Investigation into the Role of Antioxidants in Tumorigenesis

By

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Abstract

The role of antioxidants in cancer has been controversial for a long time. Although the public’s belief is that antioxidants prevent and/or inhibit cancer, there is increasing evidence to suggest the opposite: that cancer cells require antioxidants to survive. We wanted to interrogate the role of antioxidants in cancer by investigating both upstream regulators and downstream effectors of antioxidant signaling. We have identified protein tyrosine phosphatase non-receptor type 12 (PTPN12) as a novel regulator of antioxidant signaling in cancer. PTPN12 reduces reactive oxygen species (ROS) levels by promoting activity of the forkhead box O (FOXO) family of antioxidant transcription factors. We have also elucidated the impact of glutathione (GSH), the most abundant antioxidant in the cell, on tumorigenesis. We have found that GSH is required for cancer initiation, yet dispensable once transformation has occurred due to compensation provided by the thioredoxin (TXN) antioxidant pathway. Together, these studies expand our knowledge of the role of antioxidants in cancer and provide numerous avenues of research for the future.
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List of Abbreviations

6-AN: 6-aminonicotinamide
ALL: acute lymphoblastic leukemia
AML: acute myeloid leukemia
ATP: Adenine triphosphate
AUR: Auranofin
BSO: Buthionine sulfoximine
CAT: Catalase
cDNA: Complementary DNA
CM-H2DCFDA: Chloromethyl 2’, 7’-dichlorofluorescin diacetate
Cul3: Cullin 3
Cys: cysteine
Cys2: cystine
DCIS: Ductal carcinoma in situ
DJ-1: Parkinson protein 7 (PARK7)
DMEM: Dulbecco's modified eagle medium
EGFR: Epidermal growth factor receptor
ER: Estrogen receptor
FBS: Fetal bovine serum
FOXO: Forkhead Box O
FTH: Ferritin, heavy chain
FTL: Ferritin, light chain
G6PD: Glucose-6-phosphate dehydrogenase
GCL: Glutamate cysteine ligase
GCLC: Glutamate cysteine ligase, catalytic subunit
GCLM: Glutamate cysteine ligase, modifier subunit
GGT1: gamma-glutamyltransferase 1
Gln: Glutamine
GLS: Glutaminase
GLUT1: Glucose transporter 1
GPX: Glutathione peroxidase
GSH: Glutathione
GSR: Glutathione reductase
GST: Glutathione-s-transferase
H&E: Hematoxylin and eosin
H6PD: hexose-6-phosphate dehydrogenase
HER2: v-erb-b2 erythroblastic leukemia viral oncogene homolog 2, neuro/glioblastoma derived oncogene homolog (avian)
HIF1α: Hypoxia-inducible factor, alpha
Hmox1: Heme oxygenase 1
I. P.: Intraperitoneal
IDC: Invasive ductal carcinoma
IDH: Isocitrinate dehydrogenase
IHC: Immunohistochemistry
KEAP1: Kelch-like ECH-associated protein 1
KO: Knockout
K-RAS: v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog
ME: Malic enzyme
MEF: Murine embryonic fibroblasts
MFI: Mean fluorescent intensity
MMTV: Mouse mammary tumor virus
MYC: v-myc myelocytomatosis viral oncogene homolog (avian)
NAC: N-acetyl-cysteine
NADP+: Nicotinamide adenine dinucleotide phosphate, oxidized
NADPH: Nicotinamide adenine dinucleotide phosphate, reduced
NQO1: NAD(P)H dehydrogenase, quione 1
NRF2: Nuclear factor (erythroid-derived)-like 2
PDK1: Phosphoinositide-dependent kinase 1
PGD: Phosphogluconate dehydrogenase
PI3K: Phosphoinositide-3-kinase
PKCδ: Protein kinase C; delta
pMEC: Primary mammary epithelial cell
PPP: Pentose phosphate pathway
PR: Progesterone receptor
PRDX: Peroxiredoxin
PTEN: Phosphatase and tensin homolog
PTP: Protein tyrosine phosphatase
PTPn12: Protein tyrosine phosphatase non-receptor 12
PyMT: Polyoma middle T antigen

qRT-PCR: Quantitative real-time polymerase chain reaction

ROS: Reactive oxygen species

RPS9: Ribosomal protein S9

SCID: Severe combined immunodeficiency

SESN3: Sestrin 3

SGK: Serum- and glucocorticoid-inducible kinase

shRNA: short hairpin RNA

siRNA: small interfering RNA

SMA: Smooth muscle actin (SMA)

SOD: Superoxide dismutase

SSA: Sulfasalazine

TCGA: The cancer genome atlas

TNBC: Triple negative breast cancer

TXN: Thioredoxin

TXNRD: Thioredoxin reductase

VEGF: vascular endothelial growth factor

WT: Wildtype

XCT: Cystine/glutamate transporter

αKG: alpha-ketoglutarate
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Chapter 1. Introduction

Parts taken from:


*These authors contributed equally.

1.1 Reaction oxygen species within the cell: a definition

One of the most studied set of molecules, both in the public’s eye and in research laboratories, is reactive oxygen species (ROS). Although best known for their role in aging and DNA damage, ROS also serve critical biological roles acting as signaling molecules. The three forms of ROS are superoxide (O$_2^-$), hydrogen peroxide (H$_2$O$_2$) and hydroxyl radicals (OH’). These reactive molecules are produced in cells as a normal byproduct of mitochondrial metabolism, peroxisome-mediated defense and protein folding in the endoplasmic reticulum$^1$. These reactive species are heterogeneous in their properties and have a plethora of downstream effects, depending on the concentrations at which they are present.
At low levels, ROS increase cell proliferation and survival through post-translational modification of kinases and phosphatases\textsuperscript{2-4}. The most well characterized molecules which ROS target are protein tyrosine phosphatases (PTPs). PTPs act on phosphorylated tyrosine residues within proteins and dephosphorylate them. Within the catalytic center of PTPs resides a cysteine amino acid which is susceptible to oxidation by ROS. Once oxidized, a conformational change occurs in the enzymes which render it inactive. Hypothetically, if PTPs were able to modulate ROS levels, they could prevent their own inactivation by ROS. Interestingly, investigation into whether PTPs control ROS levels has never been conducted.

At moderate levels, ROS induce the expression of stress-responsive genes such as $HIF1\alpha$, which in turn trigger expression of proteins providing pro-survival signals, such as the glucose transporter GLUT1 and vascular endothelial growth factor (VEGF)\textsuperscript{5,6}. ROS can also cause DNA damage and potentially induce neoplastic mutagenesis in cells. However, at high levels, ROS can induce activation of protein kinase C\(\delta\) (PKC\(\delta\)), triggering senescence\textsuperscript{7,8}; and/or cause permeabilization of the mitochondria, leading to the release of cytochrome \(c\) and apoptosis\textsuperscript{9,10}. Cells prevent a buildup of ROS and subsequent oxidative stress by producing a defined set of molecules termed antioxidants.

Cancer cells arise from activation of oncogenes and/or inactivation of tumor suppressors\textsuperscript{11}. Key hallmarks of cancer cells are evasion of apoptosis and unrestrained growth. Since ROS is a byproduct of ATP generation and protein folding, processes necessary for proliferation and tumor growth, cancer cells sustain a much higher level of ROS generation compared to normal cells. Therefore, to avoid the detrimental effects of oxidative stress, cancer cells actively upregulate multiple antioxidant systems\textsuperscript{12-14}. By buffering ROS levels, cancer cells are able to maintain a level of ROS which promotes survival and tumor progression (Figure 1.1).
**Figure 1.1 Relationship between ROS levels and cancer.** The effect of ROS on cell fate depends on the level at which ROS are present. Low levels of ROS (yellow) provide a beneficial effect, supporting cell proliferation and survival pathways. However, once levels of ROS become excessively high (purple), they cause detrimental oxidative stress that can lead to cell death. To counter such oxidative stress, a cell uses antioxidants that prevent ROS from accumulating at high levels. In a cancer cell, aberrant metabolism and protein translation generate abnormally high levels of ROS. Through additional mutations and adaptations, a cancer cell exerts tight regulation of ROS and antioxidants in such a way that the cell survives and the levels of ROS are reduced to moderate levels (blue). This extraordinary control of ROS and the mechanisms designed to counter it allow the cancer cell to avoid the detrimental effects of high levels of ROS, but also increase the chance that the cell will experience additional ROS-mediated mutagenic events and stress responses that promote tumorigenesis.

### 1.2 Antioxidant pathways which reduce ROS

The most abundant antioxidant within the cell is glutathione (GSH)\(^{15}\). Elimination of ROS by GSH occurs through GSH peroxidase (GPX) and GSH-s-transferase (GST). Upon elimination of ROS, GSH becomes oxidized and must be regenerated. This is accomplished by GSH reductase (GSR), which utilizes the metabolite nicotinamide adenine dinucleotide phosphate (NADPH), to reduce GSH and render it active again. During this process, NADPH itself is oxidized and therefore it must be produced through metabolic processes (discussed later).
De novo synthesis of GSH occurs in two steps; the first is driven by a two subunit enzyme known as glutathione-cysteine ligase complex (GCL), which is composed of a modifying unit (GCLM) and catalytic unit (GCLC). GCL catalyzes the reaction of glutamate with cysteine, the rate-limiting step in GSH synthesis. In the second step, glycine is added by glutathione synthetase (GSS) to form GSH. The metabolite precursor glutamate is produced from glutamine through glutaminolysis via glutaminase (GLS). Glutamate can become either GSH, for antioxidant purposes, or α-ketoglutarate (aKG), where it can enter the tricarboxylic acid (TCA) cycle and produce energy in the form of ATP. Interestingly, glutaminolysis activity is upregulated by oncogenes and is essential for cancer cell survival\textsuperscript{16, 17}, yet most investigation have focused on the ATP-generating nature of glutamine\textsuperscript{18}. As previously mentioned, cancer cells require an increased production of antioxidants in order to alleviate the buildup of ROS. Indeed, detoxification of ROS by GSH has been implicated in promoting tumorigenesis\textsuperscript{12, 13}. Surprisingly, there has been very little evidence to demonstrate the essentiality for the rate limiting enzymes controlling synthesis of GSH; namely GCLM and GCLC, in cancer initiation and progression.

The second most important antioxidant pathway is centered around thioredoxin (TXN) (Figure 1.2). Unlike GSH, which is a metabolite, TXN is a protein whose expression is primarily controlled at the transcriptional level. The amino acid cysteine is required for TXN generation, thereby linking cellular metabolism to this antioxidant pathway. TXN acts as an antioxidant by reducing peroxiredoxin (PRDX), which directly eliminates ROS and in the process becomes oxidized. TXN is required to regenerate PRDX so that it can further reduce ROS levels. Similar to GSH,
Figure 1.2 GSH and TXN antioxidant pathways drive ROS detoxification. The main antioxidant pathways involve GSH and TXN. TXN, a protein which acts as an antioxidant intermediate to reduce peroxiredoxins (PDXs), can be regenerated by TXNRD by using the metabolite NADPH. GSH, a metabolite derived from glutamate, cysteine and glycine, directly reduces ROS levels and also requires NADPH for regeneration.

TXN is oxidized upon reducing its target molecule. Oxidized TXN is regenerated by TXN reductase (TXNRD), and again, similar to GSH, NADPH is required for this process. Both TXN and PRDX have been shown to detoxify ROS in cancer cells and promote tumor survival\textsuperscript{19, 20}. The relationship between GSH and TXN antioxidant pathways, in a malignant setting, has never been explored.

