REGULATION of xCT BY NRF-2 IN BREAST CANCER CELLS
NRF-2 TRANSCRIPTIONALLY UPREGULATES xCT IN BREAST CANCER CELLS

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TITLE: REGULATION of xCT BY NRF2 IN BREAST CANCER CELLS

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Abstract

Cancer cells adapt to high levels of oxidative stress in order to survive and proliferate, making the transcription factors involved in antioxidant defence regulation targets of interest. The transcription factor NF E2 Related Factor 2 (NRF-2) regulates cellular defence genes including those encoding intracellular redox-balancing proteins such as enzymes involved in glutathione metabolism. Glutathione in particular is an important intracellular antioxidant molecule. NRF-2 binds to the Antioxidant Response Element (ARE) in the promoter of its target genes. Under basal conditions, Kelch-like ECH-associated protein 1 (KEAP1) acts as an inhibitor that targets NRF-2 for ubiquitination. During oxidative stress, NRF-2 dissociates from KEAP1 and enters the nucleus to bind to the ARE sequence.

It is hypothesized that the elevated Reactive Oxygen Species may be depleting the glutathione levels within the cancer cell. System x_c is a cystine/glutamate antiporter that exports glutamate while importing cystine to synthesize glutathione. In response to oxidative stress, the cells increase system x_c activity in order to provide cystine for glutathione synthesis. There is evidence that expression of xCT, the specific subunit of system x_c, is regulated by NRF-2. However this has not yet been demonstrated in human breast cancer cells, which is the focus of this project.

Basal expression of NRF-2, KEAP1 and xCT was characterized in three breast cancer cell lines (MDA-MB-231, MCF-7 and T47D) and compared to two non-cancer cell lines (184B5 and MCF10A). Basal protein levels of NRF-2 and KEAP1 showed no differences between cell lines. Basal protein levels of xCT were increased in MCF10A cells than T47D cells. MCF-7 cells were treated with hydrogen peroxide (H_2O_2) resulting in NRF-2 protein accumulation in the nucleus. With H_2O_2 treatment, xCT mRNA levels increased in MCF-7 cells. Additionally, transient overexpression of NRF-2 increased extracellular glutamate levels in MCF-7 cells. These data support a model that under oxidative stress, NRF-2 is localized to the nucleus and transcriptionally upregulates xCT. This is the first study in which the regulation of xCT has been linked to oxidative stress via NRF-2 in human breast cancer cells.

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<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>4F2hC</td>
<td>4F2 heavy chain</td>
</tr>
<tr>
<td>8-OH-dG</td>
<td>8-Hydroxy-2’-deoxyguanosine</td>
</tr>
<tr>
<td>A/A</td>
<td>antibiotic/antimycotic</td>
</tr>
<tr>
<td>AARE</td>
<td>Amino Acid Response Element</td>
</tr>
<tr>
<td>AKT</td>
<td>protein kinase B</td>
</tr>
<tr>
<td>AMPK</td>
<td>5’-AMP-activated Protein Kinase</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of Variance</td>
</tr>
<tr>
<td>AP-1</td>
<td>Activator protein 1</td>
</tr>
<tr>
<td>ARE</td>
<td>Antioxidant Response Element</td>
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<tr>
<td>ARPE</td>
<td>Retinal Pigment Epithelial cell</td>
</tr>
<tr>
<td>ASK1</td>
<td>ApoptosisSignal-regulating Kinase 1</td>
</tr>
<tr>
<td>ATCC</td>
<td>American Type Culture Collection</td>
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<tr>
<td>ATF4</td>
<td>Activating Transcription Factor 4</td>
</tr>
<tr>
<td>ATM</td>
<td>Ataxia Telangiectasia Mutated kinase</td>
</tr>
<tr>
<td>Bach 1</td>
<td>BTB and CNC homology 1</td>
</tr>
<tr>
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<td>BTB and CNC homology 2</td>
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<tr>
<td>BHK21</td>
<td>Bronchoalveolar lavage</td>
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<tr>
<td>BPE</td>
<td>Syrian Hamster kidney cells</td>
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<tr>
<td>BSA</td>
<td>Bovine Pituitary Extract</td>
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<tr>
<td>BSO</td>
<td>Bovine Serum Albumin</td>
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<tr>
<td>BTB</td>
<td>Broad complex, Tramtrack and Bric-a-brac</td>
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<td>CDDO-Im</td>
<td>2-cyano-3,12-dioxooleana-1,9(11)-dien-28-oic acid imidazolides</td>
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<td>cDNA</td>
<td>Complementary DNA</td>
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<tr>
<td>CEF</td>
<td>Ceftriaxone</td>
</tr>
<tr>
<td>c-fos</td>
<td>FBJ murine osteosarcoma viral oncogene homolog</td>
</tr>
<tr>
<td>c-jun</td>
<td>JUN proto-oncogene</td>
</tr>
<tr>
<td>CO₂</td>
<td>Carbon dioxide</td>
</tr>
<tr>
<td>COS-7</td>
<td>CV-1 Origin carrying SV40 genetic Material</td>
</tr>
<tr>
<td>COPD</td>
<td>Chronic Obstructive Pulmonary Disease</td>
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<tr>
<td>Cul3</td>
<td>Cullin-3</td>
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<tr>
<td>CuZnSOD</td>
<td>Copper Zinc Superoxide Dismutase</td>
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<tr>
<td>CXCL14</td>
<td>Chemokine (C-X-C motif) ligand 14</td>
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<tr>
<td>DaDs</td>
<td>Diallyl-Disulfide</td>
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<td>dbCAMP</td>
<td>Bucladesine</td>
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<tr>
<td>DEM</td>
<td>Diethyl maleate</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s Modified Eagle’s Medium</td>
</tr>
<tr>
<td>DMF</td>
<td>Dimethylformamide</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl Sulfoxide</td>
</tr>
<tr>
<td>Term</td>
<td>Description</td>
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<tr>
<td>---------------------------</td>
<td>---------------------------------------------------------------</td>
</tr>
<tr>
<td>ECL</td>
<td>Enhanced luminol-based Chemiluminescent substrate</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EEAT3</td>
<td>Excitatory Amino Acid Transporter</td>
</tr>
<tr>
<td>EGF</td>
<td>Epidermal Growth Factor</td>
</tr>
<tr>
<td>eIF2α</td>
<td>Eukaryotic translation initiation factor 2 subunit 1</td>
</tr>
<tr>
<td>EpRE</td>
<td>Electrophile Response Element</td>
</tr>
<tr>
<td>ER</td>
<td>Estrogen Receptor</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular-Regulated Kinase</td>
</tr>
<tr>
<td>ESCC</td>
<td>Esophageal Squamous Cell Carcinoma</td>
</tr>
<tr>
<td>ETC</td>
<td>Electron Transport Chain</td>
</tr>
<tr>
<td>ETS</td>
<td>Protein C-ets-1</td>
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<tr>
<td>FADH₂</td>
<td>Flavin Adenine dinucleotide</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal Bovine Serum</td>
</tr>
<tr>
<td>FMN</td>
<td>Flavin Mononucleotide</td>
</tr>
<tr>
<td>Fyn</td>
<td>Proto-oncogene tyrosine-protein kinase Fyn</td>
</tr>
<tr>
<td>GCL</td>
<td>Glutamate Cysteine Ligase</td>
</tr>
<tr>
<td>GCLc</td>
<td>Glutamate Cysteine Ligase catalytic unit</td>
</tr>
<tr>
<td>GCLm</td>
<td>Glutamate Cysteine Ligase modifier unit</td>
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<tr>
<td>GM-CSF</td>
<td>Granulocyte Macrophage Colony-Stimulating Factor</td>
</tr>
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<td>GPE-1</td>
<td>GST P enhancer 1</td>
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<td>GPx1</td>
<td>Glutathione Peroxidase 1</td>
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<td>Glutathione Peroxidase 4</td>
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<tr>
<td>GR</td>
<td>Glutathione Reductase</td>
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<td>Glutathione</td>
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<tr>
<td>GSSG</td>
<td>Glutathione disulfide</td>
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<tr>
<td>GST</td>
<td>Glutathione S-Transferase</td>
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<tr>
<td>H₂O₂</td>
<td>Hydrogen peroxide</td>
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<tr>
<td>hEGF</td>
<td>Human endothelial growth factor</td>
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<td>HePTP</td>
<td>Hematopoietic Tyrosine Phosphatase</td>
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<td>HIF-1α</td>
<td>Hypoxic Inducible factor 1 alpha</td>
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<tr>
<td>HMOX-1</td>
<td>Heme oxygenase-1</td>
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<tr>
<td>HRP</td>
<td>Horse radish Peroxidase</td>
</tr>
<tr>
<td>HUVEC</td>
<td>Human Umbilical Vein Epithelial cells</td>
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<td>IFN-γ</td>
<td>Interferon Gamma</td>
</tr>
<tr>
<td>IGF-1</td>
<td>Insulin-like growth factor 1</td>
</tr>
<tr>
<td>IGF-IR</td>
<td>Insulin-like growth factor 1 receptor</td>
</tr>
<tr>
<td>IκBα</td>
<td>nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha</td>
</tr>
<tr>
<td>IκBβ</td>
<td>nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, beta</td>
</tr>
<tr>
<td>IKKα</td>
<td>IKB Kinase alpha</td>
</tr>
<tr>
<td>IL-1B</td>
<td>Interleukin 1B</td>
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<tr>
<td>IL-6</td>
<td>Interleukin 6</td>
</tr>
<tr>
<td>IL-8</td>
<td>Interleukin 8</td>
</tr>
<tr>
<td>Inos</td>
<td>Nitric oxide Synthase</td>
</tr>
<tr>
<td>IP-10</td>
<td>C-X-C motif Chemokine 10</td>
</tr>
</tbody>
</table>

X
IRS-1.......................................................Insulin Receptor substrate 1
IVR............................................................intervening region
JAK2..............................................................Janus Kinase 2
JNK.............................................................Jun N-Terminal Kinase
KC.............................................................Keratinocyte chemoattractant
KEAP1.......................................................Kelch-like ECH-Associated Protein 1
LCK..........................................................lymphocyt-specific protein tyrosine Kinase
LPS...........................................................lipopolysaccharide
MaF.......................................................musculoaponeurotic fibrosarcoma oncogene homolog K
MAPK......................................................Mitogen Activated Protein Kinase
MCL-1....................................................myeloid leukemia cell differentiation protein
MCP..........................................................Membrane Cofactor Protein
MEF.........................................................Mouse Embryonic Fibroblasts
MG132......................................................carbobenzyox-Leu-Leu-leucinal
mGDPH..................................................mitochondrial glycerol-3-phosphate dehydrogenase
MIP 2α......................................................Macrophage inflammatory protein 2-alpha
MMP........................................................Matrix Metalloproteinases
MnSOD.....................................................Manganese Superoxide Dismutase
MRP........................................................Multi-drug Resistance Protein
MtDNA..................................................mitochondrial DNA
NADPH...............................................Reduced Nicotinamide Adenine Dinucleotide Phosphate
NaH₂PO₄................................................Monosodium Phosphate
ND6............................................................NADH dehydrogenase 6
NF-E2......................................................Nuclear Factor (Erythroid-derived 2)
NFκB.......................................................Nuclear Factor kappa B
NIH........................................................National Institute of Health
NOX.........................................................NADPH Oxidase
NOX-1.....................................................NADPH Oxidase 1
NOX-4.....................................................NADPH oxidase 4
NRF-1......................................................NF-E2 related factor 1
NRF2......................................................NF-E2 related factor 2
NRF3......................................................NF-E2 related factor 3
NRP/B......................................................Nuclear Restrict Protein in Brain
NQO1.....................................................NAD(P)H Quinone Oxidoreductase 1
OA-NO₂..................................................Nitro-oleic Acid
OCT-1...................................................Octamer Binding Protein 1
p21/CIP1..................................................cyclin-dependent kinase inhibitor 1
p38..........................................................P38 mitogen activated protein Kinase
p53..........................................................Tumour protein p53
PBS..........................................................Phosphate buffered saline
PCB29-pQ..................................................2,3,5-trichloro-6-phenyl-[1,4]benzoquinone
PCR..........................................................Polymerase Chain Reaction
PDGF......................................................Platelet-derived Growth Factor
PI3K......................................................Phosphatidylinositol 3-kinase
PI3P......................................................Phosphatidylinositol (3,4,5)-trisphosphate
PKC..........................................................Protein Kinase C
PKD1 .................................................. Protein Kinase Cµ
PLC ..................................................................Phospholipase C
PPP ............................................................Pentose Phosphate Pathway
Prxl ............................................................. Peroxiredoxin 1
PTEN .......................................................... Phosphatase and tensin homolog
PTP .............................................................. Protein Tyrosine Phosphatase
PVDF .......................................................... Polyvinylidene fluoride
RAC ............................................................. Receptor Adenylate Cyclase protein
rBAT ................................................................ related to b0,+ amino acid transporter
RBX1 .......................................................... ringbox protein 1
RCS .............................................................. Reactive Chloride Species
RIPA ........................................................... Radioimmunoprecipitation assay
RNA ........................................................... Ribonucleic Acid
RNS ........................................................... Reactive Nitrogen Species
ROS ............................................................ Reactive Oxygen Species
RSS ............................................................ Reactive Sulphur Species
RT PCR ....................................................... Real Time Polymerase Chain Reaction
(S)-4-CPG .................................................... (S)-4-Carboxyphenylglycine
SDS ............................................................. Sodium Dodecyl Sulfate
SFN ............................................................. sulforaphane
SHP-1 ........................................................ Src homology region 2 domain-containing phosphatase
SNAP ........................................................ soluble NSF attachment-proteins
SOD ............................................................. Superoxide Dismutase
Syk ............................................................. spleen tyrosine kinase
TBHQ ........................................................ tert-butylhydroquinone
TBST ........................................................ Tris Buffered Saline with Tween 20
TGF-B ........................................................ Transforming Growth Factor B
TNBC ........................................................ Triple Negative Breast Cancer
TNF-α ........................................................ Tumour Necrosis Factor Alpha
TPA ............................................................. 12-o-tetradecanoylphorbol-13-acetate
TRE ............................................................. TPA responsive element
TRIS ........................................................ Tris(hydroxymethyl)aminomethane
Trx ............................................................. Thioredoxin
UGT ............................................................. UDP-glucoronsyltransferase
UV ............................................................... Ultraviolet Radiation
VEGF ........................................................ Vascular Endothelial Growth Factor
VSMC ........................................................ Vascular Smooth Muscle cells
xCT/SLC7a11 ................................................ solute carrier family 7, membrane 11
y-GCS ........................................................ y-glutamylcysteine synthetase
Introduction

