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Research Paper

Expression of xCT and activity of system x_c^- are regulated by NRF2 in human breast cancer cells in response to oxidative stress

Eric Habib, Katja Linher-Melville, Han-Xin Lin, Gurmit Singh *

Department of Pathology and Molecular Medicine, McMaster University, Hamilton, Ontario, Canada L8S 4L8

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ABSTRACT

Cancer cells adapt to high levels of oxidative stress in order to survive and proliferate by activating key transcription factors. One such master regulator, the redox sensitive transcription factor NF E2 Related Factor 2 (NRF2), controls the expression of cellular defense genes including those encoding intracellular redox-balancing proteins involved in glutathione (GSH) synthesis. Under basal conditions, Kelch-like ECH-associated protein 1 (KEAP1) targets NRF2 for ubiquitination. In response to oxidative stress, NRF2 dissociates from KEAP1, entering the nucleus and binding to the antioxidant response element (ARE) in the promoter of its target genes. Elevated reactive oxygen species (ROS) production may deplete GSH levels within cancer cells. System x_c^-, an antiporter that exports glutamate while importing cystine to be converted into cysteine for GSH synthesis, is upregulated in cancer cells in response to oxidative stress. Here, we provided evidence that the expression of xCT, the light chain subunit of system x_c^-, is regulated by NRF2 in representative human breast cancer cells. Hydrogen peroxide (H_2O_2) treatment increased nuclear translocation of NRF2, also increasing levels of xCT mRNA and protein and extracellular glutamate release. Overexpression of NRF2 up-regulated the activity of the xCT promoter, which contains a proximal ARE. In contrast, overexpression of KEAP1 repressed promoter activity and decreased xCT protein levels, while siRNA knockdown of KEAP1 up-regulated xCT protein levels and transporter activity. These results demonstrate the importance of the KEAP1/NRF2 pathway in balancing oxidative stress in breast cancer cells through system x_c^-. We have previously shown that xCT is upregulated in various cancer cell lines under oxidative stress. In the current investigation, we focused on MCF-7 cells as a model for mechanistic studies.

Introduction

Oxidative stress contributes to intracellular signaling and patterns of aberrant gene expression in numerous cancers [1]. Importantly, by affecting cellular redox balance, reactive oxygen species (ROS) may trigger the synthesis of one of the major antioxidant molecules, glutathione (GSH). This process requires adequate maintenance of intracellular cysteine levels. It has been shown that a variety of cell types including cells of the central nervous and immune systems, as well as fibroblasts and breast cancer cells [2–7], maintain intracellular cysteine levels by importing cystine in exchange for glutamate. The equimolar transport of these 2 amino acids is mediated by the cell surface antiporter, system x_c^- [8,9]. Neurons selected for resistance to oxidative stress express higher levels of the light chain component of system x_c^-, xCT [10], which is encoded by the *SLC7A11* gene and confers transporter activity [11,12]. Elevated xCT expression and glutamate release are commonly observed in cancer cells [7,11]. Transcripts of xCT are highly expressed in glioma cells [13], and the expression of xCT is negatively correlated with survival in invasive breast cancers [5] and esophageal squamous cell carcinomas [14]. Inhibiting xCT may sensitize estrogen receptor-positive (ER+) breast cancers to anti-insulin-like growth factor 1 receptor (anti-IGF1R) therapy, and it is thought that xCT is upregulated to protect breast cancer cells from ROS damage [5]. We have shown that in ovarian cancer cells under oxidative stress, xCT expression and the function of system x_c^- increase to maintain levels of GSH [15]. In addition, we determined that various cancer cell lines, including human and mouse breast cancer and mouse melanoma cells, express high levels of xCT and release glutamate into the culture medium [11].

There is preexisting evidence that xCT expression is controlled by the redox sensitive transcription factor, NF E2 Related Factor 2 (NRF2). A correlation between NRF2 and xCT has been established in response to oxidative stress in a mouse model [16], and

* Corresponding author.
E-mail address: singhg@mcmaster.ca (G. Singh).

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electrophiles and other NRF2 activators have been correlated with increases in xCT in glioma stem cells [17], retinal epithelial cells [18], RGC-5 cells [19], rat primary astrocytes [20], mouse microglial cells [18], and human bronchial epithelial cells [21]. More directly, NRF2 has been shown to upregulate xCT protein levels in astrocytes and HEK293 cells [22]. In rat glial cells, overexpression of Nrf2 confers neuroprotection by up-regulating xCT expression [23], and in rat cardiomyocytes, NRF2-mediated induction of xCT protects against reperfusion injury [24]. Diethyl Maleate (DEM), an inducer of ROS, increases glutamate transport and cystine uptake in human fibroblasts, and this transport requires xCT mRNA and protein synthesis [2]. DEM also increases xCT mRNA levels [8]. Another study found that DEM increases murine xCT mRNA levels in an Nrf2-dependent manner, requiring its binding to an antioxidant response element (ARE), also known as an electrophile response element (EpRE), in the proximal promoter region of the xCT gene [4]. Tert-butyl hydroquinone, an NRF2 inducer, increases cystine uptake by 2-fold in mouse embryonic fibroblasts (MEFs) [25], and system x_c^- activity is lost in Nrf2 −/− MEFs [4]. In macrophages [26], endothelial cells infected with Kaposi's Sarcoma-Associated Herpesvirus [27], and rat microglial cells [28], xCT upregulation is dependent on NRF2. However, in SH-SY5Y cells, NRF2 does not directly mediate the upregulation of xCT [29]. In MCF10A normal breast epithelial cells, herbal compounds induce nuclear translocation of NRF2 and increase xCT mRNA levels, also inducing ARE reporter activity in MCF-7 breast cancer cells [30]. NRF2 binds to the mouse xCT promoter in MEFs [31], and in mouse brain, GSH depletion increases NRF2 accumulation and xCT mRNA levels, but the same effects were not observed in the liver or kidneys [32].

NRF2 has been referred to as the master regulator of antioxidant defenses [33]. It is part of the “cap ‘n’ collar” subgroup of basic region leucine zipper transcription factors, a family of six transcription factors that includes NF-E2, NRF1, NRF2, NRF3, Bach 1, and Bach 2 [34]. Knockdown of NRF2 has been shown to be compensated by overexpression of NF-E2, suggesting that this protein family may share binding sequences [37]. NRF2 transcriptionally regulates the expression of groups of cellular defense genes encoding intracellular redox-balancing proteins such as glutamate cysteine ligase (GCL), glutathione peroxidase (GPX), thioredoxin (TRX), and heme oxygenase 1 (HMOX1), as well as phase I and II detoxifying proteins including glutathione S-transferase (GST), multi-drug resistance protein (MRP), NAD(P)H quinone oxidoreductase (NQO1), and UDP-glucoronosyltransferase (UGT) [35]. NRF2 has been shown to reprogram metabolic signaling in cancer cells, including through the pentose phosphate pathway, NADPH production, glutamine metabolism, and glycolysis [36]. Binding sites for NRF2 overlap with those utilized by other transcription factors. For example, the NRF2 binding sequence in the NQO1 promoter also contains two AP1 sites [38], and the NRF2 and AP1 binding sites in the HMOX1 promoter share significant homology [39]. The subunits of AP1, c-JUN and c-FOS, have both been demonstrated to enhance NRF2 binding [40]. Of relevance, a recent study has shown that the human xCT promoter contains a proximal ARE/AP1 site that is bound by NRF2 in T24 bladder carcinoma cells [41].

NRF2 is regulated through several mechanisms including its phosphorylation at multiple sites [42,43] in response to intracellular signaling through the MAPK [42] and PI3K-AKT pathways [44], although its predominant regulator is Kelch-like ECH-associated protein 1 (KEAP1). KEAP1 senses cellular ROS and directly inhibits NRF2 in the cytoplasm [34]. By acting as a substrate adaptor, it binds NRF2 with the cullin 3 (CUL3)-ringbox protein 1 (RBX1) E3 ligase, resulting in NRF2 ubiquitination and subsequent proteasomal degradation [45]. Under basal conditions, the KEAP1/NRF2 complex is either targeted for ubiquitination or is shuttled back and forth between the cytoplasm and nucleus. In the nucleus, the DNA binding activity of NRF2 is repressed by KEAP1 [34]. Under conditions of oxidative stress or chemical induction of NRF2, KEAP1 undergoes a post-translation modification in its linker region that conformationally alters KEAP1/CUL3/RBX1, resulting in NRF2 stabilization and target gene transactivation [45,46].