A key molecule which acts as a lynchpin between both GSH and TXN antioxidant pathways is cysteine. Cysteine can be generated within the cell, from methionine, or imported from...
extracellular sources as its oxidized form cystine. Once imported into the cell, cystine is reduced to cysteine, permitting it to carry out its antioxidant functions. Import of cystine is controlled by the antiporter XCT (encoded by the gene \textit{SLC7A11})\textsuperscript{21}. XCT exports glutamate in exchange for the import of cystine, after which it is reduced to form cysteine. Import of cystine via XCT can support antioxidant expression within cancer cells and promote tumorigenesis\textsuperscript{22}. Furthermore, the tumor-associated antigen CD44 stabilizes XCT to support cystine uptake and tumor growth\textsuperscript{23}. An investigation into the requirement for cystine import to support GSH and Figure 1.3 Mechanisms of redox control and their alterations in cancer. The production of two of the most abundant antioxidants, NADPH and GSH, has been shown to be modulated in cancer. PKM2, which is overexpressed in many cancers, can divert metabolic precursors away from glycolysis and into the PPP to produce NADPH. IDH1/2 and ME also contribute to NADPH production. The oncogene c-Myc increases glutamine uptake and glutaminolysis, driving \textit{de novo} synthesis of GSH. Additionally, c-Myc indirectly contributes to NADPH generation by promoting expression of PKM2\textsuperscript{24}. Together, NADPH and GSH buffer the increased levels of ROS caused by increased cell proliferation. G6P, glucose-6-phosphate.
TXN antioxidant pathways in cancer cells has not been conducted.

NADPH functions as a critical regenerator of both GSH and TXN antioxidant systems (Figure 1.3) There are only three ways in which a cell can produce NADPH: the pentose phosphate pathway (PPP), which consists of glucose-6-phosphate dehydrogenase (G6PD) and phosphogluconate dehydrogenase (PGD) acting on glucose-6-phosphate (G6P); the conversion of pyruvate to malate by malic enzyme (ME); and the conversion of isocitrate to α-ketoglutarate by isocitrate dehydrogenase (IDH1/2) isoforms. Similar to the previously mentioned antioxidants, NADPH and its production plays a significant role in promoting tumorigenesis.\(^{25, 26}\) For example, glycolytic enzymes specific to cancer, such as pyruvate kinase M2 (PKM2), increase NADPH generation by slowing glycolysis down and diverting metabolites away and into the PPP.\(^{27, 28}\) Additionally, ME-mediated NADPH production has been shown to support cancer cell survival (Figure 1.4). The specific need for NADPH in cancer cells, and its relative impact on oxidative stress compared to other antioxidant pathways, has not been fully explored.

### 1.3 Nrf2: the master regulator of antioxidant expression

The most important regulator of antioxidant expression within the cell is arguably nuclear factor (erythroid-derived 2)-like factor 2 (NFE2L2, or Nrf2)\(^ {30}\). Under resting conditions, the transcription factor Nrf2 is constitutively degraded by cullin-3 (Cul3) due to its binding to kelch-like ECH-associated protein 1 (Keap1). Under conditions of oxidative stress, Keap1 is oxidized and modified so that it cannot bind to Nrf2, stabilizing this transcription factor in the nucleus.\(^ {31}\) Once in the nucleus, Nrf2 is permitted to increase transcription of multiple antioxidant genes.
Figure 1.4 Metabolic pathways coordinate to promote GSH synthesis and regeneration. PKM2 slows glycolysis and allows for metabolite diversion into NADPH (via the PPP) which can regenerate GSH. NADPH can also be produced from IDH and ME enzymes. Metabolites diverted by PKM2 also promote serine biosynthesis by PHGDH, which leads to glycine production and ultimately de novo GSH production. Cystine import, which is also required for GSH production, is promoted by tumor associated antigen CD44 due to its stabilization of Xct. Glutamate, which is the final component of GSH, is derived from glutamine through glutaminolysis via GLS.

Nrf2 is the primary regulator of GCLM and GCLC mRNA expression within the cell, and therefore, is a major determinant of GSH synthesis levels. Nrf2 supports not only GSH synthesis but also GSH utilization, since this transcription factor controls expression of glutathione peroxidase 2 (GPX2) and several glutathione s-transferases (GSTa1/2/3/5;
GSTm1/2/3, GSTp1) which use GSH to reduce ROS levels. Furthermore, Nrf2 directly increases the transcription of all enzymes involved in NADPH synthesis, along with GSH reductase; linking Nrf2 intrinsically to GSH regeneration. Along with the GSH antioxidant pathway, Nrf2 controls key players in the TXN antioxidant pathway, including TXN1, TXNRD1 and PRDX1. Nrf2 further supports both GSH and TXN production by promoting expression of the glutamate/cystine transporter XCT and increasing cellular cysteine levels.

In addition to its direct involvement in ROS detoxification via GSH and TXN metabolism, Nrf2 indirectly helps to modulate ROS by regulating free iron (II) homeostasis. Iron (II) catalyses the Fenton reaction, which converts H₂O₂ to the highly reactive OH⁻. A major source of free iron (II) is the breakdown of heme by heme oxygenase (HO-1), whose transcription is greatly upregulated after Nrf2 stabilization. At first glance, upregulation of HO-1 by Nrf2 would seem to be counterintuitive, since uncontrolled release of iron (II) into a cell would promote the Fenton reaction. However, in conjunction with HO-1 upregulation, Nrf2 boosts transcription of genes encoding elements of the ferritin complex, namely ferritin light chain (FTL) and heavy chain (FTH). The ferritin complex first detoxifies iron (II) by converting it into iron (III) and then stores it within its own structure where it cannot be utilized for the Fenton reaction. Thus, Nrf2 reduces the production of harmful OH⁻ radicals from ROS by promoting the release of iron from heme and its subsequent sequestration (Figure 1.5).

In the past, Nrf2’s roles in antioxidant gene expression and ROS detoxification were believed to be facets of a function in tumor suppression. It was believed that by reducing ROS levels, Nrf2 would prevent mutagenesis and subsequent malignant transformation in cells. More recent research, however, has shown quite the opposite to be true. Nrf2 targets such as GCLM and HO-1 have been implicated in promoting cancer cell survival because they neutralize...
Figure 1.5 Nrf2 is the master regulator of antioxidant responses. Nrf2 controls four primary areas of antioxidants: 1) GSH synthesis and reduction (GCLM/C, SLC7A11, GSR); 2) GSH utilization (GSTa1/2/3/5, GSTm1/2/3, GSTp1, GPX2); 3) TXN synthesis and reduction (TXN1, TXNRD1, PRDX1); 4) NADPH synthesis (G6PD, PGD, ME1, IDH1). Both GSH and TXN utilize NADPH to regenerate themselves once they have reduced ROS.

the toxic effects of oxidative stress\textsuperscript{\textcircled{12, 42}.} In addition, evidence is accumulating that oncogenes, such as K-Ras, c-Myc and PI3K, can help promote Nrf2 stability and activity\textsuperscript{14, 25}. Even more convincing are mutations in Nrf2 or its regulators that have been identified in tumor samples from cancer patients. For example, mutations in Nrf2’s Keap1-binding domain prevent Keap1 from modifying Nrf2\textsuperscript{43, 44}. Inactivating mutations in Keap1 itself have also been identified\textsuperscript{45}. All of these mutations lead to constitutive stabilization of the Nrf2 protein in the nucleus. Nrf2-related mutations have been found primarily in squamous cell carcinomas but also occur less
frequently in a broad spectrum of tumor types (lung, skin, esophagus, ovarian and breast)\textsuperscript{43}. In summary, Nrf2 is the intrinsic controller of oxidative stress in the cell, yet numerous questions regarding its role in cancer remain to be addressed.

1.4 The FOXO family of transcription factors

After Nrf2, the forkhead box O (FOXO) family of transcription factors (FOXO1 and FOXO3a) are the second most predominant controllers of antioxidant gene expression in the cell. Although primarily known as inducers of cell cycle arrest and cell death, FOXOs help to prevent oxidative stress by inducing antioxidant gene expression\textsuperscript{1}.

The activation of FOXO transcription factors is controlled by phosphorylation, mediated by Akt as well as serum- and glucocorticoid-inducible kinase (SGK)\textsuperscript{46-48}. Phosphorylation of FOXOs leads to their nuclear exclusion and cytoplasmic sequestration by binding to the protein 14-3-3\textsuperscript{49}. Although FOXOs have roles in regulating GSH-mediated detoxification by inducing transcription of GPX1 and GSTm1\textsuperscript{50, 51}, and in iron homeostasis by inducing transcription of HO-1\textsuperscript{52}, one could argue that FOXOs primarily exert their antioxidant effects through their regulation of superoxide dismutases (SODs), catalase and sestrin 3\textsuperscript{53}. SODs act on \( \text{O}_2^- \) and convert them into \( \text{H}_2\text{O}_2 \). Similar to HO-1, the role of SODs seems to go against the process of antioxidant detoxification since \( \text{H}_2\text{O}_2 \) is produced instead of consumed. Yet it is only in this form that ROS can be reduced to \( \text{H}_2\text{O} \) by catalase, GSH or TXN. Interestingly, catalase is regulated in combination with SODs by FOXOs, similar to ferritins being upregulated in combination with HO-1 by Nrf2\textsuperscript{54, 55}. Another antioxidant system regulated by FOXOs involves sestrin-3\textsuperscript{53}. If, during the ROS detoxification process the catalytic center of PRDX becomes overoxidized, these
enzymes are rendered inactive and useless\textsuperscript{56}. Members of the sestrin family can reduce overoxidized PRDX and regenerate their catalytic activity\textsuperscript{57}.

Interestingly, FOXOs function synergistically with Nrf2. For example, Nrf2 promotes NADPH- and GSH-mediated detoxification, while FOXOs control SODs and catalase, allowing for both antioxidant pathways to be activated. Similarly, Nrf2 controls TXN-mediated detoxification (TXN1, TXNRD1, PRDX1), while FOXOs control the sestrins that regenerate PRDX and increase the activity of this pathway. Additionally, FOXOs upregulate p21, which promotes Nrf2 stabilization\textsuperscript{58, 59}. One of the only directly overlapping roles for Nrf2 and FOXOs is inducing HO-1 expression\textsuperscript{38, 52}, highlighting the critical role for iron homeostasis to regulation of oxidative stress (Figure 1.6).

Like Nrf2, FOXOs were considered for many years to act exclusively as tumor suppressors, mainly due to their role in inducing cell cycle arrest and apoptosis. FOXO transcription factors were initially discovered as promoters of organism longevity, and therefore it is somewhat unsurprising that it plays a role in supporting tumorigenesis\textsuperscript{49}. Cancers in which the Akt signaling pathway is hyperactivated such that FOXOs are inhibited, are particularly sensitive to changes in oxidative stress\textsuperscript{53}. In addition, oncogenic factors such as β-catenin and TGFβ can conspire to aid FOXOs in their tumor-supporting activities\textsuperscript{60, 61}. For example, in acute myeloid leukemia (AML), it is activated FOXOs and not the traditional oncogene AKT that drive survival of AML leukemia cells\textsuperscript{62}. Intriguingly, FOXOs participate in chromosomal translocations leading to alveolar rhabdomyosarcomas and acute lymphoblastic leukemias (ALL)\textsuperscript{63}. In rhabdomyosarcomas, the PAX3-FOXO1 translocation product is resistant to inhibition by Akt, indicating that Akt can promote proliferation while also permitting antioxidant
expression driven by FOXOs\textsuperscript{64}. Novel regulators of FOXO signaling and their impact on tumorigenesis still remains to be elucidated.

\textbf{Figure 1.6} Nrf2 and FOXOs are synergistic in support of antioxidant pathways. While NRF2 mainly target GSH, TXN and NADPH related responses, FOXOs target SODs, Catalase and sestrins.