Cancer research is multi-faceted. The primary interest is in identifying potential therapeutic targets and seeking a cure. However while we continue seeking a cure we need to develop strategies that could improve quality of life symptoms such as pain, depression and fatigue for patients living with the disease. My research project is focused on developing an understanding on why cancer induces pain. In my laboratory we have identified a potential culprit, namely the secretion of glutamate from cancer cells. Cancer-induced pain has been demonstrated to be a common debilitating symptom in breast cancer metastasis (Coleman et al., 2006). However, the underlying molecular mechanism of this pain remains to be understood. Oxidative stress has long been associated with cancer, and has been described as a factor in some of the aberrant gene expression seen in certain cancers (Balendrin et al., 2004). In addition, one of the suggested factors underlying this cancer pain is the extra cellular release of glutamate from tumour cells (Ungard et al., 2013). It has been hypothesized that this extracellular release of glutamate may be driven by the reactive oxygen species in cancer cells. The mechanism between the extracellular release of glutamate and the oxidative stress inside the cell is not yet elucidated, although it is hypothesized that it may be facilitated by the membrane transporter System $\text{x}_c^-$. My project will focus on investigating a redox sensitive transcription factor that regulates the gene expression of a number of antioxidant enzymes including the membrane transporter System $\text{x}_c^-$ in the context of breast cancer.
Forms of Oxidative Stress in Cancer

Oxidative stress occurs in cells that undergo oxidative metabolism, and can exist in different forms including reactive nitrogen species (RNS), reactive oxygen species (ROS), reactive sulphur species (RSS), and reactive chloride species (RCS) (Bannister, 2002). ROS is the most abundantly produced form of oxidative stress and includes hydrogen peroxide, ozone, hydroxide ions and singlet oxygen (Simic et al., 1989). ROS is produced by metabolic processes like the electron transport chain (ETC) (Veskoukis et al., 2012). ROS acts a signaling molecule between the mitochondria and nucleus; in healthy cells small amounts of ROS are beneficial (Scarpulla, 2002).

Although at low levels ROS is required for healthy cell signaling, higher levels of ROS can promote proliferation and initiate carcinogenesis (Preston et al., 2001; Policastro et al., 2004; Balendirn et al., 2004; Brigelius-Flohe et al., 2009). Typically high levels of ROS can induce apoptosis through mitochondrial release of cytochrome C and nitrogen oxidase synthase activity (Nazarewics et al., 2007). However, it seems that in cancer cells ROS levels may be high enough to promote proliferation but not induce apoptosis. Additionally, that altered signaling pathways may allow cancer cells to evade apoptosis (Mochizuki et al., 2006).

In cancer cells, ROS levels are elevated above normal levels and can damage lipids, protein, and DNA (Veskoukis et al., 2012). Various types of cancer cell lines have been demonstrated to produce large amounts of H2O2, including pancreatic, melanoma, colon, ovarian and breast cancer cell lines (Szatrowski et al., 1991). In breast cancer tissues, higher concentrations of 8-hydroxy-2’-deoxyguanosine (8-OH-dG), 2,6 diaminio-4 hydroxy-5-formamidopyrimidine, and 8-hydroxyadenine modifications were found in
ductal carcinoma tissue, taken from women compared to adjacent non-cancerous control tissues (Malins et al., 1991). ROS induced DNA lesions such as 8-OH-dG, have been demonstrated to be more abundant in colon, lung, stomach, ovary and brain tissues compared to cancer free adjacent tissue controls (Olinski et al., 1992; Jaruga et al., 1994).

Elevated ROS in normal cells increase tumourigenicity (Policastro et al., 2004). Elevated ROS in cancer cells have also been demonstrated to result in a more aggressive phenotype more prone to metastasis (Arbiser et al., 2002). Treating breast cancer cells (PDV, PB, MCF-7) with catalase, an H₂O₂ detoxifying enzyme, inhibited proliferation and anchorage independent growth (Policastro et al., 2004). Mitochondrial function was required for tumourigenesis in vivo and ROS generated from the Qₒ site of Complex III is required for anchorage independent growth in osteosarcoma 143B cells (Weinberg et al., 2010). ROS can signal in a number of ways to promote tumourigenicity and proliferation. In addition to creating genome instability (both nuclear and mitochondrial) through mutations, ROS can signal through various signaling Mitogen Activated Protein Kinase (MAPK) pathways and affect transcription factor activity such as tumour protein p53 (p53), Nuclear Factor kappa B (NFκB), and hypoxic inducible factor 1 alpha (HIF-1α) (Verschoor et al., 2010).

A number of growth factors can signal through ROS to induce cell proliferation. In rat vascular smooth muscle cells (VSMCs), platelet derived growth factor (PDGF) induced cell proliferation through H₂O₂ mediated MAPK signaling (Sundaresan et al., 1995). In COS-7 and NIH3T3 cells, PDGF, vascular endothelial growth factor (VEGF) and epidermal growth factor (EGF) transactivation required mitochondrial electron transport chain function to activate jun n-terminal kinase (JNK) and protein kinase B
(AKT) signaling pathways through H$_2$O$_2$ production (Chen et al., 2004). ROS derived from mitochondria signaled through the extracellular-regulated kinase (ERK) pathway in colon cancer cells (Weinberg et al., 2010).

In COS-7 cells treatment with copper, a metal demonstrated to produce ROS, induced JNK and P38 mitogen activated protein kinase (p38) activity (Mattie et al., 2008). In prostate carcinoma cells, H$_2$O$_2$ activated ERK, p38 and JNK (Jung et al., 2008). In rat fibroblasts, H$_2$O$_2$ scavenging catalase inhibited ERK, JNK and p38 MAPK phosphorylation and inhibited cell proliferation (Preston et al., 2001). The proposed mechanism for the activation of MAPK through ROS is the inactivation of protein tyrosine phosphatases. In Jurkat cells, H$_2$O$_2$ induced phosphorylation of all three MAPK (ERK, p38 and JNK), by inhibiting 3 protein tyrosine phosphatases (PTPs): protein tyrosine phosphatase receptor type c (CD-45), Src homology region 2 domain-containing phosphatase (SHP-1) and hematopoietic tyrosine phosphatase (HePTP) (Lee et al., 2002). SHP-1 targeted ERK and JNK but not P38 phosphorylation (Lee et al., 2002). HePTP targeted ERK and P38 phosphorylation but not JNK (Lee et al., 2002). This is corroborated in rat brain tissue where H$_2$O$_2$ and glutathione disulfide (GSSG), the oxidized form of glutathione, inhibited MAPK phosphatase activity (Foley et al., 2004). There is also evidence for other kinases involved in ROS induced MAPK activity. ROS induced phosphorylation of all 3 MAPK signaling required lymphocyte-specific protein tyrosine kinase (LCK) and proto-oncogene tyrosine-protein kinase Fyn (Fyn) (Lee et al., 2002). H$_2$O$_2$ induced ERK phosphorylation was dependent on phospholipase c (PLC) and protein kinase c (PKC) (Lee et al., 2002). Protein kinase c$_{\mu}$ (PKD1) also inhibits p38 phosphorylation by H$_2$O$_2$ (Song et al., 2008).
In HT1080 fibrosarcoma cells, H$_2$O$_2$ activates AKT through inhibition of phosphatase and tensin homolog (PTEN), an antagonist of phosphatidylinositol 3-kinase (PI3K), the upstream activator of AKT (Connor et al., 2005). Activation of AKT increased VEGF expression and angiogenesis in vivo (Connor et al., 2005). In NIH3T3 cells, EGF stimulation increased ROS levels, which oxidized PTEN inducing PIP3, and downstream AKT (Kwon et al., 2004).

Activator Protein 1 (AP-1), is a transcription factor composed of FBJ murine osteosarcoma viral oncogene homolog (c-fos) and JUN proto-oncogene (c-jun) subunits that controls the regulation of cell stress response and growth signaling. Hydrogen peroxide induced AP-1 activation resulting in increased chemokine (C-X-C motif) ligand 14 (CXCL14), a protein that enhanced migration and cell motility in weakly metastatic MCF-7 breast cancer cells through Ca$^{2+}$ signaling (Pelicano et al., 2009). Copper (Cu$^{2+}$), a metal able to produce ROS, induced AP-1 sequence binding in COS-7 cells (Mattie et al., 2008). H$_2$O$_2$ increased AP-1 upregulation of GADD153, a gene important for cellular response and growth (Guyton et al., 1996). In MCF-7 breast cancer cells that were resistant to Tamoxifen, an ROS inducing anticancer drug, AP-1 transcription was increased (Schiff et al., 2000). c-Jun was required for hypoxia induced resistance to etoposide, an anticancer drug that acts on redox sensitive transcription factors (Cosse et al., 2009). In contrast, a lower ratio of reduced to oxidized glutathione inhibited c-Jun DNA binding through disulfide formation with a cystine residue on c-Jun (Klatt et al., 1999). In human hepatoma HepG2 cells, c-Fos and c-Jun binding was enhanced in reducing environments through cysteine residues in the DNA binding regions (Abate et al., 1990).
NFκB is a stress response protein that can be induced by a number of oxidative stressors (as reviewed by Paul, 1999). In human myeloid KBS-5 cells, H$_2$O$_2$ increased NFκB phosphorylation and nuclear translocation through spleen tyrosine kinase (Syk) phosphorylation of the tyrosine residue of nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha (IκBα) (Takada et al., 2003). H$_2$O$_2$ could not activate NFκB in Syk deficient cells such as MCF-7 cells and HT1299 cells (Takada et al., 2003). In MCF-7 cells, ROS induced NFκB activity and translocation through IKB Kinase alpha (IKKα) and IkBα phosphorylation (Li et al., 2006). In mouse epidermal cells, antioxidants inhibit NFκB and subsequent cancer cell transformation through nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, Beta (IκBβ) phosphorylation at Serine 32 residue (Nomura et al., 2000). Once IκBβ is phosphorylated, it is signaled for degradation and releases NFκB, which translocates from the cytosol into the nucleus (Nomura et al., 2000). In RIE-1 small intestine cells, H$_2$O$_2$ induced NFκB activity through phosphorylation of IκBα, dependent upon PKD1 (Song et al., 2008). The cysteine 62 residue is a key residue for DNA binding, alterations resulted in DNA dissociation and altered binding site recognition of the κB motif (Matthews et al., 1993).

Another redox sensitive transcription factor is the tumour suppressor p53, which has proapoptotic effects (Cosse et al., 2009). In HepG2 cells hypoxia decreased binding activity and target gene expression (Cosse et al., 2009). Oxidation of p53 reduced its ability to form tetramers potentially through oxidizing the cysteine residues (CYS182) on its surface resulting in reduced capacity to bind to DNA and upregulate proapoptotic genes (Sun et al., 2003). In human umbilical vein epithelial cells (HUVECs), oxidative
stress through hydrogen peroxide or UV radiation, increased phosphorylation of Ser-15 and subsequent p53 activation and cell cycle apoptosis, through the ataxia telangiectasia mutated kinase (ATM), independently of MAPK, ERK, PI3K (Chen et al., 2003).

HIF-1α is a transcription factor that is activated by low oxygen levels and controls the cells survival response to hypoxia (Guzy et al., 2005). In human 143B, osteosarcoma cells, H$_2$O$_2$ generated from mitochondria stabilized HIF-1α in hypoxic conditions (Guzy et al., 2005). In human hepatoma 3B cells, stabilization of HIF-1α required H$_2$O$_2$ production by mitochondria (Chandel et al., 2000). Exogenous H$_2$O$_2$ was also able to stabilize HIF-1α in normoxic conditions (Chandel et al., 2000). Antimycin A, an ROS inducer, and other glutathione depleting reagents also induced HIF-1α in normoxic conditions (Chandel et al., 2000). In prostate carcinoma cells (DU145), ROS prevented ubiquitination of HIF-1α resulting in stabilization and upregulation of downstream target VEGF, mediated by JNK, Janus Kinase 2 (JAK2), and 5’-AMP-activated protein Kinase (AMPK) signaling (Jung et al., 2008).