Altered NRF2 expression and function have been reported in tissue derived from tumors [47,48] and multiple cancer cell lines [49,50], and NRF2 is highly expressed in MCF-7 breast cancer cells [50]. Its knockdown by siRNA decreased tumor growth mediated by the pentose phosphate pathway [36]. High NRF2 activity also led to increased resistance of breast cancer cells to doxorubicin and paclitaxel [48]. In addition, somatic mutations in KEAP1 are present in breast, colorectal, gastric, hepatocellular, lung, and prostate carcinomas [51]. In breast and lung cancer cells, KEAP1 mutations impaired NRF2 repression [52,53]. A recent meta-analysis found that 213 somatic mutations in KEAP1 were present in 17 different cancer types, including breast, colorectal, gastric, kidney, liver, lung, and squamous cell carcinomas, and ovarian and breast cancers, several of which were associated with abolishing the suppression of NRF2 or enhancing its DNA binding activity [54].

While the role of NRF2 and its association with KEAP1 have been examined in diverse cell types, to our knowledge, the current investigation is the first to mechanistically link the regulation of xCT and the KEAP1/NRF2 pathway in human breast cancer cells. Our findings provide insights into gaining a better understanding of the mechanisms that underlie redox balance in human breast cancer cells. Here, we show that intracellular oxidative stress, represented by acute hydrogen peroxide (H_2O_2) treatment, upregulates system x_c^- through NRF2 in MCF-7 cells. We report that treating MCF-7 cells with H_2O_2 leads to nuclear translocation of NRF2, elevated xCT expression, and increased transporter activity. Overexpression of NRF2 upregulates human xCT promoter activity and protein expression, while overexpressing KEAP1 downregulates promoter and transporter activity, with knockdown of KEAP1 producing the inverse effect. It will be of therapeutic interest to examine the effect of small molecule inhibitors targeting the KEAP1/NRF2 pathway, especially given that system x_c^- plays a role in drug- and radiation-resistant cancers.

Materials and methods

Cell culture

MCF-7 human breast cancer cells were cultured according to ATCC guidelines in a humidified incubator at 37 °C with 5% CO_2. Complete culture media was comprised of Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS) and 1% antibiotic (penicillin, streptomycin)/antimycotic (amphotericin B) (Life Technologies).

H_2O_2 treatment

MCF-7 cells were plated at either a density of 2×10^5 cells per well in 6-well plates or 2.2×10^6 cells in a 10-cm dish and incubated overnight to attain optimal attachment. Media was then aspirated and cells were washed with PBS. Cells were then treated with complete DMEM supplemented with various concentrations, ranging from 50 to 200 μM, of stabilized H_2O_2 (Sigma) for 1 h. Media with H_2O_2 was then replaced with complete media for 1 h for recovery prior to collecting the cells or media for additional analyses.

Real time RT-PCR

Total RNA was isolated by the Trizol method following the manufacturer's protocol (Life Technologies). cDNA was synthesized using Superscript III (Life Technologies) and $oligo_{(dt)}$ primer. Primers for xCT were 5′-CCTCTATTCGGACCCATTTAGT-3′(forward) and 5′-CTGGGTTTCTTGTCCCATATAA-3′(reverse), and primers for β-Actin, used as a housekeeper, were 5′-GATGGGCGGCGGAAAATAG-3′(forward) and 5′-GCGTGGATTCTGCATAATGGT-3′(reverse). Real time RT-PCR reactions were carried out in a Biorad Plex PCR machine in a final volume of 12.5 μL consisting of 1 μL of cDNA, 6.25 μL SYBR Green premix (Takara), 3.25 μL RNAse-free water, and 1 μL each of 10 pmol/μL forward and reverse primers. PCR amplification was initiated for 1 min at 95 °C followed by 40 cycles of denaturation at 95 °C for 10 s, annealing at 60 °C for 25 s, and elongation at 72 °C, with subsequent photodetection and quantification of relative mRNA levels according to the $2^{-[\Delta]\ [\Delta]Ct}$ method.

Whole cell lysates

Cells were washed with PBS and lysed directly in wells using 500 μL of RIPA buffer supplemented with protease inhibitors (Roche) by scraping. Lysates were incubated on ice for 30 min, transferred to 1.5 mL Eppendorf tubes, and sonicated at an amplitude of 40 for 15 pulses. Following centrifugation at 15,000*g* for 15 min at 4 °C, supernatants containing the cytoplasmic protein sample were transferred to new tubes and stored at 70 °C prior to use.

Nuclear lysates

Cells were seeded at 10^6 cells per 10-cm dish. At the time of harvest, cells were washed with PBS and treated with 0.25% EDTA/Trypsin to promote detachment. Following a brief centrifugation, cell pellets were resuspended in a small volume of PBS and transferred into 1.5 mL Eppendorf tubes, which were then centrifuged for 5 min at 500*g*. The supernatant was discarded, and each pellet was processed using the NE-PER Nuclear and Cytoplasmic Extraction Kit (Thermo Scientific) to collect both the cytoplasmic and nuclear fractions. Protein samples were stored at −70 °C prior to use.

Western blotting

Protein content in lysates was measured using the Bradford assay. Cell lysates were boiled with loading buffer containing β-Mercaptoethanol at 95 °C for 5–10 min. Approximately 50 μg of protein were loaded onto 10% SDS-polyacrylamide gels and transferred onto PVDF membranes for Western blotting. Membranes were blocked in 5% non-fat dry milk in TRIS–buffered saline containing 0.1% Tween (TBST) for at least 1 h at room temperature, then incubated at 4 °C overnight in primary antibody: NRF2 (Santa Cruz sc-722, 1:1000), KEAP1 (Cell Signaling 546C, 1:1000), ETS-1 (Abcam ab26096, 1:1000), or xCT (Abcam ab37185, 1:1000). Membranes were washed 3 × 10 min with TBST and incubated with horseradish peroxidase (HRP)-conjugated secondary antibody targeted to rabbit (Cell Signaling 7074S, 1:5000) for 1 h at room temperature. Following washes with TBST, blots were developed using an enhanced chemiluminescence kit (ECL plus, Amersham Biosciences). Either staining with amido black (Sigma) or reprobing stripped blots with calnexin (Santa Cruz H-70, 1:1000) or actin (Cell Signaling 13E5, 1:1000) were used as loading controls. Western Blots were densitometrically quantified using ImageJ software (NIH). Normalized protein levels were then compared by fold change.

Amplex red assay

Media was collected from untreated and treated cells after 48 h and transferred into 1.5 mL Eppendorf tubes. The media was then immediately analyzed or stored at −20 °C. Extracellular glutamate levels were measured using the Amplex Red Glutamic Acid/Glutamate Oxidase Assay Kit (Life Technologies). A standard curve was prepared using serial dilutions of glutamate stock solution with final concentrations ranging between 0 and 25 μM. Media samples were diluted 1:5 in 1 × Reaction Buffer (0.1 M TRIS pH 7.5). 25 μL of diluted sample was added in triplicate to a 96-well plate. An assay mix was prepared consisting of 2429 μL of 1 × 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). 25 μL of assay mixture was added to each well containing diluted media samples, and the plate was incubated at 37 °C for 30 min in the absence of CO_2. Fluorescence was measured using a Cytofluor luminometer with an excitation wavelength of 530 nm and emission wavelength of 590 nm, and values for μM of glutamate minus media only (background) were calculated based on the standard curve. These values were subsequently normalized to total cell number determined by the crystal violet assay.

Crystal violet assay

After media was collected for the glutamate assay, cells were washed with PBS and fixed for 20 min with either 200 μL or 2 mL of formalin for wells in either a 96-well or 6-well plate, respectively. After complete removal of formalin, plates with the attached cells were stained with Crystal Violet (0.25% in methanol) for 10 min, washed with cold water, and allowed to air-dry. Solubilizer (0.5 M NaH_2PO_4 in 50% ethanol) was added to the stained cells (100 μL or 1 mL for wells in either a 96-well or 6-well plate). Optical absorbance was read on a Biotek PowerWave plate reader at 570 nm following 1 s of agitation. Values were measured against standard curves created by fixing known cell concentrations 6 h after seeding.

