The Effect of Redox Environment on Function of the *Drosophila* PRL protein

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# TABLE OF CONTENTS

INTRODUCTION ........................................................................................................................................... 1

I. CANCER DEVELOPMENT .......................................................................................................................... 1

II. REDOX ENVIRONMENT AND CANCER ................................................................................................. 4

III. IMPLICATIONS OF PHOSPHATASE OF REGENERATING LIVER (PRL) IN CANCER PROGRESSION

   (a) PRL structure and function .............................................................................................................. 8
   (b) Association of increased PRL expression with cancer invasion and metastasis ...................... 12
   (c) PRL as a tumor suppressor in vivo ............................................................................................... 15

IV. INVESTIGATION OF PRL FUNCTION IN ALTERED CELLULAR REDOX STATES ............................. 16

RESULTS ....................................................................................................................................................... 22

DISCUSSION .................................................................................................................................................. 31

MATERIALS AND METHODS ..................................................................................................................... 42

REFERENCES .................................................................................................................................................. 44
**Introduction**

I. Cancer Development

   Cancer is a broad, complex group of diseases characterized by abnormal cell growth and invasion of other tissues. Typically, regulatory mechanisms ensure normal cell proliferation and homeostasis, which allow for the production of new cells to replace dead cells and maintain general stability in the body. However, in cancerous cells, these regulatory circuits become defective, resulting in unchecked growth and, ultimately, tumorigenesis (the formation of tumors).

   The history of cancer research has been fraught with challenges, largely because of the breadth of diseases characterized under the label of “cancer.” There are over one hundred distinct types of cancer, and specific organs can even host subtypes of tumors [Hanahan and Weinberg, 2000]. What, then, are the underlying mechanisms that govern the development of cancer in the body? And how can we develop widely applicable treatments for a disease that is so inherently individualized? Thanks to rapid advances in the latter half of the 20\(^{th}\) century, scientists have developed a more thorough understanding of how normal human cells transform into malignant cancer cells. And by understanding how and why cells become cancerous, we can pioneer more targeted, effective therapies aimed towards their elimination from the body.

   Cancer is a fundamentally genetic disease. In normally functioning cells, there are a series of regulatory checks ensuring against excessive proliferation; for example, apoptosis (programmed cell death) acts as a “kill switch” to ensure normal cell turnover and maintain cell populations in homeostasis [Elmore, 2007]. Cells become malignant when mutations accumulate in key genes, which include two main classes: oncogenes and tumor suppressors [Hanahan and Weinberg, 2000]. Oncogenes are typically associated with gain-of-function mutations; these are
genes whose products promote cell proliferation, inhibit apoptosis, or both, and are found to be overactive in cancerous cells [Hanahan and Weinberg, 2000; Croce, 2008]. Conversely, tumor suppressors are negative regulators of cell proliferation. Loss-of-function mutation in tumor suppressors removes constraints on proliferation, thereby allowing for unchecked growth and consequent tumorigenesis [Weinberg, 1989]. It should be emphasized that oncogenes and tumor suppressors are not inherently cancerous genes, and are actually necessary at certain levels to regulate cell growth. Rather, cancer is attributable to mutations that disrupt the homeostatic balance between these genes by either overactivating oncogenes or impeding the function of tumor suppressors.

Mutations occur randomly during processes such as cell division, during which the complete set of genetic information is replicated and is therefore susceptible to copying errors; or due to mutagens such as reactive oxygen species within the cell. Factors that put you at an increased risk for cancer, such as smoking, often do so because they expose your cells to heightened levels of mutagens. An extensive array of checks and repair mechanisms guard against these mutations, so accumulating enough mutations for a cell to become cancerous occurs over an extended period of time. Notably, cancer cells are characterized by genomic instability. It’s often unclear whether genomic instability contributed to the development of cancer, whether the cancer resulted in genomic instability, or both. However, this instability has been defined as an “enabling characteristic” of cancer [Hanahan and Weinberg, 2011]. For example, a mutation could prevent a cell’s main DNA damage repair mechanism from functioning properly, leaving this cell highly susceptible to accumulating further mutations. Genomic instability and other hallmarks of cancer are further discussed below. It is possible that there are other mechanisms that more specifically drive the accumulation of multiple mutations.
in cancer cells, but experimental evidence for proposed mechanisms are limited, and the topic’s complexity is beyond the scope of this study.

The accumulation of multiple mutations in is an important characteristic of the development of cancer; very rarely is cancer attributable to a single mutation in a single gene, as convenient as this would be for treatment. The multistep nature of tumorigenesis and its connection to oncogenic mutations was first noted in the 1980s, when researchers found that transfecting fibroblasts (a type of cell) with a single oncogene was insufficient to induce cancerous growth, and instead required at least two cooperating oncogenes [Land et al., 1983; Weinberg, 1989]. Cells have a multiplicity of defense mechanisms to sustain normal homeostatic growth, so overcoming these defenses is a multistep process. For example, Rb and p53 are members of two highly-studied tumor suppressor pathways. Though these pathways are distinct, findings suggest that Rb and p53 each operates in a redundant network. If one of these tumor suppressors becomes nonfunctional, other redundant mechanisms take over to constrain excessive growth and proliferation [Lipinski and Jacks, 1999; Ghebranious and Donehower, 1998]. Thus, cancerous growth requires accumulation of multiple mutations in oncogenes and tumor suppressors in order to gain the capabilities required for unrestrained growth.

Based on decades of groundbreaking advances in cancer research, Hanahan and Weinberg (2000) synthesized these required capabilities into six distinct hallmarks of cancer: self-sufficiency in growth signals, insensitivity to growth-inhibitory signals, evasion of programmed cell death, limitless replicative potential, sustained angiogenesis, and tissue evasion and metastasis. In revising these hallmarks a decade later, the authors included two additional emerging hallmarks: active evasion from destruction by the immune response, and reprogramming of cellular energy metabolism to support rapid growth and proliferation. These
biological capabilities are acquired during the multistep development of human tumors, and it seems that all eight capabilities are shared in common by most solid human tumors. Development of these hallmark capabilities is attributable to two enabling characteristics. First, genomic instability in cancer cells can accelerate the generation of random mutations that additively produce these cancerous phenotypes. Second, the immune system has been implicated in promoting tumor progression, as the immune response promotes inflammation and can therefore contribute to excessive cell growth and proliferation [Hanahan and Weinberg, 2011].

In short, the factors that contribute to cancer development are numerous and complex. While we can implicate specific genes in tumorigenesis and progression, it is imperative that we take into account the way that these genes function within a broader network of intra- and extracellular interactions. My research therefore focuses on the way in which cellular redox environment potentially alters the structure and function of the protein PRL, in an effort to elucidate how PRL contributes to cancer progression.

II. Redox Environment and Cancer

Oxidative metabolism is a process that generates energy within the mitochondria of eukaryotic cells. This process produces low concentrations of reactive species, which can be classified into four groups: reactive oxygen species (ROS), reactive nitrogen species (RNS), reactive sulfur species (RSS), and reactive chloride species (RCS) [Bannister, 2007]. ROS are the most abundantly produced of these compounds, followed by RNS. At low concentrations, reactive species provide useful and necessary functions within the cell, serving as secondary messengers in processes such as signal transduction, enzyme activation, gene expression, and control of caspase activity necessary for apoptosis [Sosa et al., 2012]. However, these
compounds can be toxic for the cell if their concentration rises. Cells therefore maintain endogenous antioxidants that scavenge reactive species, and disruption of the balance between pro-oxidants and anti-oxidants generates oxidative stress, leading to damage of important cell components such as proteins, lipids, and DNA [Schraufstatter et al., 1988].

