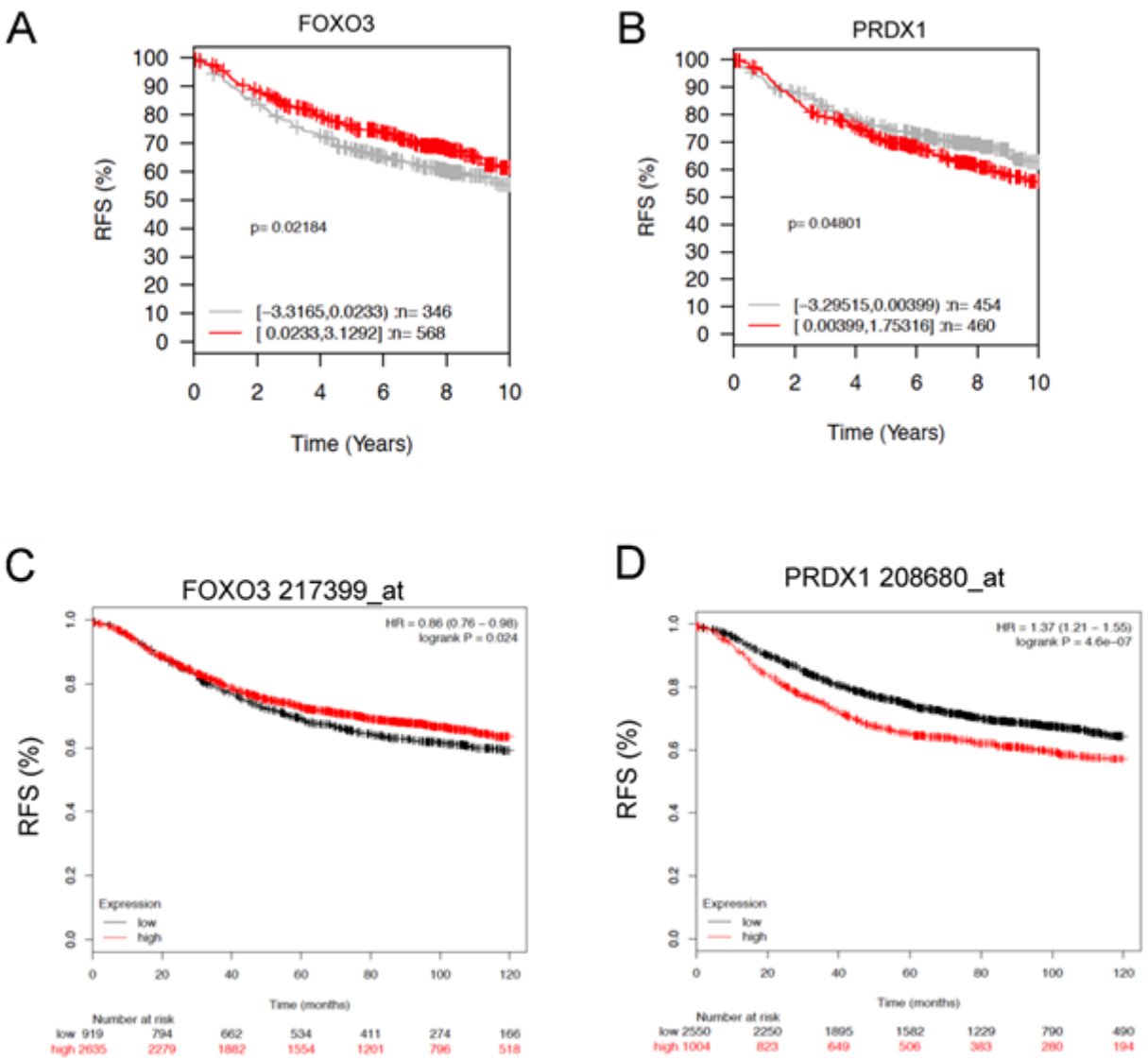

Figure 3.31: Patient survival versus PRDX1 and FOXO3 expression levels.
(A and B) Expression levels of FOXO3 and PRDX1 correlated with relapse free survival (RFS) of breast cancer patients from "Gene expression based Outcome for Breast Cancer Online (GOBO)" Fredlund E, *et al. Breast Cancer Research*. 2012;14(4):R113 **(C and D)** Expression levels of FOXO3 and PRDX1 correlated with relapse free survival (RFS) of breast cancer patients from "Kaplan Meier Plotter for Breast Cancer". Gyorffy B *et al. Breast Cancer Res Treatment.* 2010 Oct;123(3):725-31

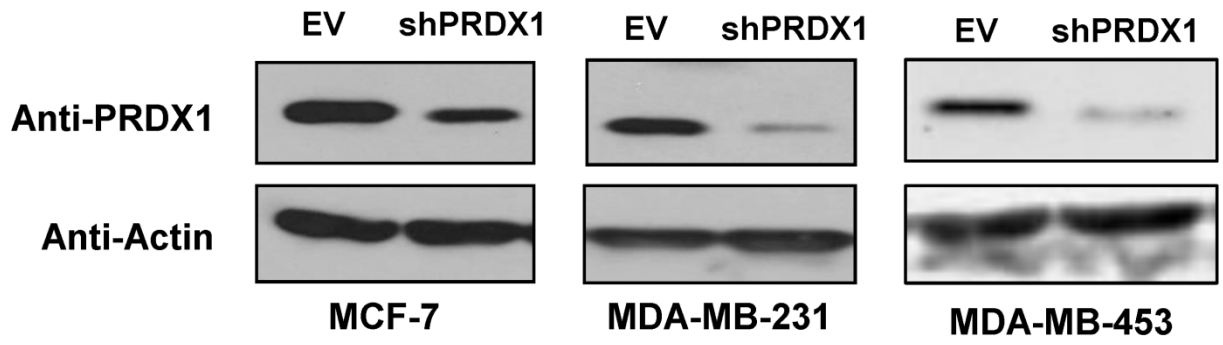

Figure 3.32: PRDX knockdown in breast cancer cell lines.
PRDX1 protein levels in MDA-MB-453 cells infected with PLKO1-shPRDX1 or EV control. Immunoblot with PRDX1 or Actin antibodies.