In contrast, overexpression of catalase (CAT), a scavenger of H$_2$O$_2$ had no effect on HIF-1α stabilization in hypoxic conditions, suggesting that HIF-1α stabilization was independent of ROS (Srinivas et al., 2001). Active receptor adenylate cyclase protein (RAC) stimulated ROS in HepG2 rat hepatocytes, which diminished nuclear HIF-1α and activity (Gorlach et al., 2003). HIF-1α induction was prevented by H$_2$O$_2$ pretreatment in HELA cells (Huang et al., 1996). In human endothelial cells, ROS reduced hypoxia induced HIF-1α stabilization (Chang et al., 2005).

Taken together this evidence demonstrates that ROS can signal in a variety of cell types to determine cell stress response and potentially promote tumourigenesis.
Sources of ROS

There are various sources of ROS in cells, including mitochondria, NOX systems, and hypoxia (Flohé et al., 2009).

In healthy cells, mitochondria containing the Electron Transport Chain (ETC) complexes oxidize reduced nicotinamide adenine dinucleotide phosphate (NADPH) and flavin adenine dinucleotide (FADH$_2$) and oxygen to H$_2$O in order to synthesize ATP, supplying the cell with energy. However, mitochondria leak oxygen radicals through complexes I and III, increasing ROS levels (Loschen et al., 1974; Gyulkhandanyan et al., 2004; Weinberg et al., 2010). The flavin mononucleotide (FMN) of Complex I is a physiologically relevant sources of ROS (Liu et al., 2002). Complex I produced ROS more readily at a lower threshold of inhibition than Complex III and Complex IV, even though they produced larger amounts of ROS at higher levels of inhibition (Sipos et al., 2003). Complex I inhibition in MCF-7 breast cancer cells increased cell motility and invasiveness through ROS production (Pelicano et al., 2009).

As mitochondria become more dysfunctional through mutations or ROS damage, they can leak more ROS (Czarnecka et al., 2006). Lung cancer carcinomas with high metastatic potential had increased ROS levels caused by reduced complex I activity resulting from mitochondrial DNA (mtDNA) mutations in ND6 (NADH dehydrogenase subunit 6) (Ishikawa et al., 2008). The metastatic phenotype was lost when the mtDNA was replaced with that from a low metastatic potential cancer cell, demonstrating a relationship between mtDNA mutations and metastasis. Highly metastatic fibrosarcoma B82M cells showed complex I defects, however it was not found in LuM1 colon adenocarcinoma cells. The study further elucidated that the metastatic potential may be
dependent on ROS levels, as pretreatment with ROS scavenging molecules abolished the metastatic phenotype. MtDNA mutations resulting in increased ROS in MDA-MB-231 breast cancer cells transferred the metastatic phenotype to low metastatic potential HeLa cells (Ishikawa et al., 2008). Both prostate and breast cancer cells became more invasive when depleted of mtDNA (Naito et al., 2008). They also demonstrated mesenchymal markers such as E-cadherin, and increased activities of MAPK, JNK and TGF-B. In p53 tumour suppressor knockout mice, mtDNA was reduced by 50% and increased ROS levels (Lebedeva et al., 2009). MtDNA mutations causing increased ROS also upregulated MCL-1, HIF-1α and VEGF, markers of invasiveness in tumours (Ishikawa et al., 2008).

In prostate cancer cell lines, mitochondrial glycerophosphate dehydrogenase (mGDPH), another source of mitochondrial ROS, was upregulated compared to normal prostate cell lines (Chowdhury et al. 2005; Chowdhury et al., 2007). It has also been demonstrated that in healthy tissues, despite low content of mGDPH, its contributions to ROS per unit were relatively large (Mráček et al., 2009).

NADPH oxidases (NOX) are expressed in a number of types of cells. Each type of the four NOXs have differential tissue distributions, and differential regulation in cancer cells has been demonstrated (Cheng et al., 2001). NOX- 4 is dysregulated in cancer colon cancer and glioblastoma cells (Cheng et al., 2001). In NIH3T3 cells, NOX1 overexpression increased ROS (Kwon et al., 2004). In human endothelial cells NOX increased ROS (Chang et al., 2005). In MCF-7 cells Interleukin 1B (IL-1B), a proinflammatory cytokine, stimulation produced ROS via NADPH oxides (Li et al., 2006). ROS derived from NOX play a role in signaling pathways in pancreatic cancer.
cells and malignant hairy cells. (Mochizuki et al., 2006; Kamiguti et al., 2005) These signaling pathways included apoptosis Signaling Kinase (ASK1) signaling pathways, VEGF and VEGF receptors, matrix metalloproteinases (MMP), and PTPs (Arbiser et al., 2002; Yoon et al., 2002; Mochizuki et al., 2006).

Hypoxia can also contribute to ROS in cells (Guzy et al., 2005). In human hepatoma 3B cells, hypoxia stimulated ROS production by mitochondria through complex III (Chandel et al., 2000). One mechanism through which hypoxia contributes to ROS formation is the inhibition of Complex III in hypoxic osteosarcoma 143B cells (Guzy et al., 2005). Another study, however, failed to find ROS in prostate cancer after hypoxia treatment for an hour (Jung et al., 2008).

**Antioxidant Molecules and Enzymes**

Oxidative stress depends on the balance between ROS and antioxidants (Veskoukis et al., 2012). Some endogenous antioxidants include glutathione, alpha lipoic acid, coenzyme Q, ferritin, uric acid, bilirubin, metallothionein, enzymatic superoxide dismutase (SOD), and CAT (Yoshida et al., 2003). These compounds among others, act to scavenge ROS and prevent cellular damage. Oxidative stress caused by imbalances between ROS and antioxidant enzymes have been found in many different types of cancer including breast cancer (Policastro et al., 2004) and prostate cancer (Arsova-Sarafinovska et al., 2009). This dysregulation in antioxidant molecules and enzymes depends on the tumour type and the antioxidant enzyme.

Antioxidant activities were measured in lung cancer patients and SOD, CAT and GPx activities were lower in cancerous tissues than non-cancerous control tissue (Pawel,
et al., 1999). In lung cancer tissues, CAT activity was lower than control non tumour tissues (Ho et al., 2001). In breast cancer tissues, CAT and GPx were negatively correlated with malignancy (Policastro et al., 2004).

In contrast, the levels of Manganese Superoxide Dismutase (MnSOD), Copper Zinc Superoxide Dismutase (CuZnSOD) and CAT were up regulated in prostate cancer cell lines (Chowdhury et al., 2007). In lung cancer tissues, SOD activity was increased (Ho et al., 2001). Additionally, MnSOD protein levels were higher in tumour tissues, while there were no significant differences in CuZnSOD protein levels (Ho et al., 2001). This dysregulation in tumours was further suggested by correlations between SOD and CAT in tumour free tissues that were not found in tumour tissues (Ho et al., 2001). Lung A549 cells treated with inflammatory cytokines such as Tumour Necrosis Factor Alpha (TNF-α), IL-1B, or Interferon gamma (IFN-γ) demonstrated increased SOD activity (Ho et al., 2001). Of the SOD enzymes, MnSOD protein expression increased, although CuZn SOD failed to increase (Ho et al., 2001). In breast cancer tissues, SOD was positively correlated with malignancy (Policastro et al., 2004). In tamoxifen resistant breast cancer cells treated with estrogen, SOD activity was increased (Schiff et al., 2000). This suggests that antioxidant enzyme balances with ROS may be specific to different cancers depending on tissue and cell types.

Tumours also have been shown to affect circulating antioxidant levels. In thyroid cancer, circulating SOD activity was unaffected but GPx activities were lower in cancer patients (Akinci et al., 2008). In patients with prostate cancer, lower erythrocyte circulating GPx and SOD activities were measured in prostate cancer patients (Arsova-Sarafinovska et al., 2009).
**Glutathione Pathway and Cancer**

The glutathione pathway is one of the key ROS scavenging pathways in a number of different tissue and cell types. The glutathione pathway is reviewed in (Balendirin et al., 2004). In brief, glutathione is a tripeptide consisting of cysteine, glutamate and glycine. The rate limiting enzyme Glutamate-cysteine ligase (GCL), ligates cysteine and glutamate into $\gamma$-glutamyl-cysteine. Glycine is then ligated by GSH synthetase (GS) producing glutathione. Glutathione can scavenge radical from organic molecules by glutathione s-transferases (GST). Glutathione peroxidase (GPx) couples the oxidation of GSH to GSSG, with the reduction of $\text{H}_2\text{O}_2$. GSSG then can be reduced back to GSH by glutathione reductase (GR), or exported by multidrug resistance proteins (MRPs). This pathway is summarized by Figure 1.1.
Dysregulation of the glutathione pathway has been implicated and investigated in many disease pathologies including Alzheimer's, Parkinson's, various other neurological disorders, various immune disorders including COPD, and cancer (Morris et al., 2014). It follows that if ROS levels are elevated in cancer cells that glutathione levels, being the main scavenger of ROS would be altered as the cell adapts in order to survive.

GSH levels have been demonstrated to be elevated in various cancer tissues from patients including leukemia (Joncourt et al., 1995), colon (Berger et al., 1994), breast...
(Perry et al., 1993) and squamous cell lung cancer (Cook et al., 1991). So it appears that although healthy cells may detoxify carcinogens using GSH, cancer cells may use high levels of GSH as a survival mechanism. Tumour cells with high levels of GSH may also become chemoresistant (Kim et al., 2008). Ovarian chemoresistant cells had ten-fold higher levels of GSH (Britten et al., 1992). Although inhibiting GSTs had no effect on cellular response to chemotherapeutic drugs, depleting GSH levels with Buthione Sulfoxamine (BSO) sensitized resistant tumours to cisplatin (Zhang et al., 2001). After long-term combination chemotherapy, GSH levels were elevated in resistant lung squamous cell carcinoma cells (Kawai et al., 2002). GSH was lower in tamoxifen resistant MCF-7 treated with estrogen compared to non-resistant cells (Schiff et al., 2000).

Glutathione Peroxidases are a group of enzymes that catalyze the reaction of GSH and $\text{H}_2\text{O}_2$ and other electrophiles. A number of different isoforms of GPx have been associated with tumour progression and promotion. Of the GPx isoforms, GPx2 and GPx4 seem to be the most important although they have opposite roles in cancer promotion (Brigelius-Flohe et al., 2009).

GPx2 has been implicated in malignant growth (Brigelius-Flohe, 2008) and advanced stages of colorectal adenomas (Chu et al., 2004). GPx2 levels have been demonstrated to be elevated in squamous cell epithelial carcinomas (Serewko et al., 2002), breast cancer (Naiki-Ito et al., 2007), and colorectal cancers (Mork et al., 2000; Lin et al., 2002; Florian et al., 2001; Murawaki et al., 2008). In colon cancer, GPx2 was transiently upregulated in the early stages of transformation (Florian et al., 2001). Nrf-2 upregulates GPx2, in addition to other genes that are required for cancer cells to
proliferate (Banning et al., 2005). In MCF-7 breast cancer cells, GPx2 overexpression was able to protect cells from oxidative stress induced apoptosis (Yan et al., 2006). GPx2 knockdown in cells decreased anchorage independent aggressive growth despite increasing the tumours ability to migrate and invade in vivo in nude mice (Banning et al., 2008).

GPx4 is a lethal knockout demonstrating its importance as an antioxidant defence (Imai et al., 2003; Yant et al., 2003). There are 3 forms of GPx4, depending on whether it is located in the cytosol, nucleus or mitochondria (Imai et al., 2003). GPx4 protects healthy cells from UV damage (Wenk et al., 2004) and cells overexpressing it were more resistant to oxidative stress induced apoptosis (Ran et al., 2007). GPx4 is inversely correlated with pancreatic cancer (Liu et al., 2006), breast tumour grade (Cejas et al., 2007), and fibro sarcomas (Heirman et al., 2006). GPx4 also decreased metastatic potential of fibrosarcoma cells (Heirman et al., 2006) and MMP1, a marker of invasion and migration, in dermal fibroblasts (Wenk et al., 2004).

In general, it is hard to make a simple statement as to whether GPx enzymes have anti tumour or tumour promoting effects as it depends on the stage of cancer, and the isoform of GPx. With GPx4 clearly being anti tumourous and GPx2 being pro cancerous.

GST is an enzyme that conjugates GSH with xenobiotics and other electrophiles. GSTs seem to play a dual role in both cancer prevention and promotion. As a chemo preventive enzyme, GST polymorphisms, such as GSTM1 and GSTM3, have been correlated inversely with lung cancer and bladder cancer risk (Nakajima et al., 1995; Inskip et al., 1995). GSTP activity has been inversely correlated with tumour development in the gastrointestinal mucosa (Peters et al., 1990), but there was no lower
correlation found in lung cancer patients (Harris et al., 1998). Individuals with increased risk of skin basal cell carcinomas also demonstrated a correlation with GST polymorphisms (Strange et al., 2001). In contrast, tumour cells have increased GST expression in multidrug resistant cancer cell lines (Meijerman et al., 2008; Schiesselbauer et al., 1990; Ban et al., 1996). GST-P1 was upregulated in hepatocellular carcinoma cells (Ikeda et al., 2004). Upon treatment of estrogen in tamoxifen resistant MCF-7 breast cancer cells, GST activity was upregulated (Schiff et al., 2000).