\textbf{1.5 Summary}

The control of antioxidants, and the overall management of ROS, is crucial to cell survival. The essentiality of this management is greatly increased for cancer cells, as they proliferate much faster than normal cells and therefore produce ROS at a significantly higher rate. Cancer cells
manage the increased ROS levels by upregulating antioxidants such as the GSH and TXN pathways. This is accomplished by increasing expression and/or activity of antioxidant transcription factors such as NRF2 and FOXOs. While numerous questions surrounding antioxidants remain, two such questions stand out; 1) what novel molecules regulate antioxidant transcription factors; 2) how does depletion of these antioxidants affect tumorigenesis. The following chapters will shed light on these inquiries.
Chapter 2. Investigation into the role of PTPn12 in tumorigenesis

Parts taken:


It is well known that protein tyrosine phosphatases (PTPs) which become oxidized due to exposure to reactive oxygen species (ROS) undergo a conformational change and are inactivated. However, whether PTPs can actively regulate ROS levels in order to prevent PTP inhibition has yet to be investigated. Here we demonstrate that PTP non-receptor type 12 (PTPN12) protects cells against aberrant ROS accumulation and death induced by oxidative stress. Murine embryonic fibroblasts (MEFs) deficient in PTPN12 underwent increased ROS-induced apoptosis under conditions of antioxidant depletion. Cells lacking PTPN12 also showed defective activation of FOXO1/3a, transcription factors required for the upregulation of several antioxidant genes. PTPN12-mediated regulation of ROS appeared to be mediated by phosphoinositide-dependent kinase-1 (PDK1), which was hyperstimulated in the absence of PTPN12. Because tight regulation of ROS to sustain survival is a key feature of cancer cells, we examined PTPN12 levels in tumors from a cohort of breast cancer patients. Patients whose tumors showed high levels of PTPN12 transcripts had a significantly poorer prognosis. Analysis of tissues from patients with various breast cancer subtypes revealed that more triple negative breast cancers (TNBC), the most aggressive breast cancer subtype, showed high PTPN12 expression than any other subtype. Furthermore, both human breast cancer cells and mouse mammary epithelial tumor cells engineered to lack PTPN12 exhibited reduced tumorigenic and
metastatic potential in vivo that correlated with their elevated ROS levels. The involvement of PTPN12 in the antioxidant response of breast cancer cells suggests that PTPN12 may represent a novel therapeutic target for this disease.

2.1 Introduction

Inefficiencies in the cellular machinery governing mitochondrial metabolism and protein translation lead to the production of free radicals known as reactive oxygen species (ROS). These molecules play important roles in numerous aspects of cellular homeostasis because they oxidize and either activate or inactivate several classes of proteins, thereby modifying multiple signaling pathways. Arguably the best-studied targets of ROS-mediated modification are protein tyrosine phosphatases (PTPs) 65. PTPs contain a conserved cysteine residue in their catalytic domain that, when oxidized, triggers a conformational change in the protein and subsequent inactivation of its enzymatic function. Thus, almost all PTPs become oxidized under conditions of oxidative stress 66. Although extensive research has been conducted to determine how ROS inactivate PTPs, very little effort has been dedicated to investigating the converse: whether PTPs can regulate ROS levels to protect against PTP oxidation and inactivation.

Although the role of ROS in mammalian cancers has been investigated for decades, it is not yet completely understood. It is clear that ROS can contribute to de novo tumorigenesis by inducing DNA damage and causing mutagenic events 67, but ROS can also hinder tumorigenesis by inducing cell senescence or cell death. Accordingly, cancer cells exercise intricate control over their ROS levels, and several studies have demonstrated that deregulating the ROS/antioxidant balance in tumor cells leads to their death 13. Key proteins used by both normal
and cancer cells to control ROS are the forkhead box O (FOXO) transcription factors. Several downstream effectors of FOXOs can neutralize ROS and thus are directly involved in counteracting oxidative stress. This reliance on FOXO-mediated ROS regulation has raised the intriguing possibility that disruption of a cancer cell’s ROS management machinery might offer a novel therapeutic approach to cancer treatment.

In this study, we investigated whether PTP non-receptor type 12 (PTPN12; also known as PTP-PEST) is crucial for controlling ROS in tumor cells and particularly in breast cancers, the most prevalent malignancy among women. Our data indicate that PTPN12 potentially has an oncogenic function (at least in breast cancers) because it regulates ROS in a manner that promotes tumor cell survival.

### 2.2 Results

#### 2.2.1 PTPN12 promotes cell survival under conditions of antioxidant depletion

To investigate whether PTPN12 promotes survival under conditions of increased oxidative stress, we utilized immortalized MEFs derived from PTPN12-null mice. To ensure that cells of an identical genetic background were studied, PTPN12-null MEFs were transfected with either empty vector (to generate cells referred to as Ptpn12-KO) or a Ptpn12 expression construct (to generate cells re-expressing Ptpn12 and referred to as Ptpn12-RE). The level of expression of PTPN12 protein in Ptpn12-RE MEFs was comparable to its expression in MEFs derived from Ptpn12-competent mice (referred to as Ptpn12-WT) (Figure 2.1a and S1), validating our approach.
Cells metabolize glucose either via glycolysis to produce adenosine-5'-triphosphate (ATP), or via the pentose phosphate pathway (PPP) to produce the antioxidant reduced nicotinamide adenine dinucleotide phosphate (NADPH) \(^1\). Without NADPH production through the PPP, cells succumb to ROS-mediated apoptosis. To create an oxidizing environment, we treated Ptpn12-RE and Ptpn12-KO MEFs with 6-aminonicotinamide (6-AN). 6-AN inhibits glucose-6-phosphate dehydrogenase (G6PD), the first enzyme in the PPP, and so blocks NADPH production and promotes ROS-mediated cell death \(^7\). We found that Ptpn12-KO MEFs treated with 6-AN showed a significantly higher rate of apoptosis than control cells (Figure 2.1b). Because one function of NADPH is the regeneration of reduced glutathione (GSH), the most abundant intracellular antioxidant \(^1\), we investigated whether direct depletion of GSH would also increase the apoptosis of Ptpn12-KO cells. To deplete GSH, we took advantage of the fact that de novo GSH synthesis is driven by the action of glutamate cysteine ligase (GCL) on the metabolites glutamate and cysteine. Buthionine sulfoximine (BSO) is a potent inhibitor of GCL activity and thus depletes a cell of GSH. We treated Ptpn12-RE and Ptpn12-KO MEFs with varying concentrations of BSO and found that, in the absence of PTPN12, cells were more sensitive to GSH depletion and underwent increased apoptosis in a dose-dependent manner (Figure 2.1c). These results suggest that PTPN12 protects cells from oxidative stress caused by deficits in antioxidants such as NADPH and GSH.

L-Glutamine (Gln) is essential for cell survival, and a common effect of many oncogenes is to increase Gln metabolism in cancer cells \(^1\). Gln undergoes glutaminolysis to generate glutamate \(^1\), which can then be processed via GCL to form GSH. It has therefore been
hypothesized that Gln deprivation leads to diminished antioxidant production and thus susceptibility to the induction of endogenous oxidative stress. To determine if PTPN12 counteracts the effects of Gln deprivation, we cultured *Ptpn12*-RE and *Ptpn12*-KO MEFs in fully supplemented (normal) or Gln-free medium. Measurement of intracellular ROS levels in *Ptpn12*-
RE and Ptpn12-KO MEFs revealed that, while ROS increased in both cell lines under Gln-free conditions, Ptpn12-KO cells contained approximately twice as much ROS as controls (Figure 2.2a). When we monitored cell viability, we observed approximately 3-fold more cell death in Gln-free Ptpn12-KO cultures compared to Gln-free Ptpn12-RE cultures (Figure 2.2b). To test if this Gln deprivation-induced death could be avoided by supplementation with an exogenous antioxidant, we cultured Ptpn12-RE and Ptpn12-KO MEFs in Gln-free medium with or without the antioxidant N-acetyl cysteine (NAC). While Ptpn12-RE cells were unaffected by NAC treatment, this antioxidant significantly reduced apoptosis in Gln-free Ptpn12-KO MEF cultures (Figure 2.2b). Next, we examined the proliferation of Ptpn12-RE and Ptpn12-KO MEFs cultured without Gln. In normal medium, MEFs of both genotypes grew at similar rates, as did Ptpn12-null MEFs transfected with a vector expressing a phosphatase-dead form of PTPn12 (R237M) (Figure 2.2c). However, in the absence of Gln, only MEFs expressing PTPN12 were able to proliferate robustly, and this proliferation defect was not rescued by expression of PTPn12-KO-R237M (Figure 2.2d). These data indicate that the phosphatase function of PTPN12 is required for cell growth in an environment lacking antioxidants, and imply that the increased apoptosis and reduced proliferation of Ptpn12-KO cells subjected to Gln withdrawal is directly related to the elevated ROS within these cells.

2.2.2 PTPN12 promotes FOXO activation by inhibiting PDK1 signaling

The results of a previously reported large-scale screen showed that PTPN12 dephosphorylates PDK1. PDK1 modulates oxidative stress, which may explain the sensitivity of Ptpn12-KO cells to ROS accumulation induced by antioxidant depletion. We serum-starved Ptpn12-RE and Ptpn12-KO MEFs overnight and restimulated them with 20% FBS over a 2 hrs
Figure 2.2 PTPN12 promotes cell survival in a Gln-free environment. (a) Intracellular ROS levels as determined by CM-H$_2$DCFDA staining in Pttn12-RE and Pttn12-KO MEFs cultured for 48 hrs in normal or Gln-free medium. Results are expressed as arbitrary units (a.u.) based on mean fluorescent intensity (MFI). (b) Apoptosis of Pttn12-RE and Pttn12-KO MEFs after 72 hrs in normal, Gln-free, or Gln-free medium supplemented with NAC (1 mM), followed by staining with Annexin V/PI. (c and d). Numbers of viable cells in cultures of Pttn12-RE, Pttn12-KO and Pttn12-KO-R237M MEFs grown for 4 days in normal (c) or Gln-free (d) medium as determined by trypan blue exclusion. Data are expressed as the fold change in cell number compared to day 1 values.

Immunoblotting revealed that more PDK1 was phosphorylated at Tyr9 and Ser241 in Pttn12-KO MEFs than in Pttn12-RE MEFs; even at time zero (Figure 2.3a). Although autophosphorylation of Ser241 in the activation loop of PDK1 is necessary for its activity, tyrosine phosphorylation at Tyr9 has been shown to promote PDK1 protein stabilization and
Figure 2.3 PTPN12 regulates PDK1 activity and downstream FOXO signaling. (a) Immunoblot to detect the indicated total and phosphorylated (P) proteins in lysates of Ptpn12-RE and Ptpn12-KO MEFs that were serum-starved overnight and stimulated with 20% FBS for the indicated times. (b) Quantitative RT-PCR analysis of Ptpn12-RE, Ptpn12-KO and Ptpn12-KO-R237M MEFs to detect mRNAs for the indicated FOXO target genes. Data are expressed as mRNA level relative to housekeeping gene Rps9. (c) Immunoblot to detect the indicated total and phosphorylated (P) proteins in lysates of Ptpn12-RE and Ptpn12-KO MEFs that were serum-starved overnight or cultured in medium with 10% FBS, and treated with or without NAC (1 mM). (d) Intracellular ROS levels in Ptpn12-RE and Ptpn12-KO MEFs transiently transfected with either vector control or FOXO3a:A3 expression vector and cultured for 48 hrs in Gln-free medium. Results are expressed as ROS levels relative to control Ptpn12-RE MEFs and based on mean fluorescent intensity (MFI).
activity\textsuperscript{73, 74}. Our data suggests that loss of PTPN12 results in excessive phosphorylation of PDK1 and thus upregulation of PDK1 activity.