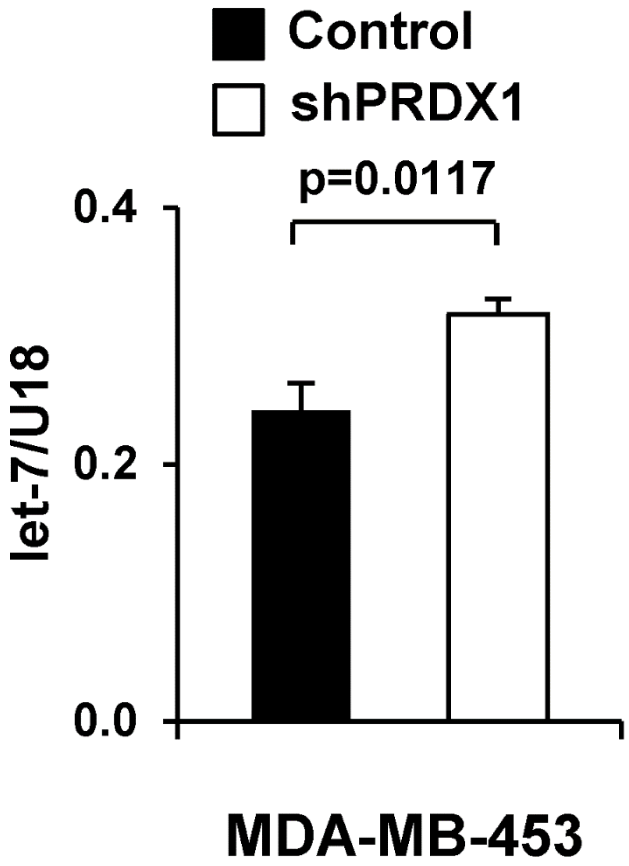

Figure 3.33: let-7 expression in PRDX1 knockdown in breast cancer cells.
EV or shPRDX1 MDA-MB-453 cells were harvested, lysed, and analyzed for miRNA expression by TaqMan assays using the delta C_T method with U18 as the internal standard (N= 3).