Transient transfections

MCF-7 cells were plated into 6-well plates at a density of 2.5×10^5 cells/well and allowed to attach overnight. The following day, 6 μL of Lipofectamine 2000 (Life Technologies) was added to 250 μL of DMEM for 5 min. This mixture was then added to 4 μg of plasmid DNA in 250 μL of DMEM, pipetted up and down several times, and allowed to incubate at room temperature for 20 min. The resulting 500 μL was added drop-wise to 1.5 mL of fresh complete media on the plated cells, which were then cultured for a further 48 h.

Human xCT promoter construct and dual luciferase assays

PCR amplification of the human xCT promoter region spanning −2329/+278 was carried out with Platinum Pfx DNA polymerase and 2X PCRx Enhancer Solution (Invitrogen), genomic DNA isolated from human breast cancer cells, and the following primers that introduced a KpnI or XhoI site, respectively: 5′-TTATGGTACCGAGGAAGCTAGGACTATTTCT-3′ (forward) and 5′-ATAACTCGAGAGTAGGGACACACGGGGGA-3′ (reverse). The resulting product was bi-directionally sequenced and cloned into the pGL3-Basic firefly luciferase reporter construct (Promega). Dual luciferase assays (Promega) were performed according to a protocol described previously [55].

Overexpression vectors

pcDNA3.1-NRF2, the vector used to overexpress NRF2, has been described previously [56], as well as the vector used to overexpress ETS1, pcDNA3.1-ETS1 [57]. The KEAP1 overexpression vector was generated by subcloning the human gene from a commercially available cDNA clone (MGC clone MHS6278-202757046, GE Dharmacon) into a modified pcDNA3.1 vector with a C9 tag using *BamH*I and *Xba*I sites. The primers for subcloning KEAP1 were 5′-AAAAACGGATCCatgcagccagatcccaggccT-3′ (forward) and 5′-ACACAC TCTAGAACAGGTACAGTTCTGCTGGTC-3′ (reverse), and the product was verified by bi-directional sequencing.

siRNAs

Four different siRNAs targeting human KEAP1 were obtained from Qiagen, K1 (Hs_KEAP1_5, SI03246439), K2 (Hs_KEAP1_6, SI04155424), K3 (Hs_KEAP1_7, SI04267886), and K4 (Hs_KEAP1_8,

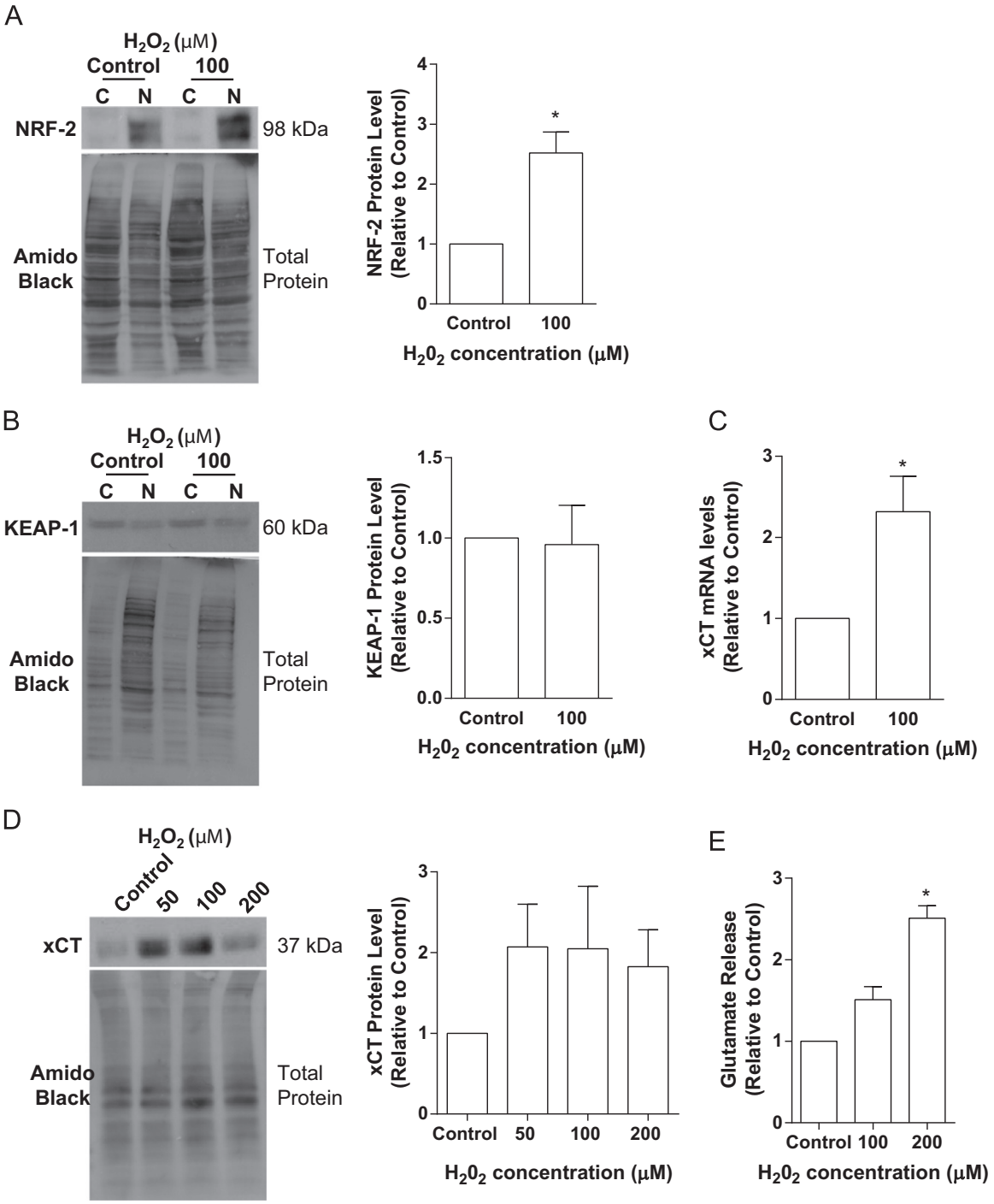

Fig. 1. H_2O_2 increased NRF2 nuclear localization, xCT expression, and the activity of system x_c^- in MCF-7 cells. Cells treated with H_2O_2 were fractioned for analysis of cytosolic (C) and nuclear (N) protein levels of (A) NRF2 and (B) KEAP1 by Western blotting, with normalized fold changes relative to control obtained by densitometric analysis showing an increase in nuclear NRF2 in response to oxidative stress. (C) Real Time RT-PCR revealed that xCT mRNA levels increased significantly by 2-fold in response to treatment with H_2O_2, which also increased (D) xCT protein levels and (E) the release of glutamate by MCF-7 cells.

SI04288844), along with a non-specific (NS) control siRNA (Ctrl Control 1, SI03650325). MCF-7 cells plated at 1.25 $\times$ 10^5 cells/well of 6-well plates were transiently transfected for 72 hours with each siRNA using Hiperfect reagent (Qiagen) following a protocol described previously [55], and cells and media were collected for further analyses.

Statistical analyses

Results were obtained from at least three independent experiments. Means and standard errors of the mean (SEM) were calculated using GraphPad Prism software, which was also used to determine statistical significance between treatment groups using *a t*-test, with a * denoting $p < 0.05$, and ** denoting $p < 0.005$.