**NRF-2**

The transcription factor NF E2 Related Factor 2 (NRF-2) has been called the master regulator of antioxidant defences. It was originally identified as a member of the ETS family of transcription factors because it responded to ETS binding sites (Viribasis and Scarpulla, 1991). It is now considered part of the “cap ‘n’ collar” subgroup of the basic region leucine zipper transcription factors, a family of 6 transcription factors including NF-E2, NRF1, NRF2, NRF3, Bach 1 and Bach 2 (Zhang, 2006). NRF1 is ubiquitously expressed, and essential, as knock mouse models were embryonic lethal (Chan et al., 1998). NRF 2 is also ubiquitously expressed but not essential, as knockout mouse models resulted in only mild anemia and excessive bleeding (Chan et al., 1996). NRF-3 is only expressed in placenta, and knockout models of mice developed with no obvious phenotypic results (Derjuga et al., 2004).

**NRF-2 target genes and pathway**

Nrf2 is an important regulator of a variety of genes, it was recently discovered that carriers of a minor allele of a NRF-2 SNP had 20% reduced mortality in an 18 year follow-up study in the general population (Figarska et al., 2014). NRF2 regulates a large
variety of genes, including groups of cellular defence genes including intracellular redox-balancing proteins such as GCL, GPx, Thioredoxin (TRX) and Heme Oxygenase-1 (HMOX-1). It also regulates Phase I and II detoxifying proteins including GST, MRP, NAD(P)H quinone oxidoreductase (NQO1), and UDP-glucuronosyltransferase (UGT).

Of particular interest to our lab, however, is its regulation of xCT, which will be discussed later (Shih et al., 2003). Additionally, NRF-2 has been demonstrated to reprogram a number of metabolic pathways in cancer cells, including enzymes in the Pentose Phosphate Pathway (PPP), NADPH production, glutamine metabolism, and enzymes involved in glycolysis (Mitsuishi et al., 2012).

Recent research has demonstrated a role for NRF-2 in the inflammation response and inflammatory pathologies such as Multiple Sclerosis and Chronic Obstructive Pulmonary Disorder (Nguyen et al., 2009; Zhang et al., 2006). Diallyl-Disulfide (dads), induced NRF-2 and downstream HMOX-1 expression and activity, which attenuated inflammation through NFκB, Nitric oxide Synthase (iNOS) and Matrix Metalloproteinases (MMP9) pathways (Shin et al., 2013). Andrographolide, an inducer of NRF-2, suppressed inflammation in oxidative lung injury. By upregulating NRF-2 target genes GCLm, GCLc, GR, GPx-2 and HMOX-1, suppressing inflammatory genes such as IL-1B, IFN-γ-inducible protein 10 (IP-10), monocyte chemoattractant protein1 (MCP), Keratinocyte chemoattractant (KC), Granulocyte macrophage colony-stimulating factor (GM-CSF), TNF-α, and Macrophage inflammatory protein 2-alpha (MIP 2α) resulting in lower inflammatory cell and neutrophil counts in bronchoalveolar lavage (BAL) fluid (Guan et al., 2012). An andrographolide derivative also increased NRF-2 and HMOX-1, in an ERK dependent manner. Treatment with the derivative attenuated liposaccharide
(LPS) induced TNF-α, IL-1B, and Interleukin 6 (IL-6) production (Zhang et al., 2013). Another study demonstrated that andrographolide induced GSH in lung oxidative stress suppressed the release of NFkB mediated inflammatory cytokines IL-6, and Interleukin 8 (IL-8) (Li et al., 2013). NRF-2 reduced the inflammatory response in lung fibroblasts exposed to oxidative stress through suppressing CCL11/Eotaxin-1, a chemokine that recruits eosinophils to the lung (Fourtounis et al., 2012).

**Antioxidant Response Element**

As a transcription factor, NRF-2 regulates gene expression by binding to the Antioxidant Response Element (ARE) also known as the Electrophile Response Element (EpRE) in the promoter of its target genes. A number of studies have confirmed variations of the ARE sequence as seen in the TABLE 1.1. The general consensus on the sequence of the ARE is TGACNNNGC, although some have suggested variations of extended sequences including RTGABnnnGCR (IUPAC) (Vollrath et al., 2006) and TMAnnRTGAYnnGCRwww (IUPAC) (Wasserman et al., 1997). The GC box at the 3’ end of the ARE was necessary for oxidative inducibility (Nguyen et al., 1994). Additionally, NRF-2 binding sites demonstrate overlap with other transcription factors. One study found that knockdown of NRF-2 was compensated for by NF-E2, suggesting that the family of proteins may share binding sequences (Chan et al., 1996). Another study found that the NRF-2 binding sequence on the NQO1 promoter contained two AP-1 sequences (Venugopal et al., 1996). The AP-1 binding site and NRF-2 binding sites on the HMOX-1 promoter have significant homology (Inamadar et al., 1996). NRF-2 was able to bind to two TPA Responsive Elements (TRE) binding sequences, typically related
to AP-1 binding, and a GPE-1 enhancer element binding sequence in the GST-P promoter (Ikeda et al., 2004). These results seem to indicate that NRF-2 may be able to activate antioxidant defence and Phase II detoxifying genes through AP-1 sequences.
Table 1.1: Experimentally determined Antioxidant Response Element sequences

The consensus ARE sequence is TGACNNNGC, although longer versions have been proposed. It also shares much similarity with the AP-1 and NF-E2 sequences, and subsequently binding can occur between the three transcription factors.
NRF-2 Regulation

NRF-2 is regulated through multiple mechanisms, including phosphorylation, signaling pathways and binding proteins.

NRF-2 can be activated through phosphorylation. It seems that the importance of phosphorylation of NRF-2 on its activity depends on which residues are being phosphorylated. One study confirmed Mitogen Activated Protein Kinases (MAPK) mediated phosphorylation of NRF-2 at multiple serine or threonine residues (S215, S408, S558, T559 and S577). Phosphorylation also was demonstrated to enhance nuclear levels of NRF-2. However this same study found that mutating those phosphorylation sites caused only slight decreases in NRF-2 transcriptional activity. Only a slight decrease in NRF-2 target gene mRNA was measured with a mutational loss of phosphorylation and it had no effect on NRF-2 protein levels (Sun et al., 2009). Another study confirmed this finding in HepG2 cells. Cells were treated with PCB29-pQ, an inducer of ROS that activated NRF-2 and caused localization to the nucleus. However this inducer failed to phosphorylate JNK, ERK and p38 MAPK, suggested their limited involvement in NRF-2 (Li et al., 2014). In contrast, NRF-2 failed to localize to the nucleus, when HepG2 cells were treated with PKC δ inhibitors (Huang et al., 2000). This was later confirmed when PKC δ phosphorylation of the serine 40 residue on NRF-2 was demonstrated to be necessary for TBHQ induced NRF-2 nuclear translocation and activity in liver (HepG2, Hep1) and breast (MCF-7 and BT549) carcinomas (Niture et al., 2009).

NRF-2 activation has also been linked to PI3K pathways. In HepG2 cells treated with PCB29-pQ, an inducer of ROS, AKT phosphorylation positively correlated with NRF-2 induction and HMOX-1 protein levels (Li et al., 2014). When the same HepG2
cells were treated with PI3K inhibitors, total and phosphorylated AKT decreased along with HMOX-1 protein levels, implying that AKT was necessary for HMOX-1 induction by NRF-2 (Li et al., 2014). In lung A549 cells, 293, Jurkat and Mouse Embryonic Fibroblasts (MEF), AKT activation was associated with NRF-2 metabolic reprogramming (Mitsuishi et al., 2012).

Although there are multiple mechanisms of NRF-2 regulation, the predominant mechanism is through Kelch-like ECH-Associated Protein 1 (KEAP1). KEAP1 has two important characteristics in NRF2 regulation, firstly it can sense ROS in the cell and secondly it directly interacts with NRF-2. As an inhibitor of NRF-2, KEAP1 acts as a cellular switch that can sense ROS and turn the NRF-2 antioxidant response on or off. The mechanism underlying this ROS sensing switch has been investigated in vitro in both mouse and human, and been demonstrated to rely on redox sensitive cysteine residues that can react with protein thiols. In mouse KEAP1, the cysteine residues C257, C273, C288 and C297 have been demonstrated to react with thiol reagents (Dinkova-Kostova et al., 2005). In human KEAP1, the cysteine residues C151, C288 and C297 have been demonstrated to react with thiol reagents (Eggler et al., 2005). The residues demonstrated to have the most functional importance in regulating NRF2 transcriptional activity were C151, C273, and C288 (Zhang and Hannick., 2003; Levonen et al., 2004). In mouse embryonic fibroblasts, the Cys151 residue was necessary for NRF-2 activation by a number of inducers including tert-butylhydroquinone (TBHQ), diethyl maleate (DEM), sulforaphane (SFN), dimethylformamide (DMF) but not 2-cyano-3,12-dioxooleana-1,9(11)-dien- 28-oic acid imidazolides (CDDO-Im), cadmium chloride (CdCl2), nitro fatty acids (OA-NO₂), suggesting that a number of KEAP1 cysteine residues may act as
sensors (Takaya et al., 2012). In NIH3T3 cells, KEAP1 required Cys 273 and Cys 288 for the ubiquitination and degradation of NRF-2 (Kobayashi et al., 2006). In zebrafish, different inducers interacted with different cysteine residues including Cys 151, Cys 273, Cys 288, Cys 297, Cys 489 suggesting multiple mechanisms for KEAP1 sensing oxidants (Kobayashi et al., 2009).

KEAP1 directly associates with NRF-2 in the cytoplasm. Acting as a substrate adaptor, it ligates NRF-2 with the Cullin 3 (Cul3)-ringbox protein 1 (Rbx1) E3 ligase resulting in NRF-2 ubiquitination and subsequent proteasomal degradation (Zhang et al., 2004). The KEAP1-NRF-2 complex was previously thought to exist exclusively in the cytoplasm, however recent evidence shows that the complex can shuttle between the cytoplasm and nucleus (Zhang et al., 2004; Eggler et al., 2005). Under basal conditions the NRF-2-KEAP1 complex is either targeted for ubiquitination or shuttling back and forth between the cytoplasm and nucleus. However, even in the nucleus, the transcriptional activity of NRF-2 is still repressed by KEAP1 (Zhang et al., 2006). Under conditions of oxidative stress or chemical induction of NRF-2, KEAP1 undergoes a post translation modification in its linker region, changing its structural conformation. This change forms a suboptimal conformation of KEAP1-CUL3-RBX1, resulting in NRF-2 stabilization (Zhang et al., 2004; Eggler et al., 2005). NRF-2 then saturates the KEAP1 E3 ligase complex, and free NRF-2 is free to translocate to the nucleus where it can induce expression of target genes. Upon treatment of TBHQ in human liver and breast cancer cell lines, NRF-2 could only dissociate from KEAP1 if it was phosphorylated at serine residue 40 (Niture et al., 2009).
The importance of translocation of NRF-2 has been debated in a variety of papers. It was previously thought that NRF-2 was bound to its inhibitor protein KEAP1 in the cytoplasm and then released under oxidative stress. However, that has been debated as Kobayashi and others (2006) demonstrated that oxidative stress produces of NRF-2 through *de novo* synthesis, rather than liberating it from KEAP1.

In addition to KEAP1, there are a number of proteins in that can bind to NRF-2 or KEAP1 to affect NRF-2 activation. The cell cycle protein, p21, was shown to stabilize NRF2 through direct interaction with residues in the DLG and ETGE domains which are critical for KEAP1 dependent ubiquitination (Chen et al., 2009). Nuclear Matrix Protein (NRP/B), a member of the kelch-related β-actin binding proteins, was overexpressed in MDA-MB-231 breast cancer cells and localized to the nucleus. It was demonstrated to co-localize with NRF-2 to the nucleus and increase binding on the NQO1 ARE reporter (Seng et al., 2007). Hydrogen peroxide treatment increased NRP/B and NRF-2 levels and co-immunoprecipitation associations (Seng et al., 2007). The subunits of AP-1, c-Jun and c-Fos, have both been demonstrated to enhance NRF-2 binding (Jeyapaul et al., 2000). NRF-2 has also been demonstrated to complex with MafK proteins in order to bind to the ARE as well (Ikeda et al., 2004).

**NRF-2 and Cancer**

Cancer cells have demonstrated aberrant expression and activity of NRF-2 in multiple cell lines and types, implicating NRF-2 in carcinogenesis and promoting the survival of cancer cells. NRF-2 has also been found to be overexpressed in MDA-MB-231 breast cancer cells (Seng et al., 2007) and in late stage cancer tissue (Wang et al., 2008). However, NRF-2 was shown to be more highly expressed in MCF-7 breast cancer
cells than MDA-MB-231 breast cancer cells (Alwi et al., 2012). NRF-2 was found to be higher in normal breast epithelial cell lines compared to 7 out of the 10 cancer cell lines that were studied, including MDA-MB-231 cell lines, MCF-7, & ZR-75 (Loignon et al., 2009). The three breast cancer cell lines that demonstrated higher NRF-2 levels compared to normal epithelial cells were MDA MB 435, BT20 and T47D cells (Loignon et al., 2009). A number of lung cancer cell lines and lung tumour tissue samples showed increased NRF-2 protein levels, and nuclear localization (Singh et al., 2006).

