Genetic Characterization of ABL1 Inhibition and Regulation by the Tumor Suppressor PRDX1

A dissertation

submitted by

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ABSTRACT

The first portion of this thesis serves to further characterize the roles of PRDX1, mainly focusing on ABL1 regulation and oxidative stress. Our laboratory first discovered PRDX1 physically binds to c-ABL1 and suppresses its kinase activity. However, the interaction is not well defined. We found imatinib-resistant ABL1 kinase mutations, which are commonly seen in BCR-ABL1 of CML patients, were also resistant to PRDX1 suppression. Other studies in our lab also demonstrated that mice lacking *Prdx1* acquire various types of age-dependent cancers. A growth delay was observed in primary *Prdx1*$^{-/-}$ MEFs, so we exploited this observation, as it may influence oncogenesis. We found that PRDX1 antioxidant activity plays a role in maintaining cell survival. In the absence of PRDX1, oxidative stress seems to contribute to the growth delay, and possibly leads to a cancerous phenotype.

In an effort to better understand BCR-ABL1 regulation and signaling, the second portion of this thesis focuses on PRDX1 activity and its possible functional role in BCR-ABL1-mediated transformation and leukemogenesis. BCR-ABL1 is a constitutively active tyrosine kinase known to cause chronic myeloid leukemia (CML) and, in some instances, acute B lymphoblastic leukemia (B-ALL). As the biomedical community strives to find better chemotherapeutic drugs to target and eliminate leukemic cells, it is of great importance to also understand what leads to leukemia development and progression at the genetic and molecular levels. Although there are currently

extensive studies attempting to understand the signaling mechanisms elicited by BCR-ABL1, the disease is still not completely understood.

Because PRDX1 was previously found to partially inhibit BCR-ABL1 kinase activity, we hypothesized it may be a candidate protein regulating leukemic transformation and leukemogenesis. Our *in vitro* data show that in the absence of *Prdx1*, BCR-ABL1 tyrosine kinase activity is elevated, leading to enhanced transformation potential, an increase in cell survival and proliferation, and stimulation of malignant growth within the lymphoid lineage. Loss of *Prdx1* also initiates disease development at a slightly faster rate in our B-ALL murine model system. Other experiments also prove PRDX1 regulation plays a role in myeloid transformation and leukemogenesis. Bone marrow cells lacking *Prdx1* show enhanced BCR-ABL1-mediated myeloid transformation. In addition, BCR-ABL1, in the absence of *Prdx1*, elicits a more aggressive leukemia phenotype in the CML murine model system. Together, the results presented in this thesis demonstrate that PRDX1 functions to suppress cell transformation and hematological malignancies induced by BCR-ABL1.

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CHAPTER 1: THESIS INTRODUCTION

THESIS BACKGROUND

The main purpose of this thesis is to provide a functional characterization of PRDX1 in oxidative stress and ABL1 regulation. We focused on the antioxidant enzymatic activity of PRDX1 and the inhibitory effect it has on ABL1 kinase activity. We also did studies to address the interaction of PRDX1 with the leukemogenic tyrosine kinase BCR-ABL1 through *in vitro* and *in vivo* genetic approaches. In this introduction, current knowledge of the biology, history, and functions of ABL1 and of peroxiredoxins will be reviewed. The role of BCR-ABL1 in the pathophysiology of Philadelphia-positive human leukemias and how it translates into a mouse model system will also be addressed.

c-ABL1: A non-receptor tyrosine kinase

Protein kinases are important in various signaling pathways, as they act to transduce crucial signals to substrates that influence a cell's fate. The main function of a kinase is to transfer a phosphate group from ATP to serine, threonine, or tyrosine residues within the respective substrate. Over 500 different kinases have been identified in the human genome. Consequently, the regulation of these proteins transmitting important information is a necessary task, as researchers have shown that de-regulation of kinase activity is a hallmark of many cancers (Nagar 2007).

Out of the 500 or so protein kinases found, about ninety are tyrosine kinases. Tyrosine kinases are broken down into two classes: receptor kinases, which are transmembrane proteins with a cytoplasmic tyrosine kinase domain, and non-receptor kinases, which variously can be found at the membrane, in the cytoplasm, and in the nucleus. c-ABL1, a non-receptor tyrosine kinase, has been found to play a role in diverse cellular processes, such as cell differentiation, cell division, DNA damage, DNA repair, and apoptosis (Nagar 2007).

c-ABL1 Function and Primary Structure

Currently, the physiological function of c-ABL1 remains unknown. However, researchers have shown it is highly expressed in the thymus, spleen, and testes (Tybulewicz, Crawford et al. 1991). Disruption of c-ABL1 function in mice leads to abnormal fetal development, runted growth with head and eye defects, infertility, and a defective lymphoid system. Although intensively studied, the mechanism of regulation of c-ABL1 kinase activity is not well understood. More information is known about the transforming variants of c-ABL1 that turn c-ABL1 into an oncoprotein. A prime example is the fusion of *ABL1* with *BCR*, leading to the production of the BCR-ABL1 protein responsible for causing chronic myeloid leukemia. c-ABL1 variants with oncogenic properties generally produce tumors of the hematopoietic system. An experiment to demonstrate the importance of c-ABL1 in

hematopoiesis was conducted by expressing a *BCR-ABL1* transgene under a non-tissue-specific promoter in mice. It was shown that even though BCR-ABL1 was present in all tissues, mice only developed leukemia (Heisterkamp, Jenster et al. 1990; Tybulewicz, Crawford et al. 1991).

c-ABL1 is well conserved among various species. Homologs of human c-ABL1 exist in the mouse (c-ABL1), sea urchin (E-Abl), fruit fly (D-Abl), and nematode (N-Abl) (Kharbanda, Yuan et al. 1998). In mammals, c-ABL1 exists as two forms: human type 1a (mouse type I) and human type 1b (mouse type IV). These two forms are expressed from distinct promoters, leading to a variance at the mRNA and protein level by virtue of different first coding exons. The resulting polypeptides differ only at their extreme N-termini, where only type 1b contains a myristoyl modification and a few additional residues (Figure 1B) (Kharbanda, Yuan et al. 1998; Van Etten 1999).

The human *c-ABL1* gene is found on chromosome 9q34 and is compromised of 11 exons. The c-ABL1 N-terminus is similar to sarcoma (Src) kinases, another class of tyrosine kinases, which are involved in cell growth regulation (Figure 1A). At the N-terminus, c-ABL1 contains the fairly conserved regions known as the Src homology 1 (SH1), also referred to as the kinase or catalytic domain, the SH2 domain, and the SH3 domain (Wen and Van Etten 1997). The catalytic domain is necessary for c-ABL1 to function as a kinase and transfer phosphate groups to substrates. The SH2 domain is important for recognition and binding to substrates with

phosphorylated tyrosine residues. The SH3 domain is important for binding proline-rich ligands. Specificity can be acquired by the interactions between non-conserved SH3 residues as well as non-proline residues in the ligand (Van Etten, Debnath et al. 1995). What sets c-ABL1 apart from the rest of the Src kinases, though, is its C-terminus, which is encoded by a single exon. Defined domains within the C-terminus include three nuclear localization signals (NLS), one nuclear export signal (NES), a DNA-binding domain, proline-rich sequences that bind to SH3-containing proteins, and F- and G-actin binding domains (Wen and Van Etten 1997).

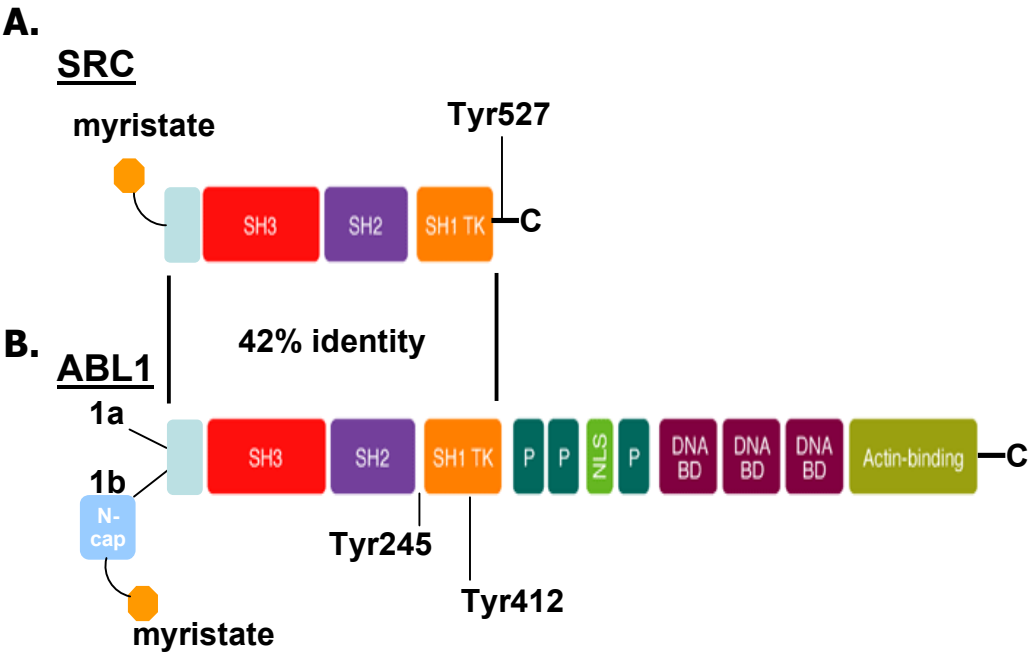


Figure 1: Primary Structures of c-ABL1 and SRC.

The N-terminus of c-ABL1 (**B**) is very similar to a typical Sarcoma (SRC) kinase (**A**), possessing the SRC homology domains SH3, SH2, and SH1 (also known as the tyrosine kinase (TK) domain). The C-terminus of c-ABL1 sets it apart from SRC kinases, as it contains various additional domains. There are two different mammalian forms of c-ABL1: human 1a (mouse type I) and human 1b (mouse type IV). Only c-ABL1b encodes for the "N-cap" residues and contains a myristoyl group (myristate). The tyrosine residues identified are phosphorylated sites involved in maintaining an autoinhibitory state (Tyr527 in SRC) or inducing kinase activation (Tyr245 and Tyr412 in c-ABL1). Adapted from Smith, Burthem et al. 2003.

c-ABL1 Localization

In fibroblasts, c-ABL1 mainly localizes to the nucleus. However, in primary human hematopoietic cells and neurons, c-ABL1 is observed largely in the cytoplasm rather than in the nucleus. In general, type 1a is mostly cytoplasmic, and type 1b is mainly found in the nucleus. Type 1b, however, contains a myristoyl group on an N-terminal glycine residue that can target it to the plasma membrane. c-ABL1 localization is an important factor in determining its function. Nuclear c-ABL1 mainly activates signals that lead to a G_1 arrest or apoptosis in response to DNA damage; cytoplasmic c-ABL1 promotes cell survival; c-ABL1 at the membrane seems to be involved in cell adhesion (Wetzler, Talpaz et al. 1993). Domains in the C-terminus play a role in localization, as NLS sequences and the DNA-binding domain target c-ABL1 to the nucleus and to DNA, respectively, and the NES sequence and the F- and G-actin domains target c-ABL1 to the cytoplasm and actin filaments, respectively (Wetzler, Talpaz et al. 1993; Taagepera, McDonald et al. 1998).

It is noteworthy to mention that all c-ABL1 mutants with transforming ability are found exclusively in the cytoplasm (Shaul and Ben-Yehoyada 2005). For example, the c-ABL1 variant BCR-ABL1 localizes only to the cytoplasm and promotes cell survival. This exclusive cytoplasmic localization, regardless of BCR-ABL1 containing three nuclear localization signals, seems to

be crucial for survival, as forcing and trapping BCR-ABL1 in the nucleus leads to apoptosis (Yoshida, Yamaguchi et al. 2005).

c-ABL1 Regulation and Inhibition

c-ABL1 is normally found in the inactive state (Shaul and Ben-Yehoyada 2005). Consequently, much research has gone into determining how c-ABL1 is kept in the inactive state. Recent work has found that c-ABL1 is capable of autoinhibition. The myristoyl group and the first eighty amino acids from the N-terminus, found only in c-ABL1 type 1b, have been proposed to play a role in c-ABL1 regulation. One group has reported that an "N-cap" is crucial for stabilizing the autoinhibitory state of c-ABL1, as removal of it leads to deregulation. In this autoinhibitory state, the N-cap interacts with the SH3/SH2 domains, forming a clasp, and the myristoyl group interacts with the C-lobe of the catalytic domain (refer to Figure 2) (Van Etten 2004; Nagar 2007). However, it is unclear how important the myristoyl group and the N-cap are because several c-ABL1 mutants as well as the c-ABL1 1a isoform that lack both the myristoyl group and the N-cap are still capable of self-regulation (Van Etten 2004).

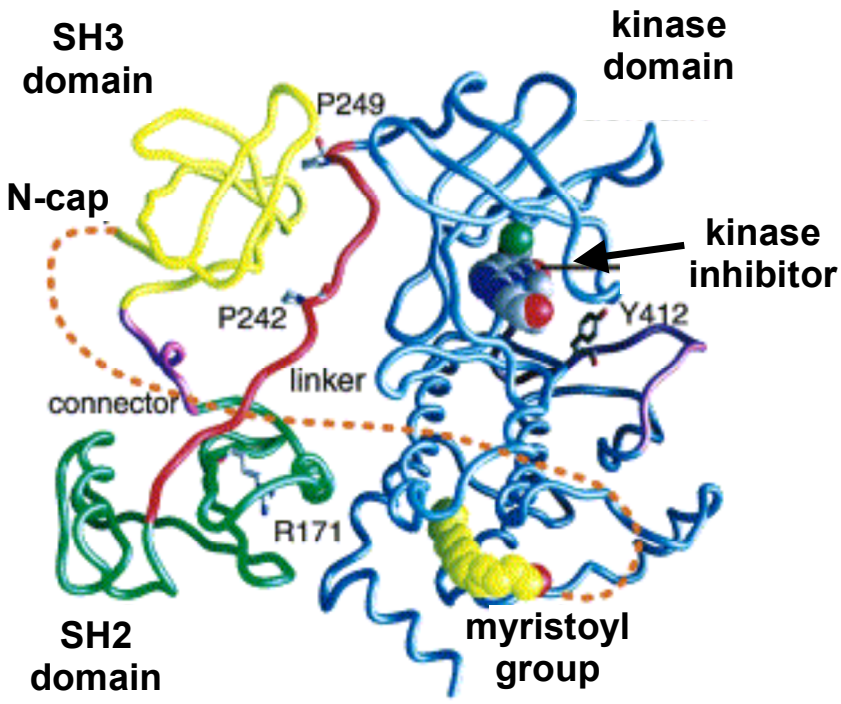


Figure 2: Crystal Structure of myristoylated c-ABL1b.

The c-ABL1b N-terminus is shown in an inhibitory form. A kinase inhibitor (PD16) is used to stabilize the inhibitory state. The dotted line signifies the "N-cap" residues. The C-terminus is not shown, as full-length c-ABL1 has proven difficult to crystallize. Figure adapted from Hantschel, Nagar et al. 2003.

Another interaction found to stabilize c-ABL1 is the strong linkage between the SH2 and the C-lobe of the kinase domain. However, this interaction seems to be observed only in the presence of the myristoyl group. Without the myristoyl group, the SH2 domain is obstructed and the kinase domain cannot interact with it. Yet another intramolecular interaction for maintaining c-ABL1 in its inactive state is the binding of SH3 to a single proline in the linker region between the SH2 domain and the catalytic domain. This interaction seems to be crucial, as proline mutations that disrupt the recognition of the SH3 domain lead to increased catalytic activity (Van Etten 2004). Although a c-ABL1 autoinhibitory structure has been reported, the possibility of interacting proteins necessary for retaining protein stability should not be overlooked (Shaul and Ben-Yehoyada 2005).

As noted before, ABL1 shares homology to c-SRC. However, its regulation differs from that of c-SRC regulation based on intramolecular interactions. The SRC SH3 domain binds a proline residue in the SH2-kinase linker region, as does ABL1, but SRC forms an additional key interaction between the SH2 domain and the phosphorylated tyrosine residue 527 in the C-terminal tail (Thomas and Brugge 1997). ABL1 lacks this interaction, although crystallographic studies suggests that the SH2 domain of ABL1 type Ib forms a similar interaction with the C-lobe of the kinase domain, keeping it in an inactive conformation (Nagar, Hantschel et al. 2003).

c-ABL1 SH3 Kinase Activity Regulation

v-Abl is an oncoprotein that has swapped the N-terminal region of c-ABL1 with viral gag sequences from the Abelson murine leukemia virus. c-ABL N-terminal deletion mutants were tested to determine if it was actually the gag sequence that contributed to transformation or if it was the loss of an unidentified regulatory sequence or domain in c-ABL1. A mutant that lacked the SH3 domain was shown to activate c-ABL1 kinase activity and transform lymphoid cells *in vitro*, as well as cause leukemia *in vivo*. This led to the discovery that the SH3 domain functions to regulate the kinase activity of c-ABL1, therefore, the gag sequences are not necessary to induce leukemia (Jackson and Baltimore 1989). Also of interest was that modest overexpression of native c-ABL1 failed to transform cells. However, high overexpression of c-ABL1 led to phosphorylation of tyrosine residues in c-ABL1 itself and other proteins. This implied that there might be a cellular protein or factor that interacts with the SH3 domain to regulate ABL1 kinase activity, as this inhibitory factor can still be present at times when c-ABL1 is being overexpressed and most likely titrated out under conditions where ABL1 is highly overexpressed (Wen and Van Etten 1997). Therefore, it is of great interest to determine how and what regulates, or suppresses, c-ABL1 kinase activity through the SH3 domain.

A yeast two-hybrid approach was used to identify a potential protein that interacts with the SH3 domain of c-ABL1 and functions to inhibit c-ABL1

kinase activity. The murine c-ABL1 SH3 domain served as the fusion bait to screen a HeLa cell cDNA library. After screening several clones that interacted with the SH3 domain, these clones were re-tested using an SH3 domain mutant containing a point mutation (P131L). This point mutation, which replaces a conserved proline residue with a leucine at the binding surface of SH3, prevents interaction between SH3 and proline-rich ligands, therefore potentially leading to the deregulation of kinase activity. The clones that failed to interact with the SH3 mutant were sequenced. The predominant clone acquired by this screen had an identical cDNA sequence to the human proliferation-associated gene, or *PAG* (Wen and Van Etten 1997). This gene is now referred to as *Peroxiredoxin 1,* or *Prdx1* (Neumann, Krause et al. 2003). PRDX1 is a known antioxidant enzyme, protecting cells from reactive oxygen species that cause oxidative stress (Rhee, Kang et al. 2005).

Subsequent studies found that PRDX1, though lacking the common PXXP motif known to bind SH3, indeed functions as an inhibitor of c-ABL1 kinase activity through SH3 regulation. PRDX1 is also highly specific to the c-ABL1 SH3 domain, as it fails to bind to other SH3 domains of c-Src, Grb-2, and the p85 subunit of phosphotidyl-inositol-3 kinase (Wen and Van Etten 1997). Several proteins have been reported to interact with c-ABL1 and bind to its SH3 domain. However, PRDX1 is the only SH3-interacting protein linked to inhibition of c-ABL1 kinase activity (Wen and Van Etten 1997; Pendergast 2002; Fan, Cong et al. 2003). Rb, the only other protein found to

suppress c-ABL1 kinase activity, acts on nuclear c-ABL1 through the ATP-binding domain (Welch and Wang 1993). In addition to its interaction with the SH3 domain, PRDX1 interacts with the N-terminus of the kinase domain. This interaction with the kinase domain is just as important as the interaction with SH3 for inhibiting c-Abl kinase activity. It has been observed, as well, that the PRDX1 C-terminus alone is enough to bind to c-ABL1. Although these domains and regions have been found to interact, it is still unknown where exactly PRDX1 binds to c-ABL1 and vice versa (Wen and Van Etten 1997).

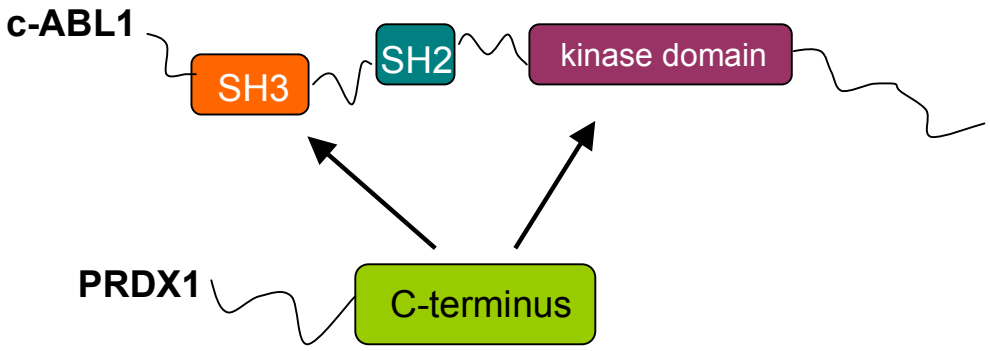


Figure 3: PRDX1 physically interacts with c-ABL1 and suppresses its kinase activity.

Through the yeast two-hybrid screen mentioned above, PRDX1 was found to interact with the SH3 domain. Further analysis identified PRDX1 as an inhibitor of c-ABL1 kinase activity. In addition to interacting with SH3, PRDX1 strongly interacts with the kinase domain of c-ABL1. It was shown that it is the C-terminus of PRDX1 that interacts with both c-ABL1 domains, and that each interaction is necessary for c-ABL1 kinase regulation.

Peroxiredoxins: Functional Role

It has been over twenty years since peroxiredoxins were discovered and cloned for characterization. The first research group to open the field into peroxiredoxins isolated a cDNA mouse clone, later found to be peroxiredoxin 3, expressed in erythroleukemia cells (Yamamoto, Matsui et al. 1989). Peroxiredoxins have a major contribution to the survival and functional roles of a cell. Activation of peroxiredoxins within cells and tissues is highly dependent on oxidative stress imposed from the exogenous environment or created from endogenous cellular reactions. Elevated expression levels of peroxiredoxins are a typical response that allows the cell to prevent or relieve oxidative damage. To provide protection, peroxiredoxins mainly target hydrogen peroxide (H_2O_2), reducing it to harmless elements. In addition to eliminating H_2O_2, peroxiredoxins are also capable of reducing peroxinitrites and removing thiyl radicals (Fujii and Ikeda 2002; Rhee, Chae et al. 2005).

Peroxiredoxins act endogenously as antioxidants, protecting cells from oxidative damage to its macromolecules by reactive oxygen species, or ROS. Various cellular enzymes exist that possess antioxidant activity. These enzymes function to control ROS levels. ROS arise from incomplete reduction of oxygen and are formed through various pathways, both by intracellular and extracellular reactions. Endogenous ROS formation is linked to mitochondria respiration, peroxisome activity, lipoxygenase action, induction

of NADPH oxidase, and active cytochrome P450. Exogenous ROS sources are ultraviolet light exposure, ionizing radiation, chemotherapeutics, inflammatory cytokines, environmental toxins, and aerobic metabolism. In most of these processes, oxygen is utilized to move a reaction forward or is reduced to water in order to obtain energy for cellular activity. However, during this reduction process, oxygen can fail to be fully reduced to water. The incomplete reduction of oxygen then leads to the formation of oxidant intermediates, or ROS. ROS include the superoxide radical, hydroxyl radical, and hydrogen peroxide. These forms of oxygen are highly reactive, unlike oxygen, and are responsible for establishing oxidative stress within the cell, which causes covalent modification of many macromolecules. It is essential that intracellular ROS levels are regulated, as a high concentration of ROS could lead to damage of many macromolecules. ROS damage proteins through protein oxidation, leading to amino acid modifications, proteolysis, and degradation. ROS also modify lipids through lipid peroxidation, causing membrane leakage and cell death. Still another damaging effect by ROS is the formation of DNA lesions through 8-oxoguanine base modifications, leading to mutagenesis and apoptosis (Finkel and Holbrook 2000). Superoxide anion radicals can arise from the electron transport chain, cytochrome P450, and NADH/NADPH oxidase. These radicals can be converted to hydrogen peroxide either spontaneously, by the enzyme superoxide dismutase, or by extracellular stimuli, such as hormones or

cytokines (Kyaw, Yoshizumi et al. 2004; Rhee, Kang et al. 2005). Hydrogen peroxide itself could be damaging to cells. However, it could also be converted into a more toxic form, a hydroxyl radical, in the presence of iron or copper (Rhee, Kang et al. 2005).

It is important to note, however, that H_2O_2 is not solely a nuisance and destructive molecule to a cell. Intriguingly, it is also required for various cell processes, such as cell proliferation, differentiation, and migration. Upon ligands binding to their membrane receptors, H_2O_2 is generated at low micromolar concentrations and is capable of functioning as an effector and signaling molecule. The peroxidase activity of peroxiredoxins is inactivated through protein modification, permitting H_2O_2 to transduce necessary signals within the cell (Rhee 2006; Woo, Yim et al. 2010).

To prevent high ROS levels that could have toxic effects, cells have established a response mechanism to combat ROS. Cells may utilize non-enzymatic compounds, such as antioxidants to reduce ROS levels. Examples of these compounds are glutathione, vitamins A, C, and E, and uric acid. Cells also utilize enzymes such as superoxide dismutases, catalase, peroxidases, and peroxiredoxins to deal with ROS (Finkel and Holbrook 2000). The enzymes and compounds are referred to as ROS scavengers, as they function to reduce or eliminate ROS to establish a nontoxic environment within the cell.

The Peroxiredoxin Family

The peroxiredoxins are a group of six enzymes that exhibit peroxidase activity, meaning they are capable of breaking down peroxides into water (Rhee, Chang et al. 2003). Other enzymes that can reduce peroxides to water are catalases, which are found exclusively in the peroxisomes, and glutathione peroxidases, which are in the cytosol and mitochondria (Rhee, Chae et al. 2005). Peroxiredoxins, however, are in their own class, as they display no homology to these other peroxidase enzymes.

Peroxiredoxins, found in all six kingdoms and displaying over 70% homology amongst all isoforms, are expressed in various locations within the cell, including the plasma membrane, mitochondrium, endoplasmic reticulum, peroxisome, and cytosol (Fujii and Ikeda 2002). They are classified into three classes: Class 2-cysteine includes PRDX1-4, class atypical 2-cysteine is defined by PRDX5, and class 1-cysteine includes only PRDX6. The 2-cysteine peroxiredoxins contain the catalytic N-terminal cysteine residue and an additional conserved cysteine residue in the C-terminus. Both the N-terminus and C-terminus cysteine residues seem to be required for proper peroxidase activity, as deleting either residue prevents Prdxs from reducing peroxides. Neither PRDX5 nor PRDX6 have the conserved C-terminal cysteine residue (Rhee, Chae et al. 2005).

Peroxiredoxin 1: History and Activity

PRDX1 was first isolated from a cDNA library generated from the human mammary epithelial cell line HBL100. It was initially termed proliferation-associated gene, or *PAG*, since its expression levels correlated with cell proliferation (Prosperi, Ferbus et al. 1993). Soon after, *PRDX1* was mapped to chromosome 1p34.1, a relative hotspot for carcinogenesis (Prosperi, Apiou et al. 1994). Several genes within this locus have been found to undergo translocations or deletions, leading to leukemias, predominantly acute lymphoblastic leukemia, breast cancer, and neuroblastoma (Mitelman 1994). PRDX1, a predominantly cytosolic 23 kDa protein that is ubiquitously and abundantly expressed, is important for various cellular functions, such as cell proliferation, cell differentiation, immune responses, and control of apoptosis (Rhee, Chang et al. 2003).

Like the other 2-cysteine enzymes, it exists as a homodimer, with a head-to-tail arrangement. Hydrogen peroxide oxidizes the conserved N-terminal active site cysteine (Cys-52), causing it to react with the other conserved cysteine residue (Cys-173) at the C-terminus of the partnered monomer. This reaction creates a stable protein due to the presence of two sulfur bridges. In doing so, PRDX1 reduces H_2O_2 into H_2O. The disulfide bonds are reduced and broken by the enzyme thioredoxin, producing the activated form of PRDX1 and allowing for further H_2O_2 reduction. When

PRDX1 becomes overoxidized, the enzyme sulfiredoxin reduces PRDX1 to cycle it back into its active form (Rhee, Kang et al. 2005).

In yeast, the PRDX1 homolog, cPRDX1, was found to function as not only a peroxidase, but also as a molecular chaperone. It seems that cPRDX1 is able to prevent ROS-mediated protein unfolding and aggregation of its substrates. At low ROS concentrations, c-PRDX1 can remove ROS and protect proteins from denaturation. Under oxidative stress, however, the structure of cPRDX1 is altered, through the use of the N-terminal cysteine residue, and seems to function exclusively as a chaperone protein (Jang, Lee et al. 2004). This modification involves oligomerization of PRDX1, allowing it to recognize and bind specific substrates, chaperoning them to a certain location within the cell to address the cell damage (Barranco-Medina, Lazaro et al. 2009).

Another interesting finding of PRDX1, besides its antioxidant activity and its chaperone ability, is its involvement in tumor suppression. It was discovered that *Prdx1*-null mice develop various types of malignant cancers, such as lymphomas, sarcomas, and carcinomas, as well as severe hemolytic anemia. This observation could possibly suggest that the role of PRDX1 in degrading ROS is important in preventing anemia and tumor formation. However, it is unclear if the antioxidant activity of PRDX1 is responsible or playing a role in suppressing the cancerous phenotype (Neumann, Krause et al. 2003). On the flipside, there have been findings that also link PRDX1

expression to tumor maintenance. Nrf2, a transcription factor, has been found to positively regulate *PRDX1* gene expression (Ishii, Itoh et al. 2000). Transcription factors Ets1 and Ets2 have also been shown to upregulate PRDX1 through H_2O_2 exposure and hypoxia in cancerous cells. The activation of these transcription factors may be responsible for promoting and maintaining high levels of PRDX1 within certain tumors, as observed in the thyroid cancer, lung cancer, oral carcinomas, and mesothelioma (Shiota, Izumi et al. 2008).

PRDX1 Localization and Function

The localization of peroxiredoxins varies significantly depending on the cell type, as well as biological and environmental conditions. Tissue distribution of peroxiredoxins within mice has been studied through immunohistochemistry experiments (Godoy, Funke et al. 2011). The nuclear form of PRDX1 is able to modulate expression of selective target genes, including *NF-kB*, *c-MYC*, and the androgen receptor (Egler, Fernandes et al. 2005; Hansen, Moriarty-Craige et al. 2007; Park, Yu et al. 2007; Chhipa, Lee et al. 2009). The cytoplasmic form of PRDX1 exhibits anti-apoptotic functions by interacting with various apoptosis regulators, such as c-ABL1, ASK1, GSTpi/JNK, and p66 (Wen and Van Etten 1997; Kim, Lee et al. 2006; Kim, Kim et al. 2008; Gertz, Fischer et al. 2009). PRDX1 is also secreted, as secretion is specifically observed in lung cells (Chang, Jeon et al. 2001;

Gharib, Nguyen et al. 2010). The extracellular PRDX1 form is found to play a role in inflammation (Riddell, Wang et al. 2010).

NUCLEAR PRDX1

1. PRDX1 and c-MYC

c-MYC is an oncogene that, when expressed, induces unregulated cell growth, cell cycle progression, and differentiation, as well as interferes with a proper apoptotic response (Dang 2012). It has been shown through a yeast two-hybrid screen and co-immunoprecipitation experiments that PRDX1 physically interacts with the highly conserved MYC Box II (MBII) domain of c-MYC. This domain is linked to cell transformation and transcription regulation. When bound to MBII, PRDX1 prevents induction of some c-MYC substrates, thereby suppressing tumor development and growth. This has been shown by overexpression of PRDX1 in 32D myeloid cells, in which genes dependent on MBII are downregulated (Mu, Yin et al. 2002; Egler, Fernandes et al. 2005). Another experiment that proved the function of PRDX1 as a tumor suppressor was done by co-expressing PRDX1 and c-MYC in rat cells, subsequently demonstrating a significant decrease in c-MYC-induced cell growth (Mu, Yin et al. 2002).

2. PRDX1 and NF-κB

Cytoplasmic PRDX1 is known to suppress NF-kB activation through the elimination of peroxides within the cell (Kang, Chae et al. 1998). Interestingly, nuclear PRDX1 has a contradictory effect, enhancing NF-κB

activity. In HeLa cells, forced localization of PRDX1 to the nucleus leads to NF-κB activation upon H_2O_2 stimulation. It was suggested in this study that nuclear PRDX1 influenced NF-κB activation by inhibiting the oxidation of a cysteine residue within the DNA binding domain of p50, the cleaved product of NF-κB1 (Hansen, Moriarty-Craige et al. 2007).