Next, we examined downstream targets of PDK1 known to influence the regulation of antioxidants, namely the FOXO1/3a transcription factors. In a resting cell, FOXO proteins are phosphorylated and sequestered in an inactive form in the cytoplasm\textsuperscript{48}. This phosphorylation is mediated by serum/glucocorticoid regulated kinase 1 (SGK1), which can be directly phosphorylated and can be activated by PDK1 independent of membrane recruitment by phosphoinositide-3-kinase (PI3K)\textsuperscript{75}. Because FOXO1/3a dephosphorylation and activation is necessary for antioxidant enzyme expression, increased PDK1 phosphorylation leading to heightened PDK1 activity could result in FOXO1/3a inactivation and decreased antioxidant activity. Accordingly, we postulated that the hyperphosphorylated PDK1 in Ptpn12-KO MEFs might result in decreased FOXO activity that could account for the increased sensitivity of these cells to oxidative stress. Indeed, we observed a marked increase in phosphorylated FOXO1/3a in Ptpn12-KO MEFs compared to Ptpn12-RE MEFs (Figure 2.3a). These data suggest that, in the absence of PTPN12, PDK1 is ‘hyperactivated’ such that FOXOs are inactivated, compromising the intracellular antioxidant response. To support our hypothesis, we assayed mRNA levels of an array of FOXO target genes in Ptpn12-KO and PTPn12-KO-R237M MEFs. Among other validated FOXO target genes, H6PD (which controls NADPH production in the endoplasmic reticulum), CD44 (promotes GSH production through cystine uptake) and HO-1 (controls iron-mediated ROS regulation) were downregulated in the absence of functional PTPN12 (Figure 2.3b and S2)\textsuperscript{23, 52, 76}. FOXO3a has been reported to be inactivated under increasing ROS levels\textsuperscript{55}. To confirm that the increased phosphorylation (and subsequent inactivation) of FOXO1/3a seen in PTPn12-KO MEFs is due to PTPn12 directly and not simply an indirect effect of high
ROS levels, we treated cells with the antioxidant NAC. While the FOXO3a phosphorylation decreased in control cells, PTPn12-KO MEFs still contained a high level of FOXO1/3a phosphorylation (Figure 2.3c). This result indicates that PTPn12 is having a direct effect on preventing the phosphorylation and inactivation of FOXO1/3a.

Finally, we modulated PDK1 and FOXO1/3a in an effort to rescue the increased ROS levels observed in PTPn12-KO MEFs. We were unable to observe any changes in ROS levels when PDK1 mRNA was reduced (Figure S3), potentially because any remaining hyperactive PDK1 protein after knockdown of PTPn12 may still inhibit FOXO1/3a activation. Alternatively, this result suggests that PTPn12 may control ROS levels in a PDK1-independent fashion. Since FOXO1/3a is the downstream target of PDK1 which ultimately could affect the ROS levels in PTPn12-KO MEFs, we decided to express in cells a constitutively active FOXO3a using a FOXO3a:A3 construct. This construct produces a FOXO3a protein which has mutations at its three sites of phosphorylation. These mutations prevent the FOXO3a:A3 protein from being phosphorylated and subsequently inactivated. Under glutamine free conditions, expression of FOXO3a:A3 was able to reduce the ROS levels in PTPn12-KO MEFs cells (Figure 2.3d). We did not see a rescue of the cell death in PTPn12-KO MEFs, yet this may be due to the fact that FOXO3a also induces expression of pro-apoptotic targets, such as Bim (Figure S4). Overall, these results suggest that deregulation of FOXO signaling may account for the increased sensitivity of PTPN12-deficient cells to oxidative stress.
2.2.3 High levels of PTPN12 mRNA predict poor prognosis in breast cancer patients

Tight regulation of oxidative stress within a cell is closely linked to its tumorigenic potential \(^1\). Significantly, previous work has shown that human breast tumors and numerous commonly-used breast cancer cell lines possess a mutated form of PTPN12 that has increased phosphatase activity \(^78\). However, whether PTPN12 confers a growth advantage on cancer cells under stress has yet to be determined. To shed light on this issue, we examined two previously published datasets of mRNA levels in human breast cancer samples \(^79,80\) and found that, in both cases, patients with high \(PTPN12\) mRNA expression had a reduced probability of relapse-free survival (Figure 2.4a, b). To further explore whether PTPN12 expression correlated with breast tumorigenesis, we used immunohistochemistry (IHC) to examine PTPN12 protein expression in 240 patient-derived primary breast tumors of various subtypes. We found that significantly more triple negative breast cancers (TNBC; ER\(^{-}\)PR\(^{-}\)HER2\(^{-}\)) showed high levels of PTPN12 compared to non-TNBC breast cancers (Figure 2.4c, d). There was no correlation between PTPN12 expression and either tumor grade or size (Figure S5). Similar results were obtained when the same samples were subjected to IHC using an alternative anti-PTPN12 antibody (data not shown). These data suggest that high levels of PTPN12 allow breast tumor cells to better combat exogenous stress, and imply that PTPN12 may promote and/or support breast cancer progression.
Figure 2.4 High levels of PTPN12 mRNA predict poor patient prognosis. (a and b) PTPN12 mRNA levels in tumors of breast cancer patients examined in the Perou2 (a) and Chin (b) datasets were plotted and correlated with the fraction of relapse-free patients. (c and d) Sections of tumors from 240 patients with TNBC or “non-TNBC” (ER\(^+\)HER2\(^+\)) breast cancers were subjected to IHC to detect PTPN12 protein. Tumors were scored as exhibiting either high (total Allred score of 7-8) or low (total Allred score of 6 or below) PTPN12 protein expression. (c) Representative images of tumors with low or high PTPN12 protein shown at low magnification (upper panels; scale bar=100 \(\mu\)m) and high magnification (lower panels; scale bar=50 \(\mu\)m). (d) Percentages of patients with TNBC or non-TNBC tumors showing high or low PTPN12 expression.
Figure 2.5 PTPN12 suppresses ROS levels and promotes tumorigenesis. (a) Immunoblot to detect PTPN12 protein in extracts of MDA-MB-231 and PyMT cell lines expressing control shRNA or shPTPN12. (b) Quantitation of ROS in MDA-MB-231 cells expressing control shRNA or shPTPN12 and grown in normal or Gln-free medium. ROS levels were determined as for Figure 2a. (c) Total tumor mass in immunodeficient NIH-III nude mice (n=10) subcutaneously injected with MDA-MB-231 cells expressing control shRNA (left flank) or shPTPN12 (right flank). After 84 days, tumors were excised and weighed. Results are the mean ± SEM of total tumor mass per mouse. D. Kaplan-Meier analysis of the survival of 129P2Ola mice injected with PyMT cells expressing control shRNA or shPTPN12 (n=10/group). Mice were monitored for 200 days.

2.2.4 Loss of PTPN12 increases ROS levels and inhibits breast cancer cell growth in vivo

TNBCs show an overlap with tumors of the ‘basal’ breast cancer subtype, which are generally aggressive breast tumors associated with very poor patient survival. To determine if
loss of PTPN12 in basal-like breast cancer cell lines would decrease their tumorigenic potential, we used the short hairpin RNA (shRNA) approach to deplete PTPN12 in MDA-MB-231 cells (Figure 2.5a, left). MDA-MB-231 cells cultured under either normal or Gln-free conditions contained higher ROS when PTPN12 expression was reduced (Figure 2.5b). This increase in ROS levels was not as large as the change observed between Ptpn12-RE and Ptpn12-KO MEFs (Figure 2.2a); potentially because the Ptpn12-KO MEFs completely lack PTPN12 protein, while shRNA against PTPN12 can never fully ablate the mRNA and subsequent protein expression. To investigate the impact of PTPN12 depletion on the tumorigenic potential of MDA-MB-231 cells in vivo, we subcutaneously injected MDA-MB-231 cells expressing control shRNA or PTPN12 shRNA into NIH-III nude mice and monitored tumor growth. Strikingly, the tumors formed by MDA-MB-231 cells lacking PTPN12 were significantly smaller than tumors derived from control cells (Figure 2.5c). IHC analysis of these tumors confirmed that PTPN12 protein expression was strongly reduced in vivo (data not shown), suggesting that the observed reduction in tumorigenicity was due to loss of PTPN12.

We bolstered these results by conducting parallel experiments in which Ptpn12 shRNA was introduced into a spontaneously immortalized mouse mammary tumor cell line (referred to as PyMT cells) derived from MMTV-PyMT mice. We injected PyMT control and PyMT Ptpn12 knockdown cells (Figure 2.5a, right) into the tail veins of 129P2Ola mice and monitored tumor development and mouse survival. We found that lung metastasis was drastically reduced and overall survival was prolonged in animals injected with PyMT cells lacking PTPN12 (Figure 2.5d). This data reinforces our contention that PTPN12 promotes and/or supports tumorigenesis and metastasis of breast cancer cells in vivo.
To investigate a potential mechanism by which PTPN12 might promote tumorigenesis and/or metastasis, we determined whether the signaling alterations we observed in Ptpn12-KO MEFs also occurred in breast cancer cells expressing PTPN12 shRNA. Indeed, like Ptpn12-KO MEFs, PTPN12-depleted MDA-MB-231 cells showed elevated phospho-FOXOs (Figure 2.6a). Taken together, our data indicate that cells lacking PTPN12 experience diminished FOXO signaling, which may be due to dysregulation of the PDK1-SGK1 axis. Such a defect could account for the increased ROS levels observed in PTPN12-deficient cancer cells and the decreased tumorigenicity of these cells in vivo. A model of this hypothetical series of events is shown in Figure 2.6b.

2.3 Discussion

ROS are known to modulate PTPs by inducing changes to their protein structure that cause their functional inactivation. We initiated our study in an effort to determine if PTPs can reciprocally regulate ROS in order to prevent this inactivation. Our investigation of the relatively poorly characterized enzyme PTPN12 has demonstrated that this PTP can function to counteract or reduce oxidative stress. When we modulated ROS levels either directly by inhibiting NADPH or GSH synthesis using 6-AN or BSO, or indirectly by Gln deprivation, we found that MEFs lacking PTPN12 were unable to cope with the resulting perturbations in ROS and ultimately underwent apoptosis. PTPN12 is thus required for effective ROS regulation.

Recent studies have indicated that cancer cells develop an intricate system of ROS control in order to survive under conditions of oxidative stress. Although a lack of NADPH production due to loss of G6PD might be predicted to inhibit tumorigenesis, women with
Figure 2.6 PTPN12 regulates ROS levels in tumor cells via FOXO signaling. (a) Immunoblot to detect the indicated proteins in lysates of MDA-MB-231 cells expressing control shRNA or shPTPN12 and cultured under normal (+) or serum starvation (-) conditions. (b) Model of alterations to FOXO signaling that might occur in the absence of PTPN12. Left: PTPN12 promotes FOXO1/3a activation (either directly or through PDK1 inhibition) and subsequently promotes the expression of FOXO1/3a target genes that combat oxidative stress. The cell is permitted to survive. Right: In the absence of PTPN12, excessive FOXO1/3a inactivation occurs and a failure to express antioxidant molecules. Oxidative stress cannot be successfully combatted and the cell is more likely to die.
inherited mutations of G6PD (mimicking the effect of 6-AN) show no decrease in breast cancer incidence\(^8^3\). In line with this observation, there is no evidence from explorations of BSO in the clinical setting that BSO treatment alone is therapeutic for breast cancer patients\(^8^4\). Similarly, Gln depletion via L-asparaginase treatment is effective only in acute lymphoblastic leukemia patients\(^1\). Considering these data, we hypothesize that these therapeutic avenues have failed in part because of oncogenic factors (like PTPN12) that promote antioxidant mechanisms and thus maintain ROS regulation. We therefore believe that targeting such molecules may leave tumor cells vulnerable to antioxidant inhibition, which in turn may ultimately force them to succumb to ROS-induced death.