NRF-2 was found to be more highly expressed in esophageal and skin squamous cell carcinomas (Kim et al., 2010). In tumour tissues from breast cancer patients, tumour tissue demonstrated lower levels of NRF-2 than the surrounding normal tissue samples (Loignon et al., 2009). NRF-2 greatly accelerated tumour growth, and NRF-2 siRNA decreased tumour growth mediated through the PPP (Mitsuishi et al., 2012). Silencing CUL3, leading to high NRF-2 activity led to increased resistance of breast cancer cells to Doxorubicin and Paclitaxel (Loignon et al., 2009).

KEAP1 has also been demonstrated to be aberrantly expressed in cancer. Proteasome inhibitor, MG132, increased NRF-2 levels in breast cancer cells in MCF-7 cells, indicating that the KEAP1 and CUL3 complex was functional (Loignon et al., 2009). CUL3 was high in cancer cell lines that were reported to have low NRF-2 expression (Loignon et al., 2009). KEAP1 mRNA was lower in 3 lung cancer cell lines (A549, H1435, and H838) (Singh et al., 2008). However in breast cancer patients, no differences were found in KEAP1 expression in tumour tissues compared to surrounding normal tissues (Loignon et al., 2009).
In addition to varying levels of NRF-2 and KEAP1, aberrant activation of this pathway can result from somatic mutations. There are more documented mutations of KEAP1 associated with cancer, than there are of NRF-2. Mutations in KEAP1 were found in gastric, hepatocellular, colorectal, lung, breast, and prostate carcinomas (Yoo et al., 2012). In breast cancer and lung cancer cells, KEAP1 mutations were found that impaired their ability to repress NRF-2 (Padmanabhan et al., 2006; Nioi et al., 2007). The mutation of the cysteine 23 (C23Y) on KEAP1 in the n-terminal allowed KEAP1 to interact with NRF-2, although KEAP1 could not facilitate the ubiquitination of NRF-2 (Nioi et al., 2007). NRF-2 activation, measured by ARE promoter activity, was not inhibited by the mutated KEAP1. This mutation, C23Y, was found in three breast cancer cell lines (MDA-MB-435, BT20 and T47D) expressing high levels of NRF-2 (Loignon et al., 2009). KEAP1 mutations were found in a number of lung cancer cell lines and tumour tissue samples (Singh et al., 2006). A number of the deletions in the tumour tissue samples showed non-conservative mutations in the IVR and BTB domains, and 3 tumours showed deletion mutations in the IVR and KELCH domains. Three cancer cell lines had mutations that could not repress NRF-2 activity.

A meta-analysis found that 213 somatic mutations in KEAP1 were present in 17 cancer types and multiple cell lines, including breast, colorectal, gastric, kidney, liver, lung, squamous cell carcinomas, ovary and breast cancers (Hast et al., 2014). Of these mutations, the 18 mutations found in squamous cell carcinomas were further investigated in vitro. Of these 18 mutations, 2 mutations resulted in a truncated protein and 16 mutations were missense mutations. None of the mutations were in the NRF-2 binding regions of KEAP1, but 3 mutations altered cysteine residues. Of the 18 mutations,
overexpression of 3 mutations (N469fs, P318fs and G333C) abolished suppression of NRF-2 activity, 3 had no effect and 11 retained partial suppression ability suggesting that KEAP1 mutations can lead to NRF-2 over activity. Of the 3 KEAP1 mutations that lost the ability to suppress NRF-2, they were expressed at lower levels, despite similar transfection efficiencies and were unable to bind to NRF-2. Seven of the mutations resulted in enhanced NRF-2 binding. Super-binding KEAP1 mutants were unable to suppress NRF-2, because the NRF-2/KEAP1 complex was stabilized resulting in increased levels of free NRF-2.

Lung cancer mutations found in G430 and G364 residues of KEAP1, showed dominant negative phenotypes that were unable to dimerize and repress NRF-2 in transgenic mice (Suzuki et al., 2011). Another study found patients with pulmonary papillary adenocarcinomas had KEAP1 truncation mutations that resulted in increased NRF-2 nuclear localization (Li et al., 2011). Mutations in KEAP1 were correlated with mutations in another tumour suppressor, p53 (Li et al., 2011). Lung cancer cell lines with a KEAP1 mutations resulting in increased NRF-2 activity and upregulated MRP1 and MRP2, showed resistance to etoposide and carboplatin (Singh et al., 2006). Although more rare, NRF-2 mutations in the DLG and ETGE regions necessary for interaction with KEAP1, were found in lung, head and neck and skin squamous cell carcinomas (Kim et al., 2010).

The role of NRF-2 activity has also been demonstrated in many cancer types through upregulated target gene expression, including HMOX-1, Prx1, GPx, GSTs, GCL, MRP, and NQO1.
As reviewed extensively earlier, enzymes involved in glutathione metabolism have been associated with cancer and drug resistance. These enzymes have been associated with cancer as a result of NRF-2 activation including GSTs (Ikeda et al., 2004; Meijerman et al., 2008; Schiesselbauer et al., 1990; Ban et al., 1996; Loignon et al., 2009; Singh et al., 2006), GPx2 (Chu et al., 2004; Brigelius-Flohe, 2008), and GCL (Iida et al., 1999; Loignon et al., 2009).

Additionally, other Phase II detoxifying genes have been associated with cancer and drug resistance as a result of NRF-2 activation. NQO1, another enzyme involved in catalyzing detoxification of highly reactive quinones has been demonstrated to be highly expressed in adrenal gland, bladder, breast, colon, liver, lung, ovary and thyroid cancer (Wang et al., 2008; Schlager et al., 1990; Malkinson et al., 1992. Siegel et al., 2000; Basu et al., 2004; Singh et al., 2006; Nioi et al., 2004). Antioxidant activities of HMOX-1 have been found in many tumours to stimulate rapid growth of cancer cells, enhance resistance to stress and apoptosis, promote angiogenesis of tumours and facilitate metastasis (Jozkowicz et al., 2007). Overexpression of HMOX-1 in melanoma cells increased cell proliferation, resistance to H2O2 induced oxidative stress and increase in endothelial cell division leading to angiogenesis seen in vivo in mice injected with cells (Was et al., 2006). Thiol specific antioxidant protein, Prx-1, is expressed in lung cancer and thyroid lesions (Yanagawa et al., 1999; Kim et al., 2007). A strong correlation exists between Prx1 and recurrence (Kim et al., 2007). Lung cancer cell lines resistant to etoposide and carboplatin showed increased NRF-2 activity with upregulated MRP1 and MRP2 levels (Singh et al., 2006). From the evidence, it appears that cancer cells undergoing oxidative stress rely on increased activity of NRF-2 and its targets for survival.
**System $x_c^-$**

System $x_c^-$ is a membrane transporter protein that is expressed on various cells and tissue types, such as brain (Sato et al., 2002; Kim et al., 2001), spinal cord, immune cells (Sakakura et al., 2007) and pancreas (Kim et al., 2001). The antiporter is a chloride dependent, sodium independent anti-porter (Takada et al., 1984; Bannai et al., 1986; Gochenauer et al., 2001; Kim et al., 2001; Patel et al., 2004). It transports one glutamate amino acid for one cystine amino acid, locked in a 1:1 molar ratio (Sato et al., 1999; Kim et al., 2001). Transport is concentration dependent, as extracellular glutamate was inhibitory to transport but intracellular glutamate was stimulatory (Bannai et al., 1986). Decreased levels of cystine import by intracellular glutamate depletion were rescued by

**Figure 1.2: System $x_c^-$ Structure and Antiporter Function**

System $x_c^-$ is composed of two subunits, a heavy subunit 4F2hc and a light specific subunit, xCT. They are bound together through a disulfide bond on the extracellular surface. The antiporter exchanges 1 Glutamate (Glu) for 1 Cystine (CySS). (Adapted from Lewerenz et al, 2013).
the addition of glutamine which was quickly converted to glutamate (Bannai et al., 1986). In glioma cells, cystine-like analogues did not inhibit transport, while glutamate like analogues did inhibit transport (Patel et al., 2004).

System $x_c^-$ consists of a heavy chain 4F2hC and a specific light chain xCT (Sato et al., 2000; Sharma et al., 2010). Additionally, system $x_c^-$-like cystine uptake was observed with co expression of xCT and rBAT (related to b0,+ amino acid transporter), which has a large degree of homology to 4F2hc (Wang et al., 2003). The xCT subunit is a protein composed of 495 amino acids (human) and is 86% homologous to the mouse xCT (Sato et al., 2000; Kim et al., 2001). The mouse cDNA isolated from macrophages encodes a protein of 502 amino acids (Sato et al., 1999). Alternative splicing in the 3’ end of the transcript may result in two different isoforms differing by 13 amino acids (Kim et al., 2001). Another study isolated a cDNA from human retinal pigment cells encoding 501 amino acids (Bridges et al., 2001). Although no x-ray crystallography has been done, xCT is predicted to have 12 transmembrane domains based on membrane topology experiments (Bridges et al., 2001; Gasol et al., 2004). In reducing conditions the molecular weight of the band on an SDS-PAGE gel is 137 kDa which is shifted to 50 kDa in non-reducing conditions, suggesting a disulfide linkage (Kim et al., 2001). Two cysteine residues in the extra cellular loop of xCT were predicted to form disulfide bonds with 4F2hc (Kim et al., 2001). The human xCT also has a possible site for N-linked glycosylation (Bridges et al., 2001).
Figure 1.3 – xCT has 12 predicted transmembrane domains (Adapted from Bridges et al., 2001).

**System $x_c$ function within cellular redox status**

It is proposed that one of the main functions of System $x_c$ is to provide cysteine for the production of glutathione through cystine import. System $x_c$ function has been linked to intracellular GSH levels in a variety of cell types including neurons, fibroblasts, neutrophils and macrophages (Bannai et al., 1984; Watanabe et al., 1987; Sasaki et al., 2002; Lewerenz et al., 2008; Yuzhe et al., 2014). System $x_c$ provided neuroprotection of neurons through increasing the GSH transport of astrocyte and meningeal cells (Shih et al., 2006). GSH levels were depleted in System $x_c$ knockout neutrophils (Sakakura et al., 2007). It was also observed that System $x_c$ is induced in culture to protect cells from oxidative stress (Sakakura et al., 2007).

Neurons that were selected for resistance to oxidative stress, both glutamate oxidative toxicity and hydrogen peroxide, expressed higher levels of xCT, catalase and GPx mRNA (Lewerenz et al., 2006). In spinal cord neurons, Ceftriaxone (CEF) increased system $x_c$ activity, intracellular GSH and GSH release from cells (Lewerenz et al., 2009).
Diethyl Maleate (DEM) and other electrophilic agents increased glutamate transport and cystine uptake in human fibroblasts (Bannai et al., 1984) and xCT mRNA (Kim et al., 2001). Expression of both xCT and 4F2hc were stimulated by either lipopolysaccharide (LPS) or DEM (Sato et al., 2000). This transport required RNA and protein synthesis, suggesting transcriptional and translational upregulation of System $x^-_c$ components in response to oxidative stress (Bannai et al., 1984). Basal ROS are lower in MDA-MB-231 cells than MCF-7 cells, potentially because of xCT levels (Yuzhe et al., 2014).

**Glutamate and Cystine**

As a gradient driven antiporter, both glutamate and cystine levels are important in regulating system $x^-_c$ function. Triple negative breast cancer cells, including MDA-MB-231 cells and a variety of other TNBC cells have high levels of dependence on glutamine metabolism, resulting in high levels of intracellular glutamate (Gross et al., 2014). Cancer cells with constitutively active NRF-2 showed a shift towards glutamine metabolism (Mitsuishi et al., 2012). It is likely that this would result in accumulation of intracellular glutamate, a product of glutamine metabolism by the enzyme glutaminase. Intracellular glutamate levels were much higher than extracellular levels, driving System $x^-_c$ to transport glutamate outside the cell in rat brain (Baker et al., 2003). Intracellular glutamate concentrations of interneurons and granule cells ranged between 4-10 mM (Kvamme et al., 1985). In NIH3T3 cells, cystine import was potently inhibited by treating cells with glutamate (Sato et al., 2004). EAAT3 was shown to aid system $x^-_c$ transport by importing glutamate back into the cell to increase the gradient across the
membrane (Lewerenz et al., 2009). So, in a number of cell types, glutamate is transported from the cell because it is in excess inside the cell.