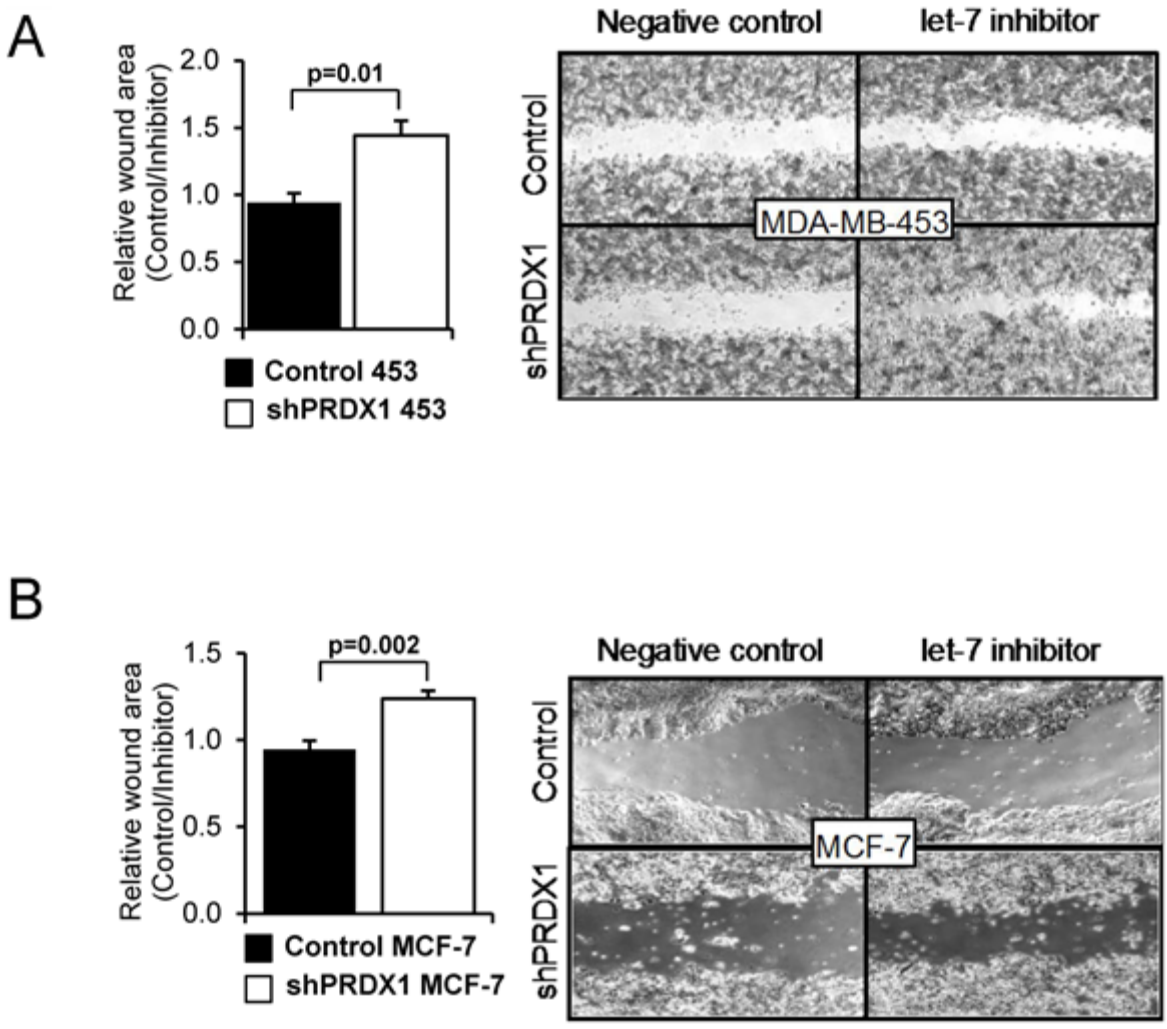

Figure 3.34: Wound healing increases following let-7 inhibition in PRDX1 knockdown breast cancer cells.

1 x 10^6 shPRDX1 **(A)** MDA-MB-453 or **(B)** MCF-7 (white bar) cells (white bar) or control cells (black bar) were transfected with a let-7 miRNA inhibitor in a 12-well plate and wound healing was assessed after 48 h (MDA-MB- 453) or 24 h (MCF-7) in the presence of mitomycin C (0.5 mg/ml). Wound area of let-7 inhibitor-treated cells was normalized to control miRNA inhibitor-treated cells (mean + SE) N= 4. Right side, representative photographs of wound healing assays.

3.5 DISCUSSION

H_2O_2 is an important second messenger in cell signaling, where it can directly induce oxidation of cysteine sulfhydryl groups in proteins thereby impacting protein activity and facilitate rapid signal transfer comparable to other post translational modifications (183). Thus oxidative equivalents can be passed on by H_2O_2-scavenging enzyme such as peroxidases (67). This reaction entails a transient disulfide exchange reaction between cysteine thiols, which requires close proximity of the peroxidase with a partnering protein (184). Peroxiredoxins have been suggested to form disulfide bridges with partnering proteins to pass oxidizing equivalents this way, thereby modulating protein activity of their binding partners (150,152). Our data suggests a similar mechanism between PRDX1 and the transcription factor FOXO3. As the disulfide exchange reaction is transient, capture of stable complexes by co-IP is low as demonstrated by weak PRDX1 staining FLAG-FOXO3 pull down experiments despite the high abundance of PRDX1 in cell lysate (Figures 3.2 and 3.3).

FOXO family members have been implicated in ROS signaling involving cysteine thiols. For example, a recent study showed p300 to form disulfide reactions with FOXO4 that result in its acetylation and nuclear localization (107). However, more examples are needed to fully understand specific roles of FOXO proteins in redox response. We present for the first time, evidence of an H_2O_2-dependent regulation of FOXO3 activity directly through the oxidative stress-signaling sensor PRDX1. Our data suggest the formation of a disulfide bond between a PRDX1-dimer and FOXO3 monomer involving the PRDX1 peroxidatic and resolving cysteines C52 and C173, respectively, as well as C71. In FOXO3, the FOXO3 C31 is a shared cysteine among FOXO family members, while C150 is unique to FOXO3, suggesting a redox regulation that is specific for FOXO3 (Figures 3.11-3.16). We further show the importance of PRDX1-

FOXO3 binding by demonstrating increased FOXO3 cytoplasmic sequestration (Figure 3.18) due to increased AKT phosphorylation of FOXO3 mutants impaired in PRDX1-binding after H_2O_2 treatment (Figures 3.19-3.21). However, a further delineation of the FOXO-PRDX1 signaling pathway is warranted to determine the role that other family members may play, as a recent proteomic profiling report found PRDX1, 2 and 5 are cysteine-dependent binding partners of FOXO3 and suggest competition among PRDX family members may be present for FOXO3 binding (153).

We show the PRDX1-FOXO3 complex that is formed in the presence of elevated H_2O_2, is a cysteine-dependent oligomer. A unique property to the PRDX1-FOXO3 axis is the utilization of C150 of FOXO3, which is exclusive to FOXO3, suggesting specificity of the PRDX1-FOXO3 interaction. Mutation of C31 or C150 inhibits FOXO3 complex formation with PRDX1 and renders these FOXO3 mutants unresponsive to oxidative insult, exhibiting cytoplasmic accumulation, which suggests PRDX1's binding of FOXO3 via disulfide bonds is critical for regulating FOXO3 in response to oxidative stress (Figures 3.11-3.21). Luciferase reporter assays indicated FOXO3 becomes unresponsive to PRDX1-induced control following treatment with high doses of H_2O_2 (Figure 3.16), suggesting over-oxidation of PRDX1 impairs FOXO3 binding and may also allow FOXO3 post-translational modifications, to induce nuclear translocation (Figure 3.8). FOXO3 C31 or C150 mutants (Figure 3.21) were unresponsive to PRDX1 regulation, but surprisingly correlated with cytosolic sequestration under H_2O_2-induced stress (Figure 3.18) compared to FOXO3WT. To determine why FOXO3 C31S and C150S mutants could not bind PRDX1, but were sequestered from the nucleus (Figure 3.18), the phosphorylation status of FOXO3 T32 and S318, which represses FOXO3 through AKT and SGK signaling (154), were probed. Interestingly, wild-type FOXO3 showed an H_2O_2 dependent

increase in T32 phosphorylation, however, FOXO3 cysteine mutants displayed much higher phosphorylation. FOXO3C31S showed an overall higher phosphorylation of T32, while FOXO3C150 T32 phosphorylation was higher than FOXO3WT but not as responsive to H_2O_2 stimulation (Fig. 3B). Analysis of S318 phosphorylation, however showed no difference between wild-type FOXO3 and the FOXO3 cysteine mutants (Figure 3.19B) suggesting that PRDX1 binding to FOXO3 is site specific, and calibrates T32 phosphorylation, but not S318.