A previous report by Sun et al. claimed that PTPN12 is a tumor suppressor because PTPN12 expression was often missing in tumor samples from patients with TNBCs\(^8^5\). However, in our study, we used two different anti-PTPN12 antibodies, including that employed by Sun et al., to perform IHC on tumor samples from 240 patients with various subtypes of breast cancer. Our results do not support the conclusion that TNBCs have diminished or absent expression of PTPN12. On the contrary, our study has demonstrated that not only is PTPN12 protein present in almost all subtypes of breast tumors, but also that more TNBCs show high expression than any other breast cancer subtype. We bolstered this finding with our analysis of two breast cancer mRNA datasets, which established that high levels of PTPN12 mRNA correlate with a less favorable prognosis. Thus, the results of our study clearly indicate that elevated PTPN12 expression is beneficial for and supportive of tumor development, and are consistent with the hypothesis that rapidly proliferating cancer cells develop mechanisms that confer a high level of resistance to cellular stress, in particular oxidative stress. Additional in-depth investigation of the impact of other PTPs on ROS control, and the delineation of alterations to these PTPs that may
be associated with tumorigenesis, will no doubt yield many useful insights into how oxidative stress is overcome by cancer cells.

2.4 Experimental Procedures

Cell lines, culture conditions and reagents

Immortalized mouse embryonic fibroblasts (MEFs) from PTPN12-null mice were transfected with either empty vector (pcDNA3.1 - referred to as Ptpn12-KO) or a wild-type mouse full length ORF Ptpn12 expression construct (referred to as Ptpn12-RE) \(^{70}\). The human breast cancer cell line MDA-MB-231 was obtained from ATCC. A cell line derived from MMTV-PyMT mice (referred to as PyMT cells) was maintained as previously described \(^{82}\). FOXO3a:A3 expression was used as previously described \(^{86}\). Unless otherwise indicated, all cell lines were maintained in Dulbecco’s Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 mg/ml streptomycin. For serum starvation, cells were cultured overnight in serum-free DMEM. For Gln deprivation, cells were cultured in DMEM without L-glutamine (GIBCO 10313). All antibodies were from Cell Signaling, except for anti-PTPN12 (Bethyl Laboratories Inc. or Sigma HPA007097) and anti-FOXO3A (Millipore). All chemical compounds were from Sigma if not otherwise stated.

RNA interference
MDA-MB-231 or PyMT cells stably expressing control shRNA or shPTPN12 were generated by lentiviral transduction, using the pGIPZ system (OpenBiosystems Cat. # RHS4531-NM_002835 and Cat.# RMM4532-NM_011203). Transduced cells were selected for minimum 7 days in culture in the presence of 1 µg/ml puromycin. Transient knockdown of PDK1 mRNA was accomplished using siRNA against mouse PDK1 (Dharmacon, L-040658-00-0020) with Lipofectomine 2000 (Invitrogen).

Cell proliferation

Replicate cultures of cells were grown for 4 days before staining with trypan blue to determine viability. Viable cells were counted on each day (day 1-4) using a Vi-Cell XR cell viability analyzer (Beckman Coulter) to establish growth curves.

Apoptosis and ROS

Cells were cultured in Gln-free medium or treated with NAC, BSO or 6-AN at the concentrations and for the times indicated in the Figures. Apoptotic cells were measured by staining with AnnexinV (BD Biosciences) plus propidium iodide (PI), followed by flow cytometric measurement of fluorescence using a FACSCalibur (Becton Dickinson). To determine relative ROS levels, cells were stained with CM-H2DCFDA (Invitrogen) and subjected to FACS analysis. ROS levels are reported as mean fluorescence intensity (MFI) expressed as arbitrary units.
Quantitative real-time PCR (qRT-PCR)

mRNA was prepared from cell extracts using the NucleoSpin RNA II kit (Macherey-Nagel). mRNA was reverse-transcribed into cDNA using the iScript cDNA synthesis kit (Bio-Rad). Real-time PCR analysis was performed using POWER SYBR Green PCR Master Mix and a 7900HT Fast Real-Time PCR System (Applied Biosystems). mRNA levels of PDK1 and the FOXO target genes BIM, TRAIL, CD44, H6PD and HO-1 were determined relative to the housekeeping gene RPS9. All experiments were performed in triplicates.

Analysis of breast cancer patient datasets

The Perou2 ⁸⁰ and Chin ⁷⁹ datasets were independently analyzed to determine PTPN12 mRNA expression. Patient survival was examined using Cox regression analysis of survival times vs. gene expression log ratios, followed by the estimation of false discovery rates using the Benjamini and Hochberg method. High expression of PTPN12 mRNA was associated with greater risks of tumor recurrence and death. These associations reached formal statistical significance with p values < 0.05. Kaplan-Meier curves were generated for each dataset for illustration purposes by rank-ordering the log ratios and splitting them into three equal groups. The curves for the highest and the lowest tertiles were displayed on the plots shown in Figure 4a and b.

Immunohistochemistry (IHC) of human breast tumor tissue array
Six in-house tissue microarrays with a total of 240 unique breast cancer cases represented in duplicate, together with appropriate control sections, were subjected to immunostaining with anti-PTPN12 antibody (Bethyl IHC-00260 or Sigma HPA007097) after obtaining UHN research ethics board approval. The TMAs contained consecutive breast cancer cases collected at a single institution (UHN) over a calendar year (2006). Receptor status (ER/PR/HER2) had been routinely assessed on all cases. Stained tissue sections were evaluated by light microscopy and scored blinded by two independent pathologists (SJD and JM) to produce an Allred score \(^87\). The total Allred score is the sum of the proportion score (percentage of cells stained: 0%=0; <1%=1; 1-10%=2; 10-33%=3; 34-66%=4; >66%=5) and the intensity score (no staining=0; weak=1; moderate=2; strong=3). Values were stratified into two groups, namely high PTPN12 expression (total Allred score of 7-8) or low PTPN12 expression (total Allred score of 6 or below) \(^88\).

**Xenografts**

For MDA-MB-231 cell analyses, immunodeficient NIH-III nude mice (5 weeks old) were subcutaneously injected in both flanks with 3x10^6 MDA-MB-231 cells expressing control shRNA (left flank) or shPTPN12 (right flank). Mice were monitored every 4 days for the presence of palpable tumors. After 84 days, the mice were sacrificed and tumors were excised. Tumor weights were recorded and tissues were photographed and processed for further analysis.

For PyMT cell analyses, control or shPTPN12 PyMT tumor cells (2.5x10^5 in 200µl PBS) were injected into the tail veins of 129P2Ola mice. Mouse survival over 200 days was evaluated using Kaplan-Meier methodology. All animal experiments were approved by the Animal Care and Use Committee of the University Health Network (Toronto, Canada).
Statistics

Kaplan-Meier survival plots and statistics were created and analyzed using GraphPad PRISM-5 software. The two-tailed student’s t-test with the standard error of the mean (S.E.M.) was used to evaluate differences in tumor cell proliferation and survival.
Chapter 3. Investigation into the role of GCLM in tumorigenesis

Parts taken from:

Harris, I. S., et al. (2013). “Glutathione and thioredoxin antioxidant pathways synergize to drive cancer initiation and progression” (submitted)

Controversy over the role of antioxidants in cancer has persisted for decades. Here, we show that GSH synthesis, driven by GCLM, is required for cancer initiation. Genetic loss of Gclm delays tumorigenesis and prevents a progression to malignancy. Intriguingly, this can be replicated using an inhibitor of GSH synthesis, yet only if delivered prior to cancer onset, suggesting that once a tumor has formed, GSH becomes dispensable due to compensatory antioxidant mechanisms. In primary cells lacking GCLM, we observed an upregulation of Nrf2 target genes responsible for promoting the thioredoxin (TXN) antioxidant pathway. Remarkably, combined inhibition of GSH and TXN antioxidant pathways leads to a synergistic cancer cell death in vitro and in vivo, suggesting the importance of these two antioxidants to tumor progression. In this study, we provide convincing evidence that antioxidants promote tumorigenesis, which is contrary to the public’s belief. We observe a synergy between GSH and TXN antioxidant pathways, both of which are driven by the transcription factor Nrf2. Interestingly, Nrf2 expression has been found to be controlled by oncogenes and tumor suppressors. Furthermore, mutations leading to constitutive Nrf2 stabilization have been identified in patient tumors. Our study provides clear scientific evidence to caution those using antioxidants or inducers of Nrf2 as a therapy for various diseases. These results also suggest that
combined targeting of multiple antioxidant pathways may prove to be a valuable therapeutic regime for cancer patients.

3.1 Introduction

Reactive oxygen species (ROS) is a normal byproduct of mitochondrial metabolism and protein folding. At low, ROS acts as a signaling molecule to activate proliferation and survival pathways. At moderately increased levels, ROS can cause damage to DNA and promote mutagenesis in a cell. At high ROS levels, however, they become an oxidative stress which can ultimately cause cell senescence or death. To inhibit oxidative stress, the cell has a set of molecules termed antioxidants which convert ROS into a benign species, such as water.

The most abundant antioxidant within this cell is glutathione (GSH). A metabolite, GSH is synthesized in a two-step process, the first of which is carried out by glutamate cysteine ligase (GCL), a heterodimer of catalytic (GCLC) and modifier (GCLM) subunits. Expression of both GCLC and GCLM is controlled by the antioxidant transcription factor Nrf2. Gene-targeted GCLC-deficient (Gclc−/−) mice lack any GSH synthesis and die during embryogenesis. In contrast, Gclm−/− mice have only 10-25% of the GSH levels of a wildtype mouse, but are completely viable. Although Gclm−/− mice have greatly reduced GSH synthesis, they lack any overt phenotype.

Cancer cells arise from activation of oncogenes and/or inactivation of tumor suppressors. Key hallmarks of cancer cells are evasion of apoptosis and unrestrained growth.
generation of ROS are byproducts of processes which drive these hallmarks, cancer cells sustain a much higher level of ROS generation compared to normal cells. Therefore, to avoid the detrimental effects of oxidative stress, cancer cells must actively upregulate multiple antioxidant systems. By buffering ROS levels, cancer cells are able to stay within the levels of ROS which are beneficial to promote tumor progression. Indeed, activation of oncogenes and loss of tumor suppressors increase Nrf2 expression and activity. Furthermore, mutations in Nrf2 and Keap1 (the inhibitor of Nrf2) which promote Nrf2 stabilization have been identified in multiple cancers. Although the importance of Nrf2 has been implied for tumorigenesis, there remain numerous questions as to which downstream targets of Nrf2 are critical for cancer survival and whether one can inhibit these processes as a form of cancer therapy.

Recent reports have suggested that mammary tumor cells rely on antioxidants, yet these studies have been completed mainly using in vitro tools and exploring non-specific antioxidant pathways. Here, we examine inhibition of the GSH antioxidant pathway, either by genetic loss of GCLM or chemical compound inhibition, and its subsequent impact on tumorigenesis.

3.2 Results

3.2.1 The role of GCLM in mammary tumorigenesis

To determine the impact of GCLM on mammary tumorigensis, we first wanted to examine whether normal mammary gland development was impaired in Gclm<sup>−/−</sup> mice. No
difference in ductal outgrowth was observed between Gclm<sup>+/+</sup> and Gclm<sup>−/−</sup> mice during mammary gland maturation (Figure 3.1A). Additionally, no gross differences in density of mammary branches were observed in Gclm<sup>−/−</sup> mice (Figures 3.1B). These results suggest that any changes in tumorigenesis observed in Gclm<sup>−/−</sup> mice would be directly linked to cancer outgrowth and not a pre-existing defect in mammary glands.