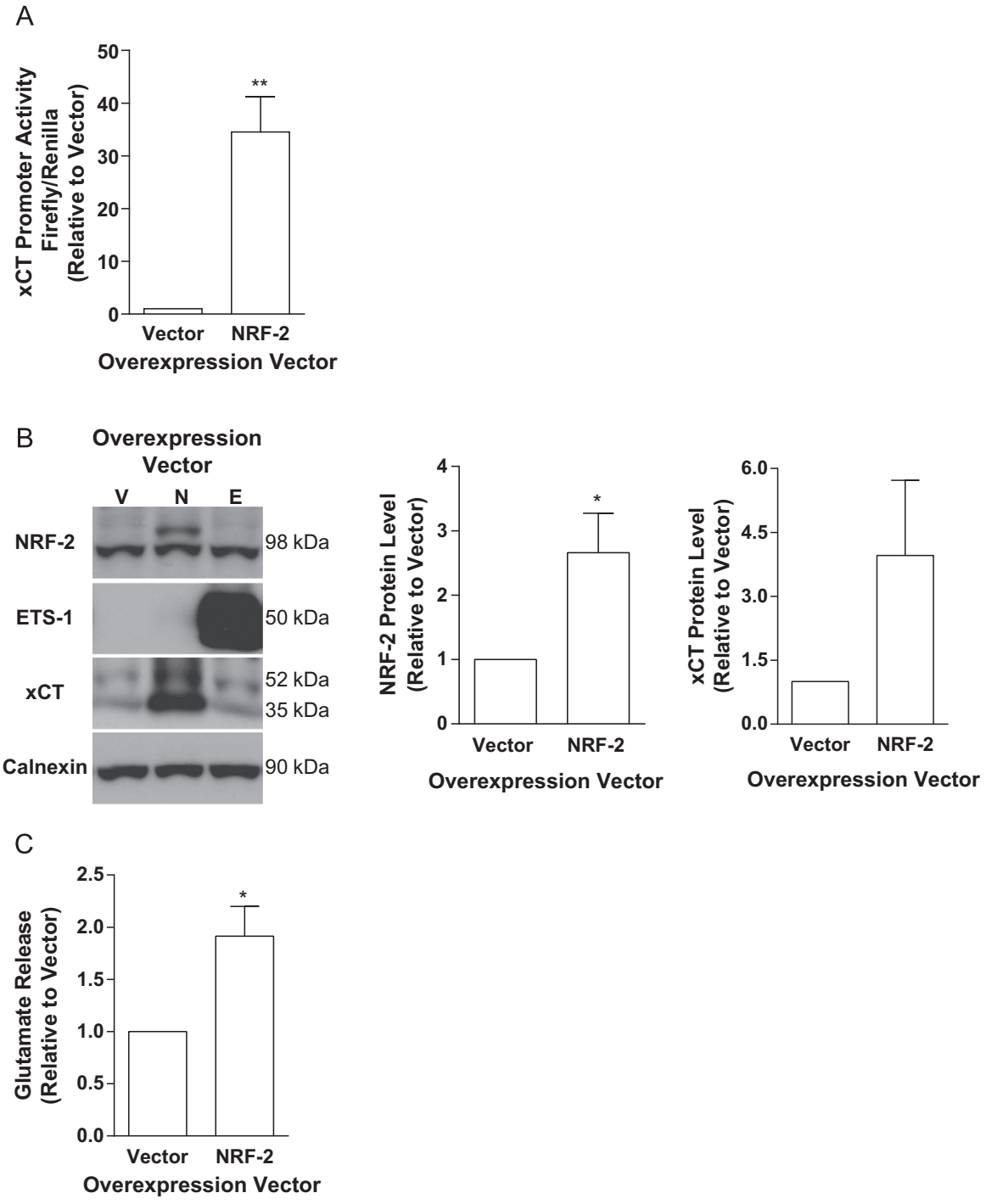

Fig. 2. Overexpressing NRF2 up-regulated xCT expression and the activity of system x_c^- in MCF-7 cells. (A) Luciferase assays were carried out on lysates isolated from MCF-7 cells transiently transfected with the human xCT promoter construct together with either an empty pcDNA3.1 vector (Vector) or an NRF2 overexpression vector (NRF2), demonstrating that NRF2 significantly up-regulated xCT at the level of transcription. (B) A representative Western blot of whole cell lysates prepared from MCF-7 cells transiently transfected with empty vector (V), or NRF2 (N) or ETS-1 (E) overexpression vectors, with densitometrically determined fold changes showing an up-regulation of xCT protein levels when NRF2 was overexpressed. (D) NRF2 overexpression also increased the release of glutamate by transiently transfected MCF-7 cells relative to Vector.

Results

H_2O_2 treatment increased NRF2 nuclear translocation, xCT expression, and glutamate release

To investigate the link between oxidative stress and the KEAP1/NRF2 pathway, MCF-7 cells were treated with 100 μM H_2O_2. Analysis of cytosolic and nuclear fractions by Western blotting with specific NRF2 and KEAP1 antibodies revealed that levels of NRF2 in the nuclear fraction increased by 2.5-fold (Fig. 1A, $p < 0.05$), whereas levels of NRF2 in the cytosolic fraction, or levels of KEAP1, remained relatively constant (Fig. 1B). In response to H_2O_2 treatment, xCT mRNA levels increased by approximately 2-fold (Fig. 1C, $p < 0.05$). In addition, following a 1 h treatment with varying concentrations of H_2O_2 ranging from 50 to 200 μM coupled with a 24 hrecovery in complete medium, xCT protein levels also increased by approximately 2-fold (Fig. 1D). Since xCT determines the transporter activity of system x_c^-, the level of

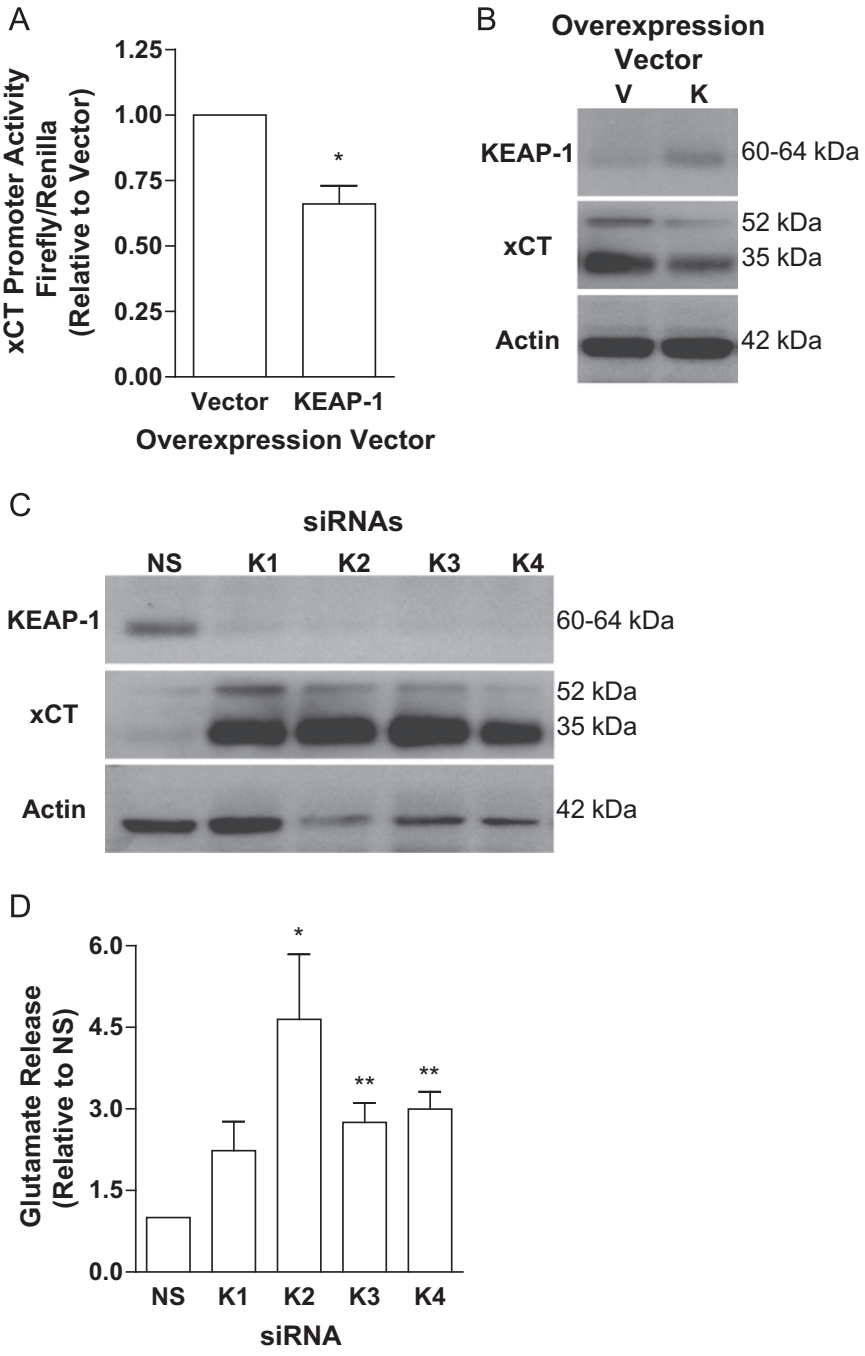

Fig. 3. Altering levels of KEAP1 in MCF-7 cells affected xCT expression and of the activity of system x_c^-. (A) Luciferase assays carried out on lysates isolated from MCF-7 cells transiently transfected with the human xCT promoter construct together with either empty pcDNA3.1 vector (Vector) or a KEAP1 overexpression vector (KEAP1) demonstrated that KEAP1 significantly up-regulated xCT at the level of transcription. (B) A representative Western blot of whole cell lysates prepared from MCF-7 cells transiently transfected with empty pcDNA 3.1 vector (V) or KEAP1 (K) overexpression vector, showing decreased xCT protein levels upon KEAP1 overexpression. (C) Knock-down of KEAP1 using four different siRNAs (K1–K4) corresponded with a concomitant increase in xCT protein levels relative to a non-specific siRNA (NS). (D) Knock-down of KEAP1 also increased glutamate release by transiently transfected MCF-7 cells relative to NS.

glutamate released by MCF-7 cells was also measured. As depicted in Fig. 1E, HO treatment significantly increased the level of extracellular glutamate ($p < 0.05$).