The damage oxidative stress can inflict on cells is rather indiscriminate, with the potential to disrupt nearly any metabolic pathway. For example, reactive species can nick DNA, cause malfunctions in DNA repair mechanisms (thus promoting genomic instability), or indirectly cause DNA mutations by generating mutagenic oxidized DNA products such as 8-hydroxy-2’-deoxyguanosine (8OHdG) [Matsui et al., 2000; Wiseman and Halliwell, 1996]. Reactive species can also increase permeability of the cell membrane due to oxidation of lipids, and alter protein structure through oxidation-induced modifications [Halliwell and Chirico, 1993; Levine, 2002]. Perhaps it’s not surprising, therefore, that oxidative stress has been associated with as many as 200 human diseases [Hybertson, 2011]. Yet the complexity of the various pathways and processes impacted by reactive species calls into question whether these diseases can then simply be treated using antioxidants.

With cancer in particular, use of antioxidants as treatment can be seen as a double-edged sword. Even if we assumed that oxidative stress was entirely “pro-cancer,” introducing malignant properties through DNA mutations and activation of certain pathways, the goal in cancer treatment is to eliminate cancerous cells from the body. Introducing high levels of antioxidants could prevent acquisition of these malignant properties in the first place. But it’s also important to consider that high levels of reactive species can quickly injure or kill cells [Zimmerman and Cerutti, 1984]. Once cells become cancerous, keeping oxidative stress in check could actually be aiding their survival by ensuring that reactive species do not reach lethal levels.
For example, Hileman et al. found that malignant cells were very vulnerable to inhibition of the antioxidant enzyme SOD due to the intrinsic oxidative stress in cancer cells, demonstrating that inhibition of antioxidants can actually be a highly effective cancer treatment [Hileman et al., 2003].

Furthermore, it’s difficult to assess whether the net effect of ROS and RNS is anti-cancer or pro-cancer due to the overwhelming range of their potential effects. ROS can promote cell-cycle stasis, senescence, apoptosis, necrosis or other types of cell death, and inhibit angiogenesis, all of which would work against excessive cell growth and proliferation. Conversely, it can also promote proliferation, invasiveness, angiogenesis, metastasis, and suppress apoptosis [Halliwell, 2007]. RNS has been shown to delay apoptosis through caspase inhibition, but also impair cell proliferation and slow tumor growth [Sawa and Ohshima, 2006; Hofseth et al., 2003]. Studies suggest that the overall effect of reactive species (RS) on cell proliferation is highly dependent on the type of cell and the level of RS. For example, excessive $O_2^{•-}$ produced by the NADPH oxidase (NOX) enzyme complex promoted abnormal fibroblast proliferation [Irani et al., 1997], but the same experiment in different cell types can result in cell senescence instead [Geiszt and Leto, 2004].

Research has suggested that lowered antioxidant defenses are associated with cancer development. One study demonstrated that knockout of CuZnSOD, a scavenger of superoxide radicals present in most parts of the cell, resulted in increased rates of liver cancer development later in life [Elchuri et al., 2005]. Knockout of another scavenger, MnSOD, has more dramatic results, as this compound is present in the mitochondrial matrix (where oxidative metabolism occurs) and is therefore the most important scavenger of $O_2^{•-}$. Complete absence of MnSOD in mice is lethal soon after birth [Li et al., 1995], and mice with 50% of the normal levels of
MnSOD show increased risk of developing lymphomas (61% incidence in mutant mice as compared to 22% incidence in wild-type mice) [Van Remmen et al., 2003]. These studies show that defects in antioxidant defense systems early in life, before any cancer-related mutations have been acquired, can contribute to tumorigenesis later in life, thus suggesting that the antioxidant response has an essential role in safeguarding against tumorigenesis.

However, a growing number of studies are calling into question the effectiveness of treating cancer using antioxidants. Results from randomized clinical trials are inconsistent, and some have indicated that administration of antioxidants may increase cancer risk [Bjelakovic et al., 2007; Heinonen et al., 1994; Goodman et al., 2011]. Despite the lack of definitive experimental evidence for anticancer properties of antioxidants, the first study to examine the impact of antioxidants on tumor growth in a mouse cancer model was not carried out until 2014. Sayin et al. (2014) administered the antioxidants N-acetyl cysteine (NAC) and vitamin E to mice with B-RAF- and K-RAS-induced lung cancer, two oncogenes that have been shown to promote tumor growth by increasing transcription of endogenous antioxidant genes. Despite being structurally unrelated, both NAC and vitamin E markedly increased tumor progression and reduced survival time at comparable levels. The tumor suppressor p53 is activated by ROS and DNA damage, and this study strongly suggested that suppression of oxidative stress actually prevented p53 activation and thereby allowed faster tumor cell proliferation [Sayin et al., 2014]. Another in vivo study found that NAC administration increased the number of lymph node metastases in a mouse model of malignant melanoma, but had no effect on primary tumors, suggesting that oxidative stress may inhibit tumor cells’ migratory ability. NAC would therefore help overcome the limitations imposed by oxidative stress to facilitate metastasis [Le Gal et al., 2015].
These conflicting findings indicate that redox environment plays an important role in cancer development and progression, but that the effect of antioxidants and RS on cancerous cells is highly complex and must be further elucidated in order to develop more effective treatments.

III. Implications of Phosphatase of Regenerating Liver (PRL) in Cancer Progression

*PRL structure and function*

Addition or removal of phosphate groups from specific proteins is a major posttranslational modification that helps control protein activity. This process is especially important in cellular regulation of signal transduction, which generally refers to the way in which external signals are received, interpreted, and acted upon by the cell. For example, the MAPK/ERK pathway begins with the extracellular binding of a signaling molecule to a cell surface receptor, and the resulting signal cascade involves phosphorylation and therefore activation of a series of intermediate proteins before finally resulting in DNA transcription and expression. Phosphorylation in these regulatory signaling pathways is regulated by two groups of proteins: protein kinases catalyze phosphorylation of specific amino acid residues (either tyrosine or serine/threonine), while protein phosphatases catalyze their dephosphorylation. Deregulation of phosphorylation alters the way in which cells respond to external signals, and has therefore been implicated in the development of a wide range of diseases including cancer [Wu et al., 2004].

Phosphatase of regenerating liver-1 (PRL-1) was first identified by Mohn et al. (1991) as being highly abundant in rat livers that regenerate after hepatectomy. A screen for its homologues in 1998 uncovered two more mammalian copies, PRL-2 and PRL-3 [Zeng et al.,
All three copies are expressed predominantly in skeletal muscle, while PRL-1 is also expressed in the brain at high levels and PRL-3 is expressed in the heart. PRL-1 and PRL-2 are also expressed more ubiquitously at low levels across tissue types, whereas PRL-3 is mainly limited to low expression in skeletal muscle and heart [Stephens et al., 2005]. These three proteins are members of the protein tyrosine phosphatase (PTPase) superfamily, specifically dual-specific phosphatases that can act upon either tyrosine or serine/threonine residues, based on their secondary structure and overall folding [Bessette et al., 2008]. Their amino acid sequences are also highly conserved across species, which suggests that PRLs have an essential role for cell function and have therefore been evolutionarily conserved [Al-Aidaros and Zeng, 2010]. Biochemical analyses have identified the following functionally significant structural features shared by all three versions of PRL:

- A conserved polybasic region, which provides a cluster of positive charges and therefore serves an important role in lipid binding [Sun et al., 2007].

- A CAAX box motif immediately following this polybasic region, which serves as a prenylation site. Prenylation at this sequence has been shown to be necessary for proper membrane association and intracellular localization of PRL [Zeng et al., 2000; Sun et al., 2005].

- Active site P-loop (CX₃R sequence) and WPD loops form catalytic or PTP domain, and are responsible for enzymatic activity where a phosphate is transferred from the substrate to the WPD loop [Bessette et al., 2008].

PRLs are unique among PTP’s due to an unusually wide, shallow active site with very few protruding loops [Kozlov et al., 2004]. This structural feature means that PRL can
accommodate a diverse, potentially redundant set of substrates, although the implications of this lowered specificity have not been fully explored. To date, the specific biologically-relevant substrates of PRL largely remain a mystery. PRL-3 has been shown to dephosphorylate ezrin [Forte et al. 2008] and keratin 8 [Mizuuchi et al. 2009] in vivo, and PRL-1 has been shown to dephosphorylate the transcription factor ATF-7 in vitro [Peters et al. 2001]. However, this is likely a very small representation of potential PRL targets, which are difficult to identify in biological systems due to the transient nature of phosphatase-substrate interaction. Not knowing what proteins PRL acts upon has therefore made it difficult to identify exactly how PRL contributes to or inhibits cancer progression (which will be discussed in-depth in the following section).