FOXO3 nuclear localization is regulated by AKT and SGK phosphorylation inducing 14-3-3 binding and cytoplasmic sequestration or kinases that induce nuclear localization. Candidate kinases include MST1, JNK or p38 (106,185) (Figure 3.35) where MST1 can induce nuclear localization of an AKT phosphorylated FOXO3 protein (82). Interestingly, all three kinases are subject to PRDX1 binding and subsequent regulation, indicating another layer of control in the PRDX1/FOXO3/let-7c axis that requires future analysis. We suspect that under high oxidative stress, MST1, an essential activator of FOXO and repressor of AKT (82,186), is activated by PRDX1 decamers (formed by ten over-oxidized PRDX1 proteins) (79) under higher oxidative stress causing phosphorylation of FOXO3 on S207. This suggests FOXO3 nuclear localization under H_2O_2-induced stress may increase in the presence of decameric (over-oxidized) PRDX1. This is in alignment with our findings that PRDX1 binding to FOXO3 does not include over-oxidized PRDX1 protein (Figure 3.5) and is dynamic as it decreases after 30 min in H_2O_2 treated cells (Figure 3.3). On the other hand, kinase activity of JNK and p38, are inhibited by PRDX1, perhaps accounting for the increased localization of FOXO3 in the nucleus in PRDX1-deficient cells compared to PRDX1 proficient cells (Figures 3.6 and 3.8). This suggests different roles for PRDX1 on FOXO3 signaling and needs further exploration in future studies. Further exploring

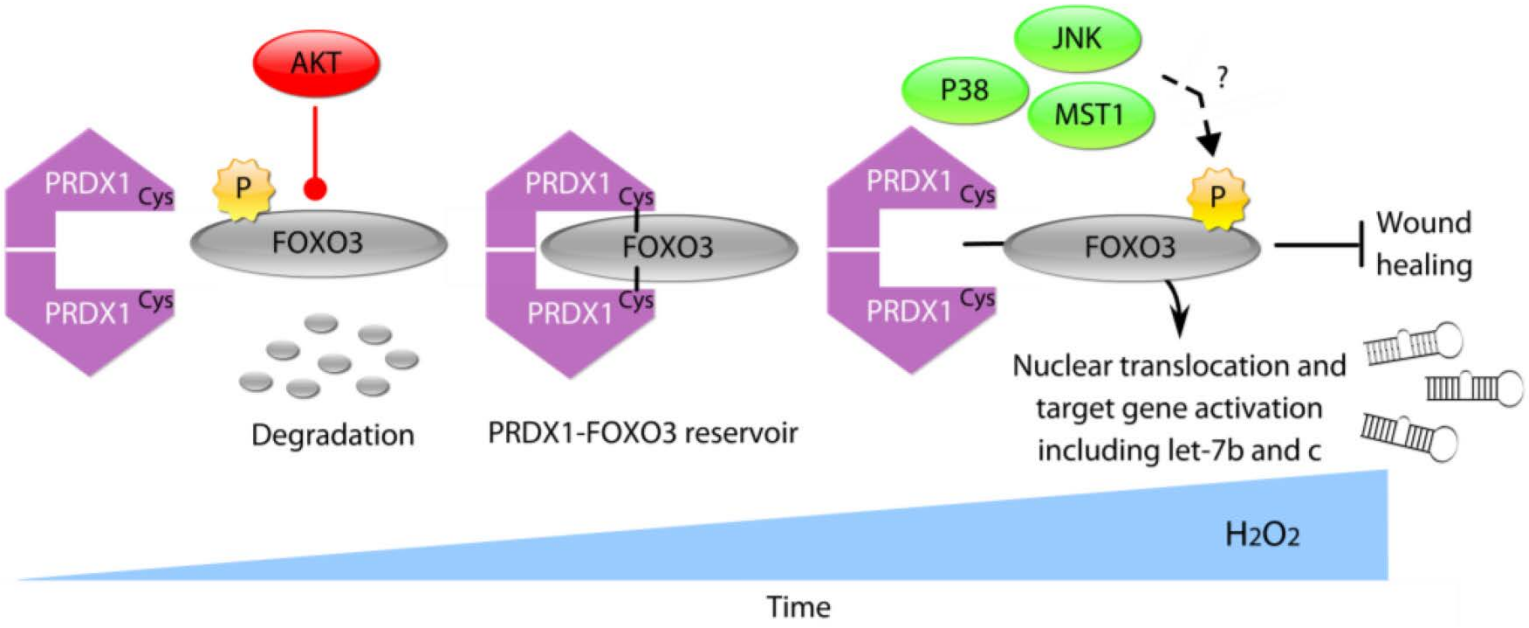

Figure 3.35: Model: Stepwise oxidation of PRDX1 regulates FOXO3 under oxidative stress. PRDX1 and FOXO3 interact in an oxidative stress-dependent way. This involves the catalytic/peroxidatic cysteine and C71 of PRDX1 and C31 and C150 of FOXO3. Our data strongly suggest disulfide bonding between PRDX1 and FOXO3 involving PRDX1 C52, C71, and C173 and FOXO3 C31 and C150 regulating the AKT-induced phosphorylation of T32 on FOXO3 and 14-3-3 binding and dissociation. Nuclear localization and 14-3-3 dissociation of FOXO3 may be promoted by monoubiquitination or phosphorylation by over-oxidized PRDX1 activating MST1 or oxidative stress activation of JNK or p38. This results in expression in let-7b and let-7c and inhibition of migration.

our findings that PRDX1 binding to FOXO3 calibrates FOXO3 nuclear translocation through modulating AKT phosphorylation, we demonstrate that PI3K inhibition rescued translocation of C31S and C150S mutant FOXO3 to FOXOWT levels following H_2O_2 exposure (Figure 3.21).