Another route through which nuclear PRDX1 induces NF-κB activation is through upregulation of p65-mediated cyclooxygenase (COX)-2 gene expression, which was found in estrogen receptor (ER) deficient human breast cancer cells (Wang, He et al. 2010). These cells demonstrate higher nuclear PRDX1 levels than cytoplasmic PRDX1 levels. A significant observation that may help explain the mechanism for nuclear PRDX1 involvement in NF-κB activation is that in ER-deficient cells, PRDX1 is phosphorylated at the threonine-90 residue, inducing PRDX1 oligomerization and promoting its chaperone activity (Jang, Kim et al. 2006). The phosphorylation may be due to Cdk2 activity, known to phosphorylate PRDX1, leading to inactivation of its peroxidase activity (Chang, Jeong et al. 2002). The phosphorylated form of PRDX1 is able to interact with NF-κB p65 and cause both proteins to associate with an upstream promoter region of COX-2 (Wang, He et al. 2010). Another finding that further supports nuclear PRDX1 phosphorylation leading to NF-κB activity is that ER-positive cancer cells do not express a phosphorylated form of PRDX1, and therefore PRDX1 fails to associate with the COX-2 promoter (Wang, He et al. 2010). The lack of

PRDX1 phosphorylation is most likely due to low levels of Cdk2 activity caused by the increased presence of Cdk2 inhibitors p27 and p21 in these cells (Zhuang and Miskimins 2008).

3. PRDX1 and Androgen Receptor Activation

PRDX1 has been shown to play a role in prostate cancer development. Increased PRDX1 expression induces androgen receptor (AR) activation, whereas *Prdx1* mRNA knockdown by short-hairpin RNA leads to suppressed AR activity. Suppression of AR activity leads to a decrease in the growth rate of androgen-dependent prostate cells. Another approach involving hypoxia/reoxygenation treatment on cells showed that PRDX1 becomes oxidated at the active site Cysteine-52 residue, leading to AR transactivation due to its association with the prostate-specific antigen gene. The antioxidant activity of PRDX1 is not required for AR stimulation, as an antioxidant activity mutant form of PRDX1 shows similar AR activation as wild type PRDX1 (Park, Yu et al. 2007). This study might present another avenue in controlling prostate cancer through disruption of the PRDX1 – AR interaction.

CYTOPLASMIC PRDX1

1. PRDX1 and ASK1

PRDX1 binds with the thioredoxin-binding domain of ASK1 in 293 cells after H_2O_2 treatment (Saitoh, Nishitoh et al. 1998). ASK1, a member of the MAPK family, activates JNK and p38 MAPK signaling cascades (Ichijo, Nishida

et al. 1997). PRDX1 overexpression after H_2O_2 exposure inactivates ASK1 by inhibiting its phosphorylation. Cells deficient for PRDX1 exhibit highly activated forms of ASK1, as well as p38 and JNK, and when incubated with H_2O_2, an apoptotic response is quickly activated (Kim, Kim et al. 2008).

2. PRDX1 and JNK

PRDX1 is able to suppress JNK activation by directly interacting with its partner, GSTpi (Kim, Lee et al. 2006). When GSTpi is bound to JNK, it functions to inhibit its activation, which can be induced by oxidative stress (Adler, Yin et al. 1999). Following gamma irradiation as a source of stress on cells, the interaction between PRDX1 and GSTpi prevents JNK release from the GSTpi/JNK complex. If PRDX1 is overexpressed in cancerous cells, JNK activation and apoptosis are suppressed after radiation exposure (Kim, Lee et al. 2006).

3. PRDX1 and p66

p66 is involved in cellular stress responses and regulating cellular lifespan. Upon exposure to oxidative stress or UV irradiation, p66 expression is increased and translocated to the mitochondrial intermembrane space, where it further generates H_2O_2 and subsequently induces apoptosis (Gertz, Fischer et al. 2009). PRDX1 negatively regulates p66, binding to it with high affinity in mitochondrial extracts and preventing initiation of apoptosis. Even though mitochondria exhibit low levels of PRDX1, its recognition of p66 is highly selective, as Prdx3, the major peroxiredoxin expressed in mitochondria,

is unable to bind p66. In this context, PRDX1 is able to suppress H_2O_2 production through regulation of p66, independent of its peroxidase activity. However, in conditions involving high levels of H_2O_2, p66 becomes phosphorylated through JNK or PKC-Beta activation, leading to the dissociation of the p66-PRDX1 complex and resulting in PRDX1 oligomer formation (Le, Connors et al. 2001; Giorgio, Migliaccio et al. 2005; Pinton, Rimessi et al. 2007). p66 is then free to translocate to the mitochondria and forms a tetramer structure, leading to ROS generation, through its interaction with cytochrome c, and eventually inducing apoptosis (Giorgio, Migliaccio et al. 2005).

4. PRDX1 and c-ABL1

As mentioned before, our lab discovered PRDX1 functioned as a physiological inhibitor of c-ABL1 tyrosine kinase activity (Wen and Van Etten 1997). c-ABL1 is known to have multifunctional roles in cell cycle regulation (Schwartzberg, Stall et al. 1991; Tybulewicz, Crawford et al. 1991). However, its primary role is in the regulation of double-stranded breaks repair system and inducing an apoptotic response following excessive DNA damage (Gonfloni 2010). Upon activation by ionizing radiation or other DNA-damaging agents, c-ABL1 in turn activates substrates leading to induction of the JNK and MAPK pathways (Kharbanda, Yuan et al. 1998). PRDX1 is thought to play a role in interfering with this cell death signaling response by sequestering c-ABL1 in the cytoplasm and preventing its activation.

Oxidative stress induces not only antioxidant activity in the cell, but also activation of DNA damage response pathways. In response to oxidative or genotoxic stress, cells stimulate stress signaling pathways such as the extracellular signal-regulated kinase (ERK), c-Jun amino-terminal kinase (JNK), p38 mitogen-activated protein kinase (MAPK), phosphoinositide-3-kinase (PI3K)/Akt, NF-kappa-B, p53, and heat-shock response pathways. Depending on which pathway(s) get activated, the cell will survive or apoptose in response to oxidative stress (Finkel and Holbrook 2000).

Both c-ABL1 and PRDX1 are involved in the oxidative stress response mechanism. In addition to DNA damage (genotoxic stress), c-ABL1 can be activated by free radicals or hydrogen peroxide, much like PRDX1. Exposure to oxidative stress abolishes the physical interaction between PRDX1 and c-ABL1 in the cytosol. The interaction is broken due to c-ABL1 phosphorylation and/or PRDX1 oxidation by ROS (Van Etten 1999; Kyaw, Yoshizumi et al. 2004). c-ABL1 activation seems to be due to upstream stress kinases such as ERK, JNK, and p38 (Kyaw, Yoshizumi et al. 2004). One group found that upon exposure to hydrogen peroxide, c-ABL1 is targeted to the nucleus in HeLa cells. It was determined that 14-3-3 proteins sequester c-ABL1 in the cytoplasm by blocking the nuclear localizing signals. However, in response to oxidative stress, JNK is activated, leading to 14-3-3 phosphorylation and release of c-ABL1 (Yoshida, Yamaguchi et al. 2005).

Another way that c-ABL1 becomes active is through the ataxia telangiectasia-mutated (ATM) kinase. ATM is usually activated by DNA double-stranded breaks, but can also be activated by oxidative stress. When ATM becomes activated, it serves to phosphorylate several proteins involved in the DNA damage checkpoint. One protein that it phosphorylates is c-ABL1. ATM phosphorylates c-ABL1 on a serine residue within the kinase domain, leading to c-ABL1 activation (Kharbanda, Yuan et al. 1998). Alternatively, phosphorylation of two tyrosines residues in c-ABL1, also stimulated by oxidative stress, can occur due to autophosphorylation or Src kinases (Kyaw, Yoshizumi et al. 2004). The first tyrosine to become phosphorylated is located in the activation loop of the kinase domain. Phosphorylation leads to an increase in kinase activity and induction of phosphorylation of another tyrosine residue in the SH2-catalytic domain linker region. This secondary phosphorylation breaks the interaction between the SH3 domain and the proline in the linker region, promoting a highly active kinase (Van Etten, 2004). Regardless of ATM, autophosphorylation, or Src activation, c-ABL1 is free and active to enter the nucleus and interact with p53 and the damaged DNA to promote growth arrest or transcription of genes involved in apoptosis (Figure 4) (Van Etten 1999).

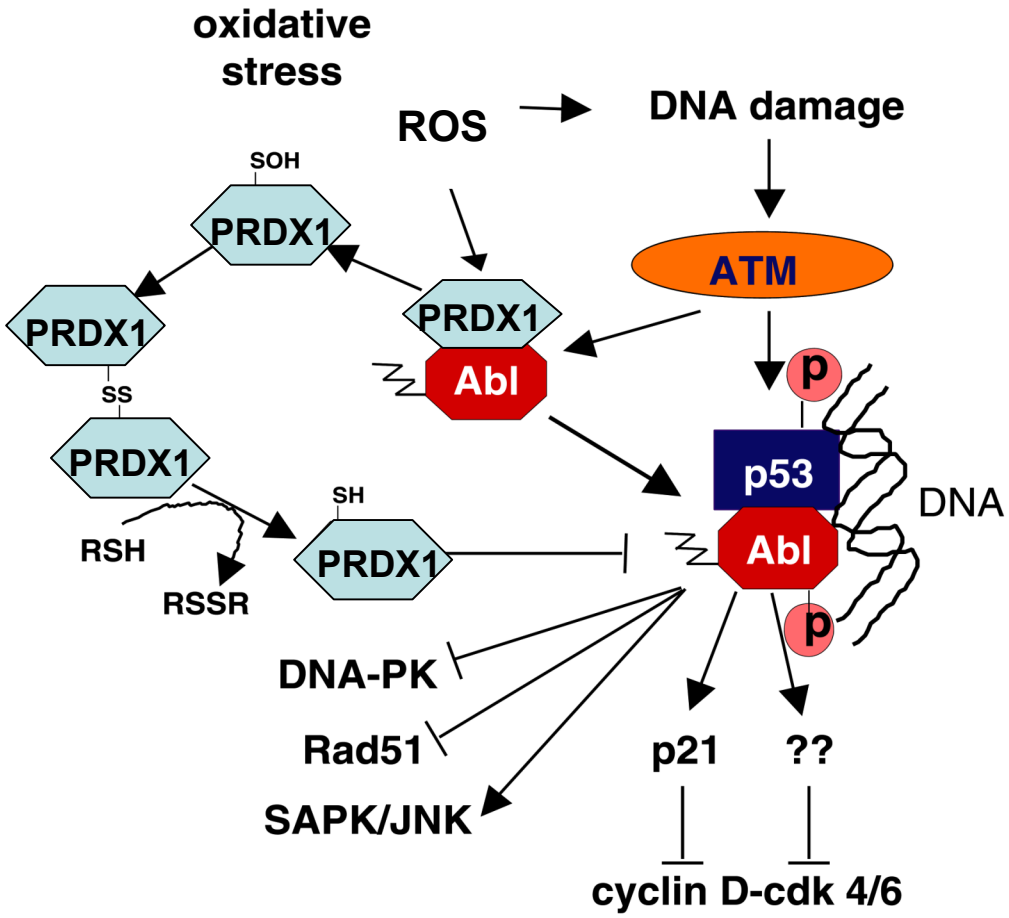


Figure 4: Oxidative Stress activates both c-ABL1 and PRDX1. Oxidative stress disrupts the interaction between PRDX1 and c-ABL1 due to PRDX1 oxidation or c-ABL1 phosphorylation. It is presumed the monomer form of PRDX1 is responsible for regulating c-ABL1 kinase activity. In high levels of oxidative stress, PRDX1 becomes oxidized and forms a homodimer structure to allow for decomposition of oxidants. In this figure, the protein ATM is shown to phosphorylate c-ABL1 upon DNA damage, which could be caused by oxidative stress. Once c-ABL1 becomes phosphorylated, it localizes to the nucleus, where it interacts with p53 and damaged DNA to induce cell cycle arrest or apoptosis. Figure adapted from Van Etten 1999.

Besides PRDX1, two other distinct antioxidant enzymes have been found to regulate stress kinases. Glutathione S-transferase P1-1 directly interacts with the c-Jun N-terminal kinase, causing its subsequent inhibition (Wang, Arifoglu et al. 2001). Thioredoxin behaves similarly to GSTP1-1, as it binds to MAPK apoptosis-signaling kinase (ASK1) and inhibits its kinase activity (Saitoh, Nishitoh et al. 1998). The observations that these two antioxidant enzymes can also inhibit stress kinases strengthen the argument that PRDX1 is able to regulate the stress kinase c-ABL1, as this may represent a general strategy for coordinate regulation of stress-related kinases by proteins that sense cellular redox levels.

PRDX1: Secreted Form

A few studies have demonstrated that PRDX1 can actually be secreted from cells. Patients with acute lung injuries show an increase in PRDX1 secretion in their bronchoalveolar lavage fluid (Gharib, Nguyen et al. 2010). Tissue samples from patients with non-small cell lung cancer showed elevated PRDX1 expression levels, with half of the samples producing PRDX1 autoantibodies and one-third of the samples secreting PRDX1 into the sera (Chang, Jeon et al. 2001; Chang, Lee et al. 2005). The mechanism of PRDX1 secretion is not entirely known, however, it is known that PRDX1 lacks a signal peptide necessary for the classical or ER/Golgi-dependent secretory

pathways. Therefore, it seems that PRDX1 secretion may be mediated through the non-classical secretory pathway.

Upon stimulation with cytokines, such as TGF-β1, IL-1, and IL-6, PRDX1 secretion can be observed (Chang, Lee et al. 2006). Lung adenocarcinoma (A549) cells secrete PRDX1, a process found to be largely dependent on TGF-β1. A549 cells have high TGF-β1 mRNA levels and high converting enzyme activity that allows for production of the active form of TGF-β1 (Chang, Lee et al. 2006). Non-cancer lung (BEAS 2B) cells do not secrete TGF-β1 PRDX1 (Chang, Lee et al. 2005). Other cancer cell lines, such as MCF7, Hep3B, and HeLa cells, also fail to secrete PRDX1 via TGF-β1, implying that PRDX1 secretion may have a cell-specific role (Chang, Lee et al. 2006). Therefore, PRDX1 could possibly be used as a biomarker or as a tool for cancer typing (Ishii, Warabi et al. 2012).

PRDX1: Inflammation and Innate Immunity

PRDX1 and TLR4

In a recent report, PRDX1 was shown to play a role in inflammation and innate immunity. Inflammation is a defense mechanism provoked by tissue injury or infection, but has also been found to be influential in cancer progression. Oxidative stress is usually involved in both situations, and so peroxiredoxins become involved in the inflammation response. Extracellular PRDX1 behaves as a pro-inflammatory agent by inducing TNF-alpha and IL-6

secretion from murine macrophages or immature bone marrow-derived dendritic cells. The stimulation of these factors is mediated through PRDX1 binding to toll-like receptor (TLR) 4, a cell surface receptor involved in the inflammatory response (Riddell, Wang et al. 2010).

PRDX1 and MIF

PRDX1 physically interacts with macrophage migration inhibitory factor (MIF) through its conserved cysteine-173 residue, partially inhibiting MIF tautomerase activity. In turn, MIF inhibits PRDX1 peroxidase activity (Jung, Kim et al. 2001). Interestingly, exposure to H_2O_2 or a hypoxic environment leads to an induction in MIF expression and secretion (Takahashi, Nishihira et al. 2001). The exact function of PRDX1 association with MIF or its possible link to MIF regulation following H_2O_2 insult or hypoxia is still unclear. However, it can be deduced that PRDX1 has a functional role in inflammation and immunity through MIF inhibition, as it has been established that MIF itself plays a pivotal role in the innate immune response as well as inflammation. MIF is known as a pro-inflammatory factor responsible for inhibiting macrophage migration in delayed-type hypersensitivity (Bloom and Bennett 1966; David 1966). It is expressed in a variety of cells, including lymphocytes, macrophages, eosinophils, epithelial cells, and endothelial cells (Conroy, Mawhinney et al. 2010). Intracellular MIF leads to suppression of apoptosis, whereas secreted MIF functions as a cytokine and amplifies the response to pathogen recognition by TLR4 upregulation (Roger, David et al.

2001; Kudrin, Scott et al. 2006). MIF overexpression can be identified in various cancers and is thought to influence cancer growth and progression through an overactive inflammation response (Conroy, Mawhinney et al. 2010). It is thought that extracellular PRDX1 binds to secreted MIF, leading to a reduction in the inflammation response (Ishii, Warabi et al. 2012). Therefore, PRDX1 may be acting as a tumor suppressor by regulating inflammation through MIF inhibition.

PRDX1 and CyPA

PRDX1 interacts with cyclophilin A (CyPA), a regulator of inflammation, leading to an increase in its thiol-specific antioxidant activity (Lee, Hwang et al. 2001). CyPA is secreted from vascular smooth muscle cells under oxidative stress conditions to initiate vascular remodeling by inducing inflammation (Suzuki, Jin et al. 2006). Extracellular CyPA stimulates signaling pathways ERK1/2, Akt, and JAK, which help in cell growth and remodeling and infiltrate immune cells. Therefore, CyPA regulation is necessary, as high CyPA expression levels can lead to various inflammatory conditions, such as rheumatoid arthritis, autoimmune diseases, and cancer (Satoh, Shimokawa et al. 2010).

PRDX1 and NK ENHANCING ACTIVITY

PRDX1 was shown to possess natural killer enhancing activity, as PRDX1 isolated from the erythrocyte cytosol compartment increased NK activity and enhanced cell cytotoxicity versus the human erythroleukemic

K562 cell line. Although it is still unknown how NK activity is enhanced, it is known that PRDX1 oligomerization is needed to induce NK activity (Shau, Gupta et al. 1993). PRDX1 could possibly induce NK activity through TLR4 in NK cells, as NK cells express a functional TLR4 receptor and can produce chemokines upon stimulation, resulting in enhanced killing activity (Ishii, Warabi et al. 2012). Another mechanism by which PRDX1 might be enhancing NK activity is through PRDX1-mediated MIF inhibition (Ishii, Warabi et al. 2012). MIF functions to suppress NK activity by inhibiting an NK cell receptor (Krockenberger, Dombrowski et al. 2008). It also contributes in the regulation of TLR4 expression levels (Roger, David et al. 2001). In support of this hypothesis, PRDX1 has stronger NK enhancing activity compared to PRDX2, which does not seem to bind to MIF (Sauri, Ashjian et al. 1996).

PRDX1: Enhancing Tumor Progression

Various studies have shown that elevated PRDX1 levels in many cancerous tissues and cancer cell lines signify a poor prognosis and diminished patient survival. These cancers include thyroid tumors, oral carcinoma, lung cancer tissue and lung cancer A549 cells, and mesothelioma (Yanagawa, Ishikawa et al. 1999; Yanagawa, Iwasa et al. 2000; Kinnula, Lehtonen et al. 2002; Kim, Chae et al. 2003). As mentioned previously, PRDX1 enhances prostate cancer growth by AR activation (Park, Yu et al.

2007). Another group found that PRDX1 could also induce prostate tumor progression through TLR4 signaling in a mouse model. PRDX1 expression levels within the cancerous tissue of this model increase during disease progression. *Prdx1* knockdown by short hairpin RNA in human (PC-3M) prostate cancer cells or mouse (C2H) prostate cancer cells led to a significant delay in PC-3M tumor growth in a mouse model, as well as the elimination of C2H prostate tumor growth in the mouse. Inoculating *PRDX1*-expressing C2H cells into TLR4-deficient mice led to a lack of tumor growth. This implied that for tumor growth to be observed, both *PRDX1* in cancer cells and TLR4 signaling in host cells are needed. In this aspect, tumor growth is possibly due to factors contributing to vasculature formation and function, such as VEGF, TNF-alpha, IL-6, and TGF-B (Riddell, Bshara et al. 2011). A functional TLR4 signaling pathway is important for maintaining tumorigenesis in various cancer types. TLR4 activation elicits cytokine and chemokine production with immunosuppressive qualities (He, Liu et al. 2007). Once TLR4 signaling is initiated, tumor cells become noticeably resistant to NK cell-mediated lysis and to apoptosis by chemotherapeutic agents, TNF-alpha, and TRAIL (He, Liu et al. 2007; Szajnik, Szczepanski et al. 2009; Szczepanski, Czystowska et al. 2009).

PRDX1: Preventing Tumor Initiation and Progression

In the other side of the spectrum, PRDX1 expression is also linked to prevention of oncogenesis. It was shown in our laboratory that mice lacking *Prdx1* spontaneously developed tumors, mainly due to high ROS levels and improper defense mechanism against oxidative DNA damage. Loss of *Prdx1* made mouse embryonic fibroblasts sensitive to DNA damage, caused hemolytic anemia in mice, and induced malignant murine cancers such as lymphomas, sarcomas, and carcinomas. It was also shown that without the function of PRDX1, natural killer cell activity was decreased, therefore reducing the tumor cell destruction ability. It is presumed that this phenotype, in addition to the inability of cells to properly eliminate ROS, leads to the development of the various types of cancers (Neumann, Krause et al. 2003).

Another study found that PRDX1 binds to PTEN, protecting its lipid phosphatase activity from inactivation by oxidative stress. Inactivating PTEN would lead to an increase in AKT activation, involved in cell growth and proliferation. Within the same study, it was proven that AKT is highly activated in *Prdx1*-deficient fibroblasts (Cao, Schulte et al. 2009). Interestingly, PTEN-deficient fibroblasts show a decrease in PRDX1 expression levels. This implies that both PRDX1 and PTEN function together to maintain antioxidant levels and suppress AKT signaling (Huo, Li et al. 2008). In hematopoietic cells, AKT activation is required for proper BCR-

ABL1-induced transformation (Skorski, Bellacosa, et al. 1997). Therefore, loss of PRDX1 would lead to higher AKT activity, possibly inducing faster BCR-ABL1 cell transformation and influencing BCR-ABL1-induced disease.

It was also shown by another group that *Prdx1* deficiency in fibroblasts and mice is shown to increase cell transformation by Ras and c-MYC (Egler, Fernandes et al. 2005). As mentioned previously, PRDX1 physically interacts with c-MYC and suppresses activation of its substrates, functioning to control cell growth (Mu, Yin et al. 2002). The reason for c-MYC activation and cell transformation is presumed to be due to a shift in intracellular ROS. *Prdx1*-deficient fibroblasts show higher ROS levels than wild type fibroblasts, suggesting the nuclear localization leads to severe DNA damage and tumorigenesis (Egler, Fernandes et al. 2005). In relation to our studies, it has been shown that c-MYC is required for BCR-ABL1 transformation. JAK2 is activated by BCR-ABL1 through the BCR-ABL1 SH2 domain, which in turn leads to the induction and stabilization of c-MYC (Sawyers, Callahan et al. 1992; Shanhai, Hui et. al. 2002). The loss of PRDX1, therefore, would reduce c-MYC regulation and could potentially induce more efficient BCR-ABL1-induced transformation and an increase in BCR-ABL1 disease onset.

BCR-ABL1: History, Discovery, and Link to Leukemia

In addition to inhibiting c-ABL1 kinase activity, PRDX1 was also found to partially inhibit the kinase activity of the fused version of c-ABL1, BCR-

ABL1 (Wen and Van Etten 1997). This particular observation is of interest because it seems to implicate PRDX1 in BCR-ABL1 regulation. The BCR-ABL1 fusion protein has been widely studied because of its discovery as the causative agent of chronic myeloid leukemia and also has been found to induce B-cell acute lymphoblastic leukemia (Quintas-Cardama and Cortes 2009).

The fusion gene *BCR-ABL1* has been extensively studied since its discovery 30 years ago (de Klein, van Kessel et al. 1982; Heisterkamp, Stephenson et al. 1983). The reason for its popularity stems from the revelation as the etiological agent that causes leukemia, primarily chronic myeloid leukemia (CML), but also, to a significant extent, B-acute lymphoblastic leukemia (B-ALL). CML is one of the first cancers to be genetically characterized, and certainly the first leukemia to be studied, as published findings describing CML date back to 1845 (Wong and Witte 2004). 115 years later, a group was able to pinpoint the cause for CML, linking the disease to an aberrantly short chromosome (Nowell and Hungerford 1960). It was not until a few years later that the aberrant chromosome was found to be a DNA fragment arising from a balanced translocation between chromosomes 9 and 22. This short chromosome was termed the Philadelphia chromosome (Ph), as named after the city in which it was discovered (Rowley 1973). Further analysis of the Ph chromosome led to the revelation that the translocation occurs between the proto-oncogene c-ABL1, on chromosome 9,

and BCR, on chromosome 22 (de Klein, van Kessel et al. 1982; Heisterkamp, Stephenson et al. 1983). Though it is presently unclear what leads to the translocation event, much has been studied about the fused gene and its functional role. The translocation produces the fused gene *BCR-ABL1*, whose product was shown to have constitutively active tyrosine kinase activity (Lugo, Pendergast et al. 1990). BCR-ABL1 expression is found in samples from CML patients, reinforcing the role of the fused protein in disease development (Shtivelman, Lifshitz et al. 1985; Stam, Heisterkamp et al. 1985).

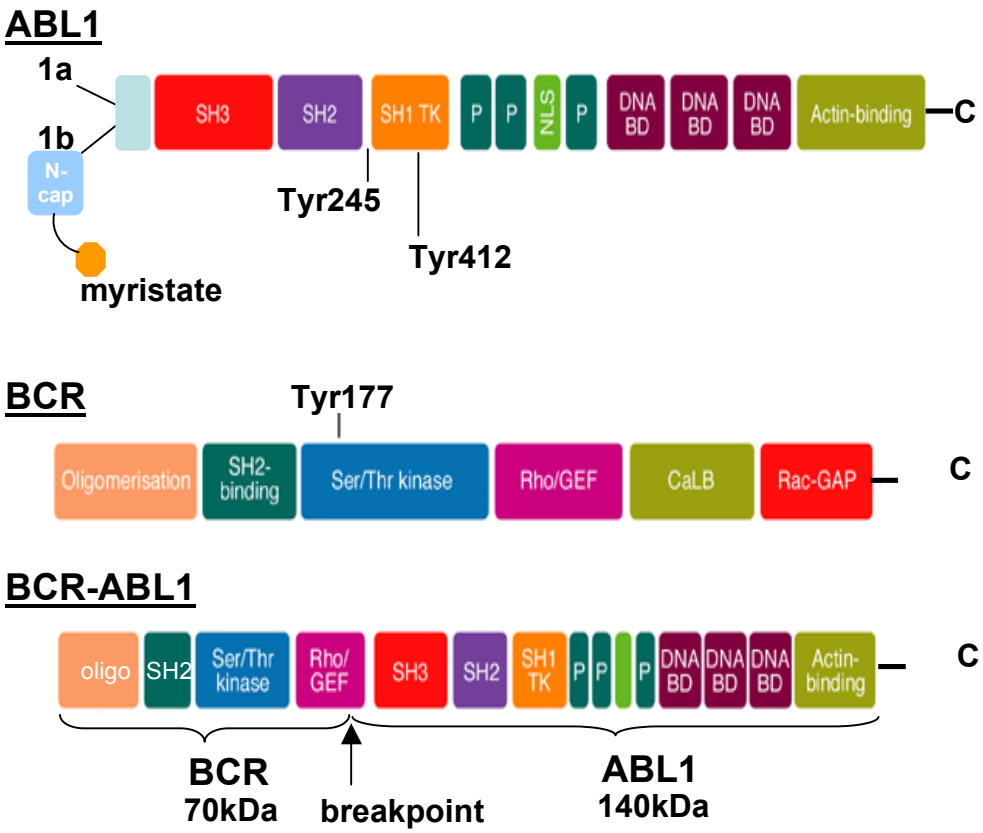


Figure 5: Primary structure of the fused protein BCR-ABL1.
BCR and ABL1 fuse to form a constitutively active tyrosine kinase. The N-terminal domains of BCR are found in BCR-ABL1, while most of ABL1 is included in the fusion. BCR-ABL1 expression in humans leads to hematological disease, most notably chronic myeloid leukemia (CML).

BCR-ABL1 Variants

To form *BCR-ABL1*, *c-ABL1* translocates and fuses to the *BCR* gene through one of several distinct breakpoints on chromosome 22. Because the *ABL1* promoter region is lost, the fusion causes *ABL1* transcription to be directed by the *BCR* promoter. Each breakpoint forms a fused gene that expresses a distinct fused protein identified by its molecular mass. One break occurs at *BCR* intron 1 within the minor breakpoint cluster region (m-bcr), forming p190 BCR-ABL1. Another break occurs in the major breakpoint cluster region (M-bcr) at the *BCR* exon 12 and 13 junction to form p210 BCR-ABL1. The last break occurs following *BCR* exon 19 in another breakpoint cluster (u-bcr) to form p230 BCR-ABL1. Each BCR-ABL1 isoform is associated with a particular type of leukemia (Advani and Pendergast 2002). B-ALL and rare cases of CML, usually proceeding from chronic phase CML to B-ALL, possess the p190 form (Li 2008). The predominant form found in CML patients is p210, although it is also seen in a few acute myeloid and lymphoid leukemias. p230 has been associated with less aggressive forms of CML, such as chronic neutrophilic leukemia (CNL) (Pane, Frigeri et al. 1996). From here on, any mention of BCR-ABL1 will refer to the p210 variant, unless otherwise specified.

BCR-ABL1: Stem Cell Disease

In order to reach a leukemic state, the Philadelphia chromosome must be active in an early stage cell within the hematopoietic system (Figure 6). Normal hematopoiesis begins with the hematopoietic stem cell (HSC). Only stem cells have the ability to self-renew, therefore, only HSCs are capable of repopulating the bone marrow. During activation of the hematopoietic system, long-term (LT) repopulating HSCs give rise to short-term (ST) HSCs, which then generate non-self-renewing multipotent progenitors (MPPs) (Kondo, Wagers et al. 2003). These progenitors differentiate into common myeloid progenitors (CMPs) or common lymphoid progenitors (CLPs). CMPs further differentiate into either granulocyte-macrophage progenitors (GMPs) or megakaryocyte-erythroid progenitors (MEPs). GMPs ultimately produce mature granulocytes and macrophages, while MEPs produce mature erythrocytes and platelets (Akashi, Traver et al. 2000). CLPs generate mature B cells, T cells, and natural killer cells. Exongenous stimuli, such as cytokines, and endogenous influences, such as transcription factors, direct cell fate and select the path to mature blood cells (Kondo, Wagers et al. 2003).

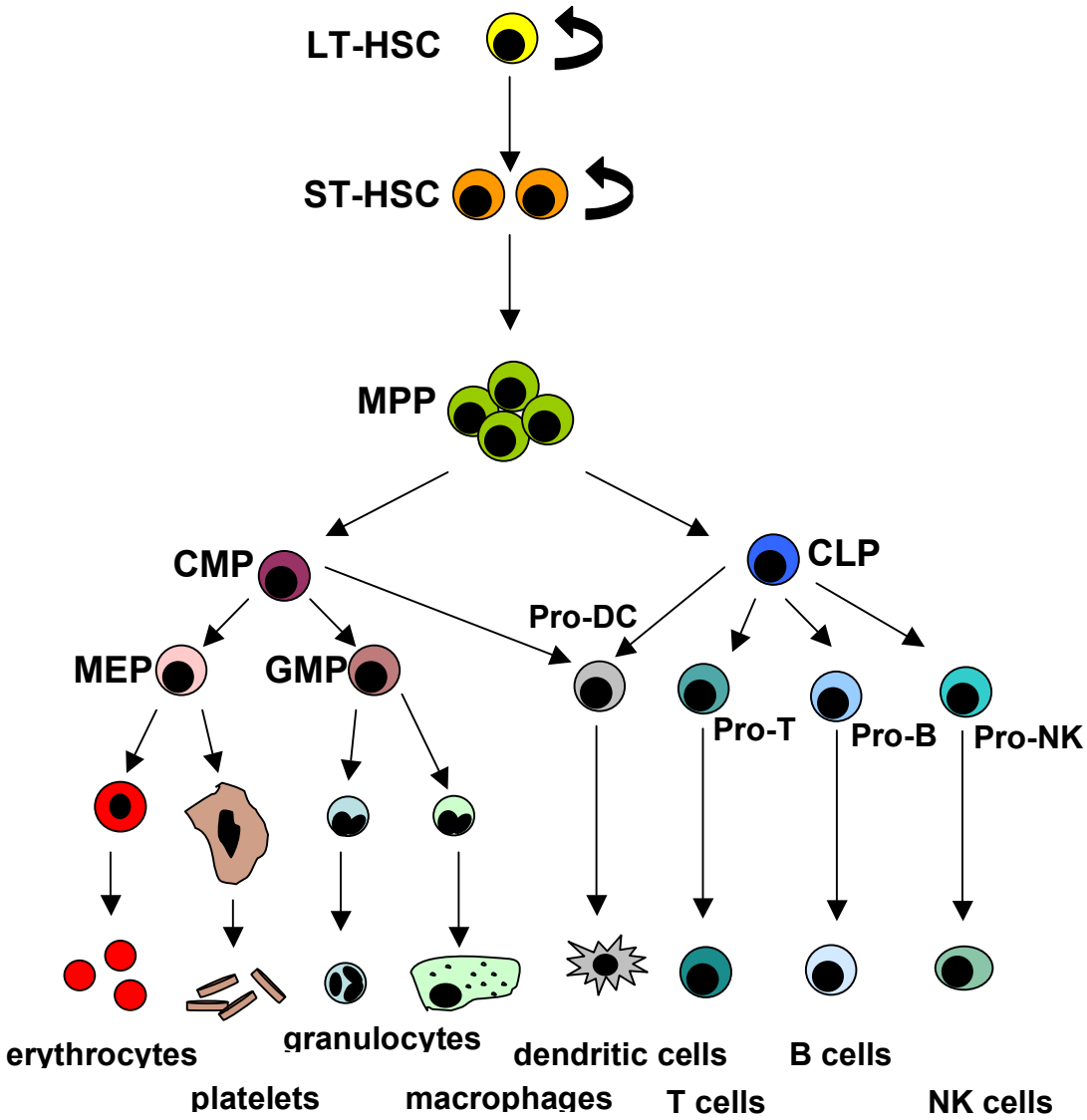

LT-HSC
ST-HSC
MPP
CMP
CLP
Pro-DC
MEP
GMP
Pro-T
Pro-B
Pro-NK
erythrocytes
platelets
granulocytes
macrophages
dendritic cells
T cells
B cells
NK cells

Figure 6: Hematopoietic system.