Overexpressing NRF2 up-regulated xCT expression and transporter activity

To confirm that NRF2 regulated xCT expression, MCF-7 cells were transiently transfected with an NRF2 overexpression vector. Using a human xCT promoter construct with a sequence that has already been shown to contain a functional ARE to which NRF2 binds in human bladder carcinoma cells [41], NRF2 overexpression was shown to significantly up-regulate xCT promoter activity in MCF-7 cells (Fig. 2A, $p=0.0075$). In addition, NRF2 overexpression, which was confirmed at the protein level with an increase in the band migrating at approximately 98 kDa, but not overexpression of ETS1, which was used as a negative control, increased xCT protein levels by approximately 3.75-fold (Fig. 2B). Overexpressing NRF2 also increased extracellular levels of glutamate released by MCF-7 cells by approximately 2-fold compared to the empty pcDNA3.1 vector (Fig. 2C, $p < 0.05$).

Altering KEAP1 expression in MCF-7 cells influenced the expression of xCT and transporter activity

To further characterize the regulation of xCT expression by the KEAP1/NRF2 pathway, MCF-7 cells were transiently transfected with a KEAP1 overexpression vector. In direct opposition to the effects obtained by overexpressing NRF2, KEAP1 overexpression led to a significant down-regulation of xCT promoter activity when MCF-7 cells were co-transfected with an xCT promoter-luciferase construct (Fig. 3A, $p < 0.05$). KEAP1 overexpression, which was confirmed at the protein level, also resulted in reduced xCT protein levels relative to empty vector (Fig. 3B). Upon knockdown of KEAP1 using four different siRNAs designed to specifically target human KEAP1, levels of xCT protein decreased relative to a non-specific siRNA (Fig. 3C). Knocking down KEAP1 also resulted in significant increases in the levels of extracellular glutamate (Fig. 3D, $p < 0.05$).

Discussion

Oxidative stress has been implicated in tumor promotion, progression, and treatment resistance through a number of different signaling pathways. One of the key pathways contributing to carcinogenesis and drug resistance is the activation of NRF2 by oxidative stress [48]. The KEAP1/NRF2 pathway is one of the major regulators of protective cellular responses that are initiated in response to oxidative and electrophilic stress, which may underlie inflammation and the development of diverse malignancies such as cancer, cardiovascular and neurodegenerative diseases, and diabetes, among many others. System x_c^- activity has also been shown to protect a number of different cell types from oxidative stress [58,59]. NRF2 regulates various antioxidant response genes that are elevated in cancers including breast [60], colon [61], leukemia [62], and squamous cell lung carcinoma [63]. Chemoresistant cancers present high levels of GSH [64], and depleting intracellular GSH sensitizes resistant tumors to cisplatin [65]. High NRF2 activity has been shown to increase the resistance of breast cancer cells to doxorubicin and paclitaxel [48], and lung cancer cell lines with increased NRF2 activity and corresponding up-regulation of target gene expression demonstrated resistance to chemotherapy [49]. Interestingly, inhibiting system x_c^- may sensitize ER+ breast cancer cells to anti-IGF1R therapy [5]. Therefore, investigating the potential link between NRF2 and system x_c^-, and whether this pathway plays a role in the oxidative stress response in human breast cancer cells, has the potential to provide insights into the development of drug targets to be used in combination therapies.

The aim of the current investigation was to examine whether modulating the KEAP1/NRF2 pathway alters system x_c^- activity by affecting the expression of xCT in response to increased oxidative stress. MCF-7 human breast cancer cells were selected to investigate the contribution of NRF2 and to measure its influence on system x_c^-, given that this particular cell line has been experimentally verified to require antiporter function as a means to mediate protection from ROS [5]. NRF2 expression has been reported to be upregulated in a number of different breast cancer cell lines, including MCF-7 cells [50]. Two approaches were therefore employed to assess whether NRF2 contributes to the regulation of system x_c^- in MCF-7 cells. The first approach focused on chemically increasing the activity of NRF2, and the second utilized the transient overexpression of this transcription factor. H_2O_2 was selected as a direct inducer of ROS and oxidative stress, given that it plays physiologically relevant roles and is endogenously produced by cells through superoxide dismutase (SOD) from reactive molecules in mitochondrial and NOX systems [66]. Indeed, significant levels of H_2O_2 are produced by breast, colon, melanoma, pancreatic, and ovarian cell lines [67].

H_2O_2 treatment increased NRF2 nuclear translocation without significantly affecting KEAP1 expression. It has been reported that KEAP1 is functional in MCF-7 cells, although no differences were found in KEAP1 expression in tumor tissues compared to surrounding normal tissues [48]. KEAP1 has been shown to shuttle between the cytoplasm and nucleus, and is able to repress NRF2 in the nucleus [34]. Electrophilic agents such as H_2O_2 modify important cysteine residues in the linker region of KEAP1, thereby inhibiting the binding of KEAP1 to NRF2, blocking NRF2 degradation by ubiquitination [68]. Given that nuclear KEAP1 levels did not significantly decrease following H_2O_2 treatment in MCF-7 cells, this latter function is likely to correspond to the underlying mechanism driving upregulation of xCT expression that follows NRF2 nuclear localization in response to H_2O_2. In the current investigation, treatment with H_2O_2 significantly increased xCT mRNA levels. In other studies, electrophilic agents have been shown to increase system x_c^- activity [2], and DEM increases xCT mRNA in an NRF2 dependent manner [4], which are consistent with the findings reported here. Use of an overexpression vector encoding NRF2 in transiently transfected MCF-7 cells significantly increased xCT promoter activity, consistent with binding of NRF2 to and transactivation of the xCT promoter in human bladder carcinoma cells [41]. Overexpression of NRF2 increased xCT protein levels and glutamate release, which is indicative of functional upregulation of system x_c^-. KEAP1 knockdown increased xCT protein levels and system x_c^- function, and further investigations aimed at simultaneously knocking down NRF2 will reveal whether the increase in xCT is reversible and therefore NRF2-dependent. The proposed mechanism underlying KEAP1/NRF2-mediated activation of human xCT in MCF-7 human breast cancer cells is diagrammatically summarized in Fig. 4.