Cystine is a rate limiting amino acid in glutathione synthesis. Intracellular glutathione levels were decreased by cystine starvation in NIH3T3 cells (Sato et al., 2004). It would seem that transport of cysteine is important for synthesis, although the same study found that inhibiting glutathione synthesis had no effect on cystine transport (Sato et al., 2004). The anionic form of cystine is transported (Takada et al., 1984), potentially explaining why cystine uptake was dependent on pH in fibroblasts (Bannai et al., 1981). Cystine uptake is inhibited by extracellular glutamate (Bannai et al., 1981; Kim et al., 2001; Sasaki et al., 2002). In a study that characterized the induction of system \( x_c^- \) activity in vitro, the rate of cystine uptake can be inhibited by a number of amino acids in addition to glutamate (Watanabe et al., 1987). The Cystine/Cysteine cycle is dependent on cellular redox environment as illustrated in Figure 1.4 (Bannai et al., 1989). In addition to synthesis of glutathione the cystine/cysteine cycle can regulate cellular redox status as well (Lewerenz et al., 2013). Cystine is brought into the cell by system \( x_c^- \), where it is reduced by Thioredoxin Reductase (TRR) to 2 cysteine molecules. The cysteine molecules can be exported back out of the cell by system ASC, where two cysteine molecules can be oxidized into cystine (Lewerenz et al., 2013).
Figure 1.4 - The Cystine/Cysteine Cycle

Cystine (CySS) is brought into the cell by system \( X_c^- \), where it is reduced by Thioredoxin Reductase (TRR) to 2 cysteine (Cys) molecules. The cysteine molecules can be exported back out of the cell by system ASC, where two cysteine molecules can be oxidized into cystine (adapted from Lewerenz et al., 2013)

Regulation of System \( X_c^- \)

A number of transcription factors have been demonstrated to transcriptionally regulate System \( x_c^- \) in response to different stressors and stimuli. Nutrient deprivation has been demonstrated to play a role in System \( x_c^- \) regulation. In NIH3T3 cells xCT mRNA was strongly induced by cystine deprivation mediated through the transcription factor ATF4 binding to the amino acid response element (AARE) on the xCT promoter (Sato et al., 2004). Glucose deprivation had no effects on the xCT promoter or on system \( x_c^- \) activity (Sato et al., 2004). ATF4 upregulation was later demonstrated to be dependent on phosphorylation of eIF2\(\alpha\) in mouse fibroblasts and neuronal cell lines (HT22, PC12), by both exogenous and endogenous forms of stress (Lewerenz et al., 2008).
Diethyl Maleate and other electrophilic agents increased glutamate transport and cystine uptake in human fibroblasts (Bannai et al., 1984). This transport required RNA and protein synthesis (Bannai et al., 1984). At the transcriptional level, xCT mRNA was induced by DEM, while 4F2hc mRNA levels were relatively constant (Sasaki et al., 2002). Mutation analysis revealed that xCT promoter activity induced by DEM required an ARE in the promoter (Sasaki et al., 2002). NRF-2 levels were increased by DEM (Sasaki et al., 2002). Deleting the NRF-2 binding motif (ARE) abolished DEM induced xCT promoter activity (Lin et al., 2013). In the 5’ flanking region of the human xCT gene Ap-1 and NF-E2 potential binding sites have been found (Sato et al., 2000).

Further evidence supporting NRF-2 transcriptional regulation of xCT was loss of System x\textsubscript{c}\textsuperscript{-} activity in NRF-2 -/- mouse embryonic fibroblasts (Sasaki et al., 2004). Co-transfection of NRF-2 and the xCT promoter showed increase in promoter activity in 2 of the 4 truncations with ARE sequences (Sasaki et al., 2004). In neuronal cells, CEF increased xCT mRNA and cystine uptake, as well as NRF-2 nuclear levels (Lewerenz et al., 2009). CEF increased xCT protein and NRF-2 protein in MEFs, which was abolished in NRF-2 knockout mice (Lewerenz et al., 2009). The exact mechanism of CEF-dependent NRF-2 activation is unknown since CEF did not increase or reduce ROS in cells (Lewerenz et al., 2009). TBHQ, an NRF-2 inducer, increased cystine uptake 2 fold in MEFs (Lewerenz et al., 2008). It seems that NRF-2 only regulates xCT in response to oxidative stress. Ethanol increased xCT mRNA and protein, through inhibition of OCT-1, independently of NRF-2, ATF-4, AP1 and NFκB (Lin et al., 2013).

In addition to regulation by transcription factors, there are a number of different stressors that up regulate System x\textsubscript{c}\textsuperscript{-}. Culture conditions have effects on System x\textsubscript{c}\textsuperscript{-}
activity. Expression of xCT and 4F2hc was significantly enhanced by oxygen (Sato et al., 2000). In Human Fibroblasts, cystine uptake was increased by exposure to hyperoxia (Bannai et al., 1989). Cystine transport was higher at lower cell density (Takada et al., 1984).

System $x_c^-$ has also been linked to signaling pathways. In Insulin Receptor Substrate 1 (IRS-1) containing cells, Insulin Growth Factor 1 (IGF-1) upregulated xCT mRNA (Yuzhe et al., 2014). IGF-1 also increased xCT mRNA and protein in MCF-7 cells, but not in tamoxifen resistant MCF-7 cells lines (Yuzhe et al., 2014). Using inhibitors, the PI3K pathway was necessary for IGF-1 upregulated xCT, while the MAPK pathway was not required for upregulation (Yuzhe et al., 2014).

**System $x_c^-$ and Cancer**

System $x_c^-$ has been implicated in a number of different cancer types. The specific subunit, xCT, was expressed in 70 primary tumour samples of esophageal squamous cell carcinomas (ESCC) and it negatively correlated with 5 year survival rate (Shiozaki et al., 2014). In a number of breast cancer cells types, xCT has been upregulated. Invasive ductal and triple negative breast cancer cells also had higher xCT mRNA than ER positive cancer cells and normal tissue, and xCT was correlated with poor overall survival (Yuzhe et al., 2014). Furthermore, inhibiting xCT sensitized ER+ breast cancer to anti IGF-IR therapy. It is likely that xCT is upregulated to protect from ROS damage as xCT expression decreased ROS mediated anchorage independent growth (Yuzhe et al., 2014). Transcripts of xCT were highly expressed in glioma cells (Patel et al., 2004). Glutamine catabolism, a source of intracellular glutamate, was necessary for colony
growth and anchorage independent growth in HCT 116 colon cancer cells, providing further evidence to support xCT expression in cancer (Weinberg et al., 2010).
Rationale

Of particular interest to our lab is the small antioxidant molecule glutathione. This molecule consists of glutamate, cysteine and glycine and acts to reduce ROS through the sulfhydryl group of the cysteine residue (Somberg et al., 2009). This molecule is of interest because our group recently demonstrated that cancer cells release large amounts of glutamate into the extracellular environment (Seidlitz et al., 2009). This finding was confirmed and it was demonstrated that the release of glutamate was via System $x_c^-$, a membrane cysteine/glutamate antiporter that consists of a heavy chain, 4F2hc and a specific light chain, xCT (Sharma et al., 2010). It is hypothesized that the ROS may be depleting the glutathione within the cell. In response to this stimulus, the cell increases system $x_c^-$ activity in order to provide cysteine for glutathione synthesis.

There is evidence that system $x_c^-$ expression is controlled by NRF-2. In Syrian Hamster Kidney (BHK21) cells, System $x_c^-$ activity was upregulated in an NRF-2 dependent manner via the ARE (Sasaki et al., 2002). In rat cells, astrocytes showed increased xCT expression and neuroprotection by overexpressing NRF-2 using transfection and chemically induced overexpression using TBHQ, tert-butylhydroquinone (Shih et al., 2003). In rat cardiomyocytes, reperfusion injury was protected against by NRF-2 mediated induction of xCT (Zhang et al., 2010). My project will focus on investigating the link between intracellular oxidative stress and extra cellular glutamate release, potentially mediated by xCT induction via the NRF2/ARE in the context of breast cancer.
**Hypothesis**

It is hypothesized that the transcription factor NRF-2, is up regulated in breast cancer cells undergoing oxidative stress, resulting in upregulation of the antioxidant response. This upregulation includes the membrane antiporter System X and results in increased expression and activity, resulting in increased glutamate release.

**Objectives**

1. A. Characterize the basal protein levels of NRF-2, KEAP1 and xCT in various healthy and cancer cell lines (MDA-MB-231, MCF-7, MCF10A, T47D and 184B5).

   B. Characterize the basal glutamate release, as a measure of activity of xCT in the same cell lines.

2. Evaluate the effects of changes in NRF-2 levels/activity on xCT content and activity in human breast cancer cells (MCF-7).
Methods

Cell culture

Cells were cultured according to ATCC guidelines. MCF-7 and MDA-MB-231 cells were cultured in Dulbecco’s Modified Eagle Medium (DMEM) with 10% Fetal Bovine Serum (FBS) and 1% Antibiotic/Antimycotic (A/A). The A/A from Life Technologies contained penicillin, streptomycin, and amphotericin B. T47D cells were cultured in RPMI with 10% FBS and 1% A/A. MCF10A cells were adapted from Mammary Epithelial Growth Medium (MEGM) to DMEM with 10% FBS and 1% A/A, over a period of two weeks. 184B5 normal breast epithelial cells were cultured in MEGM with supplements at final concentrations of 13 mg/mL bovine pituitary extract (BPE), 0.5 mg/mL hydrocortisone, 10 µg/mL human epidermal growth factor (hEGF), and 5 mg/mL insulin. All cells were cultured in humidified incubators at 37°C with 5% CO₂.

Chemicals

Hydrogen peroxide (H₂O₂) was bought from Sigma Aldrich (Oakville, Ontario). MCF-7 cells were plated at either a density of 2x10⁵ cells per well in a 6 well plate or 2.2x10⁶ cells in a 10 cm dish. Cells were left overnight to attach. Media was then aspirated and cells were washed with PBS. Then cells were treated with DMEM with 10% FBS and 1% A/A with various doses of H₂O₂ for 1 hour. Media with H₂O₂ was then replaced with complete media for 1 hour of recovery before pelleting for additional analyses.
**pcDNA3-Nrf2 plasmid**

The plasmid used to overexpress NRF-2 was generously donated by Dr. Leigh Wilson (Wilson, 2005). The plasmid encoded mammalian NRF-2 (mouse), which is highly conserved with human NRF-2. It is 80% homologous to the human NRF-2.

**Transient transfections**

MCF-7 cells were plated in 2mL of DMEM (with 10% FBS, 1% A/A) overnight in 6 well dishes at a density of 2.5x10⁵ cells/well. The following day the cells were washed with PBS. Then 6 µL of Lipofectamine (Sigma) was added to 250 µL of DMEM for 5 minutes. It was then added to 4 µg of DNA in 250 µL of DMEM and allowed to mix at room temperature for 20 minutes. The 500 µL of solution was then added to 1.5 mL of media with 10 % FBS and 1% A/A. Cells were then treated with the 2 mL of the mixture for 48 hours.

**Isolation of mRNA**

At the time of harvest, media was collected for further analysis or aspirated and discarded. Cells were then washed with PBS and then aspirated. Cells were then lysed in the dish using 1 mL of Trizol. Cells were scraped and transferred to a 1.5 mL Eppendorf tube for 5 minutes at room temperature. Then 200 µL of chloroform was added to the Trizol. The tubes were inverted 10 times fast and briefly vortexed 3 times. The tubes sat at room temperature for 3 minutes. The tubes were then centrifuged at 12000 x g for 15 minutes at 4°C. The Supernatant was carefully removed and added to a new Eppendorf tube with 500 µL of ice cold 100% isopropanol and 1 µL of glycerol to aide in
precipitation. The tubes were incubated at room temperature for 10 minutes. The tubes were then moved to a -20°C freezer for 2 hours to precipitate. The tubes were then centrifuged at 12000 x g for 10 minutes at 4°C. The supernatant was carefully removed and discarded. The remaining pellet was washed with 1 mL of 75% ethanol for every 1 mL of Trizol originally added. The tube was then inverted several times and briefly vortexed 3x. The samples were then centrifuged at 7500 x g for 6 minutes at 4°C. The supernatant was carefully removed. Residual ethanol was left to evaporate off in the culture hood for 15 minutes. The pellet was dissolved in 100 µL of RNA free water (or 50 µL for small pellets) and incubated for 10 minutes at 60°C in the heat block. The RNA quality was then measured using UV Spectroscopy at 260/280 nm readings on samples diluted 1:50. The RNA samples were stored at -70°C until further use.

**Polymerase Chain Reaction**

Isolated RNA was reverse transcribed into cDNA using Superscript III enzyme and oligo(dT) primers. The cDNA was then amplified using the primers for xCT and β-Actin. β-Actin was used as a housekeeper for xCT. Transcribed DNA was used in 12.5 µL Real Time PCR mixtures, consisting of 6.25 µL SYBR Green mix (Takara), 3.25 µL Milli-Q RNAse free water, 1 µL forward primer, and 1 µL reverse primer. The reaction was quantified in a Biorad Plex machine. PCR amplification was initiated for 1 min at 95°C followed by 40 cycles of denaturation at 95°C for 10 seconds, annealing at 60°C for 25 seconds, and elongation at 72°C with photodetection.
Preparation of whole cell lysates

At the time of harvest, media was collected for further analysis or aspirated and discarded. Cells were then washed with PBS, which was then aspirated. Cells were lysed in the well or plate using 500 µL of RIPA buffer. Cells were scraped and lysis was transferred to a 1.5 mL Eppendorf tube on ice for 30 minutes at room temperature. The lysis was then sonicated at an amplitude of 40 for 15 pulses. The samples were then spun at 15 000 x g for 15 minutes at 4°C. The supernatant was transferred to a new centrifuge tube and the pellet was discarded. The protein samples were stored at 70°C.

Nuclear lysate preparation

Cells were seeded at $10^6$ cells per 10 cm dish. At the time of harvest, media was aspirated and discarded. Cells were then washed with PBS, which was then aspirated. The cells were then placed in 0.25% EDTA/Trypsin until detached. Cells were pelleted in a 15 mL falcon tube. The pellet was then resuspended in PBS in 1.5 mL Eppendorf tubes. The samples were then spun for 5 minutes at 500 x g. The supernatant was discarded. The remaining pellets were then processed using a NE-PER Nuclear and Cytoplasmic Extraction Kit (Thermo Scientific). The kit was used to lyse cells into cytosolic and nuclear fractions. The protein samples were stored at -70°C.
Protein quantitation

Protein content in lysates (both whole and nuclear/cytosolic fractions) were measured using the Bradford assay. Briefly, 10 µL of homogenate was added in triplicate to wells in a 96 well plate. Then 240 µL of 4:1 mix of double distilled H₂O and the Bradford assay reagent was added to each well. The solution was allowed to mix for 5 minutes and measured in a Biotek PowerWave XL plate reader at 570 nm. The values were converted to mg/mL by comparing them to BSA standards in serial dilutions from 2.0 mg/mL to 0.0313 mg/mL. Samples were diluted to fall within the linear range of the standard curve.