The stem cell population within the hematopoietic system consists of long-term hematopoietic stem cells (LT-HSCs) and short-term hematopoietic stem cells (ST-HSCs). These are the only cell types to have self-renewal capability. ST-HSCs give rise to multipotent progenitors (MPPs). MPPs become divided into committed myeloid progenitors (CMPs) or committed lymphoid progenitors (CLPs). CMPs become further differentiated into megakaryotic erythroid progenitors (MEPs) and granulocyte macrophage progenitors (GMPs). These progenitors then give rise to mature cells. CLPs give rise to progenitors for T, B, and NK cells. Both CMPs and CLPs are thought to contribute to the dendritic population. Figure adapted from Passague, Jamieson et al. 2003.

Many clues pointed to CML arising from an early hematopoietic progenitor cell. In CML patients, the Ph chromosome is present across most hematopoietic cell lineages, including granulocytes, macrophages, B lymphocytes, erythrocytes, megakaryocytes, and at times T lymphocytes (Whang, Frei et al. 1963). Through studies of glucose-6-phosphate dehydrogenase isozymes found in different cell lineages of CML patients, it was shown that CML must be a HSC disease (Fialkow, Gartler et al. 1967). In support of this hypothesis, purified murine HSCs, identified by the expression pattern of cell surface markers ($Lin^{-}c\text{-}kit^{+}Sca\text{-}1^{+}$) (Hu, Swerdlow et al. 2006), were able to induce CML in mice when transduced with *BCR-ABL1*. However, *BCR-ABL1* transduction of committed myeloid progenitors failed to establish disease (Huntly, Shigematsu et al. 2004). In addition, it was found that HSCs expressing BCR-ABL1 isolated from the bone marrow of CML mice induced disease in secondary recipients. This observation implicates HSCs functioning as leukemia-initiating stem cells, since progenitors are unable to self-renew and repopulate the bone marrow (Hu, Swerdlow et al. 2006).

It is possible, however, for hematopoietic progenitor cells to become transformed during leukemogenesis and acquire the capacity to self-renew, therefore behave as leukemic stem cells. The process is not entirely understood, but one group found that through β-catenin activation in human CML myeloid blast crisis, progenitors for granulocyte and macrophage formation acquire the stem cell behavior and are seen to self-renew

(Jamieson, Ailles et al. 2004). β-catenin activation has previously been shown to be involved in self-renewal of normal HSCs (Reya, Duncan et al. 2003; Willert, Brown et al. 2003).

BCR Contributions to Leukemogenesis

Chromosomal breakpoints leading to a balanced translocation with chromosome 9 were localized to a limited region within chromosome 22. Due to this observation, the gene identified to undergo these breakpoints was termed the breakpoint cluster region gene, or *BCR* (Groffen, Stephenson et al. 1984). It encodes for a protein of molecular weight 160 kDa, and is ubiquitously expressed, and mostly localized to the cytoplasm (McWhirter, Galasso et al. 1993; Wetzler, Talpaz et al. 1993). To date, the physiological significance of *BCR* remains unknown. *BCR* knockout mice are for the most part healthy and show no defects, especially within the hematopoietic system, where *BCR-ABL1* exhibits its detrimental effects (Diekmann, Brill et al. 1991).

Even though there are limited findings on BCR itself, there are a few interesting reports that justify the importance of BCR in BCR-ABL1-mediated leukemogenesis. The BCR coiled-coil domain forms a homotetramer structure, inducing BCR-ABL1 oligomerization. It was found that the coiled-coil domain is essential for cell transformation, as well as for induction of CML in a mouse model (McWhirter, Galasso et al. 1993; He, Wertheim et al.

2002). BCR-ABL1 oligomerization through the BCR dimerization domain is a major factor in BCR-ABL1 dysregulation. It phosphorylates the tyrosine residue 245 adjacent to the SH3 auto-binding site, disrupting the autoinhibited form of the kinase induced by ABL1 SH3 and SH2 (Smith and Van Etten 2001; Smith, Yacobi et al. 2003). The loss of an autoinhibitory conformation helps explain why PRDX1 may only partially inhibit BCR-ABL1, as we believe it recognizes an inactive conformation of ABL1. The BCR kinase domain seems to play a role in BCR-ABL1 kinase activity. Eliminating BCR kinase activity at its serine/threonine domain enhances BCR-ABL1-induced transformation and oncogenesis (Perazzona, Lin et al. 2008). The pleckstrin homology domain in BCR also may seem to play a functional role in BCR-ABL1-mediated leukemogenesis. It was found that various proteins involved in cell proliferation and migration interact with this domain. Some of these interacting proteins induced BCR-ABL1 localization to the lipid membrane, possibly influencing further signaling pathways leading to leukemogenesis (Miroshnychenko, Dubrovska et al. 2010). The BCR Dbl homology domain in BCR-ABL1 is responsible for activating Rho GTPases, which are proteins involved in cytoskeleton arrangement, proliferation, apoptosis, and myeloid differentiation. Activation of the Rho GTPases seem to influence cell transformation in myeloid cells (Harnois, Constantin et al. 2003).

Certain residues have also been found to be crucial for influencing signaling pathways generated by BCR-ABL1. A tyrosine residue (Y177) that becomes a phosphotyrosine site is found in the BCR portion of BCR-ABL1 and is a necessary contact point for the adaptor protein growth factor receptor-bound protein 2, or GRB2 (Pendergast, Quilliam et al. 1993). Deactivating mutations at Y177 lead to impaired CML development and diminished lymphoid-induced leukemogenesis (Sattler, Mohi et al. 2002). Also, distinct phosphoserine and phosphothreonine residues in the N-terminus of BCR are responsible for inducing recruitment of proteins harboring an SH2 domain (Pendergast, Muller et al. 1991).

ABL1 Significance in Leukemogenesis

The ABL1 portion of BCR-ABL1 also has its own significant roles in leukemogenesis. The ABL1 ATP/kinase domain is arguably the most important portion of BCR-ABL1, as it is what defines BCR-ABL1 as a constitutive kinase. The ATP domain and the previously identified tyrosine-1294 residue are essential to induce autophosphorylation and activation of BCR-ABL1 (Pendergast, Gishizky et al. 1993). The ABL1 protein also contains proline motifs of the sequence PXXP that serve to attract proteins through their SH3 domains. These proteins are linked in signal transduction and are usually activated by BCR-ABL1 to transfer signaling for processes such as cell survival or proliferation, as is seen by CRK substrate activation (Feller,

Knudsen et al. 1994). The actin domain found in the C-terminus has been linked to cell adhesion and migration that can be important factors in transformation and carcinogenesis (Quintas-Cardama and Cortes 2009).

Surprisingly, not many direct BCR-ABL1 substrates known to be crucial for leukemogenesis have been identified. Two substrates were mentioned in the previous segment, in which GRB2 binds to the BCR portion of the oncoprotein and CRK recognizes the PXXP motif in ABL1 (Pendergast, Quilliam et al. 1993; Feller, Knudsen et al. 1994). GRB2 recruits the GRB2-associated binding protein 2 (GAB2), leading to the activation of the PI3K/AKT cell survival pathway (Sattler, Mohi et al. 2002). GRB2 also recruits a guanine nucleotide exchange factor known as son of sevenless (SOS), which in turn causes RAS activation and induces the MAPK cell survival pathway (Tauchi, Boswell et al. 1994). Another substrate that becomes directly phosphorylated and activated by BCR-ABL1 is signal transducer and activation of transcription 5, or STAT5 (Ilaria and Van Etten 1996). It was shown that STAT5 deletion in a mouse model eliminated CML induction, showing the importance of the substrate in BCR-ABL1-induced leukemogenesis (Walz, Ahmed et al. 2012).

Chronic Myeloid Leukemia (CML)

As previously mentioned, the presence of the Ph chromosome and the subsequent expression of *BCR-ABL1* lead to CML disease development.

However, the underlying cause for the creation of the Ph chromosome has yet to be determined. An interesting finding shows that exposure to a high amount of radiation increases the risk factor for CML, leading to believe that genotoxic stress on cells may induce the formation of *BCR-ABL1* (Preston, Kusumi et al. 1994; Goldman and Melo 2003).

CML is characterized by an overproduction of mature myeloerythroid cells in the bone marrow and peripheral blood. It was classified as a myeloproliferative disorder due to the fact that it arises from leukemic-initiating stem cells, is caused by the constitutive activity of tyrosine kinase signaling, and exhibits abnormalities in hemostasis and thrombosis (Dameshek 1951).

CML is defined by three stages signifying disease progression and severity. In the chronic phase, which can persist for months or even years, pluripotent stem cells express BCR-ABL1 and divide at a high rate. However, cell differentiation is not affected, and normal neutrophil maturation is observed. There are symptoms present, though, such as a high white blood cell (WBC) count in the peripheral blood, a high platelet count, splenomegaly, fatigue, weight loss, and anemia. If left untreated, the disease eventually progresses to an acute form, making it difficult to treat (Van Etten and Shannon 2004). The accelerated phase shows impairment of neutrophil differentiation, an increased presence of blast cells (<20%) in the bone marrow and peripheral blood, and resistance to chemotherapeutic agents. At

this stage, median survival rate is one to two years (Cortes, Talpaz et al. 2006). The last phase is blast crisis, and is defined by the inability of myeloid or lymphoid blast cells to undergo differentiation, contributing to over 20% of the population of bone marrow cells. Treatment is usually ineffective, and median patient survival is six months (Druker, Sawyers et al. 2001; Perrotti, Jamieson et al. 2010).

Progression of the disease from one stage to another involves additional genetic mutations besides BCR-ABL1 expression that together function to induce disease severity. Some of the mutations known to be involved are found in tumor suppressor genes, such as Rb, p16, and p53 (Feinstein, Cimino et al. 1991; Towatari, Adachi et al. 1991; Sill, Goldman et al. 1995). However, it is neither clear how the tumor suppressors acquire mutations nor what is necessary to transition from one phase to another phase.

It has been found that BCR-ABL1 expression increases as disease progresses. There is a possibility that BCR-ABL1 transcription activation plays a part in inducing disease progression, as leukemic cells in blast crisis have higher *BCR-ABL1* mRNA levels compared to chronic phase cells (Gaiger, Henn et al. 1995). Also in support of this theory, a group found that myeloid progenitors from patients in blast crisis have five times more *BCR-ABL1* transcription activity than progenitors from patients in chronic phase (Jamieson, Ailles et al. 2004).

There have been a few studies showing that *BCR-ABL1* itself may be enough to induce genetic instability that could lead to other genetic mutations functioning in disease progression. BCR-ABL1 expression influences inhibition of DNA repair enzymes, such as DNA-PKcs (Deutsch, Dugray et al. 2001). There is a possibility that BCR-ABL1 may also impair the DNA repair function of the xeroderma pigmentosum group B protein (Takeda, Shibuya et al. 1999). BCR-ABL1 is further shown to deregulate DNA-break repair enzymes BRCA1 and RAD51 (Canitrot, Lautier et al. 1999; Slupianek, Schmutte et al. 2001). BCR-ABL1 can affect transcriptional mutagenesis by inducing and activating the error-prone polymerase Beta (Canitrot, Lautier et al. 1999). Inactivation of the DNA damage response system can also be achieved through inhibition of the DNA repair rad 3-related protein (ATR) by BCR-ABL1 (Dierov, Dierova et al. 2004). The increase in cell survival and decrease in DNA damage repair both induced by BCR-ABL1 may function in combination to influence secondary genetic mutations leading to disease progression (Calabretta and Perrotti 2004).

Not only can BCR-ABL1 cause mutations in other genes, but it also seems that BCR-ABL1 can cause self-mutagenesis in the presence of oxidative stress. It was found that BCR-ABL1 actually stimulates ROS levels, resulting in mutations within its kinase domain. These mutations prove to be detrimental, as they potentially lead to resistance of drugs that recognize the kinase domain (Koptyra, Falinski et al. 2006).

B-cell Acute Lymphoblastic Leukemia (B-ALL)

In CML, the target cell for the Ph translocation event is the hematopoietic stem cell (Jamieson, Ailles et al. 2004; Hu, Swerdlow et al. 2006). In patients with Ph^+ B-ALL having no prior CML symptoms, the target cell is likely an early B-progenitor cell (Castor, Nilsson et al. 2005; Hu, Swerdlow et al. 2006).

In a BCR-ABL1-induced mouse model for B-ALL (described in detail later), BCR-ABL1 is expressed only in immature B lymphoid cells. These leukemic cells express typical B cell markers and show immunoglobin heavy chain gene rearrangement characteristically found in the pro- and pre-B stages. These findings in the mouse model recapitulate human Ph+ B-ALL, as both diseases arise from lymphoid transformation at the pro- or pre-B cell stage (Li, Ilaria et al. 1999).

Similar to CML, B-ALL arises from leukemic transformation of progenitor cells that in turn exhibit self-renewal and behave as stem cells (Hu, Swerdlow et al. 2006). Patients with B-ALL present with a high count of immature lymphoid blast cells (over 25%) in the bone marrow. In addition, lymphoid cell infiltration into the spleen, lymph nodes, and thymus is observed (Wong and Witte 2004).

The lymphoid blast crisis stage of CML and de novo Ph^+ B-ALL are pathologically similar, and approximately 20% of adults and 5% of children

are afflicted with B-ALL. 50% of adults and 20% of children from these patients carry the p210 version of BCR-ABL1. The remaining patients show the p190 BCR-ABL1 form (Sawyers 1999; Druker, Sawyers et al. 2001).

Selectivity of Disease and BCR-ABL1 Signaling

BCR-ABL1 elicits a number of signaling pathways involved in cell growth and survival (Wong and Witte 2004). It also stimulates cytokine production, inducing proliferation and allowing for growth factor independence (Hariharan, Adams et al. 1988; Anderson and Mladenovic 1996). In addition, BCR-ABL1 inhibits apoptotic pathways (Wong and Witte 2004).

Development of B-ALL or CML not only depends on the transformation of a specific cell type (early B lymphoid progenitor cells for B-ALL and hematopoietic stem cells for CML) (Jamieson, Ailles et al. 2004; Castor, Nilsson et al. 2005; Hu, Swerdlow et al. 2006), but also on the distinct activation of signaling pathways by BCR-ABL1 required for each to achieve disease progression. For example, three Src kinases, Lyn, Hck, and Fgr, are necessary for B-ALL development, but are dispensable for BCR-ABL1-induced CML, even though BCR-ABL1 functions to activate the Src kinases in both lymphoid and myeloid cells (Hu, Liu et al. 2004). CML development, on the other hand, requires phosphorylation of tyrosine 177 in BCR-ABL1 and subsequent recruitment of GRB2-GAB2, PI3K/SHP2, and STAT5 (Sattler, Mohi

et al. 2002; Walz, Ahmed et al. 2012). Another finding shows that the SH2 domain of BCR-ABL1 is essential for proper CML induction; however, it fails to play a role in B-ALL development (Roumiantsev, de Aos et al. 2001). This domain could serve to activate a specific substrate in a particular pathway not necessary for establishing B-ALL. Therefore, disease induction and development is based largely on the cell type origin and the distinct cell-specific signaling pathways involved. To address these issues, therapeutic treatments must possess a selective nature in order to attain proper drug efficacy and control of disease.

Disease Diagnosis

In order to determine if patients have CML or B-ALL, diagnostic tests are implemented. Patients may be rapidly diagnosed through initial testing involving peripheral blood smears or a bone marrow biopsy. Upon identifying pathological hallmarks of the disease, verification to determine if the Philadelphia chromosome is present is done by cytogenetic analysis (karyotyping of chromosomes). Alternatively, the *BCR-ABL1* fusion gene can be identified by fluorescent in situ hybridization (FISH) analysis, and the level of *BCR-ABL1* mRNA transcripts quantitated by reverse transcription polymerase chain reaction (RT-PCR) (Radich 2011).

Treatments

In order to identify the effectiveness of treatments for Ph^+ leukemias, patient responses are categorized by the following criteria. The first response to treatment is complete hematologic remission (CHR), which is defined by the restoration of normal peripheral blood counts after therapy. The next level of response is cytogenetic remission, where karyotyping analysis finds that there is a >65% reduction (partial cytogenetic remission, PCyR) or absence (complete cytogenetic remission, CCyR) of the Ph chromosome in a bone marrow sample. The last response level is the molecular response, which utilizes the most current sensitive technique, quantitative reverse transcriptase PCR, to quantitate the level of *BCR-ABL1* transcripts (Radich 2011). A 1000-fold (3 log) decrease in *BCR-ABL1* transcripts from diagnosis is called a major molecular response (MMR), while a reduction in *BCR-ABL1* transcripts below the level of detection (about 4.5 log decrease) is called a complete molecular response (CMR).

Historically, the treatments for CML involved administration of the myelosuppressive compounds busulfan or hydroxyurea. However, these agents fail to selectively target leukemic cells, and though patients can attain CHR, they never reach CCyR (Sawyers 1999). Therefore, these compounds merely serve to treat the symptoms of Ph^+ leukemias, and are considered palliative in nature.

The first drug treatment that was shown to prolong survival in CML was the cytokine interferon-alpha (IFN-a). There are a few patients who are able to acquire CCyR with this treatment. However, the percentage of individuals responding well to IFN-a treatment is fairly low, and the toxicity is significant, has many patients experience serious side effects (Bonifazi, de Vivo et al. 2001).

The only therapy for Ph+ leukemia that is known to be curative is allogeneic bone marrow transplantation (BMT). However, this treatment option is difficult to administer, as compatible donors and patient age need to be taken into consideration. Younger patients who have a human leukocyte antigen (HLA)-matched donor respond well to the BMT and can have long-term disease-free survival. However, the procedure is risky, with up to 25% of patients succumbing to complications related to the procedure. One complication is the inability of patients to combat infections when their immune system has been suppressed for the procedure. The other main side effect of the transplant is patients developing graft-versus-host disease (GvHD). GvHD is a condition when the donor immune cells recognize the patient's cells as foreign and began attacking those cells (Gratwohl, Brand et al. 2005), causing dysfunction of the skin, liver, and gastrointestial tract that can prove fatal.

Cells expressing BCR-ABL1 are characterized by an increase in proliferation, the ability to undergo transformation, and an acquisition of

growth independent of growth-factor stimuli. BCR-ABL1 has high tyrosine kinase activity that could be due to the absence of the myristoyl group (Nagar 2007). Another possibility is the influence of the BCR fused protein. A domain in the N-terminus of BCR causes oligomerization and autophosphorylation of the kinase, altering the inhibitory conformation (Fan, Cong et al. 2003; Van Etten 2004). Though high tyrosine activity is observed, BCR-ABL1 is still able to form an inactive structure. The inactive and active forms of BCR-ABL1 seem to be in equilibrium in both normal and leukemic cells. In the monomeric and unphosphorylated form, BCR-ABL1 is inactive, as the SH3 domain is bound to the SH2-catalytic domain linker (Van Etten 2004). This inactive state of BCR-ABL1, actually, is necessary for the primary anti-leukemic drug, imatinib, to recognize and repress its kinase activity (Nagar 2007).

The chemotherapeutic agents that target BCR-ABL1 are known as tyrosine kinase inhibitors (TKIs). Patients with Ph^+ leukemias are treated with oral TKIs, which attempt to selectively target and inhibit the tyrosine kinase ABL1. The first tyrosine kinase inhibitor to be approved for treatment of CML patients in the chronic phase was imatinib, which binds to the kinase domain of BCR-ABL1 and inactivates the kinase activity. Imatinib is also capable of inhibiting platelet derived growth-factor receptor, c-KIT, and c-ABL1 activity (Druker, Talpaz et al. 2001). It functions as an ATP-competitive inhibitor and therefore interferes with the binding of ATP. Specifically, it

binds to the ATP-cleft between the N- and C-lobes of the kinase domain (Nagar 2007). Imatinib is actually still the principal drug utilized for CML treatment, as it has proven to be very effective. The majority of patients with chronic phase CML respond well to imatinib, showing a complete hematological cytogenetic response (Druker, Talpaz et al. 2001).

There are still, however, major obstacles involved with imatinib treatment. Of important interest is that imanitib only recognizes the inactive (type II) conformation of kinases. Therefore, once BCR-ABL1 becomes tyrosine phosphorylated in the activation loop, leading to the active conformation, imanitib is no longer able to bind to the kinase. The tyrosine phosphorylation leads to binding of the phosphorylated residue to an aspartate residue in the activation loop. This interaction mimics the structure taken when c-ABL1 is bound to a substrate, therefore blocking substrates, such as imatinib, from being recognized (Nagar 2007). Also, even though patients responsive to imatinib frequently show a cytogenetic response, a complete molecular remission, in which levels of *BCR-ABL1* transcripts are undetectable, is difficult to achieve (Sawyers, Hochhaus et al. 2002; Drummond, Lush et al. 2003). In addition, there are many patients who fail to respond to imatinib with initial treatment. Further, patients in the accelerated phase or blast crisis do not respond well to imatinib treatment (Jabbour, Cortes et al. 2006).

Even patients with an initial response to imatinib can show a relapse in disease and eventually develop a more advanced stage of the disease. BCR-ABL1 signaling is actually reactivated and patients no longer become responsive to imatinib, especially when patients enter the blast crisis stage (Druker, Sawyers et al. 2001; Gorre, Mohammed et al. 2001). The failure of imatinib to eradicate CML may be a consequence of the observation that imatinib can target and eliminate highly proliferating leukemic cells, but is unable to eradicate leukemic-initiating stem cells in CML and B-ALL mice (Graham, Jorgensen et al. 2002; Hu, Liu et al. 2004). Indeed, when imatinib therapy is stopped in CML patients who have been in complete molecular remission for over 2 years, the disease relapses quickly in the majority of patients (Mahon, Rea et al. 2010).

Several mechanisms for acquired resistance to imatinib in CML have been described, including *BCR-ABL1* mutations, gene amplification, or signaling alterations that cause drug resistance. Mutations within BCR-ABL1 that confer imatinib resistance are mainly localized to the ABL1 kinase domain of BCR-ABL1 (Jabbour, Cortes et al. 2006). Amplification of *BCR-ABL1* has also been shown to contribute to imatinib resistance (Gorre, Mohammed et al. 2001). Activation of certain signaling pathways can also permit cells to bypass the effects of imatinib on BCR-ABL1 (Hu, Swerdlow et al. 2006). Because of these complications, other therapies to treat leukemia are

necessary to attempt to completely obliterate disease progression and cure Ph^+ leukemias when imanitib alone is not enough to treat patients.

In order to treat patients who are unable to respond to imatinib, there has been much research focused on finding alternative TKIs. Currently, there are two approved second-generation TKI drugs used in addition to or in place of imatinib. Dasatinib, in contrast to imatinib, binds to the active form of BCR-ABL1, and has proven to be effective in inhibiting imatinib-resistant mutants (Shah, Tran et al. 2004). Nilotinib binds to the inactive form of BCR-ABL1 and is useful in treating patients with imatinib-resistant mutations (Weisberg, Manley et al. 2005). Both drugs are more potent than imatinib. Unfortunately, neither of these drugs is able to inhibit a prominent and very detrimental BCR-ABL1 kinase mutation, referred to as the T315I 'gatekeeper' mutation (Shah, Tran et al. 2004; Weisberg, Manley et al. 2005). Hence, new approaches are needed in CML to treat resistant disease, and in order to eliminate CML "stem cells" and permanently cure patients.

Recently, a novel BCR-ABL1 kinase inhibitor was discovered through a screening process for new compounds. This inhibitor, known as GNF-2, is an attractive candidate for BCR-ABL1 kinase regulation, as its mode of action is distinct from that of ATP-competitive ABL1 kinase inhibitors like imatinib. This drug was of interest to our studies, as some of the findings by the group who discovered GNF-2 suggested that PRDX1 could act as a co-factor for GNF-2 recognition and inhibition.

There are some obstacles that exist in developing and administering potential kinase inhibitors for use against cancer. A major obstacle is directing the inhibitor to a specific kinase. An added barrier is that many kinases exhibit similar active conformations. Therefore, it would be difficult to target an inhibitor in the active state. A promising area for drug design would involve production of drugs that could deal with mutants resistant to current drugs on the market, overcome mutants that affect the autoinhibitory structure, and development of inhibitors that could recognize the active state of the kinase, as well as the inactive state (Nagar 2007).

A research group performed a screen to identify novel compounds that function to target BCR-ABL1 kinase activity. This screen was incorporated to find compounds that do not bind to the ATP-binding pocket, the common region were the majority of current kinase inhibitors bind. The screen tested various heterocyclic compounds, which are defined by carbon ring structures with elements such as oxygen or nitrogen as part of the ring. These compounds, imatinib being one of them, have been proven to possess anti-cancer activity. The library of compounds was applied to BCR-ABL1-dependent cell lines, and proliferation was tested using a fluorescence-based proliferation assay. After obtaining compounds that could inhibit cell proliferation, they were tested to monitor their ability to inhibit ABL1 kinase activity and BCR-ABL1 autophosphorylation. Compounds known to target the ATP-binding kinase domain, such as imatinib, were discarded. The remaining

compounds were modified to increase the antiproliferative activity. Through this screening, a new BCR-ABL1 inhibitor, known as GNF-2, was synthesized. This compound shows great promise, as it selectively inhibits proliferation and induces apoptosis of BCR-ABL1-expressing cells (Adrian, Ding et al. 2006).

Even though GNF-2 was found to interact with the ABL1 kinase domain, this sole interaction does not seem to be enough to inhibit cell proliferation of BCR-ABL1-expressing cells. It is unclear what other structures, such as Bcr, SH2, or SH3, GNF-2 utilizes to act as an inhibitor of Abl and BCR-ABL1. There is evidence, however, to suggest that the myristoyl pocket of the C-lobe kinase domain is necessary for GNF-2 binding. Mutations introduced in the myristoyl cleft failed to bind GNF-2, preventing suppression of cell growth (Adrian, Ding et al. 2006).

Murine Disease Models

Tyrosine kinase dysregulation is now known to be the pivotal factor in establishing certain leukemias, including several myeloproliferative neoplasms. To reach this conclusion, a mouse model was created to determine the actual role of tyrosine kinases in human leukemia. This *in vivo* approach allows for the identification of the impact an overactive tyrosine kinase has on disease progression and pathogenesis within the hematopoietic system. Much information can be gained from an in vivo mouse model upon disease induction, such as discovering specific genetic abnormalities

influencing disease development, identifying a clinical course of malignancy, and assessing responses to drug or immunotherapy treatments (Van Etten 2002).

Three main strategies to express a dysregulated tyrosine kinase in the mouse hematopoietic system are 1) by producing a transgenic mouse, 2) adapting an approach of xenotransplantation of human leukemia cells in immunodeficient mice, or 3) through a bone marrow retroviral transduction/transplantation technique (Van Etten 2001). A main obstacle with the transgenic model is that dysregulated tyrosine kinases, especially BCR-ABL1, lead to toxicity and gene silencing, making it difficult and unreliable to attain a diseased state (Heisterkamp, Jenster et al. 1991; Jaiswal, Traver et al. 2003). It was found that p210 *BCR-ABL1* transgene driven by the BCR promoter leads to embryonic lethality (Heisterkamp, Jenster et al. 1991). The p190 BCR-ABL1 variant does not cause lethality, however, the main disease to arise is lymphoid-derived, such as B-ALL, and not CML. Also, maintaining a transgenic colony and the fact that there is a long latency in establishing disease make it difficult to conduct many studies in a timely manner. These difficulties have been recently addressed, however, by the development of conditional transgenic models of BCR-ABL1 leukemia (Koschmieder, Gottgens et al. 2005).

The xenotransplantation model is a good system to study the effects of human CML cells within a murine background. This approach involves

purifying $CD34^+$ cells from bone marrow or peripheral blood of CML patients and transplanting the cells into sublethally-irradiated NOD/SCID or NOD/SCID/*Il2gr*$^{-/-}$ mice. Although cells engraft well and cells from the different CML stages can be studied, the mice themselves fail to develop a progressive myeloproliferative disease (Wang, Lapidot et al. 1998). Also, obtaining human CML cells for studies can be difficult and expensive.

Due to the various obstacles mentioned in the previous mouse models for CML studies, our laboratory has focused on utilizing the retroviral transduction/transplantation model. Bone marrow transduction allows for targeting of cells in the myeloid and lymphoid lineages. These cells then carry the retroviral provirus and are able to express BCR-ABL1. BCR-ABL1 expression leads to increased levels of selective phosphorylated proteins at tyrosine residues and activation or inhibition of multiple cell signaling pathways (Li, Ilaria et al. 1999; Roumiantsev, de Aos et al. 2001). The first model of this kind was reported by Daley et al., with subsequent modifications applied to the original murine model to improve on the efficiency of disease induction and make it a more robust model (Daley, Van Etten et al. 1990; Zhang and Ren 1998; Li, Ilaria et al. 1999; Hu, Liu et al. 2004). Donor mice are pretreated with 5-fluorouracil (except in the B-ALL model) to eliminate differentiated bone marrow populations and stimulate stem cells and progenitors. The bone marrow is harvested and transduced with a *BCR-ABL1-GFP* retrovirus in the presence of myeloid cytokines for CML,

or lymphoid-inducing medium for B-ALL. Wild type recipient mice are irradiated to make space in the bone marrow niche for the transplanted cells injected through the tail vein. Mutant recipient mice are usually only used to study the effects of the bone marrow microenvironment and its role in disease development (Li, Ilaria et al. 1999; Van Etten 2002).

Mice develop disease similar to human leukemia within 4 weeks of transplantation, with CML being induced more rapidly than B-ALL. In murine CML, mice develop similar symptoms as found in human CML patients, such as an elevated white blood cell count, splenomegaly, and expansion of granulocytes and macrophages in the bone marrow, spleen, and peripheral blood. Recipient mice that acquire CML-like disease succumb to the disease because *BCR-ABL1*-transduced transplated cells reconstitute the bone marrow. This leads to granulocytosis, cell infiltration in hematopoietic organs, and eventually organ failure (Li, Ilaria et al. 1999; Van Etten 2002). In murine B-ALL, mice also mimic some symptoms of human B-ALL, such as fatigue, enlarged lymph nodes, and an increase in the pro-B cell population within the bone marrow, lymph nodes, spleen, and peripheral blood (Wong and Witte 2004).

If patients with CML are not treated at an early stage, the disease progresses to acute myeloid or lymphoid leukemia, also known as blast crisis (Van Etten and Shannon 2004). In the murine transplant model, blast crisis is not observed, as primary recipient mice succumb to symptoms before

disease can progress to that stage. However, acute myeloid and lymphoid leukemias can be seen upon serial transplantation of CML-like cells from primary recipient mice into secondary or tertiary recipient mice (Daley, Van Etten et al. 1991; Gishizky, Johnson-White et al. 1993; Pear, Miller et al. 1998).

Upon reaching the endpoint of the diseased state, DNA from hematopoietic organs or cells is analyzed by Southern blotting to identify a proviral integration pattern. This procedure reveals that CML-like disease in the mouse model is polyclonal, as many clones are seen to arise during disease progression within a mouse. The same clones are observed in various cell types, such as neutrophils, macrophages, erythrocyte progenitors, B lymphocytes, and T lymphocytes. The integration pattern demonstrates that *BCR-ABL1*-transduced bone marrow cells that are initiating CML must have multi-lineage repopulation ability. This finding strengthens the idea that CML is actually a pluripotent stem-cell disease (Li, Ilaria et al. 1999; Million, Aster et al. 2002).