This study has several limitations. As system x_c^- is induced to protect cells in culture [59], it is possible that culture conditions may present a confounding factor. Another important in vitro condition that may have an effect on glutamate release is cell density, given that cystine import has been shown to increase at lower densities [69]. Furthermore, we only examined one breast cancer cell line, and it will be important to extend our findings in MCF-7 cells to other human and murine lines. To our knowledge, this is the first study to link system x_c^- and KEAP1/NRF2 in human

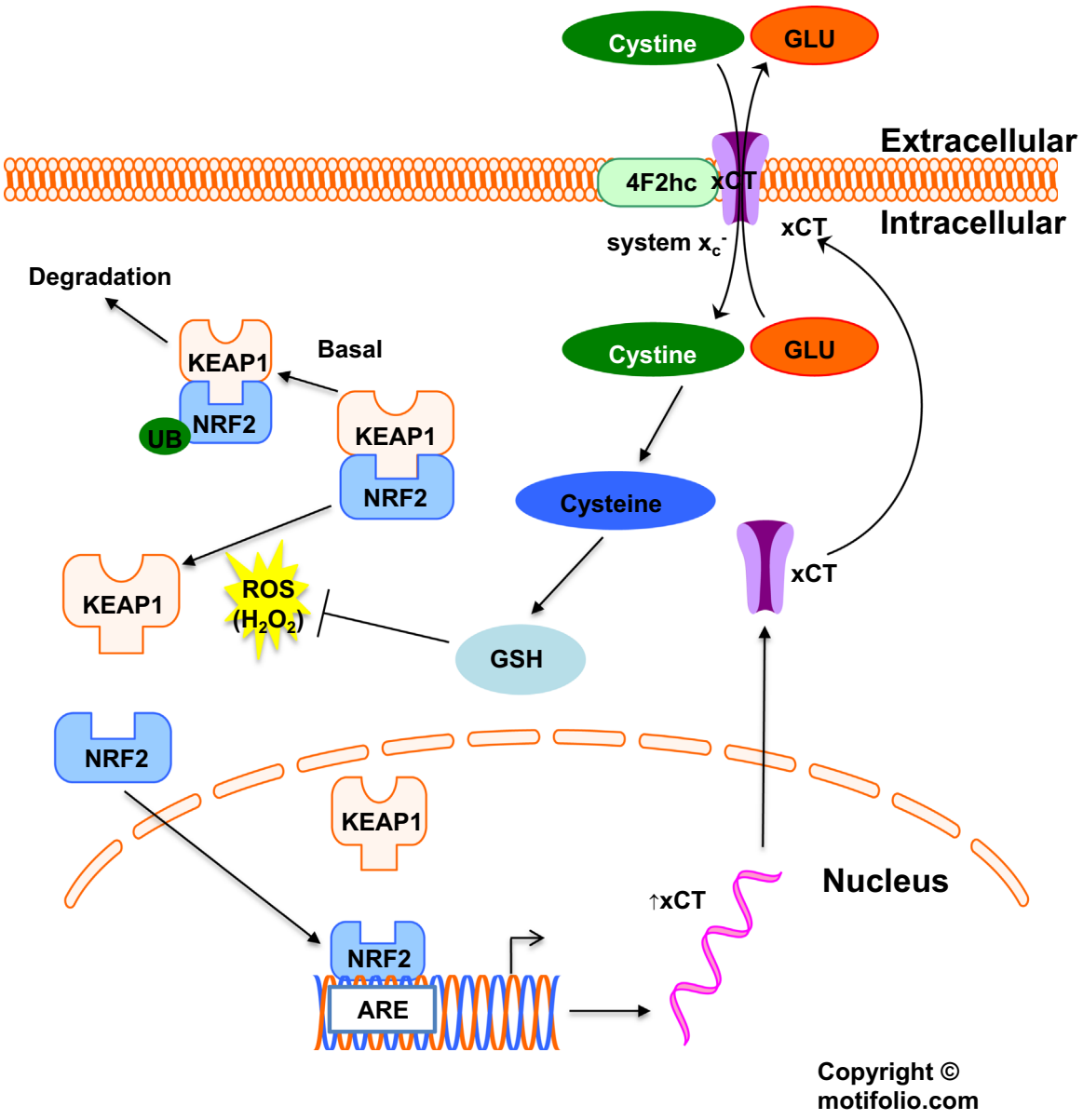

Fig. 4. A summary of the KEAP1/NRF2 pathway regulating xCT expression and system x_c^- activity in MCF-7 breast cancer cells. In the current investigation, NRF2 upregulates xCT transcription, as well as mRNA and protein levels, in human MCF-7 breast cancer cells. Under basal conditions, KEAP1 facilitates the ubiquitination (UB) and proteasomal breakdown of NRF2, while in response to oxidative stress (ROS), NRF2 dissociates from KEAP1, enabling NRF2 to translocate to the nucleus. Nuclear NRF2 binds to the antioxidant response element (ARE) in the xCT promoter, activating transcription. xCT protein is then transported to the membrane, where it couples to 4F2hc, which together comprise system x_c^-. The antiporter functions to export glutamate (GLU) and import cystine. Cystine is then reduced to cysteine, which is utilized in the production of glutathione (GSH), thereby protecting MCF-7 cells from oxidative stress.

breast cancer cells, expanding upon the current understanding of how cancer cells may protect themselves from oxidative stress. System x_c^- is emerging as a potential therapeutic target in drug- and radiation-resistant cancers. It will be of interest to evaluate the effect of small molecule inhibitors that target the KEAP1/NRF2 protein–protein interaction (reviewed in 70) with respect to system x_c^- in breast cancer cells in future studies.

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References

[1] G.K. Balendiran, R. Dabur, D. Fraser, The role of glutathione in cancer, Cell Biochemistry and Function 22 (2004) 343–352. http://dx.doi.org/10.1002/cbf.1149.
[2] S. Bannai, Induction of cystine and glutamate transport activity in human fibroblasts by diethyl maleate and other electrophilic agents, Journal of Biological Chemistry 259 (1984) 2435–2440.
[3] H. Watanabe, S. Bannai, Induction of cystine transport activity in mouse peritoneal macrophages, Journal Experimental Medicine 165 (1987) 628–640.
[4] H. Sasaki, H. Sato, K. Kuriyama-Matsumura, K. Sato, K. Maebara, H. Wang, M. Tamba, K. Itoh, M. Yamamoto, S. Bannai, Electrophile response element-mediated induction of the cystine/glutamate exchange transporter gene expression, Journal of Biological Chemistry 277 (2002) 44765–44771. http://dx.doi.org/10.1074/jbc.M208704200.
[5] Y. Yang, D. Yee, IGF-I regulates redox status in breast cancer cells by activating the amino acid transport molecule xC, Cancer Research 74 (2014) 2295–2305. http://dx.doi.org/10.1158/0008-5472.CAN-13-1803.