Collectively, these data suggest an oxidative stress dependent mechanism of FOXO3 cytoplasmic loading, a protection and release that is directed through PRDX1 by binding to FOXO3 and coordinating its post-translational modifications (Figure 3.35). Accordingly, PRDX1-dependent protection of FOXO3 from repressive phosphorylation and degradation pathways would enable a FOXO3 reservoir not reliant on *de novo* synthesis of FOXO3 or T32 dephosphorylation. The PRDX1-FOXO3 axis therefore is more than simply an inhibitory pathway, but rather, a redox-dependent signaling modulator enabling controlled bursts of FOXO3 nuclear translocation and gene expression. Based on our data shown here, we propose that PRDX1 builds a stepwise and redox-dependent FOXO3 cytoplasmic reservoir that is readily available once high H_2O_2 levels stimulate FOXO3 nuclear function.

miRNAs are direct targets of transcription factors that are known as important tumor suppressors or oncogenic proteins. Yet, remarkably little is understood of miRNAs that are responsive to FOXOs. Consensus FOXO DNA binding motifs are present in the 5' UTR of let-7b and let-7c in several species (Figure 3.22). Interestingly, let-7b and let-7c-2 in *mus musculus* and *rattus norvegicus* are located in tandem and separated by 621 and 319 nucleotides, respectively. Our results establish for the first time that let-7b and let-7c are up-regulated by FOXO3 (Figures 3.24-3.27) directly to both of let-7c promoter regions (the distal host gene and the proximal intronic promoter) considered functional in let-7c transcription (147) (Figure 3.26). In addition, FOXO3 promoter binding is enhanced following 100 μM H_2O_2 treatment as compared to no treatment (Figure 3.26). These data define a new facet of FOXO3-mediated

responses to oxidative stress, as our findings are consistent with prior reports that show let-7 members are upregulated by ionizing radiation, genotoxic stress and peroxide (158,159). Intriguingly, we identified a novel FOXO3 target with direct tumor suppressive activity, as let-7 miRNAs bind to 3' UTRs of target oncogenes and cell cycle regulators and that reduced let-7 expression has been shown to correlate with the development of cancers (187).

Systemic deletion of FOXOs results in cancer in murine models *in vivo*, underscoring their role as bona fide tumor suppressors (188). Additionally, inactivating genetic mutations and reduced expression of FOXOs occur in human cancers (110,189) and suppression of FOXO function is critical in promoting evasion of apoptosis and is a significant occurrence in several hematological malignancies including Bcr-Abl+ leukemia (154,190). Notably, a more specific role has been identified for FOXO3 as an inhibitor of cancer cell motility (178,180) and wound healing (191), suggesting that FOXO3 may regulate cell motility.

Similar to FOXOs, PRDX1 protects from tumor initiation (87), however, in contrast to FOXO3, several reports indicate poor prognosis for cancers with high expression of PRDX1 (192-194). This is in line with our TCGA and survival analyses showing PRDX1 expression elevated in breast cancer patients with decreased survival (Figures 3.30 and 3.31). Importantly, these data support our findings that a PRDX1-FOXO3-let-7 axis exists in tumorigenesis. Furthermore, PRDX1-deficient MDA-MB-453 cells showed increased levels of let-7c (Figure 3.33) and let-7 inhibition increased cell motility of MDA-MB-453 and MCF-7 breast cancer cells, both of which show low motility (195), with decreased PRDX1 expression (Figure 3.34). This finding is consistent with earlier studies showing that let-7 miRNAs inhibit motility of breast cancer cells by regulating genes in the actin cytoskeleton pathway (196). Given the findings of these studies and those presented here, it would be interesting in future studies to

determine let-7 miRNA targets that are controlled by PRDX1 and FOXO3 in cancer development.

The free radical theory of aging posits that accumulated damage from oxidative events shortens lifespan. The fundamental importance of PRDX1 in protecting from the deleterious effects of oxidative stress is best illustrated by the multiple cancers and shortened lifespan in *Prdx1*-deficient mice (87). Moreover, hyperactivation of FOXO proteins have been implicated in extending life-span (197). Our data suggest that FOXO3 fine-tunes cellular homeostasis in response to cellular redox through integration with PRDX1. Because ROS are fundamental in driving the aging process and in the development of cancers, this new mechanism we describe here adds to our understanding of how the evolutionarily conserved FOXO C31 in conjunction with C150 may be instrumental in the regulation of aging and tumor suppression.