It has been proven to be very beneficial to study leukemia in a murine transduction/transplantation model, as all aspects of disease observed in the mouse closely resemble human CML or B-ALL (Daley, Van Etten et al. 1990; Kelliher, McLaughlin et al. 1990; Li, Ilaria et al. 1999). The major similarities crucial in disease pathogenesis include cell origin of disease, disease transplantability, disease progression, and responses to kinase inhibitors

treatment and immunotherapy (Wolff and Ilaria 2001; Hu, Liu et al. 2004; Krause and Van Etten 2004). There are, however, differences in clonality and latency of disease between disease in the mouse model and human disease. Murine CML-like disease shows a polyclonal nature with a shorter latency, whereas human leukemia is a monoclonal and chronic disease. The reason for these differences is due to the fact that the hematopoietic system becomes overloaded with the retrovirus during transduction, allowing for many progenitors to be targeted by *BCR-ABL1*. Also, the proviral LTR domains induce high BCR-ABL1 expression, typically leading to higher BCR-ABL1 levels than seen in human CML cells (Li, Ilaria et al. 1999; Barnes, Schultheis et al. 2005). A high number of these transduced cells are then transplanted into each mouse, therefore leading to a polyclonal pattern and shorter latency. The discrepancies can be improved or eliminated by lowering the viral titer or limiting the number of transplanted transduced cells. These alterations help to establish an oligo- or monoclonal disease and lengthen the latency of disease in the murine model (Daley, Van Etten et al. 1990; Jiang, Stuible et al. 2003). Other main discrepancies include the hallmark observation of pulmonary hemorrhages, or blood clots within lung tissue, in the murine CML transplant model, whereas this is a rare occurrence in human CML. Pulmonary hemorrhages are thought to be the cause of death in mice inflicted with CML (Jiang, Stuible et al. 2003). Also, mice in the B-ALL transplant model commonly exhibit pleural effusion, which is lymphoid

cell infiltration within the pleural cavity of the lungs. While this can be seen in human B-ALL, it is not the most frequent cause of death, where this is the source of morbidity or death in the mouse B-ALL model (Roumiantsev, de Aos et al. 2001).

OBJECTIVE OF THESIS

PRDX1 is a multifunctional protein that has been shown to be involved in various cell processes. One main role of PRDX1 is that it functions as a peroxidase, mainly degrading hydrogen peroxide to regulate intracellular ROS levels. Its antioxidant activity may seem to have a significant contribution in cancer prevention, development, and maintenance. Another interesting role of PRDX1 is its regulation of ABL1. Our laboratory identified PRDX1 as a c-ABL1 interacting protein and candidate inhibitor of ABL1 kinase function.

We focused to further understand PRDX1 function and regulation, as there seems to be mounting evidence that peroxiredoxins may be crucial components in oncogenesis. Both oxidative stress and deregulation of ABL1 have been linked to cancer susceptibility. Reactive oxygen species (ROS) can induce cellular damage, introducing stable mutations that could particularly lead to transformation or cancer. *c-ABL1* can fuse to another gene, *BCR*, to express the fusion protein BCR-ABL1. BCR-ABL1 becomes a constitutively active kinase and is the pathological factor leading to Ph^+ leukemia.

Interestingly enough, as mentioned above, PRDX1 functions to regulate both oxidative stress and ABL1 through suppressive measures. More work is needed to identify mechanisms of how PRDX1 could prevent or delay transformation and oncogenesis. The first portion of this thesis aims to further characterize PRDX1 peroxidase activity and PRDX1 regulation of c-ABL1. The second portion of this thesis focuses to identify the significance of

PRDX1 in BCR-ABL1 regulation within the lymphoid and myeloid hematopoietic lineages.

CHAPTER 2: PRDX1 PEROXIDASE ACTIVITY AND ABL1 REGULATION

Introduction

In this chapter, we focus on the antioxidant activity of PRDX1 and its regulation of ABL1. The first set of studies exploit the proliferation defect present in *Prdx1*$^{-/-}$ MEFs and link the phenotype to a deficiency in an oxidative stress response mechanism. The second set of studies target the PRDX1 – ABL1 interaction and show that PRDX1 is unable to suppress kinase activity of ABL1 kinase mutants. Finally, the third sets of studies characterize PRDX1 regulation of BCR-ABL1 and reveal a strong inhibitory and possible localization role of PRDX1 on BCR-ABL1.

RESULTS

I. Prdx1 Regulation in Proliferation

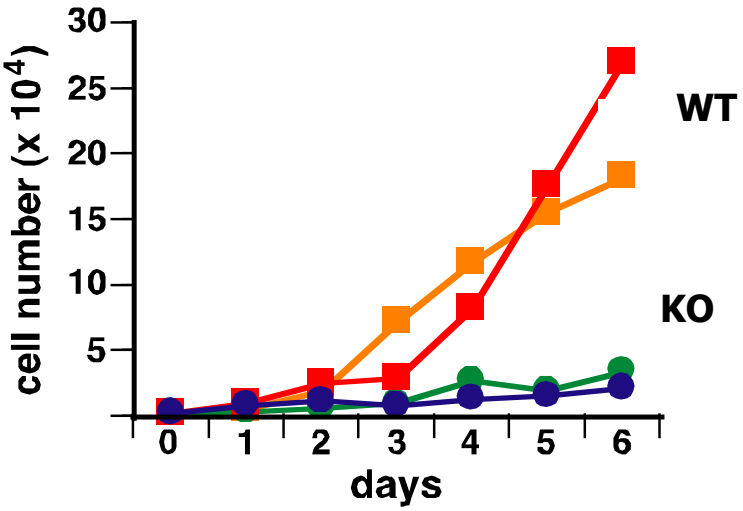
Prdx1$^{-/-}$ mice develop age-dependent fatal hemolytic anemia as well as multiple cancers. It was also observed that mouse embryonic fibroblasts (MEFs) from *Prdx1*$^{-/-}$ mice have a proliferation defect and exhibit increased sensitivity to ionizing radiation (Neumann, Krause et al. 2003). We decided to utilize the growth defect to our advantage by studying various factors that could contribute to the phenotype. The defect itself could provide a deeper understanding about the significance of PRDX1 and give us insight into a physiological function, as well as an explanation for the carcinogenesis

outcome. To unravel the mechanism of the growth delay in the absence of *Prdx1*, we considered three different biochemical causes: c-ABL1 kinase deregulation, oxidative stress, and cell senescence.

Prdx1$^{-/-}$ MEFs have a proliferation defect *in vitro*

We first worked to reproduce the proliferation phenotype in primary MEFs that was previously observed and published. Indeed, we were able to see a significant delay in growth in the *Prdx1*$^{-/-}$ cells (Figure 7B), although the difference was not as prominent as that observed in the original findings (Figure 7A) (Neumann, Krause et al. 2003). We then wanted to verify that *Prdx1*$^{-/-}$ cells were sensitive to oxidative stress. To determine if *Prdx1*$^{-/-}$ MEFs had a defective scavenging ability for ROS, we exposed cells to hydrogen peroxide. Since PRDX1 functions to specifically degrade hydrogen peroxide, *Prdx1*$^{-/-}$ MEFs would presumably accumulate hydrogen peroxide, causing growth arrest or apoptosis (Rhee, Chang et al. 2003). Similar to what was found in the Neumann, Krause et al. study (Figure 8A), *Prdx1*$^{-/-}$ cells were more sensitive to hydrogen peroxide compared to wild type cells, as the *Prdx1*$^{-/-}$ cells demonstrate a significant difference in cell viability (Figure 8B) (Neumann, Krause et al. 2003).

A.


cell number (x 10^4)
30
25
20
15
10
5
0
1
2
3
4
5
6
days
WT
KO

B.

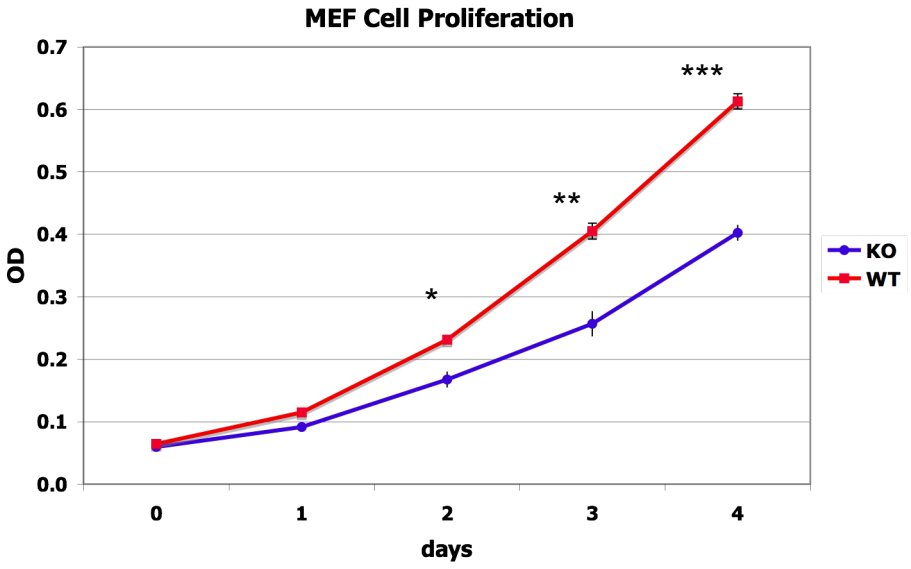
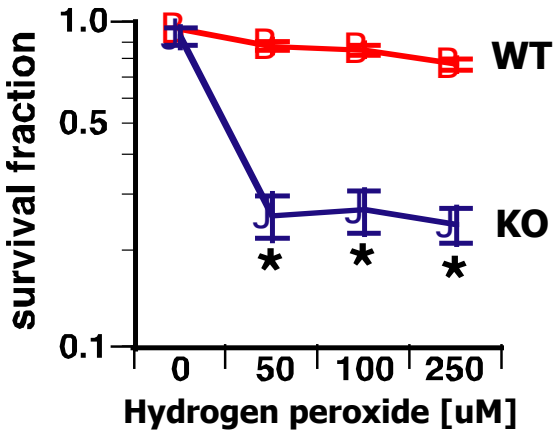

MEF Cell Proliferation
OD
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
0
1
2
3
4
days
*
**
***
KO
WT

Figure 7: Verification of proliferation phenotype observed in MEFs. A. Figure adapted from Neumann, Krause et. al. 2003. Primary MEFs were cultured and monitored for proliferation. The *Prdx1*$^{-/-}$ MEFs (labeled KO) show a distinct delay in growth compared to wild type MEFs. B. Reproducing the experiment to verify the growth defect in *Prdx1*$^{-/-}$ MEFs before continuing with further studies. Primary MEFs were cultured at $1x10^4$ cells/ml in 96-well plates in triplicate. Cells were incubated in MTS solution and cell viability was determined by spectrophotometry. The significant difference in cell survival between MEFs is reproduced, as the *Prdx1*$^{-/-}$ MEFs display decreased proliferation. (p values: * = 0.001; ** = < 0.0005; *** = <0.0001)

A.


1.0
0.5
0.1
survival fraction
WT
KO
*
*
*
0
50
100
250
Hydrogen peroxide [uM]

B.

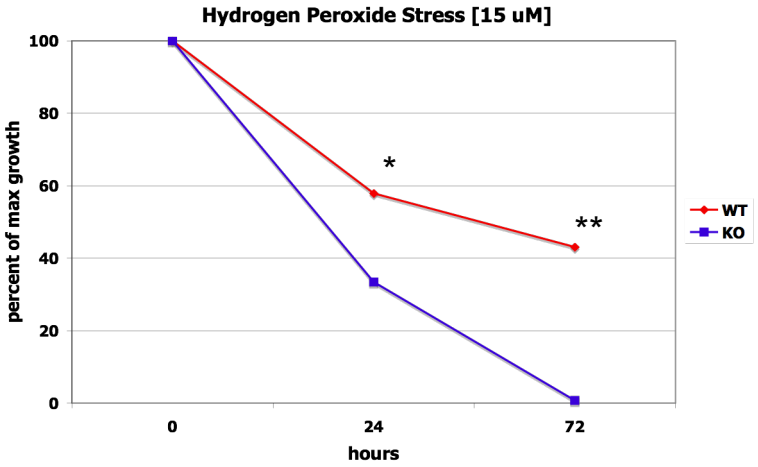

Hydrogen Peroxide Stress [15 uM]
100
80
60
40
20
0
percent of max growth
*
**
WT
KO
0
24
72
hours

Figure 8: Verifying diminished antioxidant activity in loss of PRDX1. A. Figure adapted from Neumann, et. al. 2003. Primary MEFs were exposed to various concentrations of hydrogen peroxide in culture and cell viability was monitored. Increasing hydrogen peroxide concentrations led to lower cell viability in *Prdx1*$^{-/-}$ MEFs. B. Similar findings were observed as we incubated primary MEFs with 15 μM of hydrogen peroxide and monitored for cell viability by the use of MTS solution and spectrophotometry. *Prdx1*$^{-/-}$ MEFs showed increased sensitivity to hydrogen peroxide, as cell viability was significantly reduced. (p values: * = < 0.05; ** = < 0.005)

c-ABL1 Kinase Regulation by Imatinib Fails to Eliminate Proliferative Defect in *Prdx1*$^{-/-}$ MEFs

Because there was a significant difference observed between primary wild type and *Prdx1*$^{-/-}$ MEFs, we sought out to determine the significance or reasoning for this phenotype. One possibility was that *Prdx1*$^{-/-}$ cells had a delayed and slow growth phenotype due to the lack of c-ABL1 regulation, a known proto-oncogene. It is possible that because PRDX1 no longer sequesters c-ABL1 in the cytoplasm, it finds its way into the nucleus to induce cell cycle arrest or apoptosis (Wen, Jackson et al. 1996).

To address this hypothesis, we targeted c-ABL1 with a kinase inhibitor, known as imatinib. Imatinib recognizes c-ABL1 and binds to it, causing inhibition of its kinase activity (Nagar, Bornmann et al. 2002). This interaction also prevents c-ABL1 from entering the nucleus and initiating arrest or apoptosis (Van Etten 1999). We tested various imatinib concentrations to determine if the delayed proliferation phenotype would be lost in *Prdx1*$^{-/-}$ MEFs. However, the results showed that the *Prdx1*$^{-/-}$ MEFs still exhibited a delayed proliferation phenotype in the presence of imatinib (Figure 9). The results suggest that c-ABL1 de-regulation does not contribute to the impaired proliferation phenotype in the *Prdx1*-deficient MEFs. It is likely that, because imatinib is highly efficient in c-ABL1 kinase inhibition, c-ABL1 is actually not responsible for inducing arrest or apoptosis in these *Prdx1*$^{-/-}$ MEFs. It could also be formally possible that imatinib requires

PRDX1 to keep c-ABL1 in its inactive conformation required for imatinib recognition and inhibition of kinase activity.

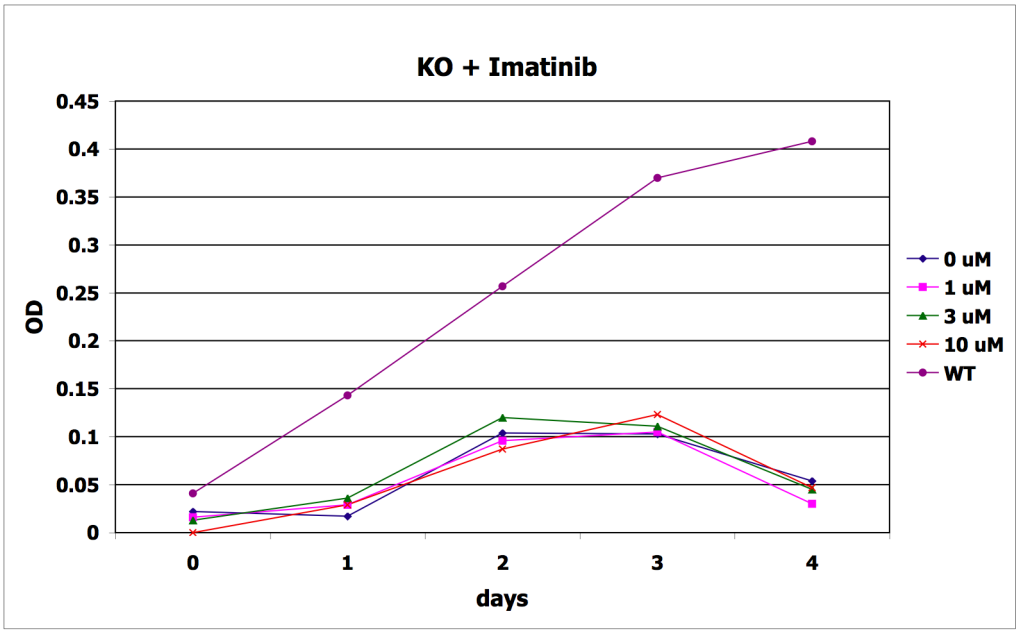


Figure 9: Imatinib fails to rescue *Prdx1*$^{-/-}$ MEFs from a delayed growth phenotype.

Primary *Prdx1*$^{-/-}$ MEFs were incubated with 1, 3, or 10 μM of the ABL1 kinase inhibitor imatinib. Cells were cultured at 1x10^4 cells/ml in 96-well plates in triplicate and monitored daily for proliferation. Cell viability was tested with MTS solution and by spectrophotometry. Exposing MEFs to increasing imatinib concentrations failed to improve cell viability in the absence of PRDX1. Therefore, c-ABL1 kinase activity does not seem to be responsible for causing the delayed growth phenotype in the *Prdx1*$^{-/-}$ MEFs.

Oxidative Stress Accumulation Affects Proliferation in *Prdx1*$^{-/-}$ MEFs

1. *Antioxidant Treatment*

We next shifted our focus to the antioxidant activity of PRDX1. We reasoned that the lack of this activity could be partly responsible for making cells more sensitive to oxidative stress, therefore contributing to the delayed growth phenotype. We proposed that if cells were treated with exogenous ROS chemical inhibitors, the proliferation phenotype might be reversed. It was expected that the compounds would clear the cells of the H_20_2 normally broken down by PRDX1, therefore helping to reduce oxidative stress. We used three different chemical antioxidant compounds: ammonium pyrrolidinedithiocarbamate (PDTC), N-tert-butyl-alpha-phenylnitrone (PBN), and 5,5-dimethyl-1-pyrroline-N-oxide (DMPO) (Nowicki, Falinski et al. 2004).

The antioxidants tested seemed to partially benefit cell growth in the *Prdx1*$^{-/-}$ background (Figure 10). The most effective concentrations for all the antioxidants were the highest tested for each antioxidant. PBN and DMPO showed proliferation reaching to near wild type levels at day 3, but then showed a reduction by day 4 (Figures 10A and 10B). PDTC seemed the most promising, showing cell growth equivalent to wild type MEFs at day 3 and sustaining at the wild type proliferation rate by day 4 (Figure 10C).

These results argue that excessive levels of ROS may mediate some of the proliferation defect observed in cells lacking PRDX1.

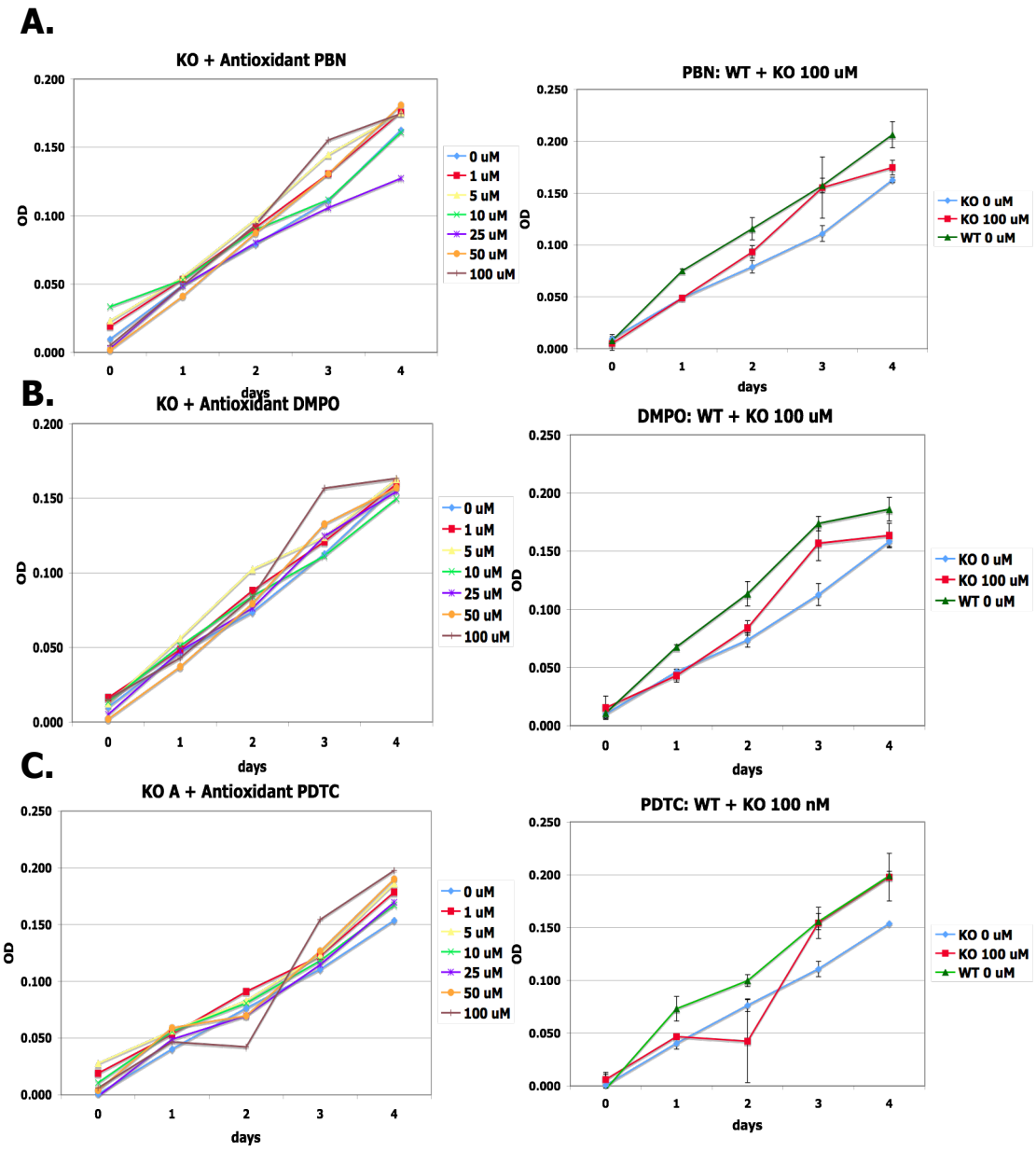


Figure 10: Eliminating ROS through antioxidants improves cell proliferation in *Prdx1*$^{-/-}$ MEFs.

Three different antioxidants, A. pyrrolidinedithiocarbamate (PDTC), B. N-tert-butyl-alpha-phenylnitrone (PBN), and C. 5,5-dimethyl-1-pyrroline-N-oxide (DMPO), were added to medium to help MEFs combat oxidative stress. Cells were cultured at $1x10^4$ cells/ml in 96-well plates in triplicate and monitored daily for proliferation by MTS and spectrophotometry to check for OD. The left panels show *Prdx1*$^{-/-}$ MEFs incubated with various concentrations of each antioxidant, and the right panels show the concentrations showing the most significant increases in cell growth compared to wild-type cells. *Prdx1*$^{-/-}$ MEFs incubated with antioxidants PBN and DMPO show increase in proliferation at day 3 that also reaches levels of wild type MEFs at concentrations of 100 μM. *Prdx1*$^{-/-}$ MEFs incubated with the antioxidant PDTC shows a nearly identical proliferation rate to that of wild type MEFs at concentration 100 nM. These results suggest that ROS levels may contribute to the delayed growth phenotype, and introducing antioxidants to reduce oxidative stress helps *Prdx1*$^{-/-}$ MEFs attain growth stability similar to wild type MEFs.

2. Hypoxia/Normoxia Conditions

Primary MEFs cultured in normoxic conditions have been reported to grow at a slow rate. During incubation at normoxic conditions, MEFs are exposed to atmospheric oxygen levels. These levels, consisting of roughly 20% oxygen, are significantly higher than oxygen levels found *in vivo*, which

range from about 0.2% to 3% oxygen. The exposure to high levels of oxygen causes serious oxidative stress in the cells and the high oxygen content increases ROS cellular levels (Parrinello, Samper et al. 2003). Therefore, MEFs that lack PRDX1, an antioxidant enzyme, are unable to efficiently eliminate ROS (Neumann, Krause et al. 2003). It is possible that ROS levels in MEFs lacking PRDX1 activity are initiating mechanisms leading to cell arrest or interfering with pathways involved in proliferation. Culturing MEFs under low oxygen conditions or under chemically induced hypoxia should lead to a decrease in ROS levels, therefore causing a reduction in oxidative stress. If ROS levels are maintained low and nontoxic, then MEFs should be able to proliferate uninterrupted, and eventually, the difference in proliferation should be eliminated between wild type MEFs and *Prdx1*-deficient MEFs.

A. HYPOXIC CHAMBER

Two approaches were utilized to test the hypothesis that the growth delay is the cause of oxidative stress produced from the environment. One approach involved culturing MEFs in a normoxic environment, as well as in a hypoxic chamber. MEFs grown in hypoxic conditions were exposed to merely 0.2% oxygen, mimicking *in vivo* oxygen levels. MEFs grown in hypoxic conditions should presumably undergo a lower degree of oxidative stress than MEFs grown in normoxic conditions. After checking for cell viability during these conditions, we noticed that the *Prdx1*$^{-/-}$ MEFs showed an improvement

in proliferation (Figure 11). The proliferation rates were indistinguishable between both the *Prdx1*$^{-/-}$ MEFs and wild type MEFs. This assay is consistent with PRDX1 playing a role in combating oxidative stress induced by the environment.

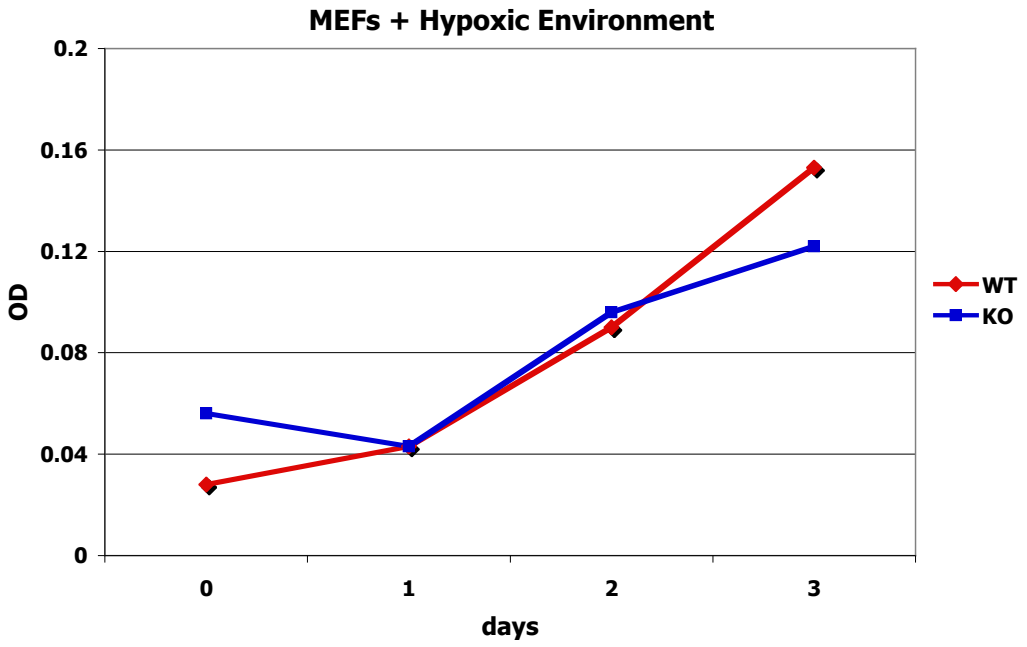


Figure 11: A hypoxic environment improves cell proliferation of *Prdx1*$^{-/-}$ MEFs.

Primary MEFs were cultured at 1x10^4 cells/ml in 96-well plates in triplicate and incubated in a hypoxic chamber to maintain ambient oxygen levels at 0.2%. Cell proliferation was monitored daily by MTS incubation and spectrophotometry. Reducing ambient oxygen levels, therefore decreases oxidative stress and formation of ROS, increasing cell proliferation in *Prdx1*$^{-/-}$ MEFs. Proliferation rates in the *Prdx1*$^{-/-}$ MEFs reach those of wild type MEFs, implying that ROS may be contributing to the delayed growth phenotype.

B. CHEMICAL HYPOXIA

The second hypoxic approach we tested to determine if ROS formation was a cause for the delayed phenotype in *Prdx1*$^{-/-}$ MEFs involved incubating cells with a chemical inducing hypoxic conditions. To achieve these conditions, we chose two different compounds: dimethyloxallyl glycine (DMOG) and cobalt chloride ($CoCl_2$).

i. DMOG

DMOG is a compound that was synthesized to readily penetrate cells and rapidly induce the transcription factor hypoxia inducible factor 1 alpha (HIF-1α), a protein that regulates processes such as apoptosis, cell metabolism, and angiogenesis during hypoxia by inhibition of HIF hydroxylases (Jaakkola, Mole et al. 2001). Various DMOG concentrations were tested to determine if it has an effect on cell growth (Loboda, Stachurska et al. 2009). DMOG slightly improves cell viability in *Prdx1*$^{-/-}$ cells, but also in wild type cells. We had hoped to notice a comparable growth rate when incubated with DMOG. However, even though DMOG did increase the growth rate in *Prdx1*$^{-/-}$ MEFs, the increase was not very considerable (Figure 12). Also, when comparing cell growth between each line at a select DMOG concentration, the wild type cells benefited more from the treatment and showed more of an increase in proliferation. Figure 6 shows the two DMOG concentrations, 5 μM and 50 μM, which were most effective at increasing proliferation. The relatively modest increase in

proliferation in the *Prdx1*$^{-/-}$ MEFs may seem to indicate that DMOG has limited efficiency in eliminating ROS from cells. It is actually unclear how effective DMOG is in mimicking hypoxia, as it has been reported that it may not properly inhibit HIF hydroxylases (Elvidge, Glenny et al. 2006). The result could also imply that other functions of PRDX1 are contributing to the delayed growth phenotype.

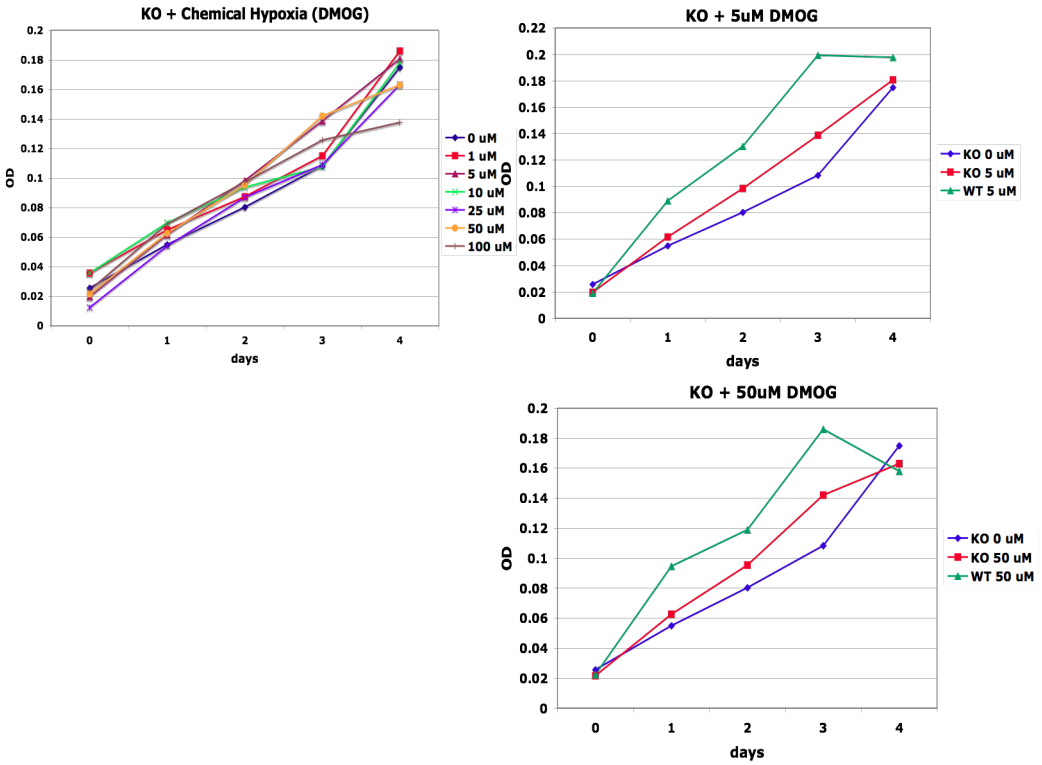


Figure 12: Promoting chemical hypoxia through dimethyloxallyl glycine (DMOG) incubation shows slight increase in *Prdx1*$^{-/-}$ MEF cell viability.