Western blot analysis

Protein content levels of NRF-2, KEAP1 and xCT were quantified using Western Blot Analysis. Cell lysates were boiled with loading buffer containing β-Mercaptoethanol at 95°C for 10 minutes. Approximately 50-100 µg of protein were loaded on to 10% SDS-polyacrylamide gels, then transferred onto PVDF membranes for blotting. Membranes were blocked with 1% milk in Tris-buffered saline with 0.1% tween (TBST) for 1 hour at room temperature, then incubated overnight in primary antibody at 4°C: NRF-2 (SC-722, 1:1000), KEAP1 (Cell Signaling 546C, 1:1000) and xCT (Abcam ab37185, 1:1000). Membranes were then washed with TBST and incubated with horseradish peroxidase (HRP) conjugated secondary antibodies targeted to rabbit at a concentration of 1:10⁴ for 1 hour at room temperature. The blots were then developed using an enhanced chemiluminescence kit (ECL plus, Amersham Biosciences, Laval, PQ, Canada). Amido black stain (Sigma) was used as a loading control.
Western Blots were quantified using the Image J software package (NIH, Bethesda, MD, USA). The bands at the molecular weight were quantified and normalized to the amido black loading control. Amido black is a whole protein stain of the gel. To quantify the amido black, the entire lane was quantified using the ImageJ software. Normalized protein levels were then compared by fold change.

**Amplex Red assay**

Media was collected from both treated and untreated cells after 48 hours. Media was collected from cells and transferred to a labeled 1.5 mL Eppendorf tubes. The media was then immediately analyzed or stored at -20°C. Extra cellular glutamate levels were measured using the Amplex Red Glutamic Acid/Glutamate Oxidase Assay Kit (Invitrogen). The standard curve for the assay was made using serial dilutions of stock solutions of Glutamate with final concentrations ranging between 25 µM and 0 µM. To analyze the samples, media was diluted 1:5 in Reaction Buffer (0.1M TRIS pH 7.5). Then 25 µL of sample was added in triplicate to a 96 well dish. An assay mix was then made composing of 2429 µL of Reaction Buffer, 6.25 µL of HRP (100 U/mL), 40 µL of L-Glutamate Oxidase (5 U/mL) and 25 µL of Amplex Red dissolved in DMSO (2.6 µg/µL). From the assay mix, 25 µL was added to each well containing media. The plate was then incubated at 37°C for 30 minutes without CO₂. The fluorescence was then measured using a Cytofluor luminometer with an excitation wavelength of 530 nm and emission wavelength of 590 nm.
Crystal Violet

After media was collected from cells and aspirated, cells were washed with PBS, aspirated and discarded. The cells were then fixed with either 200 µL or 2 mL formalin in 96 well/6 well plates respectively, for 20 minutes. After the formalin was removed, cells were stained with Crystal Violet (0.25% in methanol) for 10 minutes. After 10 minutes, cells were washed with cold tap water and allowed to dry. Solubilizer (0.5 M NaH₂PO₄ in 50% ethanol) was added to the stained cells (100 µL for 96 well plates/1 mL for 2 mL plates) in each well. Optical absorbance was read on the Biotek PowerWave at 570 nm after 1 second of agitation. Values were measured against standard curves created by fixing serial dilutions of cell concentrations 6 hours after being seeded to allow for cell adherence.

Data analysis using Graphpad software

All results were taken from at least three individual experiments. Means and standard deviations were calculated using Graphpad Prism software. This software was used to calculate t-tests and one-way ANOVAs.
Results

Basal comparisons of Tumour and Non-tumour Breast cell lines

To measure protein levels of NRF-2, KEAP1, and xCT, different cell types (MDA-MB-231, MCF-7, MCF10A, 184B5, T47D) were seeded at densities of $3 \times 10^6$ cells per well in 6 well dishes for 48 hours. Western blots were used to compare protein from levels of NRF-2 and KEAP1 protein from whole cell lysates. The NRF-2 antibody (Santa Cruz), demonstrated many bands and high levels of non-specific binding. The band at 98 kDa was quantified. The western blots revealed that there were no significant differences between cell lines in NRF-2 protein, as seen in Figure 3.1 ($P>0.05$, $n=3$). The KEAP1 antibody probed a protein doublet at 60 kDa. This doublet was quantified. There were no significant differences in KEAP1 protein levels between cell lines Figure 3.2 ($P>0.05$, $n=3$). The xCT antibody, probes for a number of bands. There appeared to be a band at 50 kDa and 37 kDa. Both were quantified, but only the 37 kDa band is shown since it represents the functional protein (Seib et al, 2011). Interesting, different cell lines showed different numbers of xCT bands around 37 kDa. This could potentially be due to different posttranslational modifications that occur in each cell. Western blots revealed that xCT was highest in MCF10A cells, and lowest in T47D cells as seen in Figure 3.3 ($n=3$, $P<0.05$). Glutamate release was highest in 184B5 cells, and lowest in MDA-MB-231 cells (see Figure 3.4 ($n=2$)), although there were insufficient replicates to perform statistical analysis.
Figure 3.1: Basal NRF-2 Protein Levels

To measure protein levels of NRF-2, different cell types (MDA-MB-231, MCF-7, MCF10A, 184B5, T47D) were seeded at densities of $3 \times 10^6$ cells per well in 6 well dishes for 48 hours. Representative western blots are shown in Figure 3.1 B. The bands with a weight of 98 kDa were quantified using Image J software. The fold changes were then normalized to the Amido Black loading control quantities shown in the chart (Quantification data shown, the stained blots not shown). Figure 3.1 A shows the normalized fold changes in protein level relative to MCF-7 levels.
To measure protein levels of KEAP1 different cell types (MDA-MB-231, MCF-7, MCF10A, 184B5, T47D) were seeded at densities of 3x10^6 cells per well in 6 well dishes for 48 hours. Representative western blots are shown in Figure 3.2 B. The fold changes were then normalized to the Amido Black loading control quantities shown in the chart (Quantification data shown, the stained blots not shown). Figure 3.2 A shows the normalized fold changes in protein level relative to MCF-7 levels.

**Figure 3.2: Basal KEAP1 Protein Levels**

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>Fold Change Relative to MCF-7</th>
<th>Amido Black Staining Loading Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>T47D</td>
<td>0.878</td>
<td>MCF-7</td>
</tr>
<tr>
<td>184B5</td>
<td>0.673</td>
<td>MDA-MB-231</td>
</tr>
<tr>
<td>MCF-7</td>
<td>1.000</td>
<td>MCF10A</td>
</tr>
<tr>
<td>MDA-MB-231</td>
<td>2.201</td>
<td></td>
</tr>
<tr>
<td>MCF10A</td>
<td>1.613</td>
<td></td>
</tr>
</tbody>
</table>
Figure 3.3: Basal xCT protein levels

To measure protein levels of xCT different cell types (MDA-MB-231, MCF-7, MCF10A, 184B5, T47D) were seeded at densities of 3X10^6 cells per well in 6 well dishes for 48 hours. Representative western blots are shown in Figure 3.3 B. The fold changes were then normalized to the Amido Black loading control quantities shown in the chart (Quantification data shown, the stained blots not shown). Figure 3.3 A shows the normalized fold changes in protein level relative to MCF-7 levels.
Figure 3.4: Basal Glutamate release from breast cells
To evaluate basal xCT activity levels in various cell types, cells were seeded at $10^5$ cells per well in 6 well dishes for 48 hours. After 48 hours, media was collected and extracellular glutamate levels were analyzed using Amplex Red assays. Glutamate release levels were normalized to cell number counted by crystal violet staining.
Hydrogen peroxide treatment localizes NRF-2 to the nucleus, upregulates xCT expression in MCF-7 Cells

To confirm NRF-2 localization in response to oxidative stress, MCF-7 cells were treated for an hour with 100 µM, then given an hour to recover in normal medium. Using western blot analysis, cytosolic and nuclear fractions were probed with specific NRF-2 and KEAP1 antibodies. Protein levels were normalized to amido black loading controls, and numbers were expressed as a ratio of Nuclear/Cytosol fold changes. Following hydrogen peroxide treatment and recovery, Nuclear protein levels of NRF-2 increased 150% as seen in Figure 3.5 (n=4, p<0.05). Interestingly, Nuclear KEAP1 Protein remained constant as seen in Figure 3.6 (n=4, p=0.87).

To measure transcriptional response to oxidative stress, MCF-7 cells were treated for an hour with 100 µM, and then given an hour to recover in normal medium. Following hydrogen peroxide treatment and recovery, xCT mRNA was measured by Real Time Polymerase Chain Reaction (RT PCR) and compared to a house keeper β-Actin. In response to hydrogen peroxide treatment, xCT was upregulated approximately 130% as seen in Figure 3.7 (n=3, p<0.05). Interestingly, following one hour treatment of H₂O₂ treatment and a 24 hour recovery in complete medium, xCT protein increased but was not significant as seen in Figure 3.8 (n=3, p=0.24) Glutamate release was measured 48 hours a 1 hour treatment with H₂O₂ at varying concentrations. As seen in Figure 3.9, H₂O₂ increased levels of glutamate release significantly at a dose of 200 µM (n=3, p<0.05).
Figure 3.5: NRF-2 localization with Hydrogen Peroxide treatment
MCF-7 cells were seeded at 2.2 x 10^6 cells in a 10 cm dish and left overnight to adhere. Cells were then treated with 100µM hydrogen peroxide for 1 hour, followed by one hour recovery in complete medium. Cells were then fractioned for analysis of nuclear and cytosolic NRF-2 protein level by western blot. Representative western blots are shown in Figure 3.5 B. The fold changes were then normalized to the Amido Black loading control quantities shown in the chart (Quantification data shown, the stained blots not shown). Figure 3.5 A shows the normalized fold changes of Nuclear/Cytosolic protein level relative to control MCF-7 levels. Data is obtained from four independently run experiments (n=4).
Figure 3.6: Keap1 Localization with Hydrogen Peroxide treatment

MCF-7 cells were seeded at 2.2 x 10⁶ cells in a 10 cm dish and left overnight to adhere. Cells were then treated with 100 µM hydrogen peroxide for 1 hour, followed by 1 hour recover in complete medium. Cells were then fractioned for analysis of nuclear and cytosolic NRF-2 protein level by western blot. Representative western blots are shown in Figure 3.6 B. The fold changes were then normalized to the Amido Black loading control quantities shown in the chart (Quantification data shown, the stained blots not shown). Figure 3.6 A shows the normalized fold changes of Nuclear/Cytosolic protein level relative to control MCF-7 levels. Data is obtained from four independently run experiments (n=4).
Figure 3.7: xCT mRNA levels with Hydrogen Peroxide treatment

MCF-7 cells were seeded at $2.2 \times 10^6$ cells in a 10 cm dish and left overnight to adhere. Cells were then treated with 100 µM hydrogen peroxide for 1 hour, followed by 1 hour recover in complete medium. Messenger RNA was isolated from cell pellets and analyzed by Real Time PCR. Analysis revealed that the xCT mRNA levels increased significantly by 1.3 fold ($p<0.05$). Results were obtained from three independently run experiments ($n=3$).
Figure 3.8: xCT protein levels with Hydrogen Peroxide treatment

MCF-7 cells were seeded at $2.2 \times 10^6$ cells in a 10 cm dish and left overnight to adhere. Cells were then treated with 100 µM hydrogen peroxide for 1 hour, followed by a 24 hour recovery in complete medium. Whole cell protein lysates were analyzed by western blot for xCT protein levels. Representative western blots are shown in Figure 3.8 B. The fold changes were then normalized to the Amido Black loading control quantities shown in the chart (Quantification data shown, the stained blots not shown). Figure 3.8 A shows the normalized fold changes of protein level relative to control MCF-7 levels. Data is obtained from four independently run experiments ($n=3$).
Figure 3.9: Glutamate Release with Hydrogen Peroxide treatment
MCF-7 cells were seeded at 1 X 10^5 cells in a 6 well plate and left overnight to adhere. Cells were then treated with 100 µM hydrogen peroxide for 1 hour, followed by a 48 hour recovery in complete medium. Media was collected after 48 hours and Amplex Red assays were used to analyze glutamate content. Crystal violet curves were used to normalize to cell number. Analysis revealed that glutamate release increased significantly at 200 µM doses. Results were obtained from three independently run experiments (n=3).
3.2.4 NRF-2 overexpression upregulates xCT activity