4.0 CONCLUSIONS

4.1 SUMMARY

Within the past decade, it has been found that, in addition to mediating the response to oxidative stress, the activity of several FOXO family members is also regulated by ROS signaling (82,93,107,108,162,198,199). This dissertation presents for the first time, evidence of a ROS-dependent regulatory pathway of FOXO3 activity through the oxidative stress signaling sensor PRDX1. In response to H_2O_2, covalent disulfide bonds were formed between dimeric PRDX1 and FOXO3. The formation of this complex controlled transcriptional activation of FOXO3 through cytoplasmic sequestration. FOXO3 nuclear translocation and target gene activation had a concentration dependent response to H_2O_2, where inhibition of FOXO3 activity at low levels of stress could be overcome at higher levels. Upon investigation into the mechanistic of such an association, it was found that following H_2O_2 treatment, cysteine mutant FOXO3 proteins were unable to bind PRDX1, and that nuclear translocation and gene expression were lower when compared to those of WT FOXO3. In addition, this work identifies let-7 miRNAs as novel FOXO3 transcriptional targets, regulated in response to H_2O_2. Altogether, the studies presented here reveal a signaling pathway that enables PRDX1 to balance FOXO3 activity in response to a gradient of oxidative stress in the form of H_2O_2.

4.1.1 A model for how PRDX1 fine-tunes FOXO3 activity under oxidative stress

The cysteine mutagenesis studies carried out establish that the peroxidatic cysteine (C52) and C71 of PRDX1 bind FOXO3 in response to oxidative stress. The data suggests thiol-disulfide exchange reactions occur between PRDX1 and FOXO3 involving dimeric PRDX1 C52, C173, and C71, which has not been previously described as participating in cell signaling, and FOXO3 C31 and C150. Under increasing oxidative stress, we propose that the catalytic cysteines of the PRDX1 dimer form a fast forming and transient inter-disulfide bond with both C31 and C150 of FOXO3. First described in yeast, catalytic mechanisms for protein thiol oxidation involve the transfer of oxidative equivalents directly from a thiol peroxidase to a specific target protein through direct protein-protein contact (200). Recently, PRDX2 was found to act as a H_2O_2 signal receptor and transmitter for STAT3, forming a redox relay between the two proteins. Upon H_2O_2 stimulation, a disulfide forms between PRDX2 and STAT3, transferring oxidation equivalents from PRDX2 to STAT3. The disulfide bond formation is shortly followed by the formation of STAT3 oxidation products, disulfide linked STAT3 dimers and tetramers, suggesting that it is the interaction with PRDX2 that triggers STAT3's subsequent oxidation (152). A similar mechanism was previously described between PRDX1 and ASK1 (150), lending support to our proposed PRDX1-FOXO3 interaction.

In addition, the covalent redox-controlled interaction between FOXO3 and PRDX1 regulates FOXO3 localization through decreasing AKT FOXO3 phosphorylation, documenting the interplay between two different post-translational modifications. FOXO3 C31 is adjacent to FOXO3's AKT phosphorylation site, T32, and the data from phosphorylation experiments show that FOXO3 C31 and C150 are highly phosphorylated at this as compared to WT FOXO3. The cysteine mutants also show no real response to changes in H_2O_2 levels. These results, in

conjunction with localization studies, lead us to conclude that FOXO3 C31 and C150 remain primarily sequestered in the cytosol, and inactive.

Under high levels of H_2O_2 14-3-3, a chaperone protein involved in cytosolic sequestration, dissociates from FOXO3, and similarly, FOXO3 translocates into the nucleus. It is possible that after a certain period of time, over-oxidation of the PRDX1 catalytic cysteine is responsible for this, releasing FOXO3 or perhaps triggering a confirmation change that allows nuclear localization kinases to phosphorylate FOXO3. This possibility has been suggested for the MST1 and PRDX1 interaction, as high oxidative stress (H_2O_2) facilitates PRDX1 decamers due to overoxidation of its catalytic cysteine, which is required to activate MST1 (79). As MST1 phosphorylation of FOXO3 leads to FOXO3 re-entering the nucleus it suggests that PRDX1 has a complex role in FOXO3 regulation, which suggests PRDX1-regulation of more than one signaling protein in the FOXO3 cascade. Clearly, further studies are needed to properly investigate this possibility.

4.1.2 The miRNA family let-7 identified as novel FOXO3 transactional targets

miRNAs are direct targets of transcription factors, and are known to be both important tumor suppressors or oncogenic proteins. Even with miRNAs importance known, little is still understood of miRNAs that are responsive to FOXOs. Consensus FOXO DNA binding motifs are found conserved in the 5’ UTR of the breast cancer associated miRNAs, let-7b and let-7c in several species. Discovered in *C.* elegans, the let-7 family of miRNAs were first identified as key regulators of developmental timing, and are highly conserved across species (201). This research establishes for the first time, that FOXO3 upregulates the expression of both let-7b and let-7c, defining a new FOXO3 target, and a facet of its responses to oxidative stress. Moreover, these

results demonstrate that let-7b and let-7c expression is enhanced in response to H_2O_2 in a FOXO3-dependent fashion, with ChIP assays showing that H_2O_2 triggers FOXO3's binding to the let-7c promoter region, findings that are consistent with previous investigations into the effect of stressors such as ionizing radiation, genotoxic stress and peroxide have on let-7 family member expression (158,159). Given that FOXOs integrate numerous cellular signals and serve as homeostatic regulators (154), the FOXO3-dependent upregulation of let-7 miRNAs in response to oxidative stress provides a founding example of the mechanistic link between FOXOs and let-7 miRNAs.

Finally, our investigations into PRDX1's effect on let-7 expression reveal let-7b and let-7c expression is enhanced in the absence of PRDXl. Additionally, let-7 expression increases in PRDX1 knockdown breast cancer cell, along with an observed decrease in cell motility and invasion. Taken together, these findings point towards the existence of a PRDX1-FOXO3-let-7 axis in tumorigenesis. PRDX1 sequesters FOXO3 in the cytosol for a period of time, and then upon release, FOXO3 translocate to the nucleus where it can to trigger the transcription of the let-7 miRNAs, and finally the newly transcribed let-7 miRNA are able to inhibit cancer cell motility and invasion, decreasing cancer metastasis (202).