Primary MEFs were incubated with various concentrations of a synthetic compound, DMOG, known to induce hypoxic mechanisms within the cell. Cells were cultured at 1x10^4 cells/ml in 96-well plates in triplicate and proliferation was monitored daily by incubation with MTS solution and through spectrophotometry. The graphs on the left show the two DMOG concentrations, 5 μM and 50 μM, that showed the most increase in cell viability in the *Prdx1*$^{-/-}$ MEFs. The difference was slightly improved, compared to no DMOG, however proliferation did not seem to match wild type rates.

ii. $CoCl_2$

Cobalt chloride ($CoCl_2$) is similar to DMOG in that it functions to mimic hypoxia in cell culture by stabilizing HIF-1α (Vengellur and LaPres, 2004). Because there should be lower ROS levels during hypoxic conditions, the knockout cell lines might display normal cell growth, such as that of the wild type cells. The *Prdx1*$^{-/-}$ cells also showed cell growth stabilization, and though growth started at an equal level as the wild type control, further exposure to $CoCl_2$ actually induced better growth stability than wild type (Figure 13). The knockout cells eventually showed a higher growth rate than the control during incubation with $CoCl_2$. These results show a promising explanation to the growth delay phenotype, linking it to an improper mechanism to deal with oxidative stress due to a loss of PRDX1.

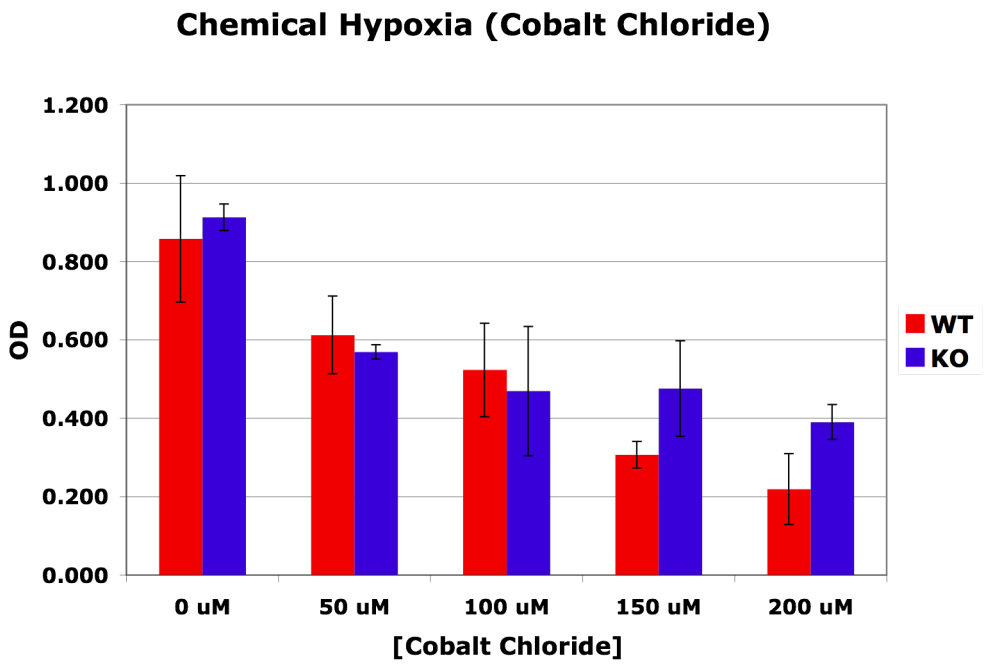


Figure 13: Cobalt chloride exposure to induce chemical hypoxia benefits *Prdx1*$^{-/-}$ MEFs.

Primary MEFs were incubated in 96-well plates at 1x10^4 cells/ml in triplicate with the noted cobalt chloride concentrations. After 72 hours, the medium was changed to fresh medium lacking cobalt chloride and cell growth was analyzed by MTS solution and spectrophotometry. Although exposure to cobalt chloride seems to be affecting proliferation in both MEF lines, the data show that *Prdx1*$^{-/-}$ MEFs attain growth stablility similar to wild type MEFs. At even higher cobalt chloride concentrations (150 μM and 200 μM), *Prdx1*$^{-/-}$ MEFs actually show better cell viability than wild type MEFs. Inducing a hypoxic response benefits *Prdx1*$^{-/-}$ MEFs, implying that oxidative stress may be playing a role in the delayed growth phenotype.

Cell Senescence Not a Factor in Growth Delay

Another plausible explanation that could be leading to the proliferation phenotype is cells entering an early growth crisis phase, or cell senescence. During cell senescence, cells fail to proliferate, as they are unable to enter the cell cycle and divide. This may be due to the accumulation of cyclin-dependent kinase (CDK) inhibitors. Because cells remain alive for prolonged periods, they become vulnerable to various damaging effects. Eventually, senescence may trigger DNA mutations and oncogene activation, as cells are forced into survival mode and are unable to induce apoptosis if a malfunction arises. To monitor for a growth crisis effect, we conducted an immortalization assay on MEFs (Parrinello, Samper et al. 2003). Figure 14 shows that both wild type and *Prdx1*$^{-/-}$ MEFs enter senescence at relatively similar times, around passage 5. This implies that senescence itself does not seem to contribute to the proliferative defect. However, interestingly enough, *Prdx1*$^{-/-}$ MEFs seem to escape senescence at an earlier passage than wild type MEFs. This observation may suggest that *Prdx1*$^{-/-}$ MEFs have increased transformation susceptibility.

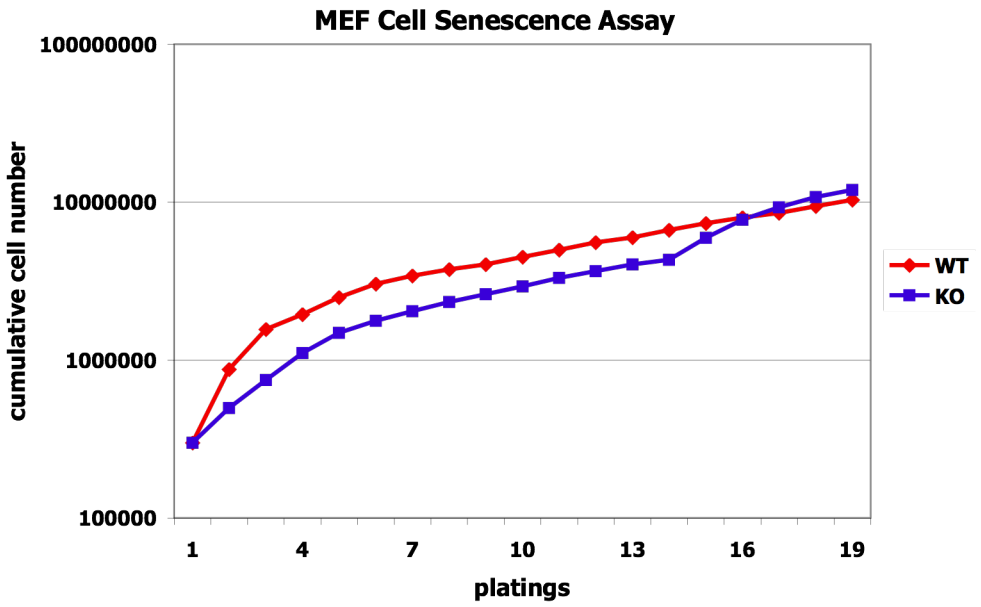


Figure 14: Cell senescence not contributing to growth phenotype in *Prdx1*$^{-/-}$ MEFs.

An immortalization assay was conducted to determine if *Prdx1*$^{-/-}$ MEFs underwent cell senescence or crisis. $3x10^4$ cells were plated in 60 mm plates for 3-4 days, and then trypsinized. Viability was determined by trypan blue exclusion and microscopy. The cumulative cell number was taken after each passage. The graph is shown in log scale. Both wild type and *Prdx1*$^{-/-}$ MEFs undergo early cell senescence. However, interestingly, *Prdx1*$^{-/-}$ MEFs exit senescence before wild type MEFs.

II. PRDX1 Regulation of c-ABL1 Kinase Mutants

Several lines of biochemical and genetic evidence indicate that c-ABL1 is regulated via both an autoinhibitory mechanism (similar to Src kinases) and through cellular inhibitors (Van Etten 2003). PRDX1 is a member of a highly conserved family of cysteine-containing antioxidant enzymes found in prokaryotes and eukaryotes. The Van Etten laboratory previously identified PRDX1 in a yeast 2-hybrid screen of proteins interacting with the SH3 domain of murine c-ABL1 (Wen and Van Etten 1997). PRDX1 was found to interact with ABL1 via binding to the SH3 domain and to the ATP-binding lobe of the kinase domain, and to inhibit the kinase activity of ABL1 in an overexpression assay in 293T cells (Wen and Van Etten 1997).

The level of phosphotyrosine (pTyr) on endogenous c-ABL1 or when c-ABL1 is exogenously expressed at modest levels is very low to undetectable by immunoblotting. However, when c-ABL1 is overexpressed from a strong promoter (CMV) following transient transfection in 293T cells, the ABL1 protein becomes strongly phosphorylated on tyrosine, and drives global phosphorylation of many other cellular proteins. This represents autoactivation of ABL1, as the increase in pTyr is prevented by treatment with imatinib or other ABL1 TKIs. One possible explanation for the autoactivation of ABL1 is that a cellular inhibitor is titrated out (Pendergast,

Muller et al. 1991). If so, co-expression of that inhibitor together with c-ABL1 should suppress pTyr levels. This is the case when PRDX1 is overexpressed with WT c-ABL1, where there is a progressive decrease in pTyr levels on ABL1 with increasing levels of exogenous PRDX1 (Figure 15A. The effect is allele-specific, as an ABL1 protein lacking the SH3 domain (ΔSH3) and hence unable to bind PRDX1 is not affected by co-expression of PRDX1 (Figure 15B).

The goal of this work is to use mutational analysis to probe the interaction between PRDX1 and ABL1. We hypothesize that PRDX1 binds ABL1 in its inactive conformation. We know it potentially binds to both the SH3 domain and the ATP-binding lobe of the kinase domain, the latter interaction inferred from a deletion mutant. Here we want to use point mutants to further characterize the interaction. It would be predicted that a point mutation that blocked PRDX1 binding but did not otherwise affect the ability of c-ABL1 to assume its autoinhibtory conformation would not affect the *in vitro* kinase activity of the purified protein, but this mutant would be at least partially dysregulated *in vivo*. This property is found in several BCR-ABL1 mutants that have been identified in patients with acquired resistance to imatinib. In particular, Y253F, T315I, Q300H, and H396P have all been implicated as potential activating mutations in c-ABL1 (Roumiantsev, Shah et al. 2002; Azam, Latek et al. 2003; Azam, Seeliger et al. 2008). Hence, we

wanted to test these mutants for their sensitivity to trans-inhibition by PRDX1 in the co-expression assay.

As previously noted, PRDX1 interacts with the N-terminal portion of the c-ABL1 kinase domain (Wen and Van Etten 1997). Therefore, it is possible that imatinib-resistant c-ABL1 mutants could also be immune to PRDX1 regulation and subsequent kinase inhibition. The experiment could possibly identify points, or amino acids, needed for recognition and interaction at the ABL1 kinase domain. It could also implicate PRDX1 in imatinib resistance. It is possible that the kinase mutations slightly alter the ABL1 kinase domain conformation due to lack of PRDX1 regulation, preventing efficient imatinib recognition. If c-ABL1 kinase mutants are unable to respond to PRDX1 suppression, it is possible that BCR-ABL1 kinase mutants would exhibit a similar effect. These observations could ultimately reveal an influential role of PRDX1 in BCR-ABL1-induced oncogenesis.

To determine if the kinase mutations affect the PRDX1 – ABL1 interaction, we utilized previously identified imatinib-resistant mutations commonly found in CML patients and introduced them into the *c-ABL1* gene by site-directed mutagenesis (Shah, Nicoll et al. 2002). One of the imatinib-resistant mutants, Y253F, has been studied and shown to deregulate ABL kinase activity. Interestingly, purified Y253F c-ABL1 showed similar kinase activity to purified wild type c-ABL1 (Shah, Nicoll et al. 2002). This

observation led us to believe that the loss of PRDX1 interaction might explain the dysregulation of this mutant kinase *in vivo*.

Only one mutation included in the study is not considered an imatinib-resistant CML mutant. The F420V mutation has been found to be located within the kinase activation loop. We chose to include this mutation because it was identified to be an activating mutation in some strains of v-Abl, and c-ABL1 F420V is able to transform fibroblasts (Jackson, Paskind et al. 1993). The amino acid site may be an important point of interaction for PRDX1, so we included it in our panel of ABL1 mutants..

As previously mentioned, the mutations were introduced into the kinase domain by site-directed mutagenesis. The mutations were verified through DNA sequencing. The *ABL1* mutations were co-transfected into 293T cells along with *Prdx1*. After transfection, whole protein lysates were obtained and ABL1 autophosphorylation was determined by immunoblot analysis. ABL1 autophosphorylation at specific tyrosine residues (Y412 and Y245) correlates with ABL1 kinase activity. Increased ABL1 autophosphorylation levels signify high ABL1 kinase activity, whereas low ABL1 autophosphorylation levels imply a low level of kinase activity (Brasher and Van Etten 2000).

After evaluating phosphotyrosine levels, it was evident that the imatinib-resistant mutants also exhibited resistance to PRDX1 suppression (Figure 15). The mutations prevent PRDX1 from properly regulating Abl

kinase activity, as phosphotyrosine levels were mostly unchanged in the presence of increasing concentrations of PRDX1. This implies that the amino acid mutations are important contact points for PRDX1 interaction or that the structure of the kinase domain has been modified enough to prevent PRDX1 recognition and/or efficient activity on ABL1 kinase activity. It is also possible that, in CML, these kinase mutants develop imatinib resistance because they lose PRDX1 binding. This could lead to a shift in BCR-ABL1 to the active phosphorylated state. This shift proves to be an issue in chemotherapy with imatinib, as imatinib can only recognize autoinhibited, unphosphorylated ABL at the ATP kinase domain (Nagar 2007).

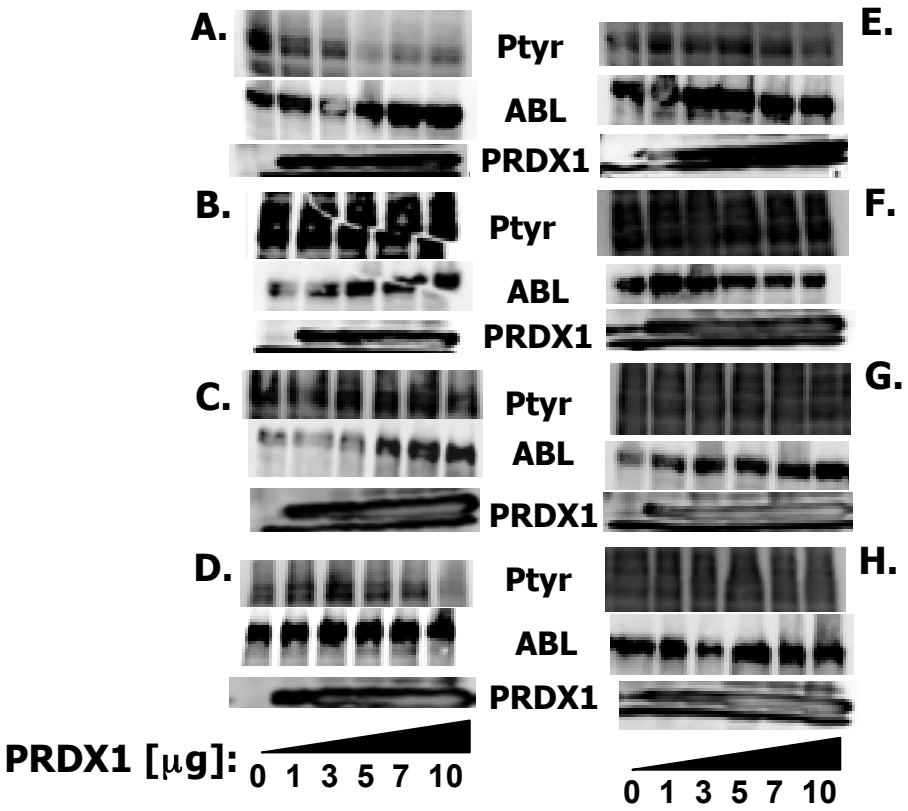


Figure 15: Imatinib-resistant ABL1 kinase mutants fail to respond to PRDX1 suppression.

Five imatinib-resistant mutations, Y253F (**C**), E255V (**D**), Q300H (**E**), T315I (**F**), and H396P (**G**), within the BCR-ABL1 kinase domain frequently found in CML patients were introduced into c-ABL1 through site-directed mutagenesis. Another mutation, F420V (**H**), is not a known imatinib-resistant mutant, but was identified as an activating kinase mutant that transforms fibroblasts. These mutations were co-transfected into 293T cells along with varying concentrations of *Prdx1* expression plasmid (0, 1, 3, 5, 7, 10 μg). After 48 hours, whole-cell lysates were prepared and samples were subjected to western blot analysis to monitor for Abl phosphotyrosine levels. **A**. WT, wild

type = negative control. **B**. SH3 deleted = positive control (PRDX1 suppresses c-ABL1 kinase activity through interacting with the SH3 domain of c-ABL1). All mutations tested show resistance to PRDX1 suppression, as kinase activity is not inhibited as seen in the wild type control. Y253F, E255V, and Q300H do seem to show partial inhibition when co-transfecting with 10 μg of *Prdx1* plasmid. These results suggest that PRDX1 may need these particular amino acids for c-ABL1 contact or that the mutations themselves change c-ABL1 conformation enough to prevent proper regulation by PRDX1.

III. PRDX1 Regulation of BCR-ABL1 Kinase

Studies of BCR-ABL1 expression in *Pdrx1* mutant MEFs

Previous studies have suggested that BCR-ABL1 can assume an autoinhibited conformation similar to c-ABL1 (Smith, Yacobi et al. 2003). PRDX1 is believed to bind to and stabilize the autoinhibited, non-phosphorylated form of ABL1, therefore regulating its kinase activity. Because it was previously determined that PRDX1 had a partial inhibitory effect on BCR-ABL1 kinase activity (Wen and Van Etten 1997), we chose to further characterize this phenotype and determine the significance of PRDX1 regulation of BCR-ABL1 kinase activity, exploiting the availabiltiy of *Prdx1* mutant mice. We initially focused on studies in primary mouse embryo fibroblasts (MEFs), but ultimately moved to experiments to reveal a possible role for PRDX1 in the hematopoietic cancers caused by BCR-ABL1.

Prdx1$^{-/-}$ MEFs are efficiently transduced by BCR-ABL1 retrovirus

Primary MEFs were transduced with a BCR-ABL1-IRES-GFP virus co-expressing BCR-ABL1 and GFP. 48 hours post-transduction, MEFs were analyzed by flow cytometry to determine transduction efficiency. There was no evidence of a difference in the ability to transduce, as similar rates of transduction between wild type MEFs and *Prdx1*$^{-/-}$ MEFs were observed (Figure 16A).

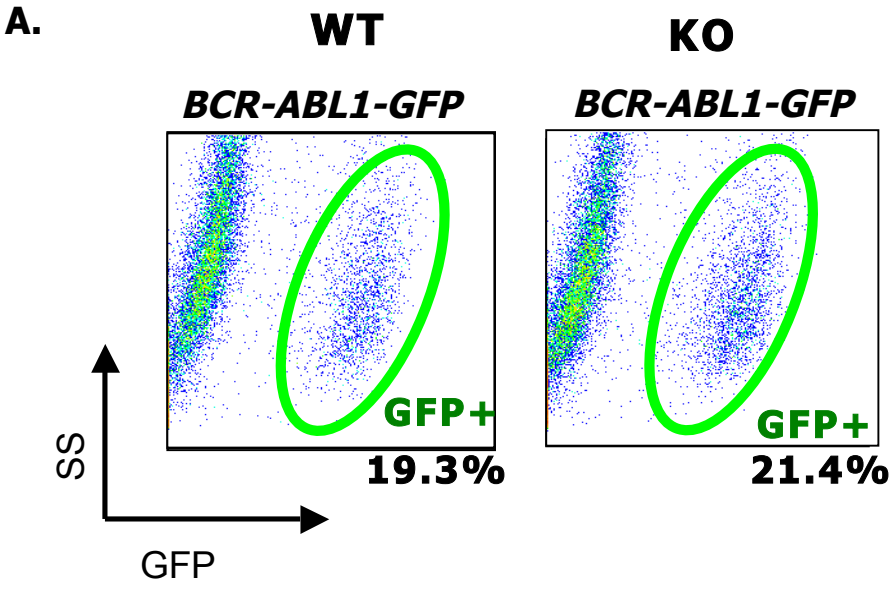
BCR-ABL1 Expression is stabilized in *Prdx1* mutant MEFs

The transduced MEFs were then expanded in culture. After numerous passages, we analyzed GFP expression by flow cytometry to quantify the *BCR-ABL1*$^{+}$ cells. Based on GFP expression, we found that wild type cells failed to retain *BCR-ABL1*, which likely reflects the cytostatic effect of overexpressed c-ABL1 and BCR-ABL1 in fibroblasts (Figure 16B) (Renshaw, McWhirter et al. 1995; Wen, Jackson et al. 1996). However, loss of PRDX1 appears to negate the cytostatic effect of BCR-ABL1 in fibroblasts. *Prdx1*$^{-/-}$ MEFs showed a dramatic increase in the number of GFP-expressing cells, suggesting that the loss of PRDX1 may somehow protect cells from the cytostatic effect of dysregulated ABL1 (Figure 16B, right panel). Because we expect increased kinase activity in *Prdx1*$^{-/-}$ MEFs due to loss of ABL1 regulation, we presumed the kinase activity would readily mediate the cytostatic effect. It is possible that PRDX1 affects nucleo-cytoplasmic shuttling of BCR-ABL1 and keeps the protein exclusively cytoplasmic, avoiding the nuclear cytostatic effect. To address this issue, it would be interesting to monitor BCR-ABL1 cellular localization by microscopy or protein lysate fractionation.

BCR-ABL1 Kinase Activity is Increased in *Prdx1*$^{-/-}$ MEFs

We then decided to monitor for BCR-ABL1 autophosphorylation in the GFP sorted MEFs, which correlates with kinase activity. Immunoblot analysis of whole cell lysates showed increased BCR-ABL1 phosphotyrosine levels in

Prdx1$^{-/-}$ MEFs compared to wild type MEFs (Figure 16C). The loss of *Prdx1* leads to a highly active BCR-ABL1, implying there must be a loss in kinase regulation. This is a promising finding, as we were hoping to link PRDX1 as a prominent inhibitor of BCR-ABL1.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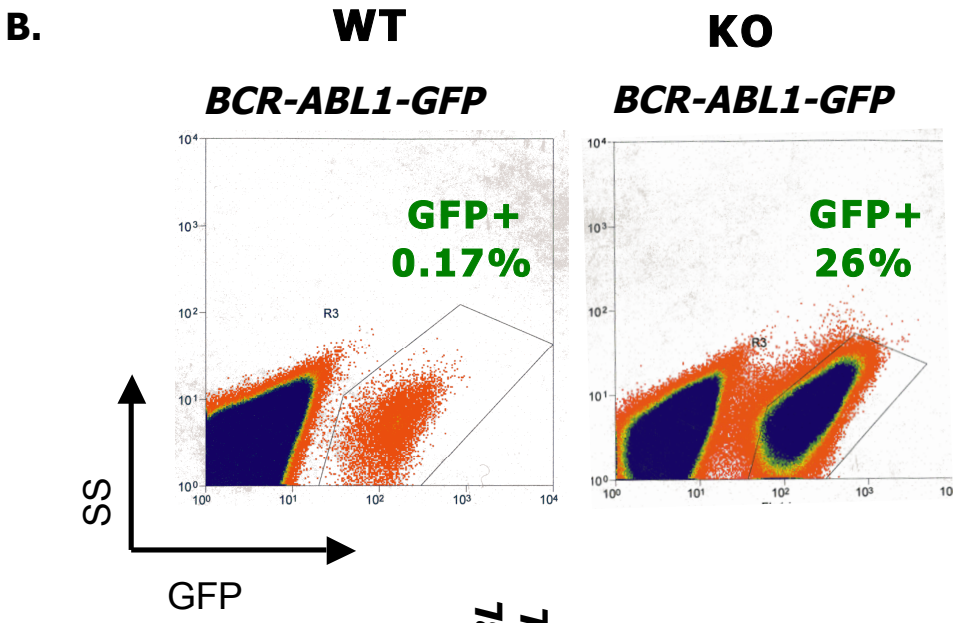
GFP+
21.4%
SS
GFP


B.
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GFP+
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R3
GFP+
26%
R3
SS
GFP

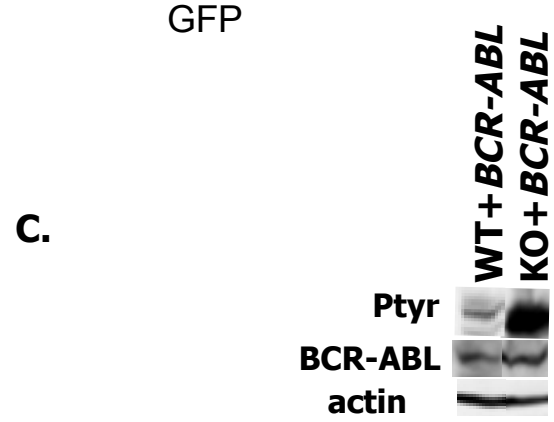

C.
WT+BCR-ABL
KO+BCR-ABL
Ptyr
BCR-ABL
actin

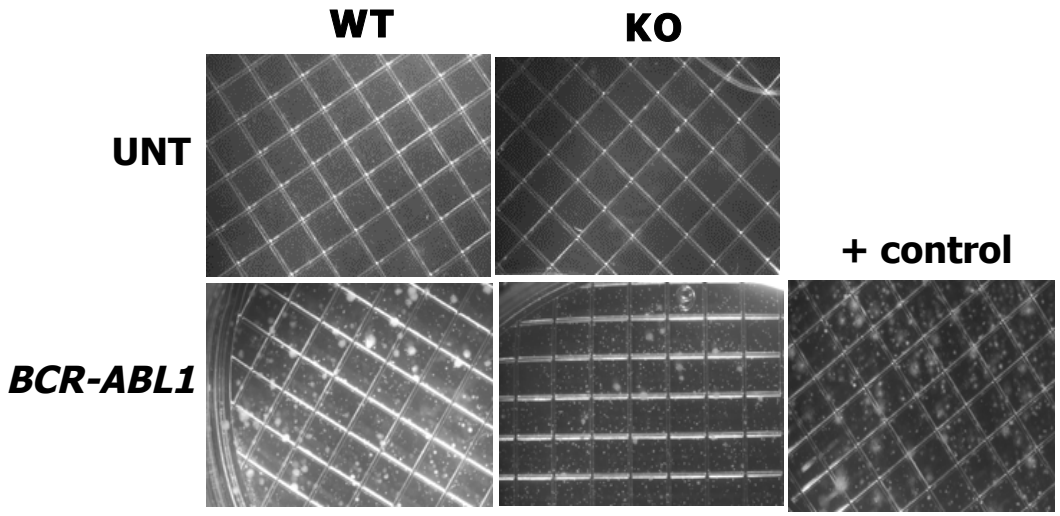
Figure 16: *Prdx1*$^{-/-}$ BCR-ABL^{+} MEFs have improved BCR-ABL1 retention and higher BCR-ABL1 autophosphorylation.

A. Primary MEFs were transduced with *BCR-ABL1-IRES-GFP* retrovirus for 4 hours. Transduction efficiency was determined 48 hours post-transduction using flow cytometry to quantify for GFP expression. The left panels are the negative controls of untransduced MEFs. The right panels are MEFs transduced with *BCR-ABL1-IRES-GFP* retrovirus. These results demonstrate transduction was successful and there are no differences in transduction efficiency. B. MEFs from panel A were expanded in culture, and the results in this figure show the percentage of MEFs retaining BCR-ABL1 based on GFP expression. Though MEFs showed similar initial transduction efficiencies, GFP expression is drastically lost in wild type MEFs. Interestingly, BCR-ABL1 has remained increasingly stable in the *Prdx1*$^{-/-}$ MEFs. C. Whole cell lysates were obtained from GFP-sorted MEFs and were analyzed by western blotting for BCR-ABL1 phosphotyrosine levels. The *Prdx1*$^{-/-}$ MEFs transduced with *BCR-ABL1* show a major increase in BCR-ABL1 autophosphorylation compared to the wild type counterpart. This result strengthens the argument that PRDX1 regulates BCR-ABL1 kinase activity, much like it functions to inhibit c-ABL1 kinase activity.

BCR-ABL1 Transformation of *Prdx1* mutant MEFs to Anchorage Independence is Impaired

We subsequently performed a quantitative transformation assay with the GFP-sorted MEFs to determine if loss of PRDX1 affected transformation by BCR-ABL1. The presence of a colony in soft agar signifies malignant growth due to transformation. We expected *Prdx1*$^{-/-}$ MEFs to have an increased transformation rate, as PRDX1 is a known tumor suppressor (Neumann, Krause et al. 2003). In addition, our MEF studies, which showed an influence on BCR-ABL1 retention and increase of BCR-ABL1 kinase activity in the absence of *Prdx1*, suggested that we would see a higher transformation potential in these cells. However, we failed to see the hypothesized outcome, and instead found the opposite effect. The *BCR-ABL1*-transduced wild type MEFs showed higher colony formation compared to *Prdx1*$^{-/-}$ *BCR-ABL1*-transduced MEFs (Figure 17B). There were also larger colonies present in the wild type *BCR-ABL1* MEF plates compared to the *Prdx1*$^{-/-}$ *BCR-ABL1*-transduced MEFs.

A.


WT
KO
UNT
+ control
BCR-ABL1

B.

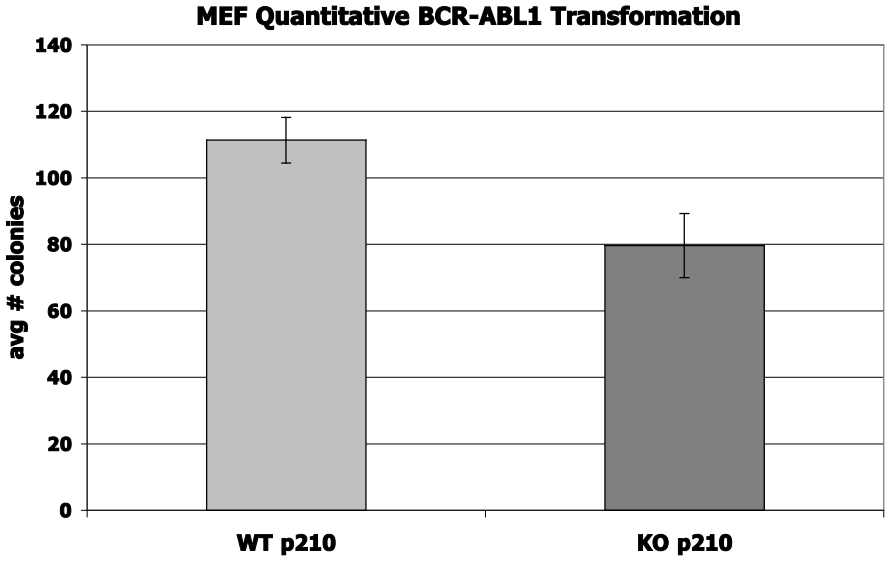

MEF Quantitative BCR-ABL1 Transformation
avg # colonies
140
120
100
80
60
40
20
0
WT p210
KO p210

Figure 17: MEF cell transformation by BCR-ABL1 not enhanced in the absence of PRDX1.

GFP-sorted MEFs were subjected to an anchorage-independence assay to test for transformation capability. MEFs were grown in soft agar and incubated for 24 days to monitor for colony formation. A. The positive control is the N54 cell line (v-Abl transformed). Top panel: untransduced MEFs. Bottom panel: *BCR-ABL1* and GFP-sorted MEFs. B. Quantification of colony formation from *BCR-ABL1* transduced MEFs. *Prdx1*$^{-/-}$ MEFs fail to show an increase in cell transformation. The wild type MEFs may have acquired an additional mutation that allowed better transformation than the *Prdx1*$^{-/-}$ MEFs.