Perhaps most significant for the purposes of this research is the presence of a reversible disulfide bond within PRL-1 [Sun et al. 2005; Skinner et al. 2009] and PRL-3 [Kozlov et al. 2004], which forms between Cys49 and the catalytic Cys104 (Figure 1). Similar biochemical studies are not available for PRL-2, although there is a cysteine (Cys46) present in PRL-2 in an analogous position to Cys49 [Bessette et al. 2008], so it’s highly likely that this reversible disulfide bond could also occur in PRL-2.
Figure 1. Protein structure of PRL-1. Catalytic domain displayed as sticks, with the WPD loop (WPFD) colored yellow and the active site P-loop (CX₅R) colored orange. Reversible disulfide bond displayed as a red line between Cys49 (colored green) and Cys104 (part of the orange P-loop). Dephosphorylation of a substrate occurs in this catalytic domain, and involves a phosphate being transferred to the WPD loop via a mechanism initiated by the catalytic Cys104. Protein PDB code 5BX1 [Liu et al., 2015], modeled using PyMOL molecular visualization system.

The catalytic Cys104 plays a crucial role in the dephosphorylation mechanism. It has an unusually low pKₐ value (~5 as compared to ~8 for most cysteine residues), rendering it deprotonated at physiological pH and extremely reactive, so that it can initiate the catalytic reaction by attacking the incoming substrate’s phosphorous [Sun et al., 2005]. However, Cys104 is also therefore highly susceptible to oxidation. Sun et al. (2005) demonstrated that PTPase activity of PRL is abolished by oxidation at Cys104, as it promotes disulfide bond formation with Cys49, rendering Cys104 unable to accomplish the nucleophilic attack necessary for
enzymatic activity. This disulfide bond formation is reversible \textit{in vitro} and \textit{in vivo} by reducing agents that expose the nucleophilic thiol [Skinner et al., 2009].

\textbf{Association of increased PRL expression with cancer invasion and metastasis}

The link between PRL and cancer was first noted in a study of metastatic colon cancer, in which Saha et al. (2001) analyzed the gene expression profile of 18 colorectal cancer metastases and found that PRL-3 was the only gene whose transcription was consistently elevated in each metastasis, as compared to nonmetastatic tumors and normal colorectal epithelium [Saha et al., 2001]. Since then, increased PRL-3 expression has been associated with colorectal, breast, gastric, ovarian, and liver carcinomas, and studies have implicated heightened PRL-3 levels as a biomarker for cancer prognosis [Bessette et al., 2008].

In colorectal cancer (CRC), primary tumors with distant metastasis were frequently associated with heightened expression of PRL-3 (84.4\% of liver metastasis cases; 88.9\% of lung metastasis cases), and this association was statistically higher than in cases without distant metastasis (liver, 35.9\%; lung, 42.3\%) [Kato et al., 2004]. The close association between PRL-3 expression and CRC liver metastasis was corroborated by a second study, which found that within their samples, PRL-3 was expressed in 23.9\% of primary CRC tumors but 66.7\% of liver metastases. Furthermore, CRC patients expressing PRL-3 had shortened overall survival time after surgical removal of the primary tumor [Peng et al., 2004]. PRL-3 has therefore been identified as a potential diagnostic tool for predicting metastasis and severity of CRC.

In breast cancer, two independent studies found that PRL-3 protein expression was increased in a subset of tumors, and that 5-year disease-free survival was significantly reduced for patients with PRL-3 positive tumors [Radke et al., 2006; Wang et al., 2006]. Researchers also
found PRL-3 expression in 75.5% of invasive breast carcinomas, and more frequent expression in lymph node metastases (91.7%) than the primary tumors (66.7%) [Radke et al., 2006]. In gastric carcinomas, PRL-3 has been implicated as a potential molecular marker for aggressive gastric cancer, as high PRL-3 expression is closely associated with tumor size, extent of lymph node metastasis, and tumor stage [Miskad et al., 2007].

There are two noted exceptions to this trend. In ovarian cancer, there is no difference in PRL-3 levels between primary tumors and metastases, but PRL-3 levels were found to be almost threefold higher in stage III tumors than stage I tumors [Polato et al., 2005]. PRL-3 therefore may be more correlated with tissue invasion rather than metastasis for ovarian carcinomas. Most surprisingly, metastatic progression of lung cancer actually appears to be associated with down-regulated PRL-3. A recent study showed that expression of PRL-3 is higher in less invasive non-small cell lung cancer. When lung cancer cell lines were transfected with catalytically inactive alleles (C104S), cell invasion ability was actually enhanced [Lin et al., 2016]. These results indicate that catalytic activity of PRL-3 inhibit metastasis for lung cancer cells, which completely contradicts data for colon cancer. The reasons for this contradiction are still unclear.

PRL-1 and PRL-2 have not been as thoroughly investigated as PRL-3, but have still been associated with cancer. In a study comparing cell lines from various tumors with untransformed cell lines, PRL-1 mRNA lines were elevated in several tumor cell lines, very consistently so in melanoma cells. Interestingly, no colon carcinoma cell lines showed elevated PRL-1 mRNA levels, despite significantly heightened PRL-3 expression [Wang J et al., 2002]. PRL-2 has also shown to be overexpressed in prostate tumor tissue [Wang Q et al., 2002].

Alongside strong associations of PRL expression with various forms of cancer, a wide range of studies have elucidated the ways in which PRL interacts with signaling pathways to
impact cell shape and mobility. Using stable Chinese hamster ovary (CHO) cell lines expressing PRL-1 and PRL-3, Zeng et al. (2003) found that PRL-1 and PRL-3 showed 5-fold greater migration and 8-fold enhanced invasive capacities. Furthermore, the authors injected these clones into mice, and found that every mouse injected with PRL-expressing cells developed lung tumors, with PRL-3 resulting in more extensive lung tumors than PRL-1. Two out of ten mice injected with PRL-3-expressing cells also had liver metastases.

Tumor formation could be attributable to PRL-induced alterations to cell morphology and resulting increased migratory abilities. Zeng et al. (2003) observed PRL-3 enrichment in membrane processes on the cell surface that are shown to play a role in cell movement, including ruffles, protrusions, and membrane extensions. In another study, overexpressed PRL-3 in mouse melanoma cells resulted in more elongated cell shape and piling of cells in layers on the cell culture dish [Wu et al., 2004]. This phenotype is similar to epithelial-mesenchymal transition (EMT), a process which endows epithelial cells with reduced cell-cell adhesion and increased motility and has been implicated in the invasion and metastasis of carcinoma cells [Boyer et al., 2000]. Migratory abilities of PRL-expressing cells in both of these studies could be significantly reduced or blocked entirely by either mutating the catalytic Cys104 [Zeng et al., 2003], using the PTPase inhibitor sodium orthovanadate, or inhibiting PRL gene expression using antisense oligodeoxynucleotides (ODNs) [Wu et al., 2004]. These results indicate that the catalytic activities of PRL were responsible for these phenotypes.

Based on the extensive evidence linking elevated PRL-3 expression with more aggressive metastatic carcinomas, it seems likely that PRL-3 has some significant role in cancer invasion and metastasis, at least in colorectal, breast, gastric, and ovarian carcinomas. Preliminary studies linking PRL-1 and PRL-2 with multiple types of cancer further supports that PRL function may
somehow be promoting excessive cell proliferation and promotion, especially as these three proteins are highly homologous in sequence and structure. However, the effects of PRL are not consistent across cancer types, which underscores that their effects on cell growth and/or mobility are influenced by other factors within the cell.