Our studies also expand upon the current knowledge that regulation of let-7 family members have been shown to be p53-dependent (158), providing another example of the functional overlap between p53 and FOXOs as tumor suppressors. let-7 miRNAs play an important role in regulating cell growth by binding to 3' UTRs of target oncogenes and cell cycle regulators. In addition, down-regulation of let-7 expression has been shown to correlate with the development of cancers (187). Consequently, let-7 miRNAs are largely viewed as tumor

suppressor miRNAs, playing important roles in inhibition of metastasis and cancer stemness, particularly seen in breast cancer (202).

4.2 FUTURE DIRECTIONS

The research presented here is only one piece of the puzzle, and more studies are necessary to understand the role of redox signaling in transcription factor control and cancer progression. We have established that PRDX1 plays an important role in regulating FOXO3's activity though cytosolic sequestration both by binding to FOXO3 and modifying FOXO3 phosphorylation by AKT. However, has discussed previously, PRDX1 also regulates the kinases MST1, JNK, and p38a, all of which are involved in the nuclear import of FOXO3, (Figure 4.1) (79,80,82,92,105,106,203). Taken together, an examination of this possible relationship between PRDX1, the nuclear entry kinases, and FOXO3, could give us a deeper understanding of FOXO3's regulation, and how it's activity is controlled.

To elucidate the role of PRDX1 in the FOXO3 signaling cascade in more detail, a series of FOXO3 kinase mutants can be used to study the proposed dynamic. For example, each kinase phosphorylation site (S7 for p38a, S209 for MST1, and S574 for JNK) will be mutated to either an aspartic acid as a gain-of-function mutation, or an alanine as a loss-of-function mutation, creating eight different mutants with various configurations of gain-of-function or loss-of-function. The kinase mutant will then be expressed in a tetracycline-inducible FOXO3 system in FOXO triple knockdown cells (204), followed by various H_2O_2 treatments (time and concentration). Differences in subcellular localization can then be analyze via western blotting

and immunofluorescence (IF), and changes in target specificity or expression will be determined through quantitative RT-qPCR (Figure 4.2).

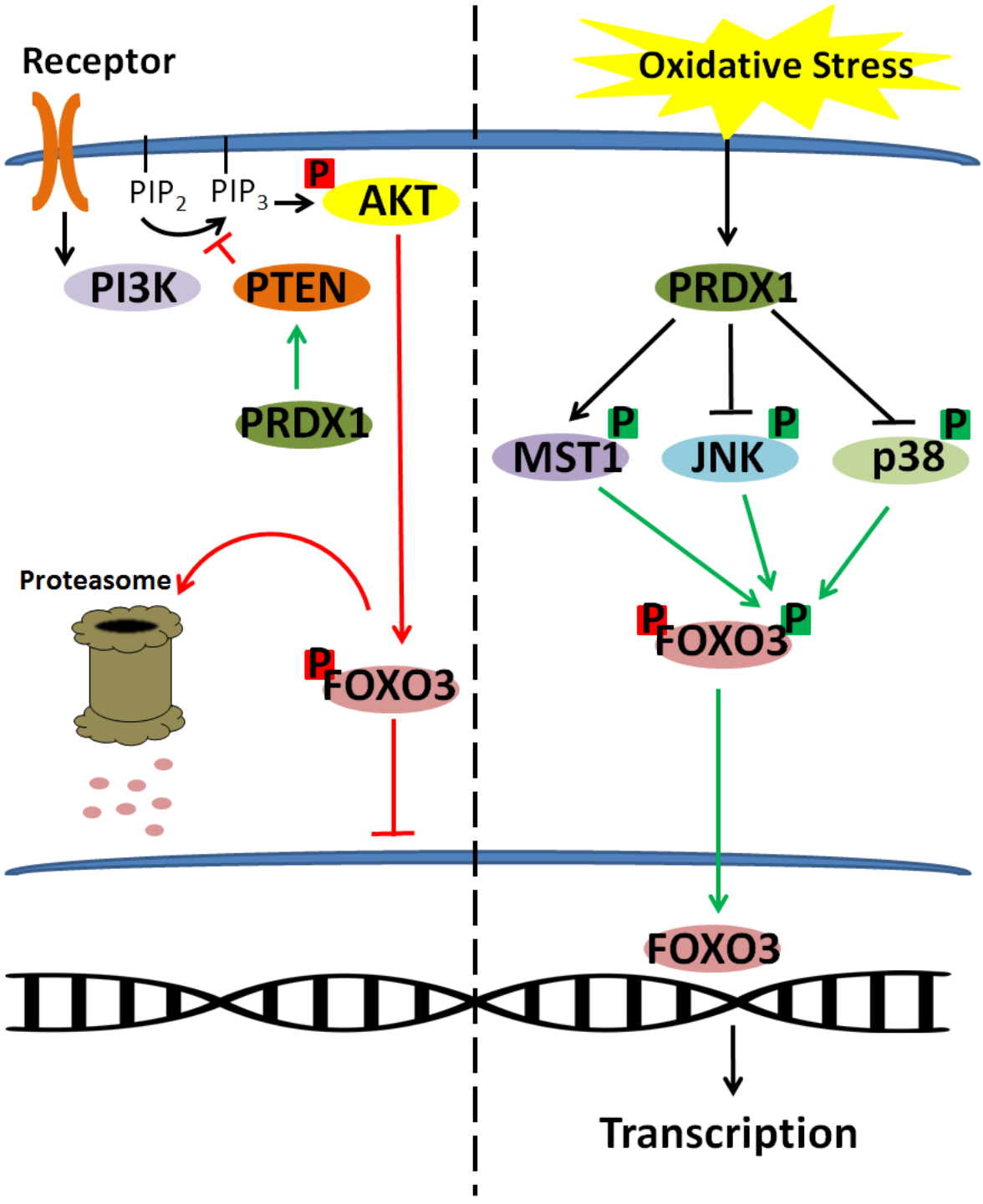

Figure 4.1: PRDX1 promotes FOXO3 localization and differentially drives transcription of gene targets, in a redox dependent manner.