Summary of BCR-ABL1 expression in MEFs

There is a clear genetic interaction of PRDX1 with BCR-ABL1, as *Prdx1*$^{-/-}$ MEFs do not exhibit a cytostatic effect of BCR-ABL1 expression, had increased cellular BCR-ABL1 kinase activity, but did not show an increase in anchorage-independent growth. The decreased transformation of *Prdx1*$^{-/-}$ MEFs is difficult to explain, but this may have something to do with membrane localization of activated ABL1 alleles. In the original studies, BCR-ABL1 was found to be defective for transformation of NIH 3T3 cells because it lacked a myristoylation group (Daley, McLaughlin et al. 1987; Daley, Van Etten et al. 1992). Later, another group found that BCR-ABL1 could transform a "permissive" subclone of 3T3 cells (Renshaw, McWhirter et al. 1995). It is likely that PRDX1 controls BCR-ABL1 localization, and in its absence, BCR-ABL1 is activated and exclusively cytoplasmic. This localization prevents it from entering the nucleus to induce a cytostaic effect or reaching the membrane to confer anchorage independence. Further experiments would need to done to explore possible explanations related to BCR-ABL1 localization in these MEFs. However, it is evident from these studies that a functional relationship between PRDX1 and BCR-ABL1 is present.

PRDX1 Role in GNF-2 Kinase Inhibition: MEF immunoblot Analysis

It was mentioned in the introductory section that a recent novel BCR-ABL1 kinase inhibitor was discovered through a screening process for new compounds. This inhibitor, known as GNF-2, is an attractive candidate for BCR-ABL1 kinase regulation, as its mode of action is distinct from that of ATP-competitive ABL1 kinase inhibitors like imatinib. Some of the results reported from the study suggested that PRDX1 could act as a co-factor for GNF-2 recognition and inhibition. Therefore, we first chose to utilize our *BCR-ABL1*-transduced MEFs to determine if we could see a difference in GNF-2 efficacy based on the presence or absence of *Prdx1*. We then moved on to the hematopoietic system and test for GNF-2 efficacy in BCR-ABL1-transformed B lymphoid cells.

An interesting discovery was that GNF-2 failed to inhibit ABL1 or BCR-ABL1 kinase activity *in vitro*. Kinase assays were performed using purified recombinant ABL1 or immunoprecipitated BCR-ABL1. Despite the lack of kinase inhibition *in vitro*, GNF-2 is still capable of binding to ABL1 and BCR-ABL1 (Adrian, Ding et al. 2006). It seems likely that GNF-2 requires a certain structure and/or another protein or co-factor that is lost in cell-free assays to suppress kinase activity. We thought a likely candidate was the kinase inhibitor PRDX1. PRDX1 is known to interact with the SH3 and kinase domains of c-ABL1 (Wen and Van Etten 1997). We therefore determined if

PRDX1 could influence GNF-2 activity and promote its efficacy as a kinase inhibitor.

To determine if we would see GNF-2 resistance in *Prdx1*$^{-/-}$ cells, we incubated *BCR-ABL1*-transduced MEFs sorted for GFP with GNF-2 in culture. BCR-ABL1-transformed MEFs were exposed to various GNF-2 concentrations for 24 hours. Whole-cell lysates were then prepared to test for kinase activity inhibition through the use of an anti-phosphotyrosine antibody. Immunoblot analysis shows that *Prdx1*$^{-/-}$ MEFs cells fail to demonstrate much resistance to GNF-2 at high drug levels (Figure 18A). There is actually no significant difference in phosphotyrosine levels between MEFs after GNF-2 incubation, as both cell types began to show inhibition at the same relative GNF-2 concentration.

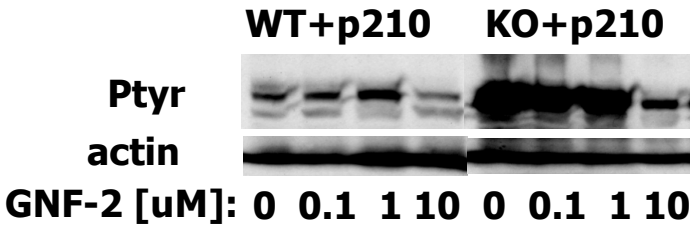
PRDX1 Role in GNF-2 Kinase Inhibition: Lymphoid Immunoblot Analysis

We then shifted our focus to begin our studies in primary hematopoietic cells. We transduced whole bone marrow from wild type or *Prdx1*$^{-/-}$ mice with *BCR-ABL1* retrovirus and cultured the cells in medium to select for transformed lymphoid cells. Once we obtained these cells, we repeated the experiment done with MEFs to monitor for GNF-2 efficacy through immunoblot analysis. However, in these experiments, the cells were incubated with GNF-2 for 90 minutes, instead of 24 hours. Similar results

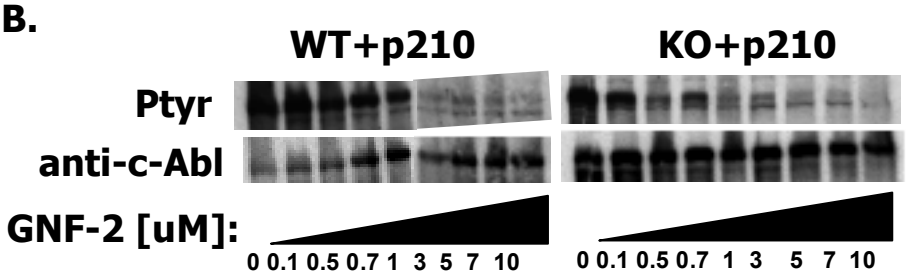
were observed as those obtained from the MEF experiment. We failed to see any GNF-2 resistance in *Prdx1*$^{-/-}$ lymphoid cells (Figure 18B), implying that PRDX1 does not play a role in kinase inhibition by GNF-2.

Even though we failed to see the expected phenotype through BCR-ABL1 autophosphorylation, we wanted to verify there was no functional defect as well. We conducted a cell viability assay to monitor for BCR-ABL1-dependent proliferation after GNF-2 incubation. We had hoped we would still see some GNF-2 resistance in the *Prdx1*$^{-/-}$ *BCR-ABL1* lymphoid cells, therefore showing more robust proliferation compared to wild type cells. However, the assay shows no significant difference in cell viability in the presence of GNF-2 (Figure 18C). The inhibitory concentration at half the max growth (IC_{50}) falls at a similar concentration for both cell lines. These results demonstrate that PRDX1 does not seem to play a functional role in the efficacy of GNF-2 as a BCR-ABL1 inhibitor.

A.


WT+p210
KO+p210
Ptyr
actin
GNF-2 [uM]: 0 0.1 1 10 0 0.1 1 10

B.


WT+p210
KO+p210
Ptyr
anti-c-Abl
GNF-2 [uM]:
0 0.1 0.5 0.7 1 3 5 7 10
0 0.1 0.5 0.7 1 3 5 7 10

C.

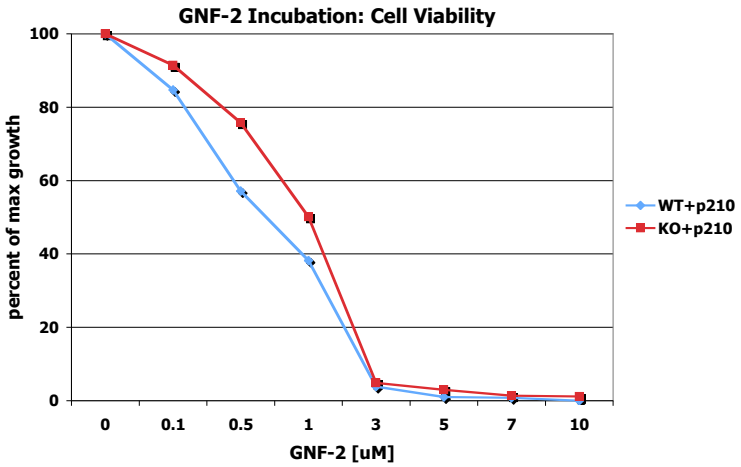

GNF-2 Incubation: Cell Viability
percent of max growth
100
80
60
40
20
0
0
0.1
0.5
1
3
5
7
10
GNF-2 [uM]
WT+p210
KO+p210

Figure 18: Allosteric kinase inhibitor GNF-2 does not require PRDX1 for efficient inhibition of BCR-ABL1.

A. *BCR-ABL1* and GFP-sorted MEFs were incubated with the ABL1 kinase inhibitor GNF-2 (0, 0.1, 1, and 10 μM) for 24 hours. Whole-cell lysates were then prepared and immunoblotted for kinase activity using an anti-phosphotyrosine antibody. *Prdx1*$^{-/-}$ MEFs failed to show GNF-2 resistance, as both wild type and *Prdx1*$^{-/-}$ MEFs seemed to be inhibited at 10 μM of GNF-2. B. Whole bone marrow was harvested from wild type or *Prdx1*$^{-/-}$ mice and transduced with *BCR-ABL1* by spinfection followed by viral adsorption. Bone marrow cells were cultured in medium promoting B lymphoid cell growth. *BCR-ABL1*-transformed B lymphoid cells were then incubated with various concentrations of GNF-2 (0.1, 0.3, 0.5, 0.7, 1, 3, 5, 7, and 10 μM) for 90 minutes. Whole-cell lysates were prepared and BCR-ABL1 phosphotyrosine levels were monitored by immunoblot analysis. ABL1 protein levels were also noted. The results show similar behavior to MEF studies, in which the loss of *Prdx1* fails to promote GNF-2 resistance. C. *BCR-ABL1*-transformed B cells were incubated with various concentrations of GNF-2 (0, 0.1, 0.5, 1, 3, 5, 7, and 10 μM) to test for cell viability. Cells were analyzed at 96 hours by incubation with MTS solution and through spectrophotometry. Both wild type and *Prdx1*$^{-/-}$ B lymphoid cell lines showed similar sensitivity to GNF-2. Therefore, it does not seem that PRDX1 is needed for GNF-2 efficacy.

CHAPTER SUMMARY

This chapter provides a better understanding of PRDX1 and its role in regulation of oxidative stress and ABL1. PRDX1 is important in maintaing cell survival, as reported by the growth delay defect observed in *Prdx1*$^{-/-}$ MEFs. In an effort to explain the proliferative phenotype, our data suggest that increased ROS levels are largely responsible for suppressing cell growth. The dysregulation of endogenous c-ABL1 does not seem to contribute to the defect. The studies focused on the ABL1 mutants suggest that there is a connection between mutations that dysregulate ABL1, alter its conformation, and confer imatinib resistance. The studies involving BCR-ABL1 implicate an interaction with PRDX1 and subsequent regulation. This interaction modulates the cytostatic effect and kinase activity of BCR-ABL1. It could also promote exclusive cytoplasmic localization of BCR-ABL1. BCR-ABL1 regulation by PRDX1, however, did not influence the activity of the allosteric BCR-ABL1 inhibitor GNF-2. Therefore, PRDX1 does not seem to be a factor necessary for GNF-2 recognition and inhibition of BCR-ABL1.

DISCUSSION

The Van Etten lab produced mice lacking *Prdx1* by homologous recombination in ES cells. The mice are viable and fertile, but develop age-dependent fatal hemolytic anemia and multiple malignant cancers. MEFs from *Prdx1*$^{-/-}$ mice have a proliferation defect and exhibit increased sensitivity to ionizing radiation (Neumann, Krause et al. 2003).

We focused on the proliferation phenotype and worked to identify the possible cause for the defect. The defect itself may provide insight into the contributions to carcinogenesis. The delay in growth in the *Prdx1*$^{-/-}$ MEFs may actually make cells susceptible to accumulation various cellular mutations triggering transformation and eventual oncogenesis. Therefore, it could be of significant value to study this defect as a model system to identify physiological function of PRDX1 involved in regulating proper cell survival. We tested two prominent roles of PRDX1 and their influence in the proliferation defect. We hypothesized that *Prdx1*$^{-/-}$ MEFs lacked c-ABL1 regulation. It was also possible that these cells had increased oxidative damage due to a loss in peroxidase activity. Another explanation we tested was possible early cell senescence in the absence of PRDX1.

To initiate the experiments and characterize the proliferation defect, we first verified the phenotype of *Prdx1*$^{-/-}$ MEFs. In agreement with previous

findings, we observed the proliferation defect, as well as the diminished response to hydrogen peroxide exposure.

c-ABL1 is a protooncogene, and when fused to BCR, it forms a constitutively active tyrosine kinase. Therefore, c-ABL1 kinase activation due to the absence of *Prdx1* would seem like an attractive explanation for the proliferation defect. In contrast to BCR-ABL1, however, c-ABL1 does not elicit proliferation pathways or suppress apoptosis. Instead, it localizes to the nucleus to induce cell cycle arrest. This behavior could be just as detrimental as BCR-ABL1 function, as the arrest could expose cells to prolonged oxidative stress and lead to an increase in cellular damage.

Our experimental approach to determine if loss of c-ABL1 regulation contributed to delayed growth in *Prdx1*$^{-/-}$ MEFs involved targeting Abl with the tyrosine kinase inhibitor imatinib. Imatinib recognizes the kinase domain of ABL1 and functions to keep it in an inactive state (Druker, Talpaz et al. 2001). Imatinib, therefore, functions in a similar fashion as PRDX1, as it suppresses ABL1 kinase activity (Wen and Van Etten 1997; Druker, Talpaz et al. 2001). We assumed that loss of PRDX1 regulation would induce the active form of c-ABL1, subsequently allowing c-ABL1 to initiate cell cycle arrest or apoptosis possibly through a nuclear interaction with p53 (Van Etten 1999). Incubation with imatinib, however, failed to abolish the growth delay defect in *Prdx1*$^{-/-}$ MEFs. This result led us to believe that a decrease in proliferation in *Prdx1*$^{-/-}$ MEFs may not actually be due to c-ABL1 kinase activity. We did consider that

maybe PRDX1 provided the necessary ABL1 conformation for imatinib recognition. However, experiments in Chapter 3 demonstrate that imatinib efficacy is not affected in the absence of PRDX1. Therefore, we believe that in the absence of PRDX1, c-ABL1 kinase is still regulated and kept in an inactive state. Unpublished experiments by Neumann and Van Etten show that PRDX3 is also capable of suppressing c-ABL1 kinase activity (Neumann, Krause et al. 2003). It is also possible that c-ABL1 kinase activity is still deregulated in the cytoplasm due to loss of PRDX1, however, once it localizes into the nucleus, it becomes regulated by retinoblastoma (Rb) protein. Rb interacts with the c-ABL1 ATP-kinase domain and inhibits its kinase activity (Welch and Wang 1993). Regulation of a signaling tyrosine stress kinase is crucial for maintaining homeostasis, so it would be evolutionary beneficial for cells to possess various levels and factors to prevent improper c-ABL1 activation. It may be interesting to determine if PRDX3 and Rb contribute to c-ABL1 regulation in the absence of PRDX1, possibly through RNA interference applications or creation of double knockout cells.

A defective mechanism in *Prdx1*$^{-/-}$ MEFs to combat oxidative stress was also considered to contribute to poor cell growth. It was shown by Neumann et. al. and verified in our studies that *Prdx1*$^{-/-}$ MEFs failed to properly degrade exogenous hydrogen peroxide, showing increased sensitivity to oxidative stress and a subsequent lower survival rate (Neumann, Krause et al. 2003). It is likely that *Prdx1*$^{-/-}$ MEFs are incapable of properly eliminating reactive

oxygen species (ROS), which may lead to cellular damage and the growth delay observed. The delay may be allowing for an accumulation of mutations induced by elevated ROS. We found that incubating *Prdx1*$^{-/-}$ MEFs with exogenous antioxidants helped regulate ROS levels and partially enhanced cell survival. The antioxidants functioned to eliminate the oxidative stress not able to be degraded due to the absence of PRDX1.

To determine if oxidative stress imposed by ambient oxygen levels could contribute to the growth delay phenotype in *Prdx1*$^{-/-}$ MEFs, we induced hypoxia. Culturing MEFs in a hypoxic environment, in which oxygen levels were maintained at 0.2%, showed an improvement in cell survival in *Prdx1*$^{-/-}$ MEFs. Chemically inducing a hypoxic response by incubation with the compound DMOG resulted in a slight increase in cell survival in *Prdx1*$^{-/-}$ MEFs. Cobalt chloride, another compound that functions to induce hypoxia, showed a better improvement in growth stability of *Prdx1*$^{-/-}$ MEFs.

Taken together, improvements in cell survival of *Prdx1*$^{-/-}$ MEFs upon antioxidant incubation or due to hypoxic conditions implicate oxidative stress in the growth delay phenotype. Antioxidants seemed to scavenge ROS and overcome the growth defect in *Prdx1*$^{-/-}$ MEFs. The oxygen levels in normoxic conditions (20% oxygen) seem to contribute to a delay in growth, as growing MEFs in a hypoxic chamber allowed *Prdx1*$^{-/-}$ MEFs to grow at a rate similar to wild type MEFs. Inducing a cellular response to mimic hypoxia by stabilizing hypoxia-inducible factor (HIF)-1alpha through DMOG or cobalt chloride also

seemed to enhance cell survival in *Prdx1*$^{-/-}$ MEFs. These results may possibly hint at a link between oxidative stress and carcinogenesis due to improper removal of intracellular ROS by PRDX1. Some studies have shown that ROS can contribute to cancer, such as Nrf2-deficient mice exhibiting an increase in cancer susceptibility. Nrf2 induces expression of antioxidants GSTp and PRDX1 (Ramos-Gomez, Kwak et al. 2001).

We also wondered if the growth delay could actually be *Prdx1*$^{-/-}$ MEFs undergoing cell senescence. It has been noted that cells can enter senescence due to various factors, including DNA damage, activation of a cell cycle checkpoint (p19Arf), or oxidative stress. It is thought that senescence could be initiated by an oncogene. However, additional mutations seem to be required to push cells out of senescence and induce cancer progression (Acosta and Gil 2012). The results from our senescence study showed that both wild type and *Prdx1*$^{-/-}$ MEFs underwent early senescence. However, it seems that *Prdx1*$^{-/-}$ MEFs overcame senescence at an earlier passage, signifying a possible phenotype in transformation susceptibility.

We had next shifted our focus to study the interaction between c-ABL1 and PRDX1 and gain insight into PRDX1 regulation and function. It is known that the PRDX1 C-terminus recognizes the SH3 and the kinase domains of c-ABL1. In doing so, PRDX1 functions to inhibit c-ABL1 kinase activity (Wen and Van Etten 1997). Regulation of kinase activity is essential, as c-ABL1 is involved in processes such as growth arrest, apoptosis, and, when behaving

as an oncoprotein, cell survival and proliferation. However, it remains unclear exactly what residues function to recognize and interact in this pair of proteins.

PRDX1 also inhibits BCR-ABL1 kinase activity in CML patient cells (Wen and Van Etten 1997). It has been reported that patients commonly acquire mutations within the ABL1 kinase domain of BCR-ABL1 that promote drug resistance to the kinase inhibitor imatinib (Shah, Nicoll et al. 2002). We thought it might be interesting to determine if these kinase mutations would interfere with PRDX1 recognition or suppression of kinase activity. We introduced frequently observed mutations with c-ABL1 and co-transfected them into 293T cells with increasing amounts of PRDX1. Our results showed that the imatinib-resistant kinase mutations also exhibited resistance to PRDX1 suppression. It is possible that PRDX1 recognizes some of these mutations for proper interaction. It could also be that ABL1 conformation is altered, preventing PRDX1 from properly binding to ABL1.

PRDX1 is a known suppressor of ABL1 kinase activity, so it would be interesting to further characterize the PRDX1 – ABL1 interaction. It may be possible to eventually engineer a form of PRDX1 that could recognize the imatinib-resistant mutants and also form a tight and stable bond to BCR-ABL1, preventing its activation.

To further study the role of PRDX1 in BCR-ABL1 regulation, we decided to introduce *BCR-ABL1* into MEFs lacking *Prdx1*. We found that although loss of *Prdx1* did not make cells more susceptible to transduction with *BCR-ABL1*, *Prdx1*$^{-/-}$ MEFs retained BCR-ABL1 at a significantly higher rate than wild type MEFs. Loss of PRDX1 overcomes the cytostatic effect induced by BCR-ABL1 in wild type fibroblasts (Renshaw, McWhirter et al. 1995; Wen, Jackson et al. 1996). *Prdx1*$^{-/-}$ MEFs also showed an increase in BCR-ABL1 tyrosine kinase activity. This led us to believe that PRDX1 has a great influence in BCR-ABL1 activation. It may be possible that PRDX1 plays a pivotal role in BCR-ABL1 localization, and in its absence, BCR-ABL1 is retained solely in the cytoplasm in a constitutively active form. The localization may prevent it from inducing a cytostatic effect by entering the nucleus or inducing transformation events by interacting with the cell membrane.

We continued our studies to understand the possible roles of PRDX1 in BCR-ABL1 regulation. A novel allosteric kinase inhibitor at the time, known as GNF-2, was reported that seemed pertinent to our research. Its mode of action is different from imatinib, as GNF-2 seems to be recognizing the myristoyl cleft of the ABL1 portion of BCR-ABL1, while imatinib recognizes only the kinase domain of ABL1. GNF-2 was found to selective inhibit cell proliferation of cells transformed by BCR-ABL1. However, interestingly enough, its activity is lost during *in vitro* conditions. In cell-free extracts, in which recombinant ABL1 or immunoprecipitated BCR-ABL1 were used, GNF-2

is not seen to inhibit kinase activity of either protein (Adrian, Ding et al. 2006). It was presumed that another protein that forms a complex or establishes a certain structure might be involved in GNF-2 efficacy. Because PRDX1 is a known inhibitor of ABL1, and because our MEF studies showed PRDX1 also significantly inhibits BCR-ABL1, it was presumed that GNF-2 possibly needs PRDX1 to form a complex with BCR-ABL1 to properly inhibit BCR-ABL1. However, we found that BCR-ABL1^{+} *Prdx1*$^{-/-}$ MEFs failed to show any GNF-2 resistance. Therefore, PRDX1 does not seem to be important in GNF-2 efficacy.

The studies in this portion of the thesis helped to elucidate the importance of PRDX1 function and regulation. PRDX1 functions to regulate oxidative stress, and when it is absent, the accumulation of ROS seem to contribute to a growth defect. PRDX1 also may delay cell transformation, as MEFs lacking PRDX1 seemed to undergo transformation at an earlier stage. In addition, PRDX1 fails to regulate c-ABL1 kinase mutations resistant to imatinib. In relation to ABL1 regulation, PRDX1 demonstrates a strong suppression of BCR-ABL1 kinase activity. All of the findings mentioned provide an important basis for understanding a possible functional role of PRDX1 in carcinogenesis, as high ROS levels, transformation susceptibility, and lack of ABL1 regulation could all lead to disease development or progression.

CHAPTER 3: PRDX1 INFLUENCE ON BCR-ABL1-INDUCED TRANSFORMATION AND LEUKEMOGENESIS

Introduction

It has been well documented that *BCR-ABL1* expression leads to cell transformation and also targets the hematopoietic system *in vivo*, inducing myeloid and lymphoid leukemias in both humans and mice (Van Etten 2002). Our laboratory found previously that PRDX1 could partially inhibit the BCR-ABL1 fusion protein in transfected cells (Wen and Van Etten 1997). In addition, the results presented in Chapter 2 further demonstrate that there is a significant genetic interaction between PRDX1 and BCR-ABL1, and that BCR-ABL1 appears to be dysregulated in MEFs lacking PRDX1. In this chapter, our studies focus on identifying the relevance of PRDX1 regulation on BCR-ABL1 activity in primary mouse hematopoietic cells. We hope to elucidate a possible role for PRDX1 in BCR-ABL1-mediated hematopoietic cell transformation and leukemogenesis. We hypothesized that loss of PRDX1 might accentuate BCR-ABL1 transformation of hematopoietic cells and also promote leukemogenesis due to lack of BCR-ABL1 regulation. Our experimental approach was to utilize *Prdx1*-null mice as donors of hematopoietic cells for retroviral expression of BCR-ABL1 in well-characterized models of myeloid and lymphoid transformation and leukemogenesis by BCR-ABL1 (Van Etten 2002).

RESULTS

I. PRDX1 Regulates BCR-ABL1-mediated B-lymphoid Cell Transformation and Cell Proliferation

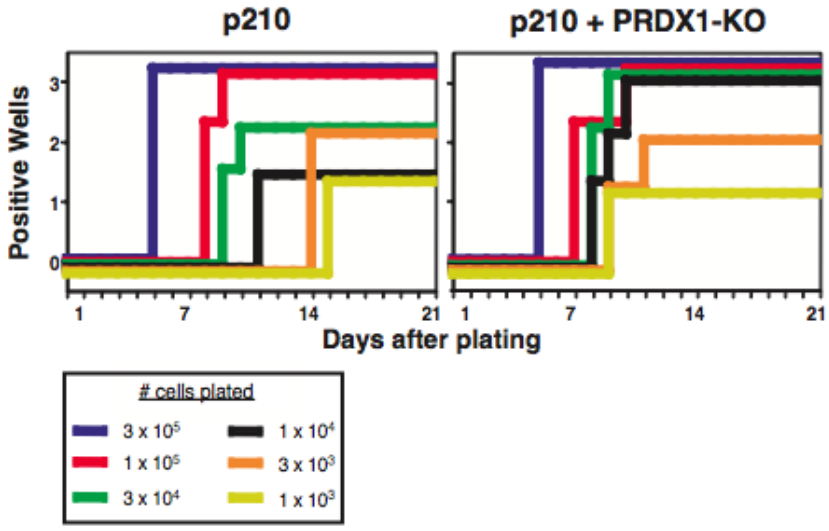
To understand the significance of PRDX1 in BCR-ABL regulation, we decided to first determine what effects would be observed within the lymphoid lineage when introducing *BCR-ABL1* in the absence of PRDX1. We took advantage of mice with the homozygous null mutations in the *Prdx1* gene ($Prdx1^{-/-}$) to conduct cell transformation and leukemogenesis experiments (Neumann, Krause et al. 2003). To address B-lymphoid transformation, we monitored stromal-dependent proliferation of immature B-lymphoid cells in a modified Whitlock-Witte-style assay after *BCR-ABL1* retroviral transduction (McLaughlin, Chianese et al. 1987). This technique was previously modified by our laboratory to introduce serial dilutions of the transduced bone marrow cells, allowing for the assay to produce semi-quantitative results (Smith, Yacobi et al. 2003). Cell transformation potency is quantified by the length of time needed for cells to reach confluency, as defined by a density of $\geq 1 \times 10^6$ cells/well (see METHODS).

In this assay, we observed that loss of PRDX1 enhanced BCR-ABL1-induced transformation, as $Prdx1^{-/-}$ cells transduced with *BCR-ABL1* were transformed more efficiently (Figure 19A). In the $Prdx1^{-/-}$ BCR-ABL1-

transduced sample, the lowest plating cell dilution that reached confluence (meaning all three wells were positive for transformation) was $1x10^4$ cells. In contrast, the lowest dilution for wild-type BCR-ABL1-transformed cells that reached confluence was $1x10^5$ cells. Hence, there was a 10-fold difference between the two donor cell types for transformation by BCR-ABL1. Also worthy to note, the lowest dilution at which cells first exhibited a lack of transformation also revealed a 10-fold difference between the wild-type (dilution $3x10^4$ cells) and *Prdx1*$^{-/-}$ (dilution $3x10^3$ cells) samples. In comparison, these differences are large, suggesting that PRDX1 acts to suppress BCR-ABL1-induced B-lymphoid transformation *in vitro*.

To extend these results, we tested whether there was a difference in cell proliferation between the established, stromal-independent transformed cell lines obtained from the Whitlock-Witte cultures in Figure 19A. As shown in Figure 19B, the *Prdx1*$^{-/-}$ cells transformed by BCR-ABL1 seemed to exhibit a significant growth advantage compared to the wild-type transformed cells. The difference in cell accumulation rates may reflect proliferation, such that the loss of BCR-ABL1 kinase regulation by PRDX1 may upregulate pathways involved in cell division. Alternatively or in addition, the cell apoptosis response might also be suppressed, allowing for the faster accumulation of the *Prdx1*$^{-/-}$ BCR-ABL1-transformed cells.

A.


p210
p210 + PRDX1-KO
Positive Wells
0
1
2
3
1
7
14
21
Days after plating
cells plated
3 x 10^5
1 x 10^5
3 x 10^4
1 x 10^4
3 x 10^3
1 x 10^3

B.

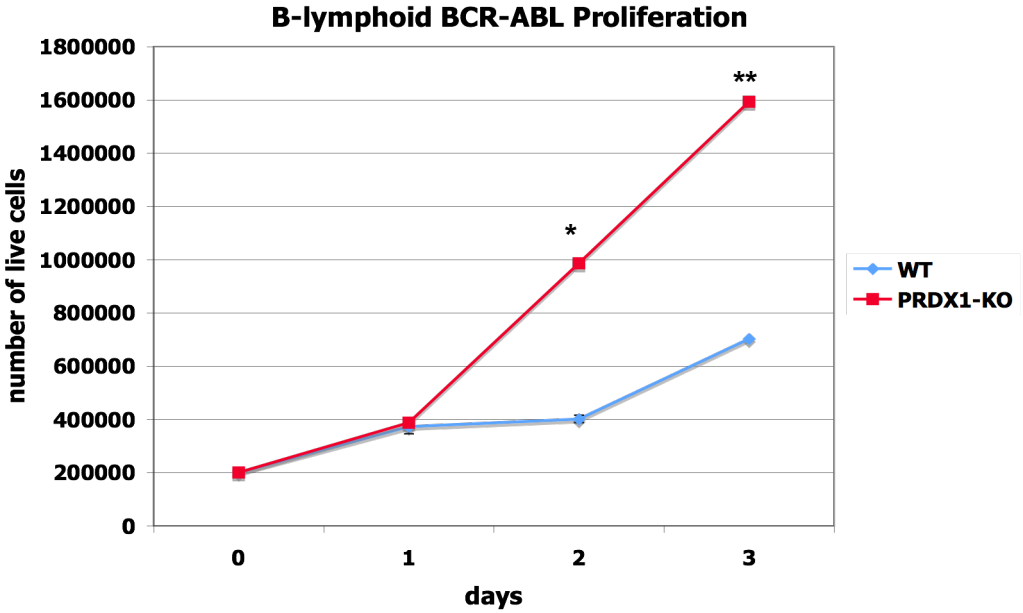

B-lymphoid BCR-ABL Proliferation
number of live cells
1800000
1600000
1400000
1200000
1000000
800000
600000
400000
200000
0
0
1
2
3
days
*
**
WT
PRDX1-KO

Figure 19: *Prdx1*$^{-/-}$ BCR-ABL^{+} B lymphoid cells exhibit a higher transformation potential and increased proliferation.

A. Donor bone marrow cells isolated from wild-type and *Prdx1*-KO mice were transduced with retrovirus expressing BCR-ABL1. Serial dilutions of the transduced cells were plated in triplicate on autologous BM stroma and cultured for B cell growth. Each line in the graphs represents a cell plating dilution. The x-axis shows the number of days it takes to reach transformation and the y-axis refers to the number of wells that have become positively transformed in each triplicate dilution ($\geq 1x10^6$ cells/well). This assay demonstrates that the *PRDX1*-KO cells have an advantage in BCR-ABL1-mediated cell transformation at lower dilutions and also seem to reach a transformed state faster in the higher dilutions, compared to the wild type cells. **B.** Transformed, stroma-independent cells obtained from the limiting dilution assay in panel A were cultured and monitored for proliferation. BCR-ABL1-transformed cells in the *Prdx1*-KO background exhibited a increased growth rate. (t-test, p values: * = 0.00002; ** = 0.000001)

II. Loss of PRDX1 Enhances BCR-ABL1-mediated pre-B Lymphoid Colony Formation in vitro

To further extend the findings from the Whitlock-Witte lymphoid transformation assay, we carried out an alternative B-lymphoid transformation assay. This assay uses semi-solid agarose medium to identify pre-B cell colonies exhibiting malignant growth due to ABL1 transformation, similar to the assay first reported by Naomi Rosenberg for v-ABL (Rosenberg and Baltimore 1976). Based on the results from the Whitlock-Witte lymphoid transformation (Figure 19), we predicted that *Prdx1*$^{-/-}$ cells transduced by *BCR-ABL1* might produce a higher number of colonies as compared to wild-type *BCR-ABL1*-transduced cells. As seen in Figure 20, the average colony number was indeed significantly higher in *BCR-ABL1*-transduced *Prdx1*$^{-/-}$ cells, compared to the wild-type cells. The results from this assay support the hypothesis that PRDX1 is functioning to regulate BCR-ABL1 transformation within the B-lymphoid lineage. Therefore, loss of PRDX1 leads to enhanced BCR-ABL1 transformation and increased malignant growth *in vitro*.

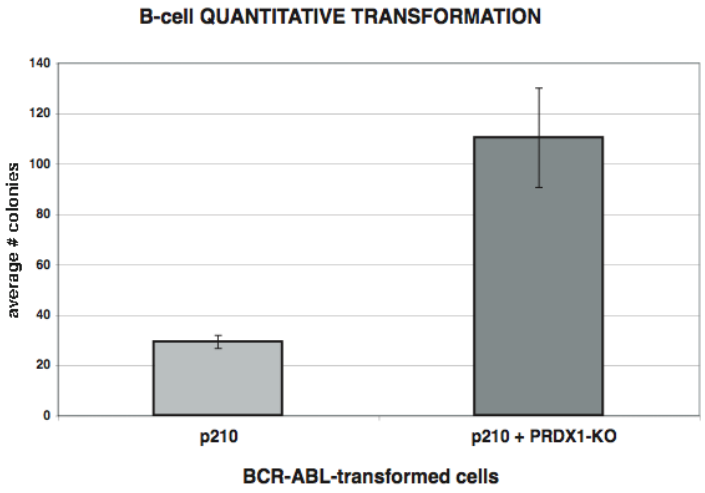


Figure 20: *Prdx1*$^{-/-}$ BCR-ABL^{+} B lymphoid cells demonstrate enhanced malignant colony formation.