**PRL as a tumor suppressor in vivo**

While studies outlined above have largely indicated a positive relationship between PRL and cancer progression, these experiments have been conducted using either already cancerous cells or immortal cultured cell lines such as CHO. The findings therefore are not entirely reliable, because these cell lines have extensive mutations that may alter PRL and the pathways with which it interacts. Characterization of PRL-1 expression in healthy developing tissue also demonstrates that PRL-1 is associated with terminal differentiation (when cells permanently cease division), which calls into question whether PRL normally functions as a growth promoter [Kong et al., 2000]. For a more reliable assessment of the “normal” function of PRL in non-cancerous environments, we can turn to studies conducted either using primary cell culture (using cells immediately after they’re derived from animal tissue), or using model organisms to examine its effects *in vivo*.

In a study conducted using mouse primary embryonic fibroblasts, Basak et al. (2008) found that PRL-3 overexpression induced cell-cycle arrest in late G1 via a p53 independent mechanism. Interestingly, the authors observed entirely different results in cancerous cells. PRL-3 overexpression did not result in cell arrest in RKO colon carcinoma cells, and actually dramatically enhanced G1/S cell cycle progression in U20S osteosarcoma cells.
In 2013, the Saucedo lab published a paper analyzing the effects of PRL overexpression using Drosophila melanogaster as a model organism. The Drosophila genome has a single copy of a PRL protein, dPRL-1, which has a 74-76% homology to all three human PRLs and conserved functional domains. Studying dPRL-1 can therefore provide insight into broad growth effects of the PRL proteins. Contrary to most previously published studies conducted using cell lines, Pagarigan et al. (2013) found that dPRL-1 functions as a growth inhibitor when overexpressed in otherwise unaltered tissue.

Together, these findings suggest that there may be additional cellular alterations associated with malignant transformation that induce the previously reported oncogenic function of PRL.

IV. Investigation of PRL function in altered cellular redox states

As previously discussed, redox environment is a major cellular alteration implicated in the development and progression of cancer. It is difficult to define whether the overall cell environment becomes more reduced or more oxidized due to the complexity and individuality of various cancers. However, pro-reduction pathways have been found to be elevated in cancer cells, such as thioredoxin (Trx) system in particular. The Trx system is one of the major components of the cellular antioxidant response, and has been suggested to contribute to several if not all of the hallmarks of cancer through various mechanisms [Arnér and Holmgren, 2006]. One study even showed that TrxR1 knockdown in tumor cells could compromise cancer cell growth and tumor development [Yoo et al., 2006], further supporting the positive relationship between elevated pro-reduction pathways and cancer. Regardless of the overall reduction potential of the cell, antioxidants such as thioredoxins and glutathiones interact with and
specifically reduce proteins, so upregulation of these reduction pathways can directly alter protein structure. In fact, Ishii et al. (2013) identified thioredoxin related protein 32 (TRP32) as a protein that strongly reduces PRL3.

Based on the structure of human PRL-1, alterations in cellular redox conditions can affect the structure of PRL-1 in a manner that impacts its catalytic activity. The catalytic cysteine Cys104 is critical for carrying out the dephosphorylation mechanism, and has been shown to be required in facilitating cell proliferation, motility, and metastasis. Skinner et al. (2013) demonstrated that Cys104, when oxidized, forms a reversible disulfide bond with Cys49, rendering PRL-1 catalytically inactive. The authors determined the reduction potential of this bond to be approximately -365 mV at pH 7.5. Reduction potentials for various “normal,” untransformed cellular environments range from -320 mV (most reducing) to -170 mV (most oxidizing), so even in the most reducing environment, PRL-1 would be primarily oxidized and catalytically inactive (>96% of PRL-1 in the cell). Because cancer cells have aberrant redox regulation, some may have sufficiently reduced cell environments to reduce the PRL-1 disulfide bond and thereby confer catalytic, oncogenic activity (Figure 2).

**Figure 2. Hypothesized model of redox-regulation of PRL.** Under oxidized conditions, PRL is catalytically inactive due to a disulfide-bond involving the catalytic cysteine residue. Specific reduction of PRL frees Cys104 to act as an electrophile, allowing catalytic function.
In order to investigate this possibility, I will be using *Drosophila melanogaster* as a model organism. *Drosophila* has proved to be very useful especially in clarifying how various genes and pathways are implicated in cancer. Most essential signal transduction pathways are conserved across organisms, so *Drosophila* can be used to gain some basic level of insight into the way these genes and pathways work: for example, dPRL-1 is homologous to all three human PRLs and has conserved functional domains. Furthermore, genetic tools such as the UAS/GAL4 driver system have been developed that can allow targeted gene expression in very specific tissues (Figure 3). In this study, dPRL-1 will be overexpressed in the eye, posterior wing compartment, and dorsal wing tissue in order to gain more specific insights into the ways in which dPRL-1 affects tissue growth at various developmental stages.

![Image](image.png)

**Figure 3. Schematic of the UAS/GAL4 driver system.** Gal4, a yeast transcription factor, is encoded on a chromosome separate from UAS and gene X (the gene of interest). Therefore, the two parts of the driver system are maintained in two separate lines of flies. Gene X will not be expressed until crossed with the line of flies expressing GAL4, as UAS is an enhancer that prevents transcription of gene X into a protein until it is bound by Gal4. In this study, apGAL4 and enGAL4 are used for wing-specific expression of gene X, while gmrGAL4 is used for expression specific to the eye tissue. The specific genes being controlled by the UAS/GAL4 driver system are dPRL-1, CncC, and Keap1. *Image taken from Johnston (2002).*
To alter cellular redox environment \textit{in vivo}, I will be changing expression levels of the master regulator of the antioxidant response, Nrf2 (CncC in \textit{Drosophila}), and Keap1. The nuclear factor erythroid 2-related factor 2 (Nrf2) is a transcription factor that belongs to the Cap ‘n’ Collar (Cnc) bZIP family of transcription factors and is highly homologous to CncC in \textit{Drosophila melanogaster}. Nrf2 binds to the consensus binding sequence of a set of antioxidant response genes, thus activating the antioxidant response to oxidative stress within the cell. Kelch-like ECH-associated protein 1 (Keap1) keeps Nrf2 in check under physiological conditions by promoting ubiquitination and degradation of Nrf2. Keap1 is a thiol-rich protein, with at least 27 reactive cysteines. When the cell is experiencing oxidative stress, the increased levels of reactive species modify these cysteines and inactivate Keap1, releasing the now-stabilized Nrf2 to activate the antioxidant response [Loboda et al., 2016]. Sykiotis and Bohmann (2008) confirmed that \textit{Drosophila} CncC and Keap1 proteins are genuine Nrf2 pathway components, which regulate transcription of antioxidant response element and detoxification genes, and are essential in defending against oxidative stress. \textit{Drosophila} can therefore be used to model alterations in antioxidant response levels, and examine the resulting effects on PRL function.
Figure 4. Keap1-CncC redox regulation system. Keap1 sequesters CncC until the cell faces oxidative stress, at which point Keap1 structure is altered and CncC is freed to transcriptionally activate the antioxidant response.

Using *Drosophila*, CncC and Keap1 can be either overexpressed, or knocked down using RNAi silencing of gene transcription. This results in four variables for oxidation state:

1. **Overexpressed CncC**, resulting in higher levels of CncC than Keap1. CncC factors that are not bound to Keap1 will activate the antioxidant response, resulting in a slightly more reduced cell environment.

2. **Overexpressed Keap1**, which will sequester most or all endogenous CncC and mitigate activation of the antioxidant response, resulting in a slightly more oxidized cell environment.

3. **CncCRNAi knockdown**, which reduces transcription of CncC to around 60% of wild-type, reducing activation of the antioxidant response and therefore resulting in a highly oxidized cell environment [Sykiotis and Bohmann, 2008].
4. Keap1\textsuperscript{RNAi} knockdown, which reduces transcription of Keap1\textsuperscript{RNAi} to around 60% of wild-type, therefore leaving a portion of CncC unrepressed. These heightened levels of active CncC will constitutively activate the antioxidant response, leading to a highly reduced cell environment [Sykiotis and Bohmann, 2008].