[6] J. Lewerenz, P. Maher, A. Methner, Regulation of xCT expression and system x (c) (-) function in neuronal cells, Amino Acids 42 (1) (2012) 171–179. 10.1007/s00726-011-0862-x.
[7] R.G. Ungard, E.P. Seidlitz, G. Singh, Inhibition of breast cancer-cell glutamate release with sulfasalazine limits cancer-induced bone pain, Pain 155 (2014) 28–36. http://dx.doi.org/10.1016/j.pain.2013.08.030.
[8] J.Y. Kim, Y. Kanai, A. Chairoungdua, S.H. Cha, H. Matsuo, D.K. Kim, J. Inatomi, H. Sawa, Y. Ida, H. Endou, Human cystine/glutamate transporter: cDNA cloning and upregulation by oxidative stress in glioma cells, Biochimica et Biophysica Acta 1512 (2001) 335–344.
[9] H. Sato, M. Tamba, T. Ishii, S. Bannai, Cloning and expression of a plasma membrane cystine/glutamate exchange transporter composed of two distinct proteins, Journal of Biological Chemistry 274 (17) (1999) 11455–11458 10206947.
[10] J. Lewerenz, M. Klein, A. Methner, Cooperative action of glutamate transporters and cystine/glutamate antiporter system Xc- protects from oxidative glutamate toxicity, Journal of Neurochemistry 98 (2006) 916–925. http://dx.doi.org/10.1111/j.1471-4159.2006.03921.x.
[11] M.K. Sharma, E.P. Seidlitz, G. Singh, Cancer cells release glutamate via the cystine/glutamate antiporter, Biochemical and Biophysical Research Communications 391 (2010) 91–95. http://dx.doi.org/10.1016/j.bbrc.2009.10.168.
[12] H. Sato, M. Tamba, K. Kuriyama-Matsumura, S. Okuno, S. Bannai, Molecular cloning and expression of human xCT, the light chain of amino acid transport system xc, Antioxidants and Redox Signaling 2 (2000) 665–671.
[13] S.A. Patel, B.A. Warren, J.F. Rhoderick, R.J. Bridges, Differentiation of substrate and non-substrate inhibitors of transport system xc(-): an obligate exchanger of L-glutamate and L-cystine, Neuropharmacology 46 (2004) 273–284.
[14] A. Shiozaki, D. Iitaka, D. Ichikawa, S. Nakashima, H. Fujiwara, K. Okamoto, T. Kubota, S. Komatsu, T. Kosuga, H. Takeshita, H. Shimizu, Y. Nako, H. Sasagawa, M. Kishimoto, E. Otsuji, xCT, component of cysteine/glutamate transporter, as an independent prognostic factor in human esophageal squamous cell carcinoma, Journal of Gastroenterology 49 (2014) 853–863. http://dx.doi.org/10.1007/s00535-013-0847-5.
[15] M.L. Verschoor, G. Singh, Ets-1 regulates intracellular glutathione levels: key target for resistant ovarian cancer, Mol. Cancer 12 (2013) 138. http://dx.doi.org/10.1186/1476-4598-12-138.
[16] W. Nandar, E.B. Neely, E. Unger, J.R. Connor, A mutation in the HFE gene is associated with altered brain iron profiles and increased oxidative stress in mice, Biochimica et Biophysica Acta 1832 (2013) 729–741. http://dx.doi.org/10.1016/j.bbadis.2013.02.009.
[17] E. Singer, J. Judkins, N. Salomonis, L. Matlaf, P. Soteropoulos, S. McAllister, L. Soroceanu, Reactive oxygen species-mediated therapeutic response and resistance in glioblastoma, Cell Death and Disease 6 (2015) e1601. http://dx.doi.org/10.1038/cddis.2014.566.
[18] T. Satoh, T. Rezaie, M. Seki, C.R. Sunico, T. Tabuchi, T. Kitagawa, M. Yanagitai, M. Senzaki, C. Kosegawa, H. Taira, S.R. McKercher, J.K. Hoffman, G.P. Roth, S. A. Lipton, Dual neuroprotective pathways of a pro-electrophilic compound via HSF-1-activated heat-shock proteins and Nrf2-activated phase 2 antioxidant response enzymes, Journal of Neurochemistry 119 (2011) 569–578. http://dx.doi.org/10.1111/j.1471-4159.2011.07449.x.
[19] A.S. Majid, A.M. Majid, Z.Q. Yin, D. Ji, Slow regulated release of H2S inhibits oxidative stress induced cell death by influencing certain key signaling molecules, Neurochemical Research 38 (2013) 1375–1393. http://dx.doi.org/10.1007/s11064-013-1034-z.
[20] T.M. Seib, S.A. Patel, R.J. Bridges, Regulation of the system x(C)- cystine/glutamate exchanger by intracellular glutathione levels in rat astrocyte primary cultures, Glia 59 (2011) 1387–1401. http://dx.doi.org/10.1002/glia.21176.
[21] Y. Shinkai, S. Nakajima, A. Eiguren-Fernandez, E. Di Stefano, D.A. Schmitz, J.R. Froines, A.K. Cho, Y. Kumagai, Ambient vapor samples activate the Nrf2-ARE pathway in human bronchial epithelial BEAS-2B cells, Environmental Toxicology 29 (2014) 1292–1300. http://dx.doi.org/10.1002/tox.21860.
[22] W. Qiang, J.M. Cahill, J. Liu, X. Kuang, N. Liu, V.L. Scofield, J.R. Voorhees, A.J. Reid, M. Yan, W.S. Lynn, P.K. Wong, Activation of transcription factor Nrf-2 and its downstream targets in response to Moloney murine leukemia virus ts1-induced thiol depletion and oxidative stress in astrocytes, Journal of Virology 78 (2004) 11926–11938. http://dx.doi.org/10.1128/JVI.78.21.11926-11938.2004.
[23] A.Y. Shih, D.A. Johnson, G. Wong, A.D. Kraft, L. Jiang, H. Erb, J.A. Johnson, T.H. Murphy, Coordinate regulation of glutathione biosynthesis and release by Nrf2-expressing glia potently protects neurons from oxidative stress, Journal of Neuroscience 23 (2003) 3394–3406.
[24] Y. Zhang, M. Sano, K. Shinmura, K. Tamaki, Y. Katsumata, T. Matsuhashi, S. Morizane, H. Ito, T. Hishiki, J. Endo, H. Zhou, S. Yuasa, R. Kaneda, M. Suematsu, K. Fukuda, 4-hydroxy-2-nonenal protects against cardiac ischemia-reperfusion injury via the Nrf2-dependent pathway, Journal of Molecular and Cellular Cardiology 49 (2010) 576–586. http://dx.doi.org/10.1016/j.yjmcc.2010.05.011.
[25] J. Lewerenz, P. Maher, Basal levels of eIF2alpha phosphorylation determine cellular antioxidant status by regulating ATF4 and xCT expression, Journal of Biological Chemistry 284 (2009) 1106–1115. http://dx.doi.org/10.1074/jbc.M807325200.
[26] L.K. Sha, W. Sha, L. Kuchler, A. Daiber, A.K. Giegerich, A. Weigert, T. Knape, R. Snodgrass, K. Schroder, R.P. Brandes, B. Brune, D.A. Knethen, Loss of Nrf2 in bone marrow derived macrophages (BMDMPhi) impairs antigen-driven CD8 T cell function by limiting GSH and Cys availability, Free Radical and Biology Medicine (2015), http://dx.doi.org/10.1016/j.freeradbiomed.2015.02.004.
[27] O. Gjyshi, S. Flaherty, M.V. Veettil, K.E. Johnson, B. Chandran, V. Bottero, Kaposi's sarcoma-associated herpesvirus induces Nrf2 activation in latently infected endothelial cells through SQSTM1 phosphorylation and interaction with polyubiquitinated Keap1, Journal of Virology 89 (4) (2015) 2268–2286. http://dx.doi.org/10.1128/JVI.02742-14.
[28] M. Ni, X. Li, Z. Yin, H. Jiang, M. Sidoryk-Wegrzynowicz, D. Milatovic, J. Cai, M. Aschner, Methylmercury induces acute oxidative stress, altering Nrf2 protein level in primary microglial cells, Toxicological Sciences 116 (2010) 590–603. http://dx.doi.org/10.1093/toxsci/kfq126.
[29] M. Qaisiya, C.D. Coda Zabetta, C. Bellarosa, C. Tiribelli, Bilirubin mediated oxidative stress involves antioxidant response activation via Nrf2 pathway, Cell Signalling 26 (2014) 512–520. http://dx.doi.org/10.1016/j.cellsig.2013.11.029.
[30] M. Liu, R. Ravula, Z. Wang, Z. Zuo, M.S. Chow, A. Thakkar, S. Prabhu, B. Andresen, Y. Huang, Traditional Chinese medicinal formula Si-Wu-Tang prevents oxidative damage by activating Nrf2-mediated detoxifying/antioxidant genes, Cell and Bioscience 4 (2014) 8. http://dx.doi.org/10.1186/2045-3701-4-8.
[31] T. Tsujita, V. Peirce, L. Baird, Y. Matsuyama, M. Takaku, S.V. Walsh, J.L. Griffin, A. Uruno, M. Yamamoto, J.D. Hayes, Transcription factor Nrf1 negatively regulates the cystine/glutamate transporter and lipid-metabolizing enzymes, Molecular and Cellular Biology 34 (2014) 3800–3816. http://dx.doi.org/10.1128/MCB.00110-14.
[32] J.H. Limon-Pacheco, N.A. Hernandez, M.L. Fanjul-Moles, M.E. Gonsebatt, Glutathione depletion activates mitogen-activated protein kinase (MAPK) pathways that display organ-specific responses and brain protection in mice, Free Radical and Biology Medicine 43 (2007) 1335–1347. http://dx.doi.org/10.1016/j.freeradbiomed.2007.06.028.
[33] C. Gorrini, I.S. Harris, T.W. Mak, Modulation of oxidative stress as an anticancer strategy, Nature Reviews Drug Discovery 12 (12) (2013) 931–947. http://dx.doi.org/10.1038/nrd4002.
[34] D.D. Zhang, Mechanistic studies of the Nrf2-Keap1 signaling pathway, Drug Metabolism Reviews 38 (2006) 769–789. http://dx.doi.org/10.1080/03602530600971974.
[35] I. Meijerman, J.H. Beijnen, J.H. Schellens, Combined action and regulation of phase II enzymes and multidrug resistance proteins in multidrug resistance in cancer, Cancer Treatment Reviews 34 (2008) 505–520. http://dx.doi.org/10.1016/j.ctrv.2008.03.002.
[36] Y. Mitsuishi, K. Taguchi, Y. Kawatani, T. Shibata, T. Nukiwa, H. Aburatani, M. Yamamoto, H. Motohashi, Nrf2 redirects glucose and glutamine into anabolic pathways in metabolic reprogramming, Cancer Cell 22 (2012) 66–79. http://dx.doi.org/10.1016/j.ccr.2012.05.016.
[37] K. Chan, R. Lu, J.C. Chang, Y.W. Kan, NRF2, a member of the NFE2 family of transcription factors, is not essential for murine erythropoiesis, growth, and development, Proceedings of the National Academy of Sciences of the United States of America 93 (1996) 13943–13948.
[38] R. Venugopal, A.K. Jaiswal, Nrf1 and Nrf2 positively and c-Fos and Fra1 negatively regulate the human antioxidant response element-mediated expression of NAD(P)H:quinone oxidoreductase1 gene, Proceedings of the National Academy of Sciences of the United States of America 93 (1996) 14960–14965.
[39] N.M. Inamdar, Y.I. Ahn, J. Alam, The heme-responsive element of the mouse heme oxygenase-1 gene is an extended AP-1 binding site that resembles the recognition sequences for MAF and NF-E2 transcription factors, Biochemical and Biophysical Research Communications 221 (1996) 570–576. http://dx.doi.org/10.1006/bbrc.1996.0637.
[40] J. Jeyapaul, A.K. Jaiswal, Nrf2 and c-Jun regulation of antioxidant response element (ARE)-mediated expression and induction of gamma-glutamylcysteine synthetase heavy subunit gene, Biochemical Pharmacology 59 (2000) 1433–1439.
[41] P. Ye, J. Mimura, T. Okada, H. Sato, T. Liu, A. Maruyama, C. Ohyama, K. Itoh, Nrf2- and ATF4-dependent upregulation of xCT modulates the sensitivity of T24 bladder carcinoma cells to proteasome inhibition, Molecular and Cellular Biology 34 (2014) 3421–3434. http://dx.doi.org/10.1128/MCB.00221-14.
[42] Z. Sun, Z. Huang, D.D. Zhang, Phosphorylation of Nrf2 at multiple sites by MAP kinases has a limited contribution in modulating the Nrf2-dependent antioxidant response, PLoS One 4 (2009) e6588. http://dx.doi.org/10.1371/journal.pone.0006588.
[43] S.K. Niture, A.K. Jain, A.K. Jaiswal, Antioxidant-induced modification of INrf2 cysteine 151 and PKC-delta-mediated phosphorylation of Nrf2 serine 40 are both required for stabilization and nuclear translocation of Nrf2 and increased drug resistance, Journal of Cell Science 122 (2009) 4452–4464. http://dx.doi.org/10.1242/jcs.058537.
[44] L. Li, H. Dong, E. Song, X. Xu, L. Liu, Y. Song, Nrf2/ARE pathway activation, HO-1 and NQO1 induction by polychlorinated biphenyl quinone is associated with reactive oxygen species and PI3K/AKT signaling, Chemico-Biological Interactions 209 (2014) 56–67. http://dx.doi.org/10.1016/j.cbi.2013.12.005.
[45] D.D. Zhang, S.C. Lo, J.V. Cross, D.J. Templeton, M. Hannink, Keap1 is a redox-regulated substrate adaptor protein for a Cul3-dependent ubiquitin ligase complex, Molecular and Cellular Biology 24 (2004) 10941–10953. http://dx.doi.org/10.1128/MCB.24.24.10941-10953.2004.
[46] A.L. Eggler, G. Liu, J.M. Pezzuto, R.B. van Breemen, A.D. Mesecar, Modifying specific cysteines of the electrophile-sensing human Keap1 protein is insufficient to disrupt binding to the Nrf2 domain Neh2, Proceedings of the National Academy of Sciences of the United States of America 102 (2005) 10070–10075. http://dx.doi.org/10.1073/pnas.0502402102.