Bone marrow derived from wild-type and *Prdx1*-KO mice was transduced with *BCR-ABL1* retrovirus and plated in triplicate in semi-solid agar media to quantify pre-B lymphoid cell transformation. Colony formation was recorded after 14 days of incubation. The results show a significant difference between wild type and *Prdx1*-KO *BCR-ABL1*-transduced bone marrow in colony formation, demonstrating that loss of PRDX1 leads to enhanced BCR-ABL1 B-lymphoid transformation *in vitro*. (t-test, p value = < 0.0005)

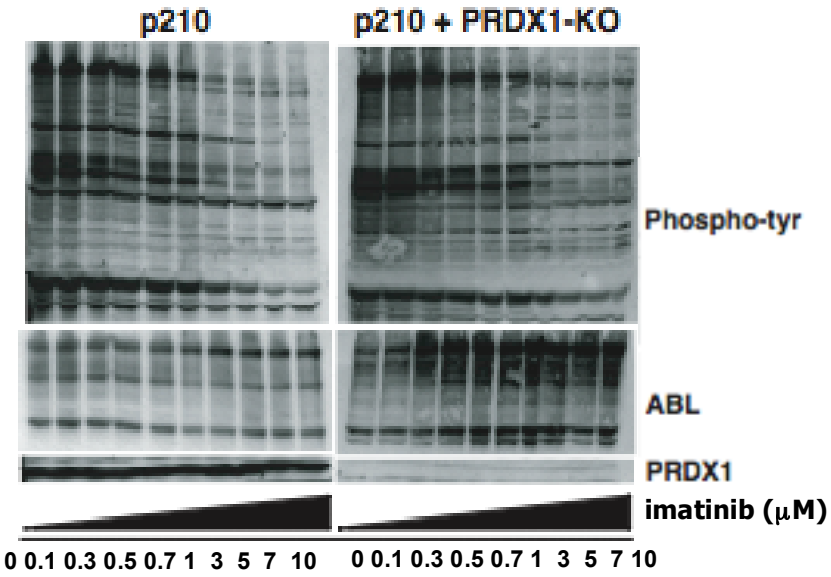
III. Loss of PRDX1 Does not Alter the Reponse to TKIs in BCR-ABL$^+$ B-Lymphoid Cells

Drug resistance is a common occurrence amongst Ph$^+$ leukemia patients undergoing chemotherapy, so we next decided to test a known BCR-ABL1 tyrosine kinase inhibitor (TKI), imatinib, on *Prdx1* wild-type and knockout B-lymphoid cells transformed by BCR-ABL1. We hypothesized that losing PRDX1 regulation might prevent proper inhibition of BCR-ABL1 kinase activity by the TKI and manifest as TKI resistance. As discussed in Chapter 1, imatinib is the most widely utilized drug for BCR-ABL$^+$ leukemias. It traps the BCR-ABL1 kinase in its inactive conformation by binding to the ATP-binding site (Quintas-Cardama and Cortes 2009).

Whole-cell lysates of BCR-ABL1-transformed B-lymphoid wild-type and *Prdx1*$^{-/-}$ cells were obtained after incubation with various concentrations of the imatinib. Phosphotyrosine levels were analyzed by immunoblotting to determine the effects of the imatinib on BCR-ABL1 autophosphorylation. Tyrosine phosphorylation is an indicator of the active form of BCR-ABL1 kinase, so that increasing concentrations of the drug should cause subsequent decreases in autophosphorylation. The trend seen in phosphotyrosine levels versus drug concentration was similar between the two cell lines after incubation with imatinib (Figure 21A). This implies that both wild-type and *Prdx1*$^{-/-}$ cells exhibit the same sensitivity to imatinib.

We also tested whether loss of PRDX1 would interfere with the inhibitory effect of imatinib on proliferation of BCR-ABL1-transformed cells. B-lymphoid wild-type and *Prdx1*$^{-/-}$ cells transformed by BCR-ABL1 were incubated with various concentrations of imatinib and relative cell proliferation was assessed 96 hours later (Figure 21B). There was no difference in cell proliferation during drug exposure, as both cell lines showed similar IC_{50}s for imatinib. Therefore, *Prdx1*$^{-/-}$ BCR-ABL1-transformed lymphoid cells do not have a TKI resistance phenotype.

A.


p210
p210 + PRDX1-KO
Phospho-tyr
ABL
PRDX1
imatinib (μM)
0 0.1 0.3 0.5 0.7 1 3 5 7 10
0 0.1 0.3 0.5 0.7 1 3 5 7 10

B.

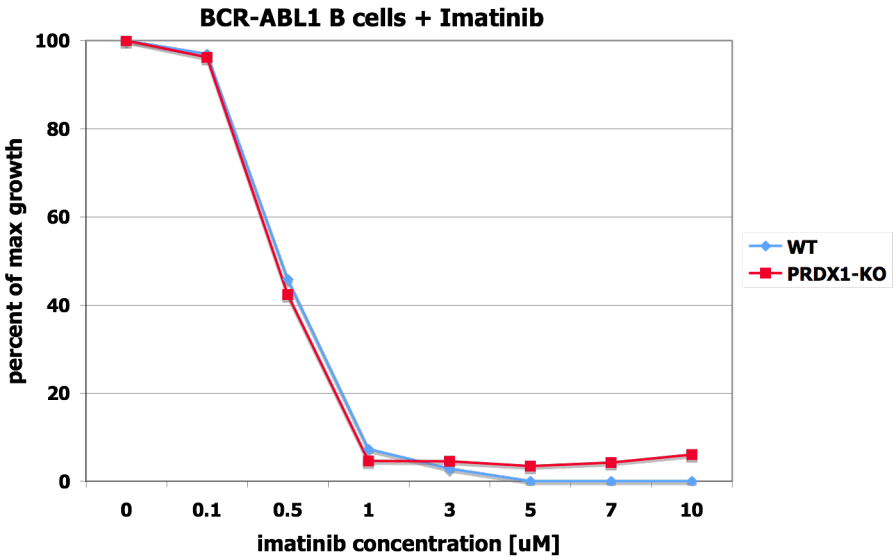

BCR-ABL1 B cells + Imatinib
percent of max growth
100
80
60
40
20
0
0
0.1
0.5
1
3
5
7
10
imatinib concentration [uM]
WT
PRDX1-KO

Figure 21: Loss of PRDX1 Does not Alter the Reponse to TKIs in BCR-ABL^{+} B-Lymphoid Cells.

A. Wild type and *Prdx1*$^{-/-}$ B cells transformed by BCR-ABL1 were incubated with the kinase inhibitor imatinib at varying concentrations for 3 hours. Whole cell lysates were then obtained and samples were subjected to immunoblotting to check for phosphotyrosine levels, ABL, and PRDX1. Western blotting analysis shows there is no significant difference in drug inhibition between wild type and *Prdx1*$^{-/-}$ cells. **B.** Wild-type and *Prdx1*-KO B cells transformed with BCR-ABL1 were incubated with imatinib at varying concentrations as in panel A. Cell proliferation was analyzed at 96 hours using an MTT assay and spectrophotometry. There was no difference in drug sensitivity between wild-type and knockout cells.

IV. Does Prdx1 Deficiency Enhance BCR-ABL1-induced B-cell Acute Lymphoblastic Leukemia in Mice?

Based on the lymphoid *in vitro* data, which showed that loss of *Prdx1* enhanced BCR-ABL1-mediated cell transformation, we hypothesized that this phenotype might also influence leukemogenesis *in vivo*. We reasoned that the increase in transformation due to a loss of *Prdx1* could potentially translate into a more severe or aggressive BCR-ABL1-induced disease development in a murine model. To test this hypothesis, we induced B-cell acute lymphoblastic leukemia (B-ALL) in mice, as previously established (Roumiantsev, de Aos et al. 2001). In this experiment, bone marrow from wild-type or *Prdx1* mutant donor mice is transduced with BCR-ABL1 without prestimulation with myeloid cytokines and transplanted immediately into sublethally irradiated recipient mice.

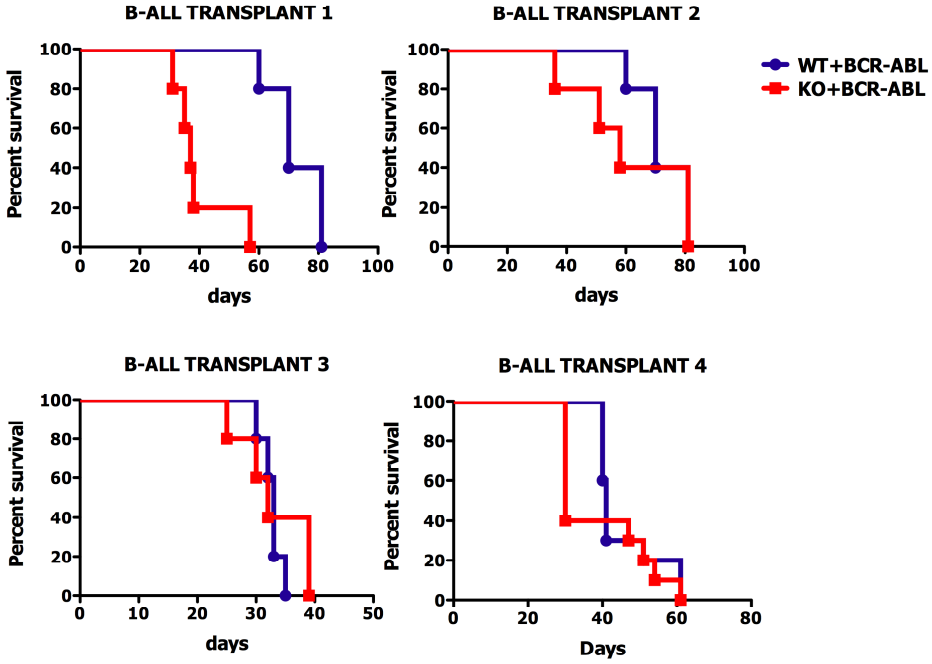
Initial studies on the B-ALL transplants showed a slight difference in leukemogenesis between the control and experimental groups (Figure 22A). The mouse cohort that was transplanted with *Prdx1*$^{-/-}$ *BCR-ABL*-transduced cells began to succumb to B-ALL sooner than the wild-type cohort. However, the effect disappeared with increasing time after transplantation, eventually making the difference in survival insignificant.

The latency of BCR-ABL1-induced leukemias in the bone marrow transduction/transplantation model is sensitive to the relative number of leukemia-initiating cells that are transplanted. To magnify a potential

difference in leukemogenesis between wild-type and *Prdx1* knockout donor cells, we modified the experimental approach by reducing the number of transduced cells injected per recipient. We believed that we might have been essentially saturating the recipient mice with transduced cells, masking any possible survival difference. A reduction in the number of *BCR-ABL1*-transduced cells that are transplanted should prolong the overall survival and possibly reveal a difference in mortality between the groups.

This modified B-ALL transplant yielded a similar result as the previous B-ALL transplants (Figure 22B). As in the B-ALL transplants with higher numbers of injected cells, the recipients of *Prdx1* mutant bone marrow initially developed fatal B-ALL before the wild-type cohort. Although we did observe an extent in survival due to a decreased number of injected transduced cells, we did not observe a significant difference in overall survival, as the majority of mice in both cohorts failed to develop B-ALL. The results suggest that PRDX1 may be involved in initiating B-lymphoid leukemogenesis, but other factors, proteins, or mechanisms may compensate for its absence upon further disease progression.

A.


B-ALL TRANSPLANT 1
B-ALL TRANSPLANT 2
B-ALL TRANSPLANT 3
B-ALL TRANSPLANT 4
Percent survival
days
Days
WT+BCR-ABL
KO+BCR-ABL

B.

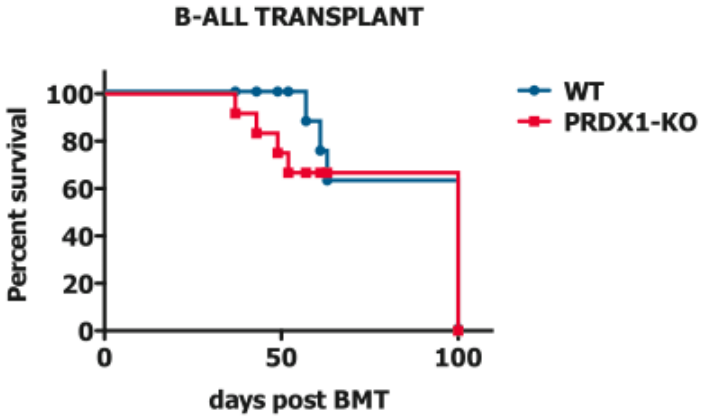

B-ALL TRANSPLANT
Percent survival
days post BMT
WT
PRDX1-KO

Figure 22: *Prdx1*$^{-/-}$ BCR-ABL^{+} recipient mice slightly more susceptible to onset of B-ALL.

A. Whole bone marrow was isolated from wild-type and *Prdx1*-KO mice and transduced with *BCR-ABL1* retrovirus. Transduced cells were then transplanted into sub-lethally irradiated wild-type recipient mice. The mice are monitored for symptoms of B-ALL (circulating GFP^{+} pre-B lymphoblasts, lymphadenopathy, splenomegaly, pleural effusion). Each survival curve represents a distinct transplant. Transplant 1 showed a prominent difference (Mantel-Cox test, p value = 0.001). However, subsequent transplantations failed to show such a pronounced phenotype. Although there is some variation amongst the survival curves and differences are not significant, each transplant shows a slight phenotype of disease onset, as B-ALL is initiated in the *Prdx1*$^{-/-}$ cohorts before the wild-type cohorts. However, the rate of disease eventually becomes similar in each group or the mice fail to succumb to disease after some time (at which point mice are sacrificed to end the study). (Mantel-Cox test, p values = 0.52, 0.58, 0.37 of transplants 2, 3, and 4 respectively) **B.** B-ALL transplant performed modifying the number of cells transplanted. A significantly reduced number of transduced bone marrow cells were injected into recipient mice. Similar to the transplants in A, the recipient mice receiving *Prdx1*$^{-/-}$ bone marrow cells initially succumb to fatal B-ALL before the wild-type cohort. However, there was no significant difference in overall survival. (Mantel-Cox test, p value = 0.7)

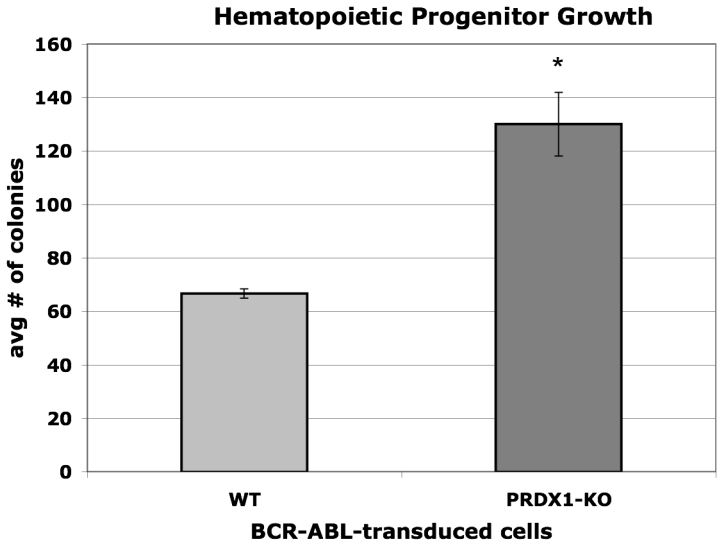
V. *Prdx1 Deficiency Enhances Transformation of Myeloid Progenitors by BCR-ABL1*

Previous studies have shown that expression of BCR-ABL1 by retroviral transduction of bone marrow allows the growth of myeloid progenitor colonies in methylcellulose in the presence of limiting concentrations of myeloid cytokines such as IL-3 (Gishizky and Witte 1992). To test the effect of *Prdx1* deficiency on myeloid progenitor transformation by BCR-ABL1, we treated wild-type or *Prdx1* mutant donor mice with 5-fluorouracil (5-FU), a chemotherapeutic agent that increases the relative abundance and induces cycling of hematopoietic stem/progenitor cells (Randall and Weissman 1997). Four days after 5-FU treatment, bone marrow was harvested and transduced with BCR-ABL1 retrovirus in the presence of myeloid cytokines, as described in METHODS. Subsequently, cells were plated in methylcellulose containing serum but lacking hematopoietic cytokines (M3234, Stem Cell Technologies). Similar to the lymphoid transformation assays in Figures 19 and 20, *Prdx1*$^{-/-}$ bone marrow myeloid progenitors were more efficiently transformed by BCR-ABL1, as they produced more than twice the colony number observed with wild-type marrow (Figure 23A). These results suggest that PRDX1 regulates myeloid progenitor population and functions to suppress myeloid BCR-ABL-mediated cell transformation.

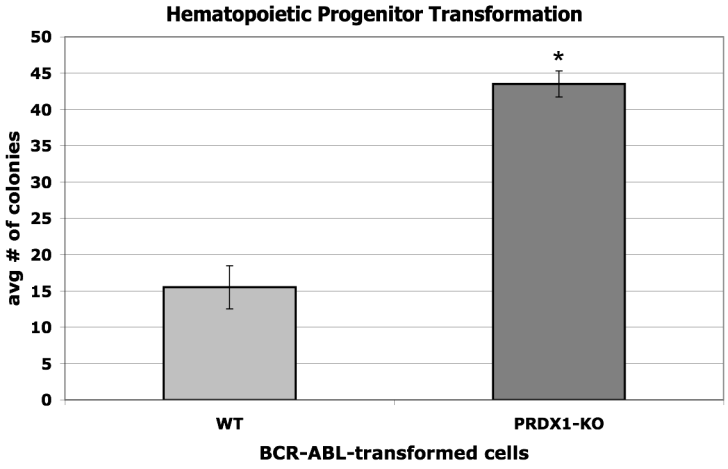
We further tested if *Prdx1* deficiency would affect the TKI response of BCR-ABL1-transformed myeloid progenitors. Transformed progenitors were

tested for drug resistance by incubating *BCR-ABL1*-transduced cells in methylcellulose with the addition of imatinib and monitored for colony formation. As in the lymphoid lineage experiments, we had postulated that *Prdx1*$^{-/-}$ BCR-ABL1-transformed myeloid progenitors might be relatively resistant to a BCR-ABL1 kinase inhibitor. However, corroborating the results with lymphoid cells, myeloid progenitors failed to show a difference in drug sensitivity (Figure 23B). Taken together, these data demonstrate that loss of PRDX1 does not make BCR-ABL1-transformed lymphoid or myeloid cells more resistant to ABL1 kinase inhibitors.

A.


Hematopoietic Progenitor Growth
avg # of colonies
160
140
120
100
80
60
40
20
0
*
WT
PRDX1-KO
BCR-ABL-transduced cells

B.


Hematopoietic Progenitor Transformation
avg # of colonies
50
45
40
35
30
25
20
15
10
5
0
*
WT
PRDX1-KO
BCR-ABL-transformed cells

C.

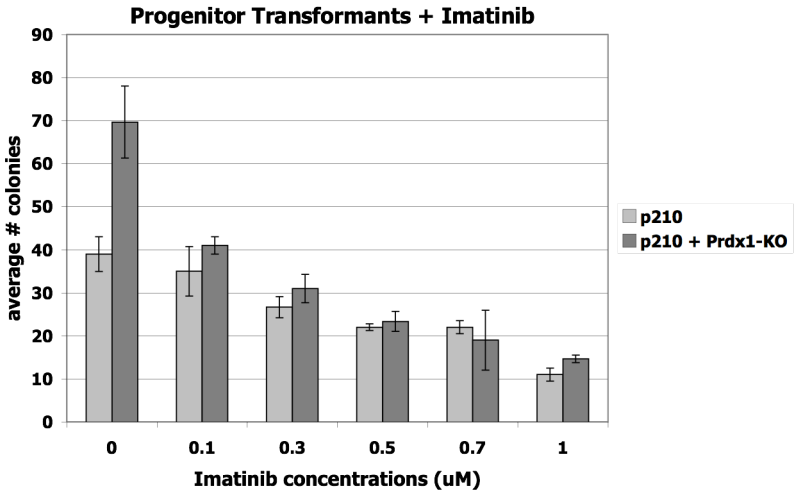

Progenitor Transformants + Imatinib
average # colonies
90
80
70
60
50
40
30
20
10
0
0
0.1
0.3
0.5
0.7
1
Imatinib concentrations (uM)
p210
p210 + Prdx1-KO

Figure 23: Myeloid transformation increased in *Prdx1*$^{-/-}$ BCR-ABL^{+}. A quantitative approach was used to determine myeloid progenitor growth (**A**) or myeloid progenitor transformation efficiency (**B**) by BCR-ABL1 in relation to PRDX1. Methylcellulose medium either containing cytokines (complete medium) or lacking cytokines (incomplete medium) was utilized to identify progenitor growth and transformation potential, respectively, of myeloid progenitor cells. Bone marrow derived from 5-FU treated wild type or *Prdx1*$^{-/-}$ mice was harvested and transduced with *BCR-ABL1*. Cells were plated in methylcellulose and colonies were identified after day 10 of incubation. Panel **A** shows an increase in myeloid progenitor proliferation in *PRDX1*-KO *BCR-ABL1* cells incubated in complete medium compared to wild type cells (t-test, p value = 0.002). The results for cells incubated in incomplete medium (**B**) also show that *Prdx1*-KO BCR-ABL-transformed cells produce more colonies. These results imply that *Prdx1*-KO mice may have an advantage in myeloid transformation due to an increased progenitor population and enhanced BCR-ABL1 progenitor transformation potential (t-test, p value = 0.0002). **C.** To determine if *Prdx1*$^{-/-}$ BCR-ABL1^{+} cells exhibited resistance to the kinase inhibitor imatinib, we grew cells in methylcellulose incomplete medium with the addition of imatinib, a drug that inhibits BCR-ABL1 kinase activity. We incubated cells in various drug concentrations and monitored for colony formation. The results show that loss of *Prdx1* does not make cells more resistant to the kinase inhibitor.

VI. Prdx1 Deficiency Accelerates BCR-ABL1-induced CML-like Myeloproliferative Neoplasia in Mice

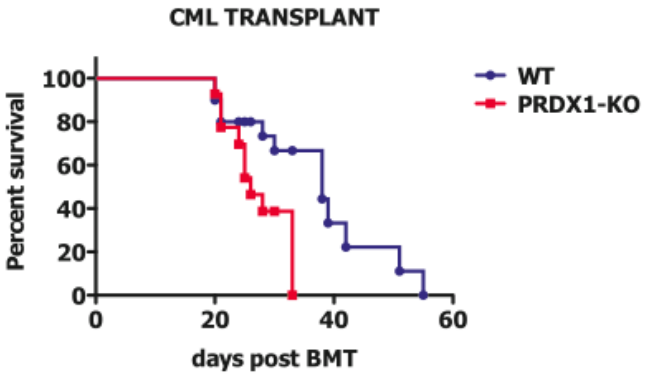
We next tested the effect of *Prdx1* loss on BCR-ABL1-mediated myeloid leukemia in the mouse model. Recipients of *BCR-ABL1*-transduced bone marrow (BM) develop a fatal CML-like myeloproliferative neoplasm (MPN) characterized by massive expansion of maturing myeloid progenitors with infiltration of spleen, liver, and lungs. Myeloid cells from these mice carry the retroviral provirus, express BCR-ABL1, and exhibit increased levels of tyrosyl-phosphorylated proteins and activation of multiple cell signaling pathways (Li, Ilaria et al. 1999; Roumiantsev, de Aos et al. 2001). Analysis of the proviral integration pattern by Southern blotting reveals that the CML-like disease originates from multiple independently engrafting clones that represent the leukemia-initiating or leukemic stem cells. Clonal analysis (Li, Ilaria et al. 1999; Million, Aster et al. 2002) and prospective BM fractionation experiments (Huntly, Shigematsu et al. 2004; Hu, Swerdlow et al. 2006) indicate that these CML-initiating cells arise from hematopoietic stem cells (HSC). The polyclonal nature and short latency of murine CML-like leukemia differ from human CML, which is monoclonal and more chronic. However, this is not an important pathophysiological difference, but in part reflects transplantation of multiple *BCR-ABL1*-transduced HSCs into each recipient. When lower titer virus is used or limiting numbers of transduced cells are transplanted, oligo- to monoclonal disease with a longer latency is observed (Jiang, Stuible et al.

2003; Lu, Gavrilescu et al. 2012). The CML-like MPN can progress to blast crisis (Daley, Van Etten et al. 1991), and is responsive to kinase inhibitor treatment (Hu, Swerdlow et al. 2006) and immunotherapy (Lu, Gavrilescu et al. 2012), representing a physiologically relevant model of the disease in primary hematopoietic cells.

We transduced BM from 5-FU-treated wild-type or Prdx1 donor mice with *BCR-ABL1* retrovirus in the presence of myeloid cytokines (see METHODS) and transplanted equal numbers of cells into lethally irradiated wild-type recipient mice. In an initial transplant where we injected a high number of transduced BM cells (5×10^5 per recipient), BCR-ABL1-transduced cells from both donor types induced fatal CML-like MPN within 4 weeks, and there was no significant difference in survival between the cohorts (data not shown). However, as with the B-ALL transplants, we reasoned that decreasing the number of transplanted transduced cells would prolong the latency of the CML-like MPN and allow a difference in leukemogenesis to be detected. Indeed, we found that when the number of transplanted *BCR-ABL1*-transduced cells was decreased to 9×10^4 cells per recipient, we observed a significant difference in survival between the two groups (Figure 24A). Under these conditions, recipients of *Prdx1*$^{-/-}$ *BCR-ABL1*-transduced BM exhibited a more aggressive CML phenotype compared to the recipients of wild-type BM. These observations suggest that PRDX1 acts as a tumor suppressor in BCR-ABL1-induced CML-like MPN.

We carried out a second CML transplant where we performed lineage depletion prior to transplantation to strengthen the CML-like phenotype and link it to the myeloid lineage. Lymphoid and myeloerythroid lineage cells were depleted from whole bone marrow of 5-FU-treated donor mice through the use of biotinylated antibodies directed against Gr-1, TER-119, B220, and CD5, and removed using streptavidin-conjugated beads and Miltenyi magnetic columns, as described in METHODS (Lu, Gavrilescu et al. 2012). This modification enriches for hematopoietic stem cells and removes committed progenitors, allowing us to inject a lower number of cells and reduce or eliminate the risk of recipient mice developing lymphoid disease. Lineage depletion to enrich for hematopoietic stem cells supported our previous CML transplant result, as a prominent CML-like phenotype was observed (Figure 24B). The recipient mice receiving *Prdx1*$^{-/-}$ *BCR-ABL1*-transduced bone marrow had significant disease susceptibility, as they developed fatal CML-like MPN much sooner than the wild-type cohort.

A.


CML TRANSPLANT
Percent survival
100
80
60
40
20
0
0
20
40
60
days post BMT
WT
PRDX1-KO

B.

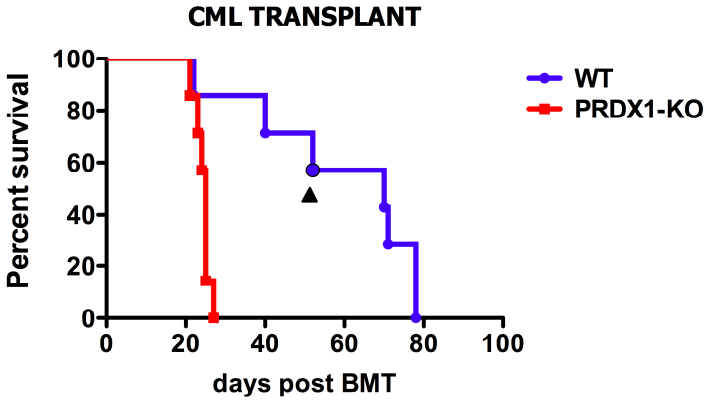

CML TRANSPLANT
Percent survival
100
80
60
40
20
0
0
20
40
60
80
100
days post BMT
WT
PRDX1-KO

Figure 24: Loss of *Prdx1* leads to an aggressive CML-like phenotype induced by BCR-ABL1.

A. Donor wild type mice and *Prdx1*$^{-/-}$ mice were injected with 5-fluorouracil to enrich for a hematopoietic stem cell population. Five days post-injection, bone marrow was isolated from the donor mice and transduced with *BCR-ABL1.* The transduced cells are then injected into lethally irradiated wild-type recipient mice. Mice are observed regularly for CML-like symptoms (leukocytosis with GFP+ myeloid cells, splenomegaly, respiratory difficulty). Recipients of *BCR-ABL1*-transduced *Prdx1*-KO BM had significantly shorter overall survival than recipients of transduced wild-type BM. (Mantel-Cox test, p value = 0.003) **B.** Whole bone marrow was harvested from 5-fluorouracil treated mice and transduced with *BCR-ABL1*. Hematopoietic stem cells were further enriched through the use of biotinylated antibodies directed against Gr-1, TER-119, B220, and CD5, and removed using streptavidin-conjugated beads and Miltenyi magnetic columns to deplete lymphoid and myeloerythroid cells. The number of cells transplanted was reduced compared to the transplant in panel A. The results reproduced the significant leukemogenic phenotype as observed in the CML transplant shown in panel A. Recipient mice receiving donor *Prdx1*$^{-/-}$ bone marrow showed accelerated disease progression compared to wild-type recipient mice. Arrowhead in WT cohort refers to mouse acquiring fatal B-ALL, and not CML-like MPN.
(Mantel-Cox test, p value = 0.003)

VII. Characterization of Leukemia Stem Cell Frequency and Phosphotyrosine Signaling in Prdx1 mutant murine CML

It is not entirely clear how the loss of *Prdx1* is contributing to an aggressive form of CML induced by BCR-ABL1. Disease clonality may be an underlying cause for cancer susceptibility in mice receiving *Prdx1*$^{-/-}$ *BCR-ABL1*-transduced bone marrow. Southern blot analysis would identify leukemic stem cell frequency and would provide information on any differences in the number of clones between donor-derived wild-type and knockout bone marrow. It may be possible that *Prdx1*$^{-/-}$ hematopoietic stem cells are prone to BCR-ABL1 transduction and activation. An increase in the number of clones or the presence of aggressive clones would partially explain the reason for a difference in disease onset. DNA samples from bone marrow, spleen, and peripheral blood are analyzed, as cells from these sources contain a high number of BCR-ABL1^{+} myeloid cells (Daley, Van Etten et al. 1990; Li, Ilaria et al. 1999; Barnes, Schultheis et al. 2005). Clonality of the diseased mice is currently being studied.

Another important cause of the CML defect to investigate is the signaling induced by BCR-ABL1. BCR-ABL1 autophosphorylation of the ABL1 domain is commonly observed in BCR-ABL1-mediated transformation, as this modification changes BCR-ABL1 to an active state and helps it maintain its constitutive activity (Pendergast, Gishizky et al. 1993; Pendergast, Quilliam et al. 1993; Brasher and Van Etten 2000). Protein lysates were obtained from

diseased samples, such as bone marrow, spleen, and peripheral blood, to identify phosphotyrosine levels of BCR-ABL1. Figure 25 shows BCR-ABL1 phosphotyrosine levels (Ptyr) are selectively higher in recipient mice which developed fatal CML from *Prdx1*$^{-/-}$ *BCR-ABL1*-transduced bone marrow. BCR-ABL1 activation leads to phosphorylation and dysregulation of signaling substrates involved in pathways linked to cell survival or apoptosis. We had questioned the involvement of selected substrates involved in cell proliferation known to be modulated by BCR-ABL1, such as CRKL, AKT, ERK, STAT5, and GAB2 (Pendergast, Quilliam et al. 1993; Feller, Knudsen et al. 1994; Ilaria and Van Etten 1996; Sattler, Mohi et al. 2002). Based on preliminary data, we were unable to see a significant difference in activation of known BCR-ABL1 target proteins (as shown in Figure 25). Because immunblot analysis for some samples was not optimal, we are currently re-testing the phosphorylation of these signaling proteins.