In order to investigate the impact of GCLM on cancer, we bred Gclm<sup>−/−</sup> mice to the MMTV-PyMT mouse line, which develops spontaneous mammary tumors (referred to as PyMT-Gclm mice)<sup>93</sup>. PyMT-Gclm<sup>−/−</sup> mice had a significantly delayed cancer onset compared to control PyMT-Gclm<sup>+/+</sup> (Figure 3.2A). Furthermore, at the experimental endpoint, the total weight of tumors isolated from PyMT-Gclm<sup>+/+</sup> mice was more than double that of tumors isolated from PyMT-Gclm<sup>−/−</sup> mice (Figure 3.2B). Interestingly, a partial gene dosage effect was evident, as cancer onset was delayed in the PyMT-Gclm<sup>+/−</sup> mice, but ultimately no difference was observed in cancer burden at endpoint (Figure S6). Histological analysis revealed that while all tumors in control mice progressed to invasive ductal carcinoma (IDC), tumors in PyMT mice lacking GCLM showed a reduced incidence of invasive progression (Figures 3.1C-E). Furthermore, when IDC was observed in PyMT-Gclm<sup>−/−</sup> mice, it was much more focal in nature. Supporting these results is prognostic data which indicates that breast cancer patients with high GCLM mRNA have a lower probability of relapse-free survival and overall survival (Figures 3.2F-H). Together, these results suggest that GSH synthesis, driven by GCLM, is not required for normal mammary gland development but necessary for breast cancer initiation and progression.
Figure 3.1 Loss of GCLM does not impact normal mammary gland development. (A) Ductal outgrowth in Gclm^{+/+} and Gclm^{-/-} mice was measured at the indicated times (n = 4-6 glands/group). (B) Representative images of whole mount mammary glands from the mice in (A). Scale bars = 6 mm. n.s., non-significant. Error bars represent ± s.e.m. here and for all figures.
Figure 3.2 GSH synthesis by GCLM is required for mammary tumor initiation. (A) Tumor-free survival curves of PyMT-Gclm<sup>+/+</sup> (n = 36) and PyMT-Gclm<sup>−/−</sup> (n = 19) mice. (B) At the endpoint (day 160), all tumors from PyMT-Gclm<sup>+/+</sup> (n = 32) and PyMT-Gclm<sup>−/−</sup> (n = 32) mice were resected and weighed. Each data point represents the combined weight of tumors from a single mouse. (C and D) Representative images of tumor sections stained for H&E (C) and smooth muscle actin (SMA) (D). (E) Pathological classifications of tumors from the mice in (B). DCIS, ductal carcinoma in situ; IDC, invasive ductal carcinoma. (F-H) Fraction of relapse-free survival (RFS) (F) and overall survival of breast cancer patients relative to high or low GCLM mRNA expression in their tumors. ***, p<0.001. Scale bars = 400 µm.
3.2.2 Early inhibition of GSH synthesis, but not upon tumor onset, impairs tumorigenesis

Buthionine-[S, R]-sulfoximine (BSO) is a potent inhibitor of GSH synthesis and oral delivery is an effective pharmacological model of continuous depletion of GSH levels \textit{in vivo} \cite{94}. In an effort to examine whether chemical inhibition of GSH synthesis could mirror genetic inhibition of GSH synthesis, we treated PyMT-Gclm\textsuperscript{+/+} mice with BSO. We chose to treat mice at two different time points: immediately after weaning (4 weeks old; “early BSO”), or upon cancer onset as assessed by palpation (approximately 6-12 weeks old; “onset BSO”). Early BSO treatment dramatically reduced the cancer burden in PyMT-Gclm\textsuperscript{+/+} mice (Figure 3.3A). Histological analysis of tumors resected from early BSO mice revealed a decreased formation of invasive carcinomas (Figure 3.3B), replicating the phenotype of PyMT-Gclm\textsuperscript{−/−} mice. Intriguingly, when BSO was delivered upon cancer onset, tumor burden was unchanged; as was tumor histology (Figures 3.3A, B). To gain molecular insight into this phenomenon, we examined the expression of nuclear factor (erythroid-derived 2)-like factor 2 (NFE2L2, or Nrf2), a major antioxidant gene transcription factor and a key factor shown to be upregulated by oncogenic factors during tumorigenesis \cite{14,25}. Analysis of tumors revealed a marked upregulation of Nrf2 target genes (\textit{Hmox1} and \textit{Nqo1}) in onset BSO mice, but not in tumors of early BSO mice (Figures 3.3C-D). These results imply that the compensation for inhibited GSH synthesis may be occurring through antioxidant gene upregulation via Nrf2 and that this compensation only occurs once malignant transformation has taken place.
Figure 3.3 BSO prevents tumorigenesis, but only if delivered prior to tumor onset. (A) At endpoint, tumors from H2O controls (n = 16) and “early BSO” (n = 8) or “onset BSO” (n = 9) treated PyMT-Gclm+/+ mice were resected and weighed. Analysis as was for Figure 1B. (B) Representative images of sections of tumors from the mice in (A) stained with H&E. (C and D) Quantitative real-time PCR (qRT-PCR) analysis of tumors from mice in (B) to detect mRNA levels for Nrf2 target genes heme oxygenase 1 (Hmox1) (C) and NAD(P)H dehydrogenase, quinone 1 (Nqo1) (D). Values for mRNAs were expressed relative to housekeeping gene ribosomal protein S9 (Rps9). *, p<0.05; n.s., non-significant. Scale bars = 400 µm.
3.2.3 GSH synthesis is dispensable in GCLM-null cells

To further investigate potential GSH-independent antioxidant responses by cancers, we isolated primary mammary epithelial cells (termed pMECs) from PyMT-Gclm^{+/+} and PyMT-Gclm^{-/-} mice. The GCLM subunit aids GCLC in producing de novo GSH; therefore loss of GCLM would greatly decrease GSH synthesis but not completely abolish it. Accordingly, we observed greatly reduced but still detectable GSH levels in PyMT-Gclm^{-/-} cells (Figure 3.4A). We postulated that PyMT-Gclm^{-/-} pMECs would be more sensitive to BSO-mediated GSH inhibition due to the decreased GSH levels. Surprisingly, however, although BSO reduced growth and induced apoptosis in PyMT-Gclm^{+/+} pMECs, it had no effect on PyMT-Gclm^{-/-} pMECs (Figures 3.4B-C); suggesting that tumor cells can compensate for complete lack of GSH synthesis. Furthermore, no significant increase in ROS levels was observed between PyMT-Gclm^{+/+} and PyMT-Gclm^{-/-} pMECs (Figure 3.4D). Interestingly, the mRNA levels of Nrf2 target genes were increased in PyMT-Gclm^{-/-} pMECs (Figure 3.4E); similar to the BSO-resistant tumors from control PyMT mice (Figures 3.3C-D). These results indicate that tumors compensate for loss of the GSH antioxidant pathway potentially through Nrf2-dependent mechanisms.

3.2.4 The TXN antioxidant pathway compensates for decreased GSH levels

Glutamate, one of the substrates used by GCLM/GCLC to produce GSH, is also required for the cellular import of cystine via the amino acid transporter XCT (also known as SLC7A11) (Figure 3.5A)^{21}. Imported cystine, once reduced to cysteine, is incorporated into a multitude of antioxidant molecules, including thioredoxins (TXN). We reasoned that if GSH synthesis was
Figure 3.4 Gclm<sup>−/−</sup> cells survive inhibited GSH synthesis. (A) Primary mammary epithelial cells (pMECs) from PyMT-Gclm<sup>+/+</sup> and PyMT-Gclm<sup>−/−</sup> mice were analyzed for total GSH levels. (B) PyMT-Gclm<sup>+/+</sup> and PyMT-Gclm<sup>−/−</sup> pMECs were cultured with or without BSO (150 µM) for the indicated times. Cell numbers expressed relative to the cell count on day 1. (C and D) Cells from (B) were stained on day 4 for apoptosis (Annexin V, 7-AAD) (C) and ROS levels (CM-H<sub>2</sub>DCFDA) (D). NT, not treated. (E) Cells from (B) were harvested on day 4 and mRNA levels of the indicated Nrf2 target genes were determined by qRT-PCR. Ggt1, gamma-glutamyltransferase 1. *** p<0.001; ** p<0.01; n.s., non-significant.
blocked, the excess glutamate that accumulated might be exported and used to import cystine, bolstering the TXN antioxidant pathway. In line with this hypothesis, overexpression of XCT has been shown to rescue the growth of cells completely deficient in GSH production, which was highly dependent upon thioredoxin reductase 1 (TXNRD1) activity. Interestingly, Txnrd1 has also been shown to be upregulated in the livers of Gclm−/− mice. Thus, increased TXN antioxidant pathway activity can apparently compensate for compromised GSH synthesis. Intriguingly, we found that the expression of the tumor-associated antigen CD44, which stabilizes XCT to promote cystine import, was controlled by Nrf2 (Figures 3.5B and S7). In PyMT-Gclm−/− pMECs, we also observed the mRNA expression levels of several Nrf2-dependent genes that promote the TXN antioxidant pathway, including Cd44, to be increased (Figure 3.5C). Upon re-examination of onset BSO treated PyMT mice (which failed to respond to BSO treatment), we observed upregulated mRNA expression of Xct, Cd44 and Txn1 (Figure 3.5D-F), implying that the TXN antioxidant pathway was increased in these malignancies. In contrast, no change in the expression of these genes was detected in tumors of early BSO mice (which did respond to BSO treatment). Next, we investigated whether a similar expression of genes within the TXN antioxidant pathway was present in human tumors. Indeed, examination the TCGA breast cancer dataset revealed XCT, TXN1 and TXNRD1 to be upregulated in breast cancer patient tissue compared to normal tissue (Figures 3.5G-I). These results suggest that in order to compensate for blockage of the GSH antioxidant pathway, cancer cells may coordinately upregulate the TXN antioxidant pathway as a means of reducing oxidative stress.
Figure 3.5 Compensation for decreased GSH synthesis occurs via TXN. (A) Schematic representation of antioxidant compensation provided by TXN antioxidant pathway upon inhibition of de novo GSH synthesis. Cys₂, cystine; Cys, cysteine. (B) Primary mouse embryonic fibroblasts (MEFs) from Nrf2+/+ and Nrf2−/− mice were harvested and mRNA expression was determined by qRT-PCR previously unknown Nrf2 target gene Cd44. (C) Cells from Figure 4B were harvested on day 4 and mRNA levels of the indicated Nrf2 target genes were determined by qRT-PCR. (D-F) Expression of mRNA in tumors from Figure 3A for Nrf2 targets Xct (D), Cd44 (E) and Txn1 (F) was determined by qRT-PCR. (G-I) Analysis of the TCGA invasive breast carcinoma data set was completed for XCT (G), TXN1 (H), and TXNRD1 (I). ***, p<0.001; **, p<0.01; n.s., non-significant.
3.2.5 GSH and TXN antioxidant pathways synergistically support cancer cell survival