By expressing these genes in specific tissues alongside overexpressed dPRL-1 and examining the \textit{in vivo} growth effects in \textit{Drosophila}, we can gain valuable insight into the effect of cellular redox environment on function of PRL, and further elucidate a potential mechanism of cancerous cell proliferation and migration.
Results

*Drosophila PRL-1 overexpression suppresses dorsal wing tissue growth in oxidized cell environments.*

To model an overly oxidized cell environment, fly lines were obtained that expressed altered levels of a redox regulator: either overexpressed Keap1, or reduced levels of CncC via RNAi knockdown [Sykiotis and Bohmann, 2008]. Both of these genotypes would result in an inhibited antioxidant response (Figure 4). The UAS/apGAL4 driver system was used to direct expression to the dorsal tissue of the wing. Through a series of genetic crosses, offspring were produced that expressed either an altered redox-regulating enzyme, or an altered redox-regulating enzyme alongside overexpressed dPRL-1, in the dorsal wing tissue.

Fly wings are composed of two layers of cells, and apGAL4 targets gene expression to the dorsal (upper) layer only. If the growth is inhibited by these genes, the dorsal layer will be smaller than the ventral layer, and the entire wing will curl upwards. If growth is promoted, the entire wing will curl downwards. Expressing genes of interest using the apGAL4 driver thereby provides a very useful qualitative screen for whether these genes are inhibiting or promoting growth.

Flies overexpressing Keap1 had wings that curled upwards slightly at the tips (Figure 5A,B), indicating that Keap1 alone produces some degree of growth inhibition. When dPRL-1 was overexpressed in a Keap1 background, the wings curled upwards to a slightly greater degree than Keap1 alone (Figure 5A,C). Because Keap1 itself has an inhibitory effect and the data is qualitative, it’s difficult to discern the extent to which dPRL-1 is inhibiting growth beyond the baseline Keap1 phenotype. However, CncC\textsuperscript{RNAi} knockdown is not associated with a growth phenotype (Figure 5D,E). When dPRL-1 is overexpressed alongside CncC\textsuperscript{RNAi}, the resulting phenotype is a distinct upwards wing curvature (Figure 5D).
Figure 5. Phenotypic effects of oxidized environment and dPRL-1 in dorsal wing tissue. Expression targeted to the top layer of the wing tissue. (A) Light microscopy comparison of wing curvature between flies with wild-type, Keap1, and Keap1+dPRL-1 overexpression in the top layer of wing tissue. Some noticeable upwards wing curvature in both Keap1 and Keap1+dPRL-1 flies, indicating growth inhibition. (B-C) SEM wing detail reveals more pronounced upwards curvature in Keap1+dPRL-1 than Keap1 alone, indicating that although there is a growth inhibition phenotype associated with Keap1 alone, addition of dPRL-1 further inhibits growth.
(D) Light microscopy comparison of wing curvature between flies with wild-type, CncC$^{\text{RNAi}}$, and CncC$^{\text{RNAi}}$ + dPRL-1 overexpression in the top layer of wing tissue. CncC$^{\text{RNAi}}$ has no apparent growth effects and produces straight wings. CncC$^{\text{RNAi}}$ + dPRL-1 produces an upwards wing curvature, indicating growth inhibition. (E) SEM wing detail confirms straight wings of CncC$^{\text{RNAi}}$ flies. (F) Wing curvature of a fly expressing dPRL-1 alone, for reference. Wings are curved upwards, indicating that dPRL-1 alone suppresses growth.

Because CncC is the protein that directly activates antioxidant transcription, knockdown of CncC will result in a lower rate of antioxidant activation and a more oxidized cell environment. Although results are not clear-cut when Keap1 overexpression is used to inhibit the antioxidant response, CncC$^{\text{RNAi}}$ still provides a reliable model of increased cellular oxidation, and this data indicates that dPRL-1 suppresses growth \textit{in vivo} in this more oxidized environment.

\textit{Overexpression of the redox regulator CncC suppresses dorsal wing tissue growth, and is further compounded by Drosophila PRL-1 overexpression.}

Heightened expression of CncC results in a constitutively activated antioxidant response, thereby increasing levels of reducing agents within the cell. UAS-CncC was therefore one of the two fly lines used to model an overly reduced cellular environment. When CncC overexpression was targeted to the dorsal wing tissue using apGAL4, the offspring had wings that were not only curled upwards, but physically smaller and shriveled-looking. The shriveling and bubbling of wings indicates that growth has been inhibited to such a large degree that the dorsal and ventral cell layers fail to fuse in places. This striking undergrowth phenotype becomes even more pronounced when dPRL-1 is over expressed alongside CncC (Figure 6A). Though growth suppression by dPRL-1 in a reduced cell environment contradicts the proposed model, seeing such a strong phenotype from overexpressed CncC alone calls into question the validity of these results. These reasons will be expanded upon in the discussion.
Figure 6. Phenotypic effects of reduced environment and dPRL-1 in dorsal wing tissue. Expression targeted to the top layer of the wing tissue. (A) Light microscopy comparison of wing curvature between flies with wild-type, CncC, and CncC+dPRL-1 overexpression in the top layer of wing tissue. CncC alone has a pronounced effect on growth, producing offspring with small, bubbling, and upwards curving wings. Addition of dPRL-1 further compounds this growth inhibition. (B) Light microscopy comparison of wing curvature between flies with wild-type, UASKeap1RNAi, and Keap1RNAi+dPRL-1 overexpression in the top layer of wing tissue. Keap1RNAi has no apparent growth effects and produces straight wings. Keap1RNAi+dPRL-1 produces downwards curving wings when expressed in the dorsal tissue, indicating growth promotion.

Drosophila PRL-1 overexpression promotes tissue growth in larval wing discs with heightened antioxidant response.

Knocking down Keap1 levels via RNAi silencing results in a more constitutively activated antioxidant response, as it lowers the level of CncC inhibition, and therefore a more reduced cell environment. Overexpression of dPRL-1 in a Keap1RNAi background produced a distinctive overgrowth phenotype, as these flies had downwards curving wing (Figure 6B). Expression of Keap1RNAi alone using the apGAL4 driver did not result in any appreciable phenotypic changes in wing size or curvature (Figure 6B), indicating that the downwards wing curvature seen with dPRL-1 overexpression was due to dPRL-1 rather than unanticipated growth effects of Keap1RNAi.

Because this was the first instance of dPRL-1 promoting growth when overexpressed in vivo, I chose to continue characterizing the way Keap1RNAi affected dPRL-1 function. I first analyzed larval imaginal wing discs, still expressing these genes of interest using the apGAL4
driver. The wing disc is a flat cluster of cells which will develop into the wing later in development. The dorsal-ventral boundary is established early in larval life, so wing discs are ideal for quantitative analysis of apGAL4 phenotypes, as they provide a flat tissue and a defined compartment where apGAL4 is expressed (Williams et al., 1993). However, due to difficulties in defining this compartment consistently for each wing disc, analysis here is conducted based on surface area of the entire disc.

Even without quantification, phenotypic differences were distinctly noticeable between wing discs overexpressing dPRL-1 and wing discs with an increased antioxidant response overexpressing dPRL-1. Visually, discs with the Keap1\textsuperscript{RNAi}+dPRL-1 genotype look to be almost twice as large as dPRL-1. They also tend to be misshapen, more rounded than wild-type discs or those expressing dPRL-1 alone, and often had growths bulging out at the edges (as exemplified in Figure 7C). These discs also appeared to be larger than those expressing Keap1\textsuperscript{RNAi} alone (Figure 7B), confirming that this phenotype is a growth effect of dPRL-1 rather than an effect of increasing the antioxidant response.