To evaluate whether NRF-2 activation increases xCT activity levels, MCF-7 cells were transfected with transient NRF-2 overexpression vectors for 48 hours. After 48 hours, cells were harvested and extracellular glutamate levels were analyzed using Amplex Red assays. Glutamate release levels were normalized to cell number counted by crystal violet staining. NRF-2 overexpression vectors increased glutamate release 2 fold versus control empty vector as seen in Figure 3.10 (n=5, p<0.05). To evaluate whether NRF-2 activation increases xCT protein levels, MCF-7 cells were transfected with transient NRF-2 overexpression vectors for 48 hours. After 48 hours, cells were harvested and protein levels were analyzed using western blot analysis. Protein levels were normalized to amido black loading controls, and numbers were expressed as fold changes. Transfections demonstrated that increased NRF-2 levels increased xCT protein levels by 3 fold, although the differences were not significant (n=4, p=0.14).
Figure 3.10: NRF-2 Overexpression
MCF-7 cells were seeded at 3 x 10^6 cells in a 6 well plate and left overnight to adhere. Cells were then transfected with empty pcDNA 3.1 or NRF-2 plasmid for 48 hours. Whole cell lysates were made for western blot analysis. Representative western blots are shown in Figure 3.10 B. The fold changes were then normalized to the Amido Black loading control quantities shown in the chart (Quantification data shown, the stained blots not shown). Figure 3.10 A shows the normalized fold changes of protein level relative to control MCF-7 levels. Results were obtained from four independently run experiments (n=4).
Figure 3.11: xCT protein levels in NRF-2 Overexpressing MCF-7 cells
MCF-7 cells were seeded at 3 X 10^6 cells in a 6 well plate and left overnight to adhere. Cells were then transfected with empty pcDNA 3.1 or NRF-2 plasmid for 48 hours. Whole cell lysates were made for western blot analysis. Representative western blots are shown in Figure 3.11 B. The fold changes were then normalized to the Amido Black loading control quantities shown in the chart (Quantification data shown, the stained blots not shown). Figure 3.11 A shows the normalized fold changes of protein level relative to control MCF-7 levels. Results were obtained from four independently run experiments (n=4).
Figure 3.12: Glutamate Release in NRF-2 Overexpressing MCF-7 cells
MCF-7 cells were seeded at 3 X 10^6 cells in a 6 well plate and left overnight to adhere. Cells were then transfected with empty pcDNA 3.1 (vector) or NRF-2 plasmid for 48 hours. Media was collected after 48 hours and Amplex Red assays were used to analyze glutamate content. Cells were counted and glutamate level was normalized to cell number. Analysis revealed that glutamate release increased significantly in cells overexpressing NRF-2. Results were obtained from five independently run experiments (n=5).
Discussion

Oxidative stress has been implicated in tumour promotion, and progression, through a number of different signaling pathways as reviewed in the introduction. One of the key pathways that may contribute to carcinogenesis and drug resistance is the activation of NRF-2 by oxidative stress (Loignon et al., 2009). NRF-2 has been implicated in a drug resistance and cancer cell survival in a number of studies. System \( \text{X}_\text{c}^- \) activity has also been shown to protect a number of different cell types from oxidative stress by increasing glutathione levels (Shih et al., 2006; Sakakura et al., 2007).

Therefore, investigating the potential link between NRF-2/System \( \text{X}_\text{c}^- \) and whether that pathway plays a role in oxidative stress response in breast cancer cells has the potential to provide drug targets.

The purpose of this study was to investigate whether the transcription factor NRF-2 regulates System \( \text{X}_\text{c}^- \) activity levels through regulating levels of system \( \text{X}_\text{c}^- \) activity. Baseline KEAP1, NRF-2 and xCT protein levels of both tumour and non-tumour cells were measured. In addition to baseline characterizations, MCF-7 cells were selected to investigate more closely NRF-2 induction and measure its influence on system \( \text{X}_\text{c}^- \).

Although there have been reports of NRF-2 being overexpressed in lung (Singh et al., 2006) and esophageal cancers (Kim et al., 2010), there are conflicting reports about the level of NRF-2 in cancer cells compared to normal epithelial cells. Seng et al., 2007 reported NRF-2 to be overexpressed in MDA-MB-231 cells. Alwi et al., 2012, demonstrated that NRF-2 was more highly expressed in MCF-7 cells than MDA-MB-231 cells. In contrast, Loignon et al., 2009, reported NRF-2 to be higher in normal epithelial cells compared to both MCF-7 and MDA-MB-231 cells, the only cell line of cell lines
compared in this experiment where NRF-2 was expressed more highly than normal epithelial cells was T47D. In that report, the findings were confirmed as NRF-2 was lower in cancer tissue samples compared to normal tissue taken from patients (Loignon et al., 2009).

No differences were reported in KEAP1 protein levels comparing tumour and surrounding normal tissues (Loignon et al., 2009). KEAP1 mRNA has been reported to be lower in cancer cell lines (A549, H1435, and H838) (Singh et al., 2009). But it seems that Keap 1 mutations play a large role in altered activity of the NRF-2/KEAP1 pathway in cancer cells. A number of KEAP1 mutations have been found in a variety of cancers (Yoo et al., 2012; Padmanabhan et al., 2006; Nioi et al., 2007; Hast et al., 2014; Suzuki et al., 2011; Li et al., 2011; Singh et al., 2006).

Differences in system $x_c^-$ have been previously reported between cancer and non-cancer cells. It has been reported that triple negative breast cancer cell lines released more glutamate through system $x_c^-$ (Sharma, et al., 2010; Timmerman et al., 2013; Ungard, 2012).

In this project, baseline measures showed no differences in NRF-2 and KEAP1 protein levels between Tumour cell lines (MDA-MB-231, MCF-7 and T47D cells) and non-tumour cell lines (184B5 and MCF10A). However there was one difference in xCT protein levels, between the MCF10A cell line and the T47D cell line. The non-tumour MCF10A cell line had significantly higher xCT protein levels than the ER, PR positive ductal carcinoma cell line T47D. The difference in xCT level did not reflect glutamate release, as there were no significant differences between glutamate levels of different cell types, although there was a trend ($p=0.06$).
One of the confounding factors of the baseline cell comparisons may have been different culture conditions between each cell line. Each cell line was grown in the ATCC recommended media, unless it could be adapted to a different medium. Initially cell lines were to be adapted to growing in DMEM, however T47D and 184B5 were unable to grow in DMEM. As a result, the T47D and 184B5 epithelial cells were grown in RPMI supplemented with 10% FBS and 1% A/A, and MEBM (MEGM and Supplementation Kit) respectively. The MCF-7, MDA-MB-231 and MCF10A cells were grown in DMEM supplemented with 10% FBS and 1% A/A. As system $\mathbf{x}_c$ is induced to protect cells in culture (Sakakura et al., 2007), it is likely that culture conditions may have been a confounding factor.

Another important in vitro condition that had an effect on glutamate release was cell density. MCF-7 cells released significantly more glutamate at lower levels (Appendix 1). System $\mathbf{x}_c$ has been demonstrated to increase cystine import at lower densities (Takada et al., 1984).

One of the difficulties with the basal cell comparisons that may have confounded the results was the apparent uneven loading on the SDS PAGE gels, as seen by the discrepancies in the amido black loading control staining. This may have been caused by interference between the RIPA buffer that was used to lyse the cells and the Bradford Protein detection reagent that was used to quantify the levels of protein in lysates. Sodium dodecyl sulfate, a component of the RIPA buffer has been demonstrated to interfere with the dye used in the Bradford Protein assay. This may have artificially increased the values of detected protein when preparing protein samples for the SDS PAGE gels. This may have resulted in less protein being loaded in one well than the
other. This confounding effect was minimized by normalizing protein values derived from quantifying developed western blots to the amido black loading control values.

MCF-7 cells were selected to measure whether NRF-2 activation induced System \( x_c^- \) activity. Two approaches were taken to measure whether NRF-2 regulates System \( x_c^- \). The first approach was to use a chemical activator of NRF-2, the second was to use an overexpression plasmid. Initially TBHQ, a well-known inducer of NRF-2, was used. However at low concentrations it did not induce NRF-2 sufficiently and at higher concentrations it had substantial effects on cell viability. As a result, hydrogen peroxide was used to induce NRF-2. This was chosen since it is a direct inducer of ROS and oxidative stress. It is also physiologically relevant since it naturally produced in cells, through SOD from Superoxide molecules from mitochondria and NOX systems (Loschen et al., 1974). \( \text{H}_2\text{O}_2 \) treatment did increase NRF-2 localization to the nucleus although it did not affect KEAP1 levels. KEAP1 can shuttle between the cytoplasm and even repress NRF-2 in the nucleus (Zhang et al., 2006). Electrophilic agents are able to modify important cysteine residues in the linker region of KEAP1, resulting in the inability of KEAP1 to bind with NRF-2 and facilitate its ubiquitination (Kobayashi et al., 2006). This could be the potential reason that System \( x_c^- \) was upregulated coincidentally with NRF-2, even though nuclear KEAP1 levels were not decreased with \( \text{H}_2\text{O}_2 \) treatment. Hydrogen Peroxide did significantly increase xCT mRNA. Protein levels were increased after 24 hours although not significantly. Using the Amplex Red assay, glutamate release was increased 48 hours post treatment. One of the limitations using the Amplex Red assay was the need to limit the exposure time of hydrogen peroxide. The Amplex Red Assay uses glutamate oxidase to produce \( \text{H}_2\text{O}_2 \) from the conversion of glutamate to \( \alpha- \)
ketoglutarate, which is then detected by the Amplex Red reagent (Appendix 4). As a result, cells were treated with hydrogen peroxide for 1 hour. The media was then changed out for complete media for 48 hours to allow glutamate to accumulate in the medium outside of the cells. While xCT mRNA was measured 1 hour after H₂O₂ exposure, protein was studied after 24 hour to allow for protein translation. The limited H₂O₂ treatment could potentially explain the lack of significant different in xCT protein levels.

The second approach used an overexpression plasmid encoding NRF-2. MCF-7 cells were transfected with NRF-2 expression vectors, which significantly increased NRF-2 protein. The NRF-2 overexpression plasmid did significantly increase xCT promoter binding in both MCF-7 and MDA-MB-231 cells (not shown, performed by Dr. Linher-Melville). The NRF-2 overexpression plasmid increased xCT protein level 4 fold, although not significantly. This is potentially due large variation in up regulation of protein expression and limited number of replicates (n=4). The NRF-2 plasmid also functionally increased glutamate release. Although protein levels of xCT did not increase significantly, it appears that System xᵮ was functionally upregulated with NRF-2 overexpression.

The antibody for NRF-2 (Santa Cruz) used evaluate NRF-2 multiple bands, confounding the results (Lau et al, 2013). Lau et al, 2013 demonstrated that the band that represented NRF-2 was around 100 kDa, rather than the often reported 55 kDa band. This study found a band around 98 kDa that was responsive to both transient NRF-2 overexpression vectors and Hydrogen peroxide treatment. These results were used to determine which band represented NRF-2, and this was used to choose which band was used in the basal cell comparisons of first objective of this study.
Conclusion

In conclusion, it appears that basal xCT activity is not correlated with NRF-2 or KEAP1 protein levels in both breast cancer and normal breast epithelial cell lines. These proteins also did not differ between the cancer and non-cancer cell lines examined. However, it does appear that NRF-2 in response to oxidative stress and overexpression, does up regulate xCT and System xᵢ⁻ activity in breast cancer cells.
Future directions

There are a number of future directions that may be valuable to explore in order to further elucidate the mechanisms studied.

As an initial follow up, further validating NRF-2 mediated upregulation of System $x_c$ would be necessary. Due to the limitations of the Amplex Red assay as discussed earlier, it would be valuable to measure cystine uptake in NRF-2 overexpressing cells. Further follow up could include measuring glutathione levels in NRF-2 overexpressing cells. Using System $x_c$ specific inhibitors, such as sulfasalazine, to test the specificity of System $x_c$ of increased glutamate release, cystine uptake, and glutathione production.

Another important follow up study would be to further pin point how NRF-2 upregulates xCT. Promoter analysis could be continued using truncations of the xCT promoter, mutation analysis of the ARE site, and a ChIP assay could be used to determine binding of NRF-2 under oxidative stress. Other transcription factors that could potentially be involved in stress response, such as Oct-1 and NFκB, could be explored for interactions with NRF-2 in xCT regulation. To expand the investigation of NRF-2 mediated upregulation, the trafficking proteins of xCT could also be studied, such as 4F2hc and CD44.

Once confident in NRF-2 regulation of xCT, it could be further tested in vivo to investigate if there is any functional or clinical significance. If a stably cell line overexpressing NRF-2 was produced, it could be xenografted into mice. Then system $x_c$ activity could be functionally measured by measuring glutamate levels in the tumour microenvironment, or glutathione levels in the tumour compared to control cancer cells.
Further investigation of the effects of NRF-2 and System \( x_c \) could provide drug targets for both anti tumour agents and sensitizing adjuvant therapies in cases of chemoresistance.
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Appendix

Appendix 1: MCF-7 Density curve of Glutamate release

Glutamate release was increased in cells seeded at lower densities in a 6 well dish (p<0.05).

Appendix 2: MDA-MB-231 Density curve of Glutamate release

Glutamate release was not significantly increased in cells seeded at lower densities in a 6 well dish (p>0.05).
Appendix 3: MCF10A Density curve of Glutamate release

Glutamate release was not significantly increased in cells seeded at lower densities in a 6 well dish (p>0.05).

Appendix 4: Amplex Red Assay Diagram

This diagram illustrates how the Amplex Red assay detects glutamate levels. Glutamate is converted to α-ketoglutarate by glutamate oxidase creating hydrogen peroxide. Hydrogen peroxide then drives the reaction that converts Amplex red into resorufin which is detected with a luminometer (adapted from Life Technologies, 2009).