To further investigate the reliance of cancer cells on the TXN antioxidant pathway for compensation, we reduced the expression of \textit{GCLM} and/or \textit{XCT} mRNAs in MDA-MB-231 human breast cancer cells using specific siRNA (Figures S8A, B). The greatest increase in cellular ROS levels was observed with a combination knockdown of both \textit{GCLM} and \textit{XCT}, indicating a potential synergistic effect when inhibiting both GSH and TXN antioxidant pathways (Figure S8C). Next, we examined the effects of inhibiting each pathway chemically, using BSO to block GSH synthesis, sulfasalazine (SSA) to inhibit the XCT transporter, and auranofin (AUR) to block activity of TXNRD\textsuperscript{98, 99}. While BSO treatment alone completely inhibited the GSH antioxidant pathway by reducing GSH levels to undetectable amounts in cancer cells, it only had a small effect on their survival and proliferation (Figures 3.6A-C and S9A). A similar outcome was obtained with inhibition of the TXN pathway alone with the inhibition of cystine import or TXNRD activity. Remarkably, we observed a powerful synergistic effect after combining BSO and SSA treatments in cancer cells, as there was increased ROS levels, apoptotic cell death and complete lack of growth (Figures 3.6A-C). This synergistic effect was even greater when the BSO and AUR treatments were combined (Figures 3.6A, C). NADPH is the sole metabolite which regenerates GSH after it is oxidized\textsuperscript{1}. Furthermore, NADPH synthesis has been linked to oncogenic modifications and tumorigenesis\textsuperscript{100} and could potentially compensate for inhibition of GSH synthesis. BSO treatment alone did not result in any changes in NADP\textsuperscript{+}/NADPH ratios and no synergy was observed when BSO was combined with an inhibitor of NADPH synthesis (Figures S9B, C). These data suggest the specificity of the synergy between GSH and TXN antioxidant pathways in supporting tumor cell
Figure 3.6 Inhibition of GSH and TXN synergistically induces cancer cell death. (A) MDA-MB-231 cells were either not treated (NT) or treated with BSO (150 µM), SSA (250 µM) or AUR (250 nM) alone or in the indicated combinations and cultured for indicated times. Cell numbers are expressed relative to cell count on day 1. (B and C) Cells from (A) were analyzed on day 3 for ROS levels (B) and apoptosis (C). (D and E) SCID mice (n=8/group) with fat pad injections of MDA-MB-231 cells were not treated (NT) or treated with BSO (20 mM) in their drinking water and/or SSA was injected daily i. p. at 250 mg/kg. Tumor volume was monitored from initiation of drug treatment until endpoint (day 12) (D). At endpoint, each tumor was resected from each animal and tumor mass was determined (E). (F) Representative images of xenograft tumors from (E). *** p<0.001; ** p<0.01; n.s., non-significant.
survival and growth. To investigate whether this synergy occurred in vivo, we injected mice with MDA-MB-231 cells and treated them with BSO, SSA or both agents once the palpable xenograft tumors had formed. While no effect on tumor growth was observed with administration of single agents, combined delivery of BSO and SSA synergistically reduced tumor growth in vivo (Figures 3.6D-F). Together, these results suggest that in malignant tumors a combined inhibition of both GSH and TXN antioxidant pathways is required for effective induction of cancer cell death.

3.3 Discussion

The role of antioxidants in tumorigenesis has been controversial for years. An overload of ROS within the cell leads to death, and the idea of inhibiting antioxidant pathways in order to kill cancer cells was first conceived long ago \textsuperscript{101}. The concept that ROS can promote de novo mutagenesis arose almost concurrently and many believed that antioxidants could prevent or inhibit tumorigenic changes in pre-neoplastic cells \textsuperscript{102}. Furthermore, later studies showed that ROS can contribute to signaling cascades which promote proliferation \textsuperscript{5}. Nonetheless, there has been a resurgence of evidence indicating that antioxidants contribute to tumorigenesis by reducing excessive ROS accumulation and subsequent oxidative stress in cancer cells \textsuperscript{89}. Our study has shown both genetically and chemically, and using a well-established spontaneous tumor model, that antioxidants are required for cancer initiation and progression. These results suggest that reduced antioxidant expression can in fact be chemopreventive and are supported by the failure of clinical trials which used antioxidant therapy to prevent cancer but instead
significantly increased cancer incidence\textsuperscript{103, 104}. Thus, caution should be exercised when using dietary antioxidant supplementation or Nrf2 inducers, as pro-tumorigenic effects may accompany these forms of treatment\textsuperscript{105}.

In numerous cancers, mutations have been identified which lead to stabilization of the antioxidant transcription factor Nrf2. Here, we show that GSH and TXN antioxidant pathways are critical downstream effectors which may mediate Nrf2-supported tumorigenesis. We hypothesized that compensation from inhibition of GSH synthesis could specifically arise from the TXN antioxidant pathway due to the excess glutamate present and subsequently increased cystine import. Accordingly, inhibition of Nrf2 in cancer cells, which decreases GCLM/GCLC expression and subsequently reduces GSH synthesis, leads to an increase in glutamate levels\textsuperscript{25}. Although Nrf2 plays a large role in NADPH generation\textsuperscript{25}, we found no compensation from these pathways upon GSH depletion. Additionally, while we focused on Nrf2, compensation for depleted antioxidants may also occur from other transcription factors such as p53 or the FOXO family\textsuperscript{53, 106}. A comprehensive examination of Nrf2-dependent and –independent mechanisms of antioxidant compensation would yield great insight towards understanding how interconnected antioxidant pathways support tumorigenesis.

Although we chose to focus on the impact of GSH synthesis, driven by GCLM, in mammary tumorigenesis, we believe that these effects translate beyond this context and into other tumors. In fact, GCLM mRNA expression was found to be increased in a wide spectrum of cancers compared to normal tissue (\textbf{Figure S5}). Examination of the loss of GCLM within other tumor models will provide fruitful information regarding whether GSH synthesis is in fact required not only for mammary tumor initiation but other malignancies.
Figure 3.7 Temporal regulation of antioxidant pathways dictates malignancy. During the pre-malignant stage, low Nrf2 levels prevent the upregulation of compensating antioxidant pathways. Accordingly, pre-malignant tumors are sensitive to inhibition of the GSH antioxidant pathway by BSO. Upon malignant transformation, activation of oncogenes and loss of tumor suppressors drive Nrf2 activation. This leads to upregulation of the TXN antioxidant pathway, which provides compensation when GSH synthesis is inhibited. Combined inhibition of both GSH and TXN antioxidant pathways, using BSO with SSA or AUR, block any compensation from occurring in malignancies and results in synergistic cancer cell death.
GSH is the most abundant antioxidant in the cell, yet clinical trials targeted at inhibiting GSH synthesis alone have been met with little success\(^8^4\). We demonstrate here the capacity of cancer cells to survive impaired GSH synthesis due to compensation by the TXN antioxidant pathway. This compensation is similar to that seen with oncogenic pathways, such as when tumors become resistant to anti-EGFR therapy by acquiring activating mutations in KRAS\(^1^0^7\). A combined therapy inhibiting both GSH and TXN antioxidant pathways may yield success when applied to cancer therapy (Figure 3.7). Interestingly, both SSA and AUR, which acted synergistically with BSO in inducing cancer cell death, are FDA-approved drugs currently being used for inflammatory diseases; potentially increasing the speed by which this combined therapy could be brought into the clinic\(^1^0^8\). Further insight into the roles of other antioxidants, and how they act both alone and together, will no doubt provide important clues into novel treatments for cancer patients.

3.4 Experimental Procedures

Mice, primary cells and cell lines

Gclm\(^/-\) and MMTV-PyMT mice were generated as previously described\(^9^2,^9^3\) and kept on the identical genetic background (C57B6 background for cancer onset/burden, 129 background for BSO studies). All mice were maintained in compliance with the regulations of the University Health Network Animal Care Committee. Isolation of pMECs was performed as previously
described\textsuperscript{109}. Each data point for experiments involving pMECs refer cells isolated from a separate mouse. All pMECs were cultured for only 1 passage in serum-free medium prior to use experiments. MDA-MB-231 cells were obtained from ATCC and cultured as specified.

**Tumor onset and burden**

Gcl\textsuperscript{m/+}MMTV-PyMT and Gcl\textsuperscript{m/-}MMTV-PyMT mice (C57B6 background) were monitored weekly for palpable tumors, commencing at an age of 60 days. The experimental endpoint was defined as 160 days of age or if a humane endpoint was reached. At endpoint, all tumors from each individual mouse were resected and weighed. Data points represent a combined weight of tumors from a single mouse. Samples were divided in half, with one half being snap frozen at -80\textdegree C and the other half being fixed in 10% buffered formalin for use in histological analyses.

**BSO delivery and tumor burden**

MMTV-PyMT mice (129 background) were used for experiments involving continuous oral delivery of BSO (Sigma; B2515), which was performed as previously described\textsuperscript{94}. The
experimental endpoint was 120 days of age or earlier if a human endpoint was reached. Endpoints are earlier than in MMTV-PyMT-C57B6 mice because the former animals develop tumors sooner. At endpoint, all tumors from each individual mouse were resected, weighed and divided into two samples for further analysis as described above. Data points represent a combined weight of tumors from a single mouse.

**Histology, immunohistochemistry and pathological analysis**

Formalin-fixed tumour samples were processed and embedded in paraffin. Tissue sections (5 μm) were deparaffinized and stained with hematoxylin and eosin (H&E), anti-Ki67 antibody (Dako; Cat. No: M7249) or anti-smooth muscle actin antibody (Dako; Cat. No: M0851). Stained tissues were evaluated by light microscopy and scored by two blinded and independent pathologists (S. J. D. and A. A.).

**mRNA isolation, cDNA synthesis and quantitation**

mRNA was prepared from cell extracts using the NucleoSpin RNA II kit (Macherey-Nagel). mRNA was reverse-transcribed into cDNA using the iScriptcDNA synthesis kit (Bio-Rad). Real-time PCR analysis was performed using POWER SYBR Green PCR Master Mix and a 7900HT Fast Real-Time PCR System (Applied Biosystems).
Tumor xenografts

Severe combined immunodeficiency (SCID) mice (n=8/group) were injected into their fat pads with 1x 10^6 MDA-MB-231 cells in PBS with Matrigel. Xenograft tumors were allowed to grow to approximately 200 mm^3 before drug treatments were commenced. Tumor volumes were measured every 3 days until endpoint (day 12). Both H₂O and BSO mice were injected i. p. with vehicle control (saline solution). At endpoint, tumors were excised, weighed and processed for further analysis as described above.

Cell counting, apoptosis and ROS determination

Cells were cultured for indicated times and stained with trypan blue to determine viability. Viable cells were counted using a Vi-Cell XR cell viability analyzer (Beckman Coulter) to establish growth curves. Apoptotic cells were measured by staining with AnnexinV and 7-AAD (BD Biosciences), followed by flow cytometric measurement of fluorescence using a FACSCalibur (Becton Dickinson). To determine relative ROS levels, cells were stained with CM-H₂DCFDA (Invitrogen) and subjected to flow cytometric analysis. ROS levels are reported as the mean fluorescence intensity (MFI) expressed relative to the control value.
RNA interference

MDA-MB-231 cells were transiently transfected with siRNA (ON-TARGETplus, SMARTpool, Thermo Scientific) against XCT (L-007612-01-0020) or GCLM (L-011670-01-0020) using Lipofectamine 2000 (Invitrogen).

Bioinformatic analysis

The NEJM295 $^{110}$ and Perou $^{111}$ datasets were independently analyzed to determine GCLM mRNA expression. Patient survival was examined using Cox regression analysis of survival times vs. gene expression log ratios, followed by the estimation of false discovery rates using the Benjamini and Hochberg method. Kaplan-Meier curves were generated for each dataset for illustration purposes by rank-ordering the log ratios and splitting them into three equal groups. The curves for the highest and the lowest tertiles were displayed on the plots shown. Analysis of the TCGA invasive breast carcinoma data $^{97}$ was conducted on processed data downloaded through UCSC Cancer Genome Browser portal. Relative mRNA expression values were identified for the genes XCT, TXN1, and TXNRD1 genes. The Mann-Whitney test was used to determine the significance of changes observed in tumor versus normal tissue, and the expression data was visualized as box plots. Oncomine datasets and box plots were obtained from www.oncomine.org $^{112}$. 
Imaging and quantification of ductal outgrowth

Mice were assessed for their stage in the estrus cycle as previously described\textsuperscript{109}. The #4 glands were excised, mounted, stained with carmine (Sigma) and visualized. Ductal outgrowth was measured as the distance from the lymph node to the leading edge of the mammary ducts.

GSH and NADP+/NADPH determination

For GSH measurements, 0.5-2x10\textsuperscript{6} cells were lysed by two freeze/thaw cycles and GSH was assayed using the Bioxytech GSH/GSSG-412 kit (OxisResearch). To determine the NADP\textsuperscript{+}/NADPH ratio, cells (2x10\textsuperscript{6}) were processed using the Fluoro NADP\textsuperscript{+}/NADPH detection kit (Cell Technology).