These qualitative observations were confirmed by quantification of the wing disc surface area, measured using a pixel count of each wing image. On average, overexpression of dPRL-1 alone resulted in a 30% decrease in size when compared to the control of Keap1\textsuperscript{RNAi} alone. However, when the antioxidant response was heightened, dPRL-1 overexpression instead resulted in an 81.6% increase in size when compared to Keap1\textsuperscript{RNAi} alone to control for any growth effects of the antioxidant response.
Figure 7. Effects of increased antioxidant activity and dPRL-1 overexpression on overall size of larval wing discs. (A) *will include image of WT wing disc for reference and compare Keap1 RNAi size to that reference (B) Expression of dPRL-1 alone suppresses growth in the larval wing disc. (C) Expression of Keap1 RNAi and dPRL-1 results in significant growth promotion. Wing disc is enlarged, and exhibits tumorous bulges even at the edge of where these genes are being expressed under apGAL4. (D) Quantification of growth effects, based on surface area of entire wing disc, confirms these qualitative trends. Found a statistically significant difference in wing disc surface area between Keap1 RNAi and dPRL-1 (30.0% decrease, p=0.0015), Keap1 RNAi and Keap1 RNAi+dPRL-1 (81.6% increase, p=0.0037), and dPRL-1 and Keap1 RNAi+dPRL-1 (159% increase, p=0.0004).
These distinct growth differences suggest that dPRL-1 acts as a growth suppressor in otherwise healthy tissue, but switches to a growth promoter when expressed in cells with heightened levels of antioxidants. Analysis of growth effects using other GAL4 drivers should also be conducted, however, to confirm whether these results can be replicated in other tissue types.

*Overexpression of dPRL-1 in eye and posterior wing tissue with a reduced environment mitigates dPRL-1-induced growth suppression.*

The Keap1RNAi knockdown alongside dPRL-1 overexpression was repeated in eye and posterior wing tissue using the gmrGAL4 and enGAL4 drivers, respectively. The distinctive overgrowth phenotype seen in the dorsal wing compartment, as described above, was not produced to the same extent in the posterior wing compartment. Growth inhibition by dPRL-1 alone was similarly limited, with dPRL-1-overexpressing wings being an average of 4.8% smaller than wild-type. Addition of the Keap1RNAi knockdown alongside dPRL-1 overexpression mitigated this growth suppression, as the wings were 3.0% larger than dPRL-1 wings. However, overexpression of dPRL-1 in conjunction with increased antioxidant levels did not increase growth when compared to either wild-type wings or increased antioxidant levels alone. Wings expressing both increased dPRL-1 and Keap1RNAi were actually 3.0% smaller than the Keap1RNAi control (p<0.0001) (Figure 8).
Figure 8. Effects of increased antioxidant activity and dPRL-1 overexpression on adult wing surface area. Expression was directed to the posterior wing compartment. An increased antioxidant response via Keap1\textsuperscript{RNAi} alone had a negligible effect on growth when compared to a wild-type control. Overexpression of dPRL-1 resulted in growth suppression, with an average of a 4.8\% decrease in posterior wing surface area compared to wild-type (p<0.0001). Wings overexpressing dPRL-1 alongside an increased antioxidant response were larger than wings overexpressing dPRL-1 alone by 3.0\% (p<0.0001), but not overgrown when compared to wild-type or the increased antioxidant response alone (3.0\% smaller than Keap1\textsuperscript{RNAi}, p<0.0001).

Gene expression in the eye tissue is not quantifiable via light microscopy due to the tissue being 3-dimensional, but can provide a qualitative check for growth effects. Growth differences can be seen both in how far out the eyes extend from the head, and in their diameter when viewed from the side. Although the effects from Keap1\textsuperscript{RNAi} and dPRL-1 are subtle, they follow the same growth trends as seen in the dorsal wing tissue. Flies overexpressing dPRL-1 alone tend to have eyes that are reduced in size, while overexpressing dPRL-1 in conjunction with a heightened antioxidant response produces eyes that are enlarged when compared to the heightened antioxidant response alone (Figure 9).
Figure 9. Effects of increased antioxidant activity and dPRL-1 overexpression on growth of adult eye tissue. Although the phenotypic differences are more subtle here than in the wing tissue, eyes overexpressing dPRL-1 appear to have a smaller surface area and occupy less space on the head than those expressing both Keap1^{RNAi} and dPRL-1.
Discussion

Thus far, data gathered from these experiments support the proposed model of redox-regulated function of PRL (Figure 2). Previous studies conducted in vitro have confirmed redox-dependent reversible formation of a disulfide bond across PRL’s active site, and demonstrated that the presence of this bond renders PRL catalytically inactive as a phosphatase. However, this is the first study that has modeled the effect of redox environment on oncogenic function of PRL in vivo. These results indicate that dPRL-1 inhibits growth when overexpressed in either wild-type tissues or tissues with an inhibited antioxidant response (resulting in a more oxidized cell environment). When the antioxidant response is upregulated via Keap1 knockdown, however, dPRL-1 output is reversed and can promote growth. It therefore appears that redox environment can act as a switch that controls oncogenic function of dPRL-1.

When examined across different tissue types, however, this trend was not as pronounced as in the apGAL4 larval wing discs or adult wings. It’s possible that different downstream targets of PRL are altering these growth phenotypes; however, if that were the case, the difference in wing phenotypes between dorsal and posterior compartments would not have been as pronounced. I suspect that the differences in magnitude between apGAL4 (dorsal wing) and enGAL4 (posterior wing) data can be attributed to differences between these two drivers, both in terms of expression magnitude and penetrance. Genes expressed using an apGAL4 driver tend to be transcribed at an approximately 2-fold higher rate than genes expressed using an enGAL4 driver [Saucedo, personal correspondence]. As for penetrance of these genes, I noted that not all flies with the apGAL4/Keap1RNAi/dPRL-1 genotype had noticeably overgrown wings, and that flies that eclosed later tended to demonstrate that phenotype at a higher rate. Eclosing later indicates that development is slowed, as the cells require extra days to divide. When analyzing larvae, I tended
Welsh

32
to dissect larvae coming out of the food at later stages as well, which may have contributed to the high rates of overgrowth (Figure 7). However, when analyzing flies expressing these genes using the enGAL4 driver, all wings were taken from adult flies and I did not select for flies that had eclosed at a later stage. Incomplete penetrance may therefore have contributed to the very mild growth phenotypes seen with enGAL4 flies.

Growth suppression and promotion was also identifiable in the eye tissue, although to a lesser extent than in the wing tissue. The gmrGAL4 driver expresses genes mostly in post-mitotic cells as well, and therefore provides a measure of cell growth rather than increased rates of cell division. Differences in growth response between gmrGAL4- and apGAL4-controlled gene expression could indicate that active dPRL-1 promotes increased cell division rather than growth in cell size, but characterization of this difference requires further experimentation. Regardless of relative magnitude of growth responses, evidence that redox environment still alters PRL function to any extent in tissues as distinct as the wing and the eye strongly supports the validity of this model (Figure 2).

It should be noted that the proposed model (Figure 2) does not take into account potential interactions of PRL with other proteins outside of its role as a phosphatase. It’s possible that redox-dependent alterations in PRL structure can achieve the observed growth effects via a mechanism distinct from its phosphatase activity, such as by promoting altered binding to a different protein partner. However, a wide range of independent studies have confirmed that the phosphatase activity is necessary for the promotion of cellular proliferation and motility [Werner et al., 2003; Sun et al., 2007; Wang et al., 2002; Zeng et al., 2003; Wu et al., 2004; Daouti et al., 2008; Guo et al., 2004]. For the purposes of understanding its role in cancerous environments, presence or absence of catalytic phosphatase activity is therefore the most relevant alteration to
Welsh

33

consider.

In analyzing this data, it’s also necessary to address the conflicting results seen between combined PRL and CncC overexpression, and overexpressed PRL alongside Keap1RNAi knockdown. Both overexpressed CncC and Keap1RNAi knockdown should result in an overly reduced cell environment by enhancing activation of the endogenous antioxidant response (Figure 4). CncC (the Drosophila homologue of Nrf2 in humans) transcriptionally activates a range of antioxidant enzymes and detoxifying proteins, but is suppressed by Keap1 when the cell is not under oxidative stress [Sykiotis and Bohmann 2008]. Activation of the antioxidant response could therefore be achieved either by overexpressing the transcriptional activator CncC or by reducing expression of the suppressor Keap1.