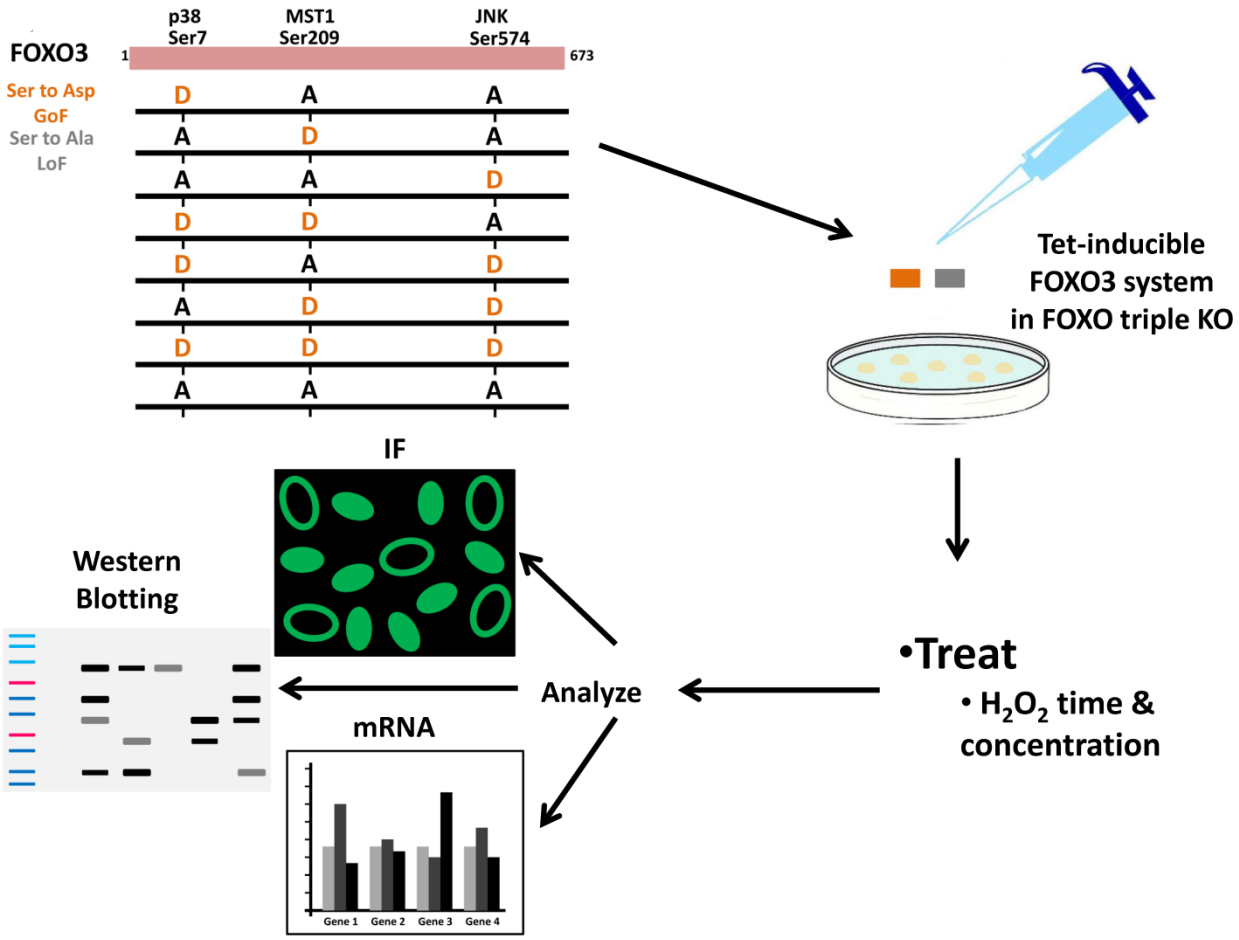

Figure 4.2: Experimental design flowchart.

This analysis, will be then combined with experiments analyzing the activities if FOXO3 phosphorylating kinases that are regulated by PRDX1: p38α, JNK and MST1. Since the oxidation of PRDX1's catalytic cysteine is dependent on pro-oxidants and regulates signal transduction of PRDX1 binding partners, we should expect that under increasing oxidative stress PRDX1 is regulating each kinase differently. With this in mind, we then predict that this will translate into different dynamics in FOXO3 phosphorylation by MST1, JNK and p38a and may

therefore promote transcription of different FOXO3 targets differentially. Along those lines, phosphorylation of different sites on FOXO3 should alter target gene specificity. The studies can also be extended to include other post-translational FOXO3 modifications such as phosphorylation by AKT, mono-ubiquitination and acetylation, which have both been shown to affect the nuclear translocation of FOXO family members (96,107). In addition, FOXO3 dephosphorylation by phosphatases may be considered, since protein phosphatase 2A (PP2A) has been show to dephosphorylate FOXO3 at S209, reversing the action of MST1 (188).

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