Statistics

Kaplan-Meier survival plots and statistics were created and analyzed using GraphPad PRISM-5 software. The two-tailed Student t-test with the standard error of the mean (S.E.M.) and 2-way ANOVA was used to evaluate differences.
Chapter 4: Future Directions

Throughout the course of my Ph. D. studies I have made an effort to explore the importance of antioxidants to tumorigenesis. Specifically, I have focused on two subjects: 1) investigation into a novel regulator of antioxidant signaling, PTPN12; 2) characterization of the inhibition of antioxidants, including GSH, in cancer cells. We have found that PTPN12 can reduce ROS levels by promoting FOXO activation, thereby supporting cancer cell survival under high oxidative stress conditions. Additionally, we have found that GSH synthesis is essential for cancer initiation, yet dispensable once malignant transformation has occurred. Compensation from inhibition of GSH synthesis is driven by the TXN antioxidant pathway, and combined inhibition of both GSH and TXN pathways leads to a synergistic cancer cell death. While these results are a strong step in the right direction for elucidating the role of antioxidants in cancer, there still remain several questions to be answered.

4.1 The role of PTPN12 and other PTPs in antioxidant regulation

We chose to investigate whether the phosphatase PTPN12 had a role in reducing ROS levels. The motivation for this investigation was that PTPs are targeted by ROS for inactivation and if they could reduce ROS levels, they could subsequently prevent their own inactivation. We chose to investigate PTPN12 and found that it promoted activation of the FOXO family of antioxidant transcription factors. By supporting FOXO signaling, PTPN12 could reduce ROS levels and support cancer cell survival under increased oxidative stress. Although we focused on PTPN12, we believe that numerous other PTPs may have a role in modulating ROS levels, especially since ROS can inactivate numerous PTPs. Furthermore, how exactly these PTPs modulate an effect
on ROS levels must also be explored. Although we found that PTPN12 has an indirect effect on ROS regulation, via FOXO signaling, other studies have indicated a more direct role for PTPs. Indeed, there has been evidence showing that phosphorylation of the antioxidant enzyme PRDX1 inhibits their activity; suggesting that dephosphorylation by PTPs could activate PRDXs and reduced ROS levels\(^{113}\). A detailed examination of both direct and indirect role of PTPs in antioxidant regulation still remains to be undertaken.

The exact role of PTPN12 in tumorigenesis also remains to be determined. As previously mentioned, an independent study suggested PTPN12 acts as a tumor suppressor, yet we provide evidence implicating PTPN12 in promoting tumorigenesis\(^{85,114}\). The role of PTPN12 may in fact be both, as has been shown with other factors such as NF-κB\(^{115}\). The more outstanding issue which remains is the expression of PTPN12 in breast cancer tissue. A previous study by Sun et al. suggested that 60% of TNBC had no detectable protein expression of PTPN12. We found that not only was PTPN12 protein expression present in all breast tumors, but TNBC had the highest frequency of elevated PTPN12 expression (greater than 60%). Our results have been substantiated by an independent finding which showed more than 60% of patients with TNBC had high PTPN12 protein expression\(^{116}\). Due to these conflicting results, a more in-depth analysis, with a greater number of patient samples and multiple PTPN12 antibodies, is required to understand the actual expression of PTPN12 in breast cancer patients.

4.2 The role of GSH synthesis, driven by GCLM, in tumorigenesis

We chose to investigate the impact of GSH, the most abundant antioxidant within the cell, on tumorigenesis. While there had been literature suggesting that GSH was crucial to cancer cell
survival, the genes promoting GSH synthesis, GCLM and GCLC, had never been explored in this context. We examined Gclm⁻/⁻ mice, which lack 75% GSH levels compared with wildtype mice, and found that loss of GCLM lead to a delay of tumor onset, reduced tumor burden and prevention from malignant transformation. We showed that once transformation has occurred though, GSH synthesis is dispensable due to compensation from the TXN antioxidant pathway. Although we demonstrated that combined inhibition of GSH and TXN antioxidant pathways could synergistically inhibit survival of transformed cancer cells \textit{in vitro} and \textit{in vivo}, there remains several interesting questions that must be addressed.

Antioxidants have been shown to have an impact on supporting stem cell self-renewal\textsuperscript{117, 118}. Notably, a recent publication specifically showed that NRF2 supports hematopoietic stem cell (HSC) self-renewal\textsuperscript{119}. While we saw normal mammary gland development in Gclm⁻/⁻ mice, we have not specifically investigated the self-renewal capacity of mammary stem cells from these mice. Furthermore, a detailed examination of the hematopoietic system from Gclm⁻/⁻ mice is also warranted.

Ideally, for an investigation surrounding GSH synthesis, one would examine GCLC and not GCLM, since the catalytic subunit drives the reaction, and loss of GCLC would prevent any GSH synthesis from occurring in the cell. Initially this was not possible, as Gclc⁻/⁻ mice, which possess systemic loss of the GCLC gene, are embryonically lethal. This prompted us to investigate Gclm⁻/⁻ mice, which are viable and have no overt phenotype. Interestingly, a Gclc\textsuperscript{0/0} mouse, which has the GCLC gene deletion under control of the CRE recombinase, has been generated so that GCLC can be deleted in a tissue-specific manner\textsuperscript{120}. Furthermore, these mice, crossed to a mouse with Cre recombinase under control of the albumin promoter, leading to a liver-specific GCLC deletion, die soon after birth, indicating that they may have a stronger
phenotype than Gclm−/− mice in respect to tumorigenesis. By crossing these mice to a mammary epithelial cell specific-Cre recombinase (i.e. MMTV-Cre), we can examine the impact of complete loss of GSH synthesis on both mammary gland formation and additionally, tumorigenesis.

It would be very interesting to examine the co-dependence of GSH and TXN antioxidant pathways using genetic models. Specifically, we would like to cross the GCLM−/− mice to a mouse which has a deficiency in cystine import and/or TXN generation. Previously, our lab generated CD44−/− mice to investigate its role in immunology. Recently, specific CD44 isoforms have been identified which promote cystine import through the stabilization of XCT glutamate/cystine transporter. Additionally, we found Cd44 mRNA expression increased in Gclm−/− cells, potentially as a means of compensating for the reduction in GSH levels. We plan to breed the CD44−/− mice to Gclm−/− mice and examine not only normal organ development, but also the impact of genetically inhibiting both GSH and TXN antioxidant pathways on tumorigenesis.

Finally, it would be a worthwhile endeavor to examine the importance of GSH synthesis and GCLM to other tumor types. We have chosen to use mammary carcinogenesis as a template for examine tumorigenesis mainly due to the previous evidence implicating oxidative stress in the regulation of mammary cell survival. Examining other tumor models, both solid tumors (pancreatic, lung) and liquid (leukemia, lymphoma), in the context of antioxidant inhibition would be very interesting.
References


Figure S1 PTPN12 levels are comparable in *Ptpn12*-RE and *Ptpn12*-WT MEFs. Immunoblot detecting PTPN12 protein in extracts of *Ptpn12*-KO, *Ptpn12*-RE and *Ptpn12*-WT MEFs. Actin, loading control.
Figure S2 PTPN12 regulates mRNA expression of known FOXO target genes. Total RNA preparations from *Ptpn12*-RE and *Ptpn12*-KO MEFs were analyzed by quantitative RT-PCR to detect mRNAs for the known FOXO target genes *Trail* and *Bim*. Data are the mRNA level housekeeping gene *Rps9* and are expressed as the mean ± SEM (*p<0.05; **p<0.01; ***p<0.001).
Figure S3 Knockdown of PDK1 does not influence ROS levels in Ptpn12-KO MEFs.
Transient knockdown of PDK1 was achieved using siRNA in Ptpn12-RE and Ptpn12-KO MEFs. (a) Total RNA preparations from Ptpn12-RE and Ptpn12-KO MEFs with siControl or siPDK1 were analyzed by quantitative RT-PCR to detect mRNA Pdk1. Data are the mRNA level housekeeping gene Rps9 and are expressed as the mean ± SEM. (b) Intracellular ROS levels in Ptpn12-RE and Ptpn12-KO MEFs with siControl or siPDK1 culture for 48 hrs in Gln-free medium. Results are expressed as ROS levels relative to siControl Ptpn12-RE MEFs and based on mean fluorescent intensity (MFI).
Figure S4 Expression of FOXO3a:A3 does not rescue cell death. (a) Total RNA preparations from Ptpn12-RE and Ptpn12-KO MEFs transient transfected with control or FOXO3a:A3 were analyzed by quantitative RT-PCR to detect mRNAs for Bim. Data are the mRNA level housekeeping gene Rps9 and are expressed as the mean ± SEM. (b) Cells from (a) were cultured in Gln-free media for 72 hrs and cell death was analyzed by PI staining.
Figure S5 Analysis of PTPN12 protein in tumor sections from breast cancer patients. Tumor samples from 240 breast cancer patients were subjected to immunohistochemistry to detect PTPN12 protein. (a) Percentages of patients with grade 1, grade 2 or grade 3 breast tumors showing high or low PTPN12 expression. (b) Percentages of patients with breast tumors of the indicated sizes showing high or low PTPN12 expression. No statistically significant differences were observed.
Figure S6 PyMT-Gclm+/− mice have delayed tumor onset but similar tumor burden. (A) Tumor-free survival curves of PyMT-Gclm+/+ (n = 36) and PyMT-Gclm+/− (n = 20) mice. (B) At endpoint (Day 160), all tumors from PyMT-Gclm+/+ (n = 32) and PyMT-Gclm+/− (n = 21) mice were resected and weighed. Each data point represents the combined weight of tumors from a single mouse. For all Supplementary Figures Error bars represent ± sem. n. s., not significant. Gclm+/− MMTV-PyMT mice show a delay in tumor onset, but no reduction in overall tumor burden. (a) Tumor-free survival curves of Gclm+/+ MMTV-PyMT (n = 36) and Gclm+/− MMTV-PyMT (n = 20) MMTV-PyMT mice. (b) At endpoint (Day 160), all tumors from Gclm+/+ MMTV-PyMT (n = 32) and Gclm+/− MMTV-PyMT (n = 21) mice were resected and weighed. Each data point represents the combined weight of tumors from a single mouse. For all Supplementary Figures: *, p<0.05; **, p<0.01; ***, p<0.001; n. s., not significant. Error bars represent ± sem.
Figure S7 Validation of Nrf2$^{+/+}$ cells. (A-B) Primary MEFs from Nrf2$^{+/+}$ and Nrf2$^{-/-}$ mice were harvested and mRNA expression was determined by quantitative real-time PCR (qRT-PCR) for validated Nrf2 target genes *Nqo1* (A) and *Hmox1* (B). mRNA expressed relative to housekeeping gene *Rps9*. *** p<0.001; ** p<0.01.
Figure S8 GCLM and XCT act together to reduce ROS levels. (A-B) MDA-MB-231 human breast cancer cells were treated with control siRNA (siControl), or with siRNA against GCLM (siGCLM), XCT (siXCT) or both. Cells were harvested and mRNA expression for GCLM (A) and XCT (B) were determined by qRT-PCR. (C) ROS levels were determined for cells from (A-B) and expressed relative to siControl. ***, p<0.001; **, p<0.01; *, p<0.05; n. s. , not significant.
Figure S9 GSH and NADPH antioxidant pathways do not act in synergy. (A-B) MDA-MB-231 cells were not treated (NT) or treated with BSO (480 µM) and total GSH levels (B) and the NADP+/NADPH ratios (B) were determined (n.d.; not detectable). (C) MDA-MB-231 cells were treated with 6-aminonicatinamide (6-an; 250 µM), an inhibitor of the pentose phosphate pathway, or BSO (150 µM) or both. Cells were stained on day 4 for ROS levels. ***, p<0.001; n. s., not significant.
Figure S10 GCLM expression is increased in multiple tumor types. Oncomine tumor datasets were examined for GCLM expression in normal and tumor tissue from (A) colon, (B) esophagus, (C) lung, (D) pancreas and (E) prostate.