However, given that reduction of Keap1 does not produce a phenotypic response while CncC overexpression results in dramatic undergrowth, I suspect that CncC overexpression leads to non-physiological levels of antioxidant enzymes. This overexpression was achieved via transcriptional upregulation of the CncC gene using the UAS/GAL4 driver system [Brand and Perrimon 1993]. Upregulation using the enGAL4 driver typically results in anywhere from 20- to 50-fold increase in transcription, and on the protein level, the apGAL4 driver is around 2-fold stronger than enGAL4 [Saucedo, personal correspondence]. Overexpressing CncC at this level (potentially around 30-fold or greater) would result in the antioxidant response being strongly and constitutively activated, as basal levels of Keap1 are not high enough to sequester the increased levels of CncC protein. Furthermore, with such high levels of CncC expression, there may be some unanticipated non-physiological growth effects. The growth suppression seen in the apGAL4 wing phenotypes for combined dPRL-1 and CncC overexpression may therefore be an artifact of these high levels of CncC, rather than an effect of dPRL-1 in a reduced cell
In contrast, the Keap1\textsuperscript{RNAi} knockdown would maintain standard physiological levels of CncC. Keap1 levels are here reduced to around 60% of wild-type, based on RT-qPCR analysis from the lab that first produced this line [Sykiotis and Bohmann, 2008]. Achieving a more reduced cell environment by limiting Keap1 levels avoids the potential off-target effects from flooding the cell with CncC protein. Instead, a portion of the basal level of CncC is left free to activate the antioxidant response. Keap1\textsuperscript{RNAi} knockdown therefore produces a higher level of antioxidants, but not to the same extent as CncC overexpression.

In characterizing Keap1 and CncC in \textit{Drosophila}, Sykiotis and Bohmann (2008) also noted phenotypic growth effects of CncC overexpression. When the authors overexpressed CncC in the eye tissue, the regular hexagonal pattern of the eye was disrupted, resulting in a rough eye appearance. In contrast, Keap1 overexpression had no effect on the wild-type eye pattern, and co-expression of Keap1 with CncC completely suppressed the CncC phenotype. Although the authors did not look at growth effects of Keap1\textsuperscript{RNAi} knockdown, the lack of phenotype for altered levels of Keap1 in general suggests that it does not directly affect growth to the same extent as CncC [Sykiotis and Bohmann, 2008].

These differences are further supported by the resulting phenotypes from apGAL4/UASCncC and apGAL4/UASKeap1\textsuperscript{RNAi}. Offspring with the apGAL4/UASCncC genotype have wings that are not only upturned, but distinctly smaller in size. Their crumpled, bubbling appearance indicates that the dorsal layer of cells (where apGAL4 expresses) has begun to separate from the ventral layer due to growth suppression. In contrast, there are no noticeable phenotypic changes associated with apGAL4/UASKeap1\textsuperscript{RNAi}. The aim of this study is to elucidate how increased antioxidant levels alter dPRL-1 function; effects of increased
antioxidant levels alone are not relevant to this question. Because CncC overexpression alone is
enough to cause an appreciable change in growth, Keap1^{RNAi} knockdown is a more reliable
model for an overly reduced cell environment for the purposes of this study. I therefore chose to
more thoroughly investigate growth effects of dPRL-1 overexpressed alongside Keap1^{RNAi}, and
not continue forward with CncC analysis.

As with CncC, overexpression of dPRL-1 using the apGAL4 driver can result in as much
as a 30-fold increase in dPRL-1 transcription. Similar arguments could be made regarding
whether this level of upregulation, and the demonstrated phenotypic responses, are
physiologically relevant due to the possibility of off-target effects. However, studies quantifying
PRL expression in tumors and metastases have demonstrated that PRL is upregulated in
cancerous environments to a far greater extent. For example, Saha et al. (2001) found that
relative expression of PRL-3 in colon cancer metastases was up to 400-fold greater than
expression in normal tissue. This number is highly variable depending on factors such as cancer
type, stage, and aggressiveness, but demonstrates that referencing physiological expression
levels is not entirely relevant when modeling cancerous environments.

Furthermore, and perhaps most importantly, the focus of this research is how altering cell
redox environment changes the way dPRL-1 affects growth. There are distinct phenotypic
differences between dPRL-1 overexpressed alone and dPRL-1 overexpressed alongside
Keap1^{RNAi}. Regardless of potential off-target effects, dPRL-1 suppresses growth in otherwise
healthy tissue but flips to a growth promoter in at least one genetically manipulated, reduced cell
environment. Because levels of dPRL-1 are kept relatively similar between these two conditions
by using the same initial stocks of flies expressing UAS dPRL-1 and the apGAL4 driver, we can
conclude that knockdown of Keap1 levels (and a resulting increase in endogenous antioxidants)
alters the growth effects of dPRL-1.

Is it possible that Keap1 interacts in a redox-independent fashion, either directly or indirectly, with dPRL-1? The results of these studies only indicate that Keap1RNAi knockdown somehow results in dPRL-1 promoting rather than inhibiting growth. Because no structural analyses were conducted, we cannot make any strong conclusions about the presence or absence of an intramolecular disulfide bond in dPRL-1 under the different redox conditions tested.

However, several converging lines of evidence from independent studies support redox-dependent alteration of dPRL-1 phosphatase activity as the most likely explanation for the switch in growth effects seen in these experiments.

One of the most compelling lines of evidence is accumulated data supporting redox modulation of PRL-1 disulfide bond formation. The first of these studies was conducted by Sun et al. (2005). The authors proposed the presence of the reversible disulfide bond based on analysis of PRL-1 crystal structures and mass spectrometry. They then tested whether redox environment regulated formation of this bond in vitro by transiently transfecting HEK293 cells with PRL-1, then incubating the cells with various concentrations of the oxidizing agent hydrogen peroxide (H$_2$O$_2$•). PRL-1 was extracted and its migration analyzed on an SDS-PAGE gel under non-reducing conditions. Two distinct bands were seen, indicating that there were two forms of PRL-1 present, and the more mobile band increased in intensity with increasing H$_2$O$_2$• concentration. Treatment of the cell lysates with the reducing agent DTT before analysis resulted in only the lower mobility band being detected. These results indicate that oxidation generates a more compact PRL-1 protein structure, and that this structural change is reversible. Furthermore, when either Cys104 or Cys49 was mutated to a serine residue, treatment of the mutant protein with H$_2$O$_2$• prevented the formation of the higher mobility band. The authors could therefore
conclude that exposure of cells to more oxidizing conditions results in the formation of a reversible disulfide bond between Cys104 and Cys49 in PRL-1 (Sun et al., 2005).

These findings were confirmed and expanded upon by two more independent studies. Yu et al. (2007) repeated a similar SDS PAGE analysis and confirmed that PRL-1 shifts to a more compact, mobile form when incubated with H$_2$O$_2$•−. Skinner et al. (2009) also took a similar approach, but noted that these previous studies were flawed in that they used truncated versions of PRL-1 and used H$_2$O$_2$•− as an oxidizing agent. H$_2$O$_2$•− rapidly oxidizes methionine residues, so it’s possible that the detected structural changes in PRL-1 were due to alterations involving methionine residues elsewhere in the protein, rather than the cysteine residues of interest. Using thiol (cysteine)-specific chemistry, with DTT as both a reducing and oxidizing agent, Skinner et al. (2009) still achieved the same results in both SDS-PAGE and mass spectrometry analysis.

In summary, three studies have independently arrived at the same result: cellular redox environment controls the reversible formation of a disulfide bond between Cys104 and Cys49 of PRL-1 in vitro. Therefore, for the purposes of this analysis, we can reasonably conclude that a significant enough alteration of the cellular redox environment would have modulated the structure of dPRL-1. One major future direction for this project is to conduct similar native gel analysis, using cells taken from different Drosophila tissues expressing these genotypes, and staining with an anti-dPRL-1 antibody. Examining migration of dPRL-1 on these gels between different tissue types and different redox-enzyme alterations would allow us to analyze relative amounts of oxidized and reduced dPRL-1 within these cells, and thereby gain a more complete understanding of the phenotypic responses.