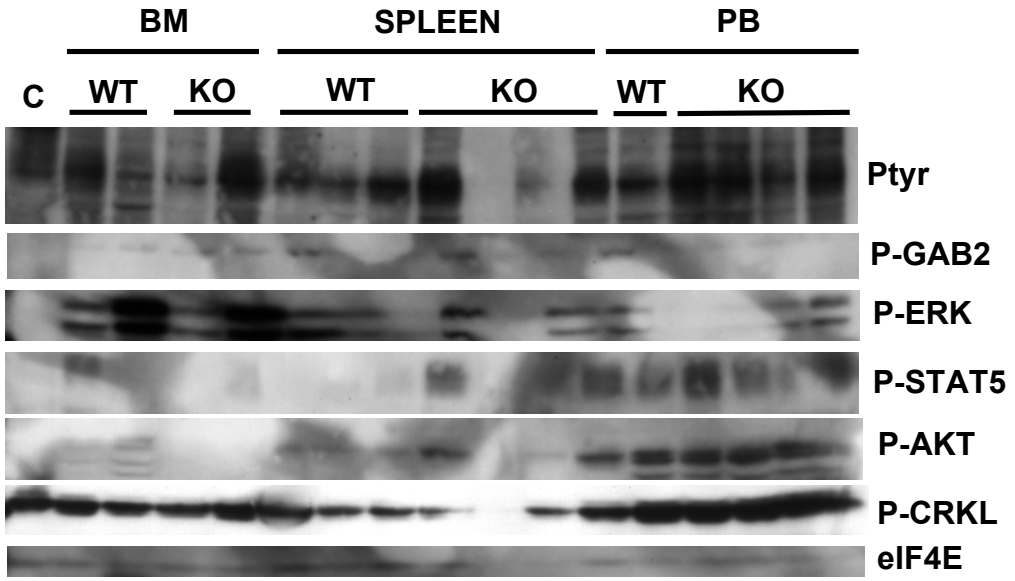


Figure 25: Phosphotyrosine Signaling in Prdx1 mutant murine CML. Protein lysates of diseased mice from CML transplant in Figure 18A were blotted to identify possible differences in signaling mechanisms leading to leukemogenesis. BM = bone marrow; PB = peripheral blood; C = control (B-lymphoid BCR-ABL1-transformed line); eIF4E = loading control. BCR-ABL1 autophosphorylation is seen by blotting for phosphotyrosine levels. Donor-derived *Prdx1*$^{-/-}$ *BCR-ABL1*-transduced bone marrow cells show in general increased BCR-ABL1 phosphotyrosine levels, signifying an increase in BCR-ABL1 kinase activity. There was no difference observed on phosphorylation of known BCR-ABL1 substrates or known pathways activated by BCR-ABL1. The samples are being analyzed further to verify any differences, as blot techniques against some of the proteins were not optimal.

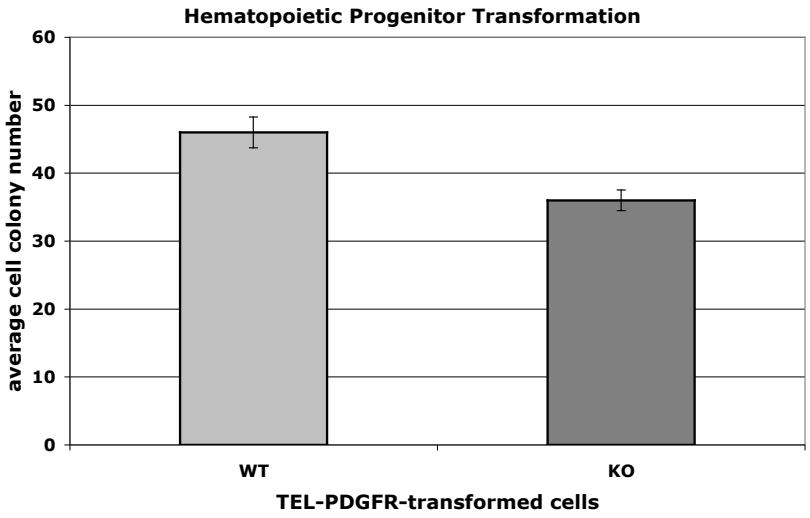
VIII. The Tumor Suppressor Function of PRDX1 in Myeloid Leukemogenesis is Specific to BCR-ABL1

We believe that the acceleration of lymphoid and myeloid cell transformation by BCR-ABL1 in *Prdx1* mutant bone marrow is a consequence of loss of the ABL1 inhibitory function of PRDX1. However, an alternative explanation is that PRDX1, an antioxidant enzyme, helps protect tyrosine phosphatases (which have a redox-sensitive catalytic cytsteine residue) from oxidative damage. In the absence of PRDX1, the unopposed action of a dysregulated tyrosine kinase on cellular phosphotyrosine levels might be enhanced. In a control experiment, we used the leukemia fusion protein TEL-PDGFRβ to determine the selectivity of PRDX1 and its role in myeloid cell transformation and leukemogenesis. Like BCR-ABL1, TEL-PDGFRβ is a constitutively active tyrosine kinase that induces myeloid cell transformation, as well as CML-like disease in murine models that is phenotypically very similar to that induced by BCR-ABL1 (Tomasson, Sternberg et al. 2000). However, unlike BCR-ABL1, PRDX1 does not interact with nor function to inhibit TEL-PDGFRβ kinase activity. We first tested the effect of Prdx1 deficiency on transformation of myeloid progenitors by TEL-PDGFRβ in the methylcellulose assay. The results demonstrated that loss of *Prdx1* does not affect myeloid transformation by TEL-PDGFRβ (Figure 26A).

We subsequently assessed induction of CML-like leukemia in the mouse transplantation using a TEL-PDGFRβ retrovirus under conditions

identical to those employed for BCR-ABL1. There was no significant difference in the overall survival of recipients transplanted with TEL-PDGFRβ-transduced BM from wild-type or Prdx1 mutant donors (Figure 26B). These results demonstrate that the acceleration of myeloid leukemogenesis in the absence of PRDX1 is specific to BCR-ABL1, and argue that the myeloid tumor suppressor activity of PRDX1 is dependent on its function as a stoichiometric inhibitor of BCR-ABL1.

A.


Hematopoietic Progenitor Transformation
average cell colony number
60
50
40
30
20
10
0
WT
KO
TEL-PDGFR-transformed cells

B.

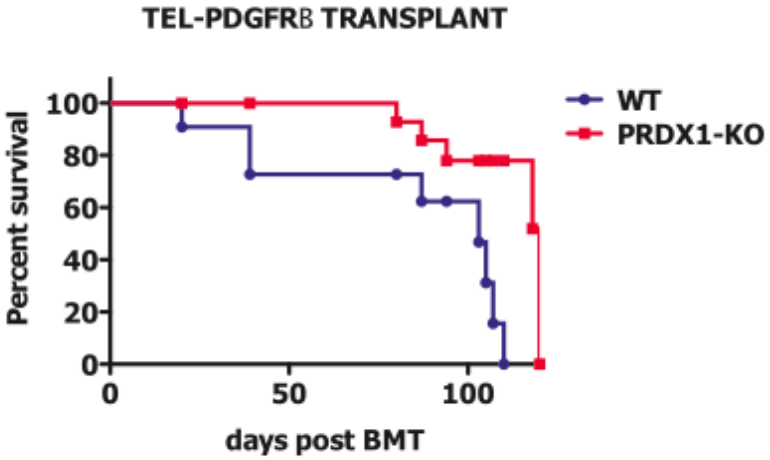

TEL-PDGFRB TRANSPLANT
Percent survival
100
80
60
40
20
0
0
50
100
days post BMT
WT
PRDX1-KO

Figure 26: The Tumor Suppressor Function of PRDX1 in Myeloid Leukemogenesis is Specific to BCR-ABL1.

To determine the importance of the PRDX1 – BCR-ABL1 interaction, experiments were conducted with a control tyrosine kinase, TEL-PDGFRβ, which induces similar disease in mice as does BCR-ABL1. Bone marrow derived from 5-FU treated wild-type or *Prdx1*$^{-/-}$ mice was harvested and transduced with *TEL-PDGFRβ*. **A.** Myeloid progenitor transformation by TEL-PDGFRβ, was assayed using a quantitative approach. Cells were plated in methylcellulose incomplete medium and colonies were identified after day 10 of incubation. The TEL-PDGFRβ results show that loss of PRDX1 fails to increase progenitor transformation potential, as in the BCR-ABL1 background. **B.** The *TEL-PDGFRβ* transplant shows the accelerated phenotype is lost, signifying the importance of the PRDX1 – BCR-ABL1 interaction in disease progression.

CHAPTER SUMMARY

This chapter provides strong evidence that the interaction between PRDX1 and BCR-ABL1 is crucial for modulation of transformation and leukemogenesis. PRDX1 regulates ABL1 kinase activity, and in an instance when this function is lost, the consequences could be detrimental for the cell or organism. In terms of BCR-ABL1, the loss of regulation due to the absence of *Prdx1* promotes more efficient lymphoid and myeloid transformation. In addition, the loss of PRDX1 regulation on BCR-ABL1 induces a slight increase in disease onset for B-ALL and leads to significant cancer susceptibility of CML-like MPN. The importance of the PRDX1 – BCR-ABL1 interaction is revealed by demonstrating loss of Prdx1 fails to enhance transformation or initiate aggressive leukemogenesis in the presence of TEL-PDGFRβ, a fusion tyrosine kinase known to cause similar transformation and disease as BCR-ABL1.

DISCUSSION

c-ABL1 is a tightly regulated tyrosine kinase that can be activated upon increased intracellular ROS levels or genotoxic stress (Van Etten 1999). Several lines of biochemical and genetic evidence indicate that c-ABL1 is regulated via both an autoinhibitory mechanism (similar to Src kinases) and through cellular inhibitors (Van Etten 2003). As mentioned before, PRDX1 was found through a yeast two-hybrid screen using the ABL1 SH3 domain as bait. PRDX1 was found to not only interact with the SH3 domain, but also the kinase domain, suppressing ABL1 kinase activity. PRDX1 had no effect on the kinase activity of an ABL1 mutant with a dysregulating mutation in the SH3 domain nor on an ABL-Src chimera containing the Src kinase domain, but did partially inhibit the BCR-ABL1 fusion protein found in chronic myeloid leukemia patients (Wen and Van Etten 1997).

BCR-ABL1 is the initiating factor in chronic myeloid leukemia development and some cases of B-cell acute lymphoblastic leukemia (Quintas-Cardama and Cortes 2009). As mentioned above, PRDX1 has an inhibitory role on BCR-ABL1 kinase activity. However, this observation had not been further analyzed to determine the impact of PRDX1 regulation on BCR-ABL1 kinase activity. Because genetic and structural studies suggest that BCR-ABL1 can assume an autoinhibited conformation similar to c-ABL1 (Smith, Yacobi et al. 2003), our working model is that PRDX1 binds to and stabilizes the autoinhibited, non-phosphorylated form of ABL1. We believe

PRDX1 binding facilitates the development of the inactive state, suppressing its kinase activity. Our prior experiments in MEFs showed that cells lacking *Prdx1* retained BCR-ABL1 at a higher frequency than wild type cells after *BCR-ABL1* retroviral transduction and culture expansion. The *Prdx1*$^{-/-}$ MEFs also showed higher BCR-ABL1 phosphotyrosine levels. These results strengthened the hypothesis that PRDX1 functions as an important factor in BCR-ABL1 regulation. The data led us to shift our focus to the hematopoietic system and further study the functional significance of PRDX1 suppression of BCR-ABL1 kinase activity.

In this portion of the thesis, we showed that loss of *Prdx1* promotes BCR-ABL1-mediated cell transformation or leukemogenesis. This conclusion is supported by experiments that involved questioning the involvement of both the lymphoid and myeloid lineages of the hematopoietic system. Lymphoid studies proved that in the absence of *Prdx1*, BCR-ABL1-induced cell transformation potential is enhanced, as fewer cells are needed to initiate a transformation event in Whitlock-Witte cultures. These *in vitro* results suggest that PRDX1 is a factor in regulating lymphoid cell transformation by BCR-ABL1. The BCR-ABL1-transformed *Prdx1*$^{-/-}$ lymphoid cells also exhibited an increase in cell growth stability. The loss of *Prdx1* significantly influenced cell proliferation, implying that BCR-ABL1 kinase may have improved activation of cell survival pathways. The loss of *Prdx1* could also influence

the acquisition of additional mutations and permit the cell to survive and transform.

In support of our findings on lymphoid cell transformation, a quantitative colony formation assay showed loss of *Prdx1* enhances a malignant phenotype in the presence of BCR-ABL1. *Prdx1*$^{-/-}$ lymphoid cells showed an increase in colony formation, implying that PRDX1 contributes to suppression of B-lymphoid transformation *in vitro*.

These results prompted us to move into *in vivo* studies and determine if loss of *Prdx1* could influence leukemogenesis mediated by BCR-ABL1. We hypothesized that based on the *in vitro* data showing enhanced BCR-ABL1-mediated cell transformation and malignancy in the loss of *Prdx1*, an aggressive development of B-cell acute lymphoblastic leukemia (B-ALL) would be observed. To address this hypothesis and monitor for B-ALL, we utilized the bone marrow transduction/transplantation murine model system.

We noticed an initial defect in B-ALL development, in which recipient mice transplanted with *Prdx1*$^{-/-}$ *BCR-ABL1* cells succumbed to disease before recipient mice transplanted with wild type BCR-ABL1 cells. However, the phenotype soon disappeared and leukemic development becomes similar to wild type. As the transplant is continued for an extended period, disease development is lost. It is unclear why mice fail to succumb to disease, but it could be related to failed engraftment of transplanted cells or the genetic strain of the recipient mice.

PRDX3 has been shown to regulate c-ABL1 kinase activity, so the presence of PRDX3 may be compensating for the loss of PRDX1 and contributing to BCR-ABL1 regulation (unpublished data, (Neumann, Krause et al. 2003)). The regulation may be enough to delay further B-ALL development upon reaching a checkpoint or threshold. We had also questioned whether the *Prdx1*$^{-/-}$ recipient cohort was undergoing cell senescence or crisis, blocking further disease development. We attempted to obtain *Prdx1*$^{-/-}$*/p19Arf*$^{-/-}$ knockout mouse strain to overcome a possible cell crisis and determine if removing a cell cycle checkpoint would improve disease development. However, several matings failed to produce litters, implying that possibly a double knockout is not viable.

Myeloid lineage studies were also interrogated, as it is well known that BCR-ABL1 induces myeloproliferative diseases. When whole bone marrow was transduced with *BCR-ABL1*, we found that *Prdx1*$^{-/-}$ myeloid progenitors demonstrated enhanced transformation in methylcellulose cultures. The *in vitro* data led us to study the PRDX1 – BCR-ABL1 interaction in the chronic myeloid leukemia (CML) transplantation model system. As expected based on the *in vitro* results, we observed cancer susceptibility in the *Prdx1*$^{-/-}$ cohort. The recipient mice transplanted with *Prdx1*$^{-/-}$ *BCR-ABL1* showed an aggressive phenotype and succumbed to CML-like MPN much sooner than the wild type cohort.

As a control for myeloid transformation and leukemia, we used *TEL-PDGFR-β*, which encodes for a tyrosine kinase that induces similar transformation and leukemogenesis as does BCR-ABL1 (Golub, Barker et al. 1994). However, PRDX1 does not contribute to TEL-PDGFR-β regulation, so *Prdx1*-loss should not promote transformation potential or disease susceptibility. The control experiment proved that PRDX1 selectively regulates BCR-ABL1 and is important in modulating cell transformation and leukemogenesis, as loss of PRDX1 failed to elevate TEL-PDGFR-β-mediated cell transformation or induce an aggressive leukemic phenotype. These control experiments may also suggest that the antioxidant activity of PRDX1 is not necessary for transformation or leukemogenesis. However, further studies need to be done to verify BCR-ABL1-induced transformation and disease is not influenced by an impaired mechanism to eliminate oxidative stress.

A rapidly increasing field of study is resistance of BCR-ABL1$^+$ cells to tyrosine kinase inhibitors. It is crucial to understand why resistance is occurring and how it can be addressed and overcome. We hypothesized that *Prdx1* mutant cells transformed by BCR-ABL1 would be resistant to the tyrosine kinase inhibitor imatinib. Imatinib recognizes the inactive conformation of c-ABL1 and BCR-ABL1. Loss of *Prdx1* could reduce the availability of inactive BCR-ABL1, therefore interfering with imatinib efficacy. Experiments testing BCR-ABL1-transformed lymphoid and myeloid cells failed

to show imatinib resistance. This was unexpected, as it implies that the inactive form of BCR-ABL1 is still readily present in the *Prdx1*$^{-/-}$ cells. As mentioned before, it is possible that PRDX3 plays a role in contributing to BCR-ABL1 inactivation, as it can inhibit ABL1 (Neumann, Krause et al. 2003). Another possible candidate for BCR-ABL1 inhibition may be glutathione peroxidase 1 (GPX1). It has been reported that GPX1 is an antioxidant that functions similarly to PRDX1, as it also binds to the SH3 domain of ABL1 and suppresses its activity (Cao, Leng et al. 2003).

As mentioned before, the role of the antioxidant activity of PRDX1 would need to be analyzed. Even though TEL-PDGFR-β data imply this activity is not necessary, there could still be some contribution to malignancy and leukemogenesis, as inactive BCR-ABL1 still remains present in *Prdx1*$^{-/-}$ cells. ROS have already been linked to cancer susceptibility, as a lack in the antioxidant GSTpi leads to increased tumorigenesis (Henderson, Smith et al. 1998). In addition, *Nrf-2* deficient mice, which are defective in the induction of the GSTpi and PRDX1, show an increase in cancer susceptibility (Ramos-Gomez, Kwak et al. 2001).

The studies presented in this chapter identify the functional significance of PRDX1 regulation on BCR-ABL1 kinase activity. PRDX1 is known to regulate ABL1 and suppress its kinase activity through physical interaction. Using a reverse genetics approach, we determined that this physical interaction is a contributing factor in controlling transformation and

leukemogenesis. PRDX1 is able to function as a regulator of BCR-ABL1 in both lymphoid and myeloid lineages. Based on our data, it seems that in *Prdx1*$^{-/-}$ bone marrow cells, the regulatory hold on BCR-ABL1 kinase activity is lost, enhancing transformation, promoting malignancy, and inducing aggressive cancer progression.

THESIS CONCLUSIONS AND FUTURE DIRECTIONS

Peroxiredoxins have increasingly become attractive to study, as they are linked to multifunctional distinct cellular roles. They were first discovered to regulate cell proliferation and function as peroxidases. The antioxidant activity serves to regulate the intracellular levels of ROS, mainly by degrading high amounts of hydrogen peroxide that could lead to cellular damage (Rhee, Chae et al. 2005). It was later found in the Van Etten laboratory that PRDX1 functions to regulate ABL1 kinase activity. PRDX1 recognizes and binds to the SH3 and kinase domains of ABL1, preventing activation of ABL1 (Wen and Van Etten 1997). This suppression helps control cell growth, as PRDX1 sequesters ABL1 in the cytoplasm and most likely seems to prevent ABL1 from entering the nucleus to induce arrest or apoptosis. Of interest, peroxiredoxins are continuously being found to play a significant role in transformation and carcinogenesis. Specific to our work, PRDX1 is linked to cancer susceptibility. However, it is not entirely clear how the absence or overexpression of PRDX1 induces the onset of disease or maintains an oncogenic state, respectively.

The functional significance of PRDX1 in carcinogenesis could be partially attributed to its peroxidase activity. It has been noted that PRDX1 seems to show contradictory roles, as it not only acts to prevent tumor formation, but also proves to be important for tumor maintenance. In

preventive measures, PRDX1 eliminates ROS, decreasing the accumulation of mutations that could initiate cell transformation (Neumann, Krause et al. 2003; Egler, Fernandes et al. 2005; Cao, Schulte et al. 2009). To help maintain tumor growth, PRDX1 is overexpressed, providing a protective microenvironment (Yanagawa, Ishikawa et al. 1999; Yanagawa, Iwasa et al. 2000; Kinnula, Lehtonen et al. 2002; Kim, Chae et al. 2003). Tumor cells harbor high ROS levels, therefore, the ROS scavenging activity of PRDX1 is crucial to maintain survival and prevent apoptosis (Neumann and Fang 2007).

As described in Chapter 2, the antioxidant activity of PRDX1 is a contributing factor for maintaing cell survival in MEFs. *Prdx1*-deficiency leads to a growth delay that can partially be attributed to a defective response for ROS elimination. In connection to this phenotype, it would be interesting to determine the impact of the antioxidant activity of PRDX1 on BCR-ABL1 transformation and leukemogenesis. The intracellular ROS levels in BCR-ABL1-transformed cells from culture or directly from diseased mice could be identified. Cells lacking Prdx1 would presumably have elevated ROS levels, and this increase in oxidative stress could contribute to oncogenesis.

Another way to approach the question of whether PRDX1 peroxidase activity is a factor in oncogenesis is to utilize an antioxidant-inactive form of PRDX1. Bone marrow cells could be transduced with *Prdx1* lacking peroxidase activity. It has been shown that the antioxidant activity is fairly dispensable for ABL1 regulation. A mutant PRDX1 with deletion of the

conserved cysteine-52 residue required for antioxidant activity is still capable of suppressing ABL1 kinase function (Wen and Van Etten 1997).

It may also be interesting to determine the effects of antioxidant treatment or induction of chemical hypoxia, as done in Chapter 2. Incubation with both types of treatments would essentially reduce ROS levels and identify their role in cell survival. The treatments would in theory mitigate or delay malignant growth and leukemogenesis in *Prdx1*$^{-/-}$ *BCR-ABL1* cells, if PRDX1 antioxidant does in fact play a role in these phenotypes.

It would be valuable to determine the antioxidant role of PRDX1 in oncogenesis, as it could provide a basis for future treatments of cancerous cells deficient in a response to oxidative stress. As noted above, PRDX1 can help prevent tumor formation, so introduction of PRDX1, or of the antioxidant activity lost due to it, into cancer cells may be effective in regulating cell growth and survival. Because PRDX1 is overexpressed in some cancers, however, it actually may be beneficial to inhibit PRDX1 in these particular cancerous cells. The targeted inhibition would essentially induce a substantial increase in intracellular ROS levels, promoting tumor cell death.

There is still much interest in learning how ABL1 is maintained in an inactive state. The PRDX1 – ABL1 interaction needs further characterization, as the results could possibly reveal a new PRDX1 consensus sequence or sequences that interact with the SH3 and the ATP domains of ABL1. PRDX1 lacks any signature motifs that recognize either of these domains. These

consensus sites could also be conserved in other potential tumor suppressor proteins that may serve to regulate the activity of other proteins or kinases. The similarities could reveal how PRDX1 is regulated and give more insight into how it regulates ABL1 kinase activity.

A genetic analysis of the PRDX1 – ABL1 interaction could also help explain disease onset. A PRDX1 mutant could be utilized that prevents ABL1 recognition and inhibition *in vivo*. This mutant would demonstrate the importance of the interaction and its involvement in disease. However, both proteins would need to remain active, so as not to elicit secondary effects related to the mutations. It may be possible to do this experiment with the ABL1 SH3 P131L point mutation, as it prevents PRDX1 from binding to ABL1 (Wen and Van Etten 1997). Further structural analysis of the PRDX1 – ABL1 interaction may also provide information about a variant form of PRDX1 that could recognize the imatinib-resistant ABL1 mutants and create a tight and stable bond to BCR-ABL1, preventing its activation.

Our data implicate PRDX1 in BCR-ABL1-mediated transformation and leukemogenesis. The lack of BCR-ABL1 regulation in the absence of *Prdx1* contributes to the cancerous phenotype. It is of interest to study BCR-ABL1-induced signaling mechanisms affected in the absence of *Prdx1*. We have yet to determine if the loss of *Prdx1* selectively activates certain pathways for cell survival and proliferation in BCR-ABL1$^+$ cells. BCR-ABL1$^+$ transformed or leukemic cells should be analyzed to identify phosphorylation events caused

by BCR-ABL1 activity (Pendergast, Gishizky et al. 1993; Brasher and Van Etten 2000). This involves BCR-ABL1 autophosphorylation, as well as substrates or targeted pathways, such CRK, STAT5, GAB2, and AKT (Feller, Knudsen et al. 1994; Ilaria and Van Etten 1996; Sattler, Mohi et al. 2002). Determining what signaling pathways are important to attain a diseased state is crucial, as treatments may be administered or developed that can specifically target that pathway. It is also of interest to determine whether disease clonality is attributed to increased transformation and the accelerated onset of disease in the absence of *Prdx1*. It is possible that bone marrow cells deficient for *Prdx1* harbor a higher number of *BCR-ABL1* clones. Alternatively or in addition, they could possess more aggressive *BCR-ABL1* clones (Daley, Van Etten et al. 1990; Li, Ilaria et al. 1999; Barnes, Schultheis et al. 2005).

It would also be informative to determine if the natural killer (NK) cell activity plays a role in cancer development, as *Prdx1*$^{-/-}$ mice display defective NK cell activity. NK activity may provide the necessary protective mechanism to modulate leukemogenesis. It is possible that because of reduced NK cell activity, the tumor surveillance system is suboptimal, leading to an increase in disease onset of *Prdx1*$^{-/-}$ *BCR-ABL1* CML mice. The NK cell population would need to be identified in the BCR-ABL1^{+} leukemic mice to verify a phenotype in NK cell development. Also, NK cytotoxic activity would need to be tested

to determine if leukemic mice transplanted with *Prdx1*$^{-/-}$ *BCR-ABL1* cells exhibit a decrease in NK cell activity.

The present thesis contributes further knowledge on PRDX1 peroxidase function and provides supporting evidence on the importance of ABL1 and BCR-ABL1 kinase regulation. The growth delay defect in *Prdx1*$^{-/-}$ MEFs was studied and we determined to be induced at least partially by the inability to modulate oxidative stress levels. Common imatinib-resistant mutations localized within the ABL1 kinase domain were studied and found to also be resistant to PRDX1 suppression. A reverse genetics approach was utilized to identify a possible pathophysiological function of PRDX1 in the presence of BCR-ABL1. We found that BCR-ABL1 dysregulation by loss of PRDX1 contributes to enhanced transformation and leukemogenesis.

METHODS

293 TRANSFECTIONS

293T cells are co-transfected with a plasmid that overexpresses c-ABL1 and at varying concentration of plasmid that expresses PRDX1 using a calcium phosphate transfection protocol. Briefly, 293T cells are plated at 3 X 10^6 cells per 60-mm plate the night before the transfection. The medium is changed to fresh medium the morning of transfection, and the plasmid DNA mix is prepared. The mix contains deionized water, plasmid DNA, $CaCl_2$, and HBS solution, which contains the phosphate. The solution is added to the medium and cells are incubated at 37°C for about seven hours. The medium is then changed and cells are incubated for 48 hours. The cells are then lysed using RIPA buffer and protease inhibitors and centrifuged to isolate the total protein. Protein content is determined using a spectrophotometer and samples are subjected to SDS-PAGE. Immunoblotting analysis is done using antibodies against c-ABL1, phosphorylation (phosphotyrosine), and PRDX1.

ANCHORAGE-INDEPENDENCE ASSAY

Single-cell suspensions of 2x104 cells were plated per 35-mm culture grid dish in 1.5 ml of DMEM containing 10% FCS and 0.36% agar and on a layer of 1.5 ml of the same medium containing 0.7% agar. Plates were fed weekly with 0.5 ml of DMEM/10% FCS. Three weeks after plating, colonies were counted by microscopy (Akagi, Sasai et al. 2003).

WHITLOCK-WITTE CULTURES

Whole bone marrow from one wild type donor mouse and one *Prdx1*$^{-/-}$ donor mouse is isolated. *BCR-ABL1* is introduced into cells by retroviral transduction by cosedimentation. Briefly, bone marrow cells incubated in RPMI-lymphoid selective medium are transduced with a *BCR-ABL1* retrovirus by centrifuging for 90 minutes at 30˚C. Immediately after, viral adsorption is achieved by incubation at 37˚C for 3-4 hours. Wild type bone marrow cells (that will serve as stromal, or feeder, cells) are collected. After the adsorption step, cells are plated at eight different dilutions (from 100 cells to 300 000 cells) in 24-well plates in triplicate (Smith, Yacobi et al. 2003). The medium promotes B-lymphoid cell development, and cell growth is analyzed daily after five days. Only the cells that have been transformed by BCR-ABL1 will grow. Wells positive for cell transformation signify an outgrowth of at least $1x10^6$ cells. Medium was changed every 3-4 days by carefully removing 0.5 mL of medium and replacing it with fresh medium without disturbing cells. After roughly 21 days, the assay is finished and lymphoid cell growth is suppressed by mast cells (McLaughlin, Chianese et al. 1987).

CELL VIABILITY

Cell proliferation is monitored by the use of Promega reagent MTS and quantified by spectrophotometry. Trypan blue analysis was also used for proliferation. MEFs are cultured at $1x10^4$ c/ml in 96-well plates and incubated

with MTS. B-lymphoid cells are seeded at an initial cell number of 5 x 10^5 cells per well in a 12-well plate. Each line is plated in triplicate and counted using trypan blue staining daily beginning at four hours post-plating and daily for 3 days.

To monitor cell proliferation of BCR-ABL1-transformed cells in medium containing kinase inhibitors, 1x10^5 c/ml were plated in triplicate in a 96-well plate. Cells were incubated with increasing concentrations of GNF-2 or imatinib. Cell proliferation was analyzed at time point 96 hours by incubating samples with the compound MTS and using spectrophotometry analysis.

QUANTITATIVE LYMPHOID MALIGNANT TRANSFORMATION ASSAY

Whole bone marrow is isolated from age-matched wild type and *Prdx1*$^{-/-}$ mice, then subjected to retroviral transduction as described above. A base layer of agarose-lymphoid medium is dispensed onto 3.5 cm plates. Transduced cells (3 x 10^6 cells) are resuspended in agarose-lymphoid medium and placed above the base agarose medium to provide for an anchorage-independence environment. The plates are incubated at 37°C, with fresh liquid lymphoid medium added every 3 to 4 days. The colony number is registered by counting the plates after 14 days (Rosenberg and Baltimore 1976).

BONE MARROW TRANSDUCTION AND TRANSPLANTATION

B-ALL TRANSPLANTS

BCR-ABL1-GFP-transduced donor bone marrow cells were obtained as in the above *in vitro* experiments. Recipient mice are sub-lethally irradiated from (<900 cGy) and transplanted with the donor transduced cells. The mice are monitored for survival and sacrificed when they become immobile due to disease-related reasons (Roumiantsev, de Aos et al. 2001). The recipient mice are bled to verify engraftment by flow cytometry for GFP expression 3 weeks post-transplant. In initial transplants, 1×10^6 cells were injected into recipient mice. In the subsequent modified transplant, the recipient mice were injected with 3×10^5 cells.

CML TRANSPLANTS

Donor wild type or *Prdx1*$^{-/-}$ mice are injected with 5-fluorouracil to enrich for a hematopoietic stem cell population, and four days post-injection, bone marrow is harvested and subjected to retroviral transduction with either *BCR-ABL1-GFP* or *TEL-PDGFR-Beta-GFP*. Recipient mice are lethally irradiated for ~900 cGy to help prevent rejection of engraftment and to make space for donor marrow. *BCR-ABL*-transduced cells are transplanted into recipients by tail vein injection. Engraftment is verified 3 weeks post-transplant by flow cytometry to monitor for GFP expression. Similar to the B-ALL assay, the recipient mice are monitored for disease and survival.

In initial transplants, 5 x 10^5 cells were injected into recipient mice. In the subsequent modified transplant, the recipient mice were injected with 9 x 10^4 cells.

For lineage depletion transplants, whole bone marrow harvested 4 days post-5-FU treatment from donor mice is depleted of lymphoid and myeloerythroid lineage cells through the use of biotinylated antibodies and streptavidin-conjugated magnetic beads. 1x10^4 cells are injected into lethally irradiated recipient mice.

METHYLCELLULOSE ASSAY

Untransduced cells are incubated in a semi-solid complete medium made of methylcellulose containing growth factors, such as myeloid cytokines.

Cells harvested from day 4 post-5-FU treated mice are transduced with *BCR-ABL1*, then incubated in complete medium containing growth factors, or in incomplete medium, lacking cytokines and growth factors. Both media promote hematopoietic progenitor proliferation, however, the incomplete medium allows for only transformed cells to form colonies. For complete medium experiments, 1x10^4 cells are plated in 3.5 cm plates and incubated for 10 days. For incomplete medium experiments, 2x10^4 cells are plated. Progenitors form colonies that can easily be counted by microscopy (Lavau, Szilvassy et al. 1997).

Drug resistance experiments involved the addition of various concentrations of imatinib to incomplete medium. Cells were incubated as in previous methylcellulose experiments and colony formation was recorded.

IMMUNOBLOT ANALYSIS

Whole cell lysates were prepared from either MEFs, B-lymphoid BCR-ABL-transformed cells, or diseased mouse organs. To monitor whether PRDX1 has an effect on the ability of GNF-2 or imatinib to inhibit BCR-ABL1 kinase activity, we incubated cells with various concentrations of the drug for 24 hours for MEFs or 90 minutes for B cells before lysis. Immunoblotting analysis was used to identify phosphotyrosine levels, as well as for PRDX1 and ABL1 expression.

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