In the absence of these native gel experiments, how do we know whether Keap1$\text{RNAi}$ knockdown would have achieved the necessary reduction potential within the cell to reduce this
disulfide bond? This is one of the major difficulties of moving from the in vitro studies to a model organism. The three authors discussed above were able to incubate cells with measurable concentrations of oxidizing or reducing agents; altering activation of the endogenous antioxidant response by partially knocking down enzyme transcription is a far less precise and measurable approach. In order to understand how Keap1RNAi knockdown affects the redox state of dPRL-1, we can examine which antioxidants are under the control of Keap1/CncC, and how those antioxidants directly interact with PRL.

The two main classes of antioxidant enzymes unregulated by CncC (Nrf2 in humans) are thioredoxins and glutathione-synthesizing enzymes [Sykiotis and Bohmann, 2008]. Glutathione (GSH) is the major determinant of cellular redox status of protein thiols, and thus is of particular interest for oxidation state of a disulfide bond [Nakamura et al., 1997]. One of the studies discussed above, besides examining redox-dependent disulfide bond formation in PRL-1, also investigated whether GSH contributed to the regulation of PRL-1 oxidation. The authors found that when GSH synthesis was inhibited, PRL-1 was more readily oxidized at lower concentrations of $\text{H}_2\text{O}_2^\bullet$- [Yu et al., 2007]. These results suggest that GSH specifically reduces PRL-1, as PRL-1 rapidly transforms into its inactive oxidized form in the absence of GSH.

Studies have shown that certain thioredoxins also specifically reduce oxidized PRL. Based on their location in the cytosol (where PRL localizes) and high levels of reductase activity, Ishii et al. (2013) identified several thioredoxin related proteins as potential regulators of PRL’s oxidative state. Incubation of PRL3 with each TRX-related protein, followed by SDS-PAGE analysis, revealed that thioredoxin related protein 32 (TRP32) strongly reduced PRL3 in a manner dependent on its active site. Furthermore, knockdown of TRP32 in vitro left PRL3 more susceptible to oxidation by $\text{H}_2\text{O}_2^\bullet$-, and pulldown assays demonstrated a direct and specific
interaction between TRP32 and PRL3 [Ishii et al., 2013].

From these combined studies, we know that (a) there is a redox-dependent reversible disulfide bond that forms across the active site of PRL in oxidized environments, (b) this bond can be specifically reduced by antioxidants such as TRP32 and GSH, and (c) that these antioxidants are directly upregulated by the Keap1/CncC redox regulation system. Therefore, I would argue that modulation of the redox environment is the only feasible reason that PRL would act as a growth promoter when levels of the oxidant Keap1 are reduced in vivo. Knocking down Keap1 levels and allowing greater transcriptional activation of antioxidants would result in PRL being directly reduced, leaving its active site accessible for its oncogenic function.

Specific reduction of PRL by TRP32 and GSH can help explain how PRL might maintain the reduced state necessary for oncogenic function, even in highly oxidized cancer cell environments. In a study that exposed rats to constant white light as an experimental model of photooxidative stress, data showed that the PRL-1 mRNA levels in the retina increased nearly 2.5-fold, yet all PRL-1 at each time point was in a reduced state [Yu et al., 2007]. These findings suggest that, while PRL protein expression levels increase in response to oxidative stress, PRL is somehow able to resist oxidation and remain in an active state. When you consider PRL’s low reduction potential as well (-365 mV, with reported reduction potentials for various cellular environments ranging from -320 to -170), it makes sense that specific reduction by antioxidants like TRP32 is necessary for producing even a baseline level of PRL activity within the cell. In fact, Skinner et al. (2009) reported that 90% of purified wild-type PRL-1 is inactive until addition of a thiol reducing agent. A cancer cell undergoing rapid metabolism and high levels of oxidative stress could trigger an increase in levels of PRL as a response, and if PRL maintains its reduced state through interaction with specific antioxidants, it may ultimately contribute to
oncogenic growth. Numerous studies have also reported heightened levels of thioredoxins in specific tumors [Arnér and Holmgren, 2006]. A synergistic relationship between PRL and TRP32, in which TRP32 maintains PRL in a reduced and active state (Figure 2), would help explain how both of these proteins are contributing to cancer progression in humans.

Future studies should interrogate this relationship using model organisms such as *Drosophila*, in order to better understand the environments under which PRL can promote cancerous growth in humans. One possible approach would be to explore the effects of overexpressing specific antioxidants in *Drosophila*, such as thioredoxins, and investigate whether co-expression of these antioxidants with dPRL-1 would result in growth promotion even in otherwise oxidized cell environments. Although *Drosophila* does not have a direct TRP32 homologue, the gene *deadhead* encodes a thioredoxin-like protein, which could be used to provide some insight into the way disulfide reducing proteins interact with dPRL-1 [Pellicena-Pallé et al., 1997].

Although experiments conducted *in vitro* provide a highly controlled environment in which to explore protein function and interactions, those findings must be confirmed *in vivo* to gain legitimacy, as the complexity of a living organism cannot be effectively modeled in a test tube. This study marks the first time that redox regulation of PRL function has been demonstrated in a model organism. Understanding the factors governing PRL function in *Drosophila* can help inform treatment for human cancers exhibiting heightened levels of PRL.

In human cancers, we see inconsistencies in the relationship between PRL and cancer progression, as explored in the introduction. For example, colorectal cancer metastases have heightened levels of PRL-3 [Saha et al., 2001], while lung cancer metastasis appears to be associated with downregulated PRL-3 [Lin et al., 2016]. These contradictory findings for
different cancers have made it difficult to define the precise role of PRL in cancer progression. However, based on the results of this study, differences in upregulation and downregulation of PRL in various cancers could be attributable to whether PRL is in a reduced state in that specific environment. Perhaps colorectal cancers typically exhibit heightened levels of antioxidants, while lung cancers do not. Furthermore, if a patient’s tumor exhibits high levels of PRL, knowing that antioxidants may activate oncogenic PRL function would caution against use of antioxidants as treatment in that particular case.

Understanding the ways in which reduction state of PRL alters its effects on growth can therefore help provide a more nuanced understanding of the role of PRL in cancer progression, and inform treatment approaches to PRL-associated cancers.
Materials and Methods

Drosophila crosses

Flies with genotypes of interest were produced by setting crosses between the following stocks.

- *Altered levels of redox enzymes:* +; +; UASKeap1, +; +; UASCncC, +; UASKeap1RNAi/CyO; +, +; UASCncC RNAi/CyO; wizC/+ (all developed by Sykiotis and Bohmann, 2008)

- *Gal4 Drivers:* w; apGal4/CyOGFP; + (Bloomington stock #3041), w; gmrGal4/CyO; + (Bloomington stock), w; enGal4/CyO; + (Bloomington stock #6356)

- *Overexpressed PRL:* w; CyO/+; UASPRL14B/Tm6 (Pagarigan et al., 2013), hsflp; UASKeap1RNAi, UASPRL14B/Sm6Tm6 (lab stock)

All animals were grown in NutriFly (Bloomington Formulation) and incubated at 28°C.

Quantitative tissue and cell growth analyses

Wing discs were dissected out of wandering third instar larvae, fixed in 4% paraformaldehyde in PBS, and mounted in a 1:1 solution of PBS and Vectashield. Adult wing samples were taken from female flies. Microscopy was conducted using the Olympus BX40 fluorescent microscope, at a magnification of 40X for adult wings and 100X for larval wing discs. Images were captured using Jenoptik ProgRes CapturePro. Posterior wing surface area and larval wing disc surface area were both quantified using the Adobe Photoshop Lasso and Histogram tools to count pixel number. Posterior compartment was measured from the L4 vein to the wing margin, and normalized to full wing surface area.

Qualitative wing and eye analyses

All analysis of adult wings and eyes was conducted using adult female flies. For eye tissue analysis, heads were separated from the body and either imaged whole or cut in half and imaged.
in profile. Light microscopy images were taken using the Leica MS5 stereomicroscope. Images were color-corrected using Adobe Photoshop.
References