[47] Y.R. Kim, J.E. Oh, M.S. Kim, M.R. Kang, S.W. Park, J.Y. Han, H.S. Eom, N.J. Yoo, S.H. Lee, Oncogenic NRF2 mutations in squamous cell carcinomas of oesophagus and skin, Journal of Pathology 220 (2010) 446–451. http://dx.doi.org/10.1002/path.2653.
[48] M. Loignon, W. Miao, L. Hu, A. Bier, T.A. Bismar, P.J. Scrivens, K. Mann, M. Basik, A. Bouchard, P.O. Fiset, Z. Batist, G. Batist, Cul3 overexpression depletes Nrf2 in breast cancer and is associated with sensitivity to carcinogens, to oxidative stress, and to chemotherapy, Molecular Cancer Therapeutics 8 (2009) 2432–2440. http://dx.doi.org/10.1158/1535-7163.MCT-08-1186.
[49] A. Singh, V. Misra, R.K. Thimmulappa, H. Lee, S. Ames, M.O. Hoque, J.G. Herman, S.B. Baylin, D. Sidransky, E. Gabrielson, M.V. Brock, S. Biswal, Dysfunctional KEAP1-NRF2 interaction in non-small-cell lung cancer, PLoS Medicine 3 (2006) e420. http://dx.doi.org/10.1371/journal.pmed.0030420.
[50] S.S. Syed Alwi, B.E. Cavell, A. Donlevy, G. Packham, Differential induction of apoptosis in human breast cancer cell lines by phenethyl isothiocyanate, a glutathione depleting agent, Cell Stress Chaperones 17 (2012) 529–538. http://dx.doi.org/10.1007/s12192-012-0329-3.
[51] N.J. Yoo, H.R. Kim, Y.R. Kim, C.H. An, S.H. Lee, Somatic mutations of the KEAP1 gene in common solid cancers, Histopathology 60 (2012) 943–952. http://dx.doi.org/10.1111/j.1365-2559.2012.04178.x.
[52] B. Padmanabhan, K.I. Tong, T. Ohta, Y. Nakamura, M. Scharlock, M. Ohtsuji, M.I. Kang, A. Kobayashi, S. Yokoyama, M. Yamamoto, Structural basis for defects of Keap1 activity provoked by its point mutations in lung cancer, Molecules and Cells 21 (2006) 689–700. http://dx.doi.org/10.1016/j.molcel.2006.01.013.
[53] P. Nioi, T. Nguyen, A mutation of Keap1 found in breast cancer impairs its ability to repress Nrf2 activity, Biochemical and Biophysical Research Communications 362 (2007) 816–821. http://dx.doi.org/10.1016/j.bbrc.2007.08.051.
[54] B.E. Hast, D. Goldfarb, K.M. Mulvaney, M.A. Hast, P.F. Siesser, F. Yan, D.N. Hayes, M.B. Major, Proteomic analysis of ubiquitin ligase KEAP1 reveals associated proteins that inhibit NRF2 ubiquitination, Cancer Research 73 (2013) 2199–2210. http://dx.doi.org/10.1158/0008-5472.CAN-12-4400.
[55] K. Linher-Melville, G. Singh, The transcriptional responsiveness of LKB1 to STAT-mediated signaling is differentially modulated by prolactin in human breast cancer cells, BMC Cancer 14 (2014) 415. http://dx.doi.org/10.1186/1471-2407-14-415.
[56] L.A. Wilson, A. Gemin, R. Espiritu, G. Singh, ets-1 is transcriptionally up-regulated by H2O2 via an antioxidant response element, FASEB Journal 19 (2005) 2085–2087. http://dx.doi.org/10.1096/fj.05-4401fje.
[57] L.A. Wilson, H. Yamamoto, G. Singh, Role of the transcription factor Ets-1 in cisplatin resistance, Molecular Cancer Therapeutics 3 (2004) 823–832.
[58] A.Y. Shih, H. Erb, X. Sun, S. Toda, P.W. Kalivas, T.H. Murphy, Cystine/glutamate exchange modulates glutathione supply for neuroprotection from oxidative stress and cell proliferation, Journal of Neuroscience 26 (2006) 10514–10523. http://dx.doi.org/10.1523/JNEUROSCI.3178-06.2006.
[59] Y. Sakakura, H. Sato, A. Shiiya, M. Tamba, J. Sagara, M. Matsuda, N. Okamura, N. Makino, S. Bannai, Expression and function of cystine/glutamate transporter in neutrophils, Journal of Leukocyte Biology 81 (2007) 974–982. http://dx.doi.org/10.1189/jlb.0606385.
[60] R.R. Perry, J.A. Mazetta, M. Levin, S.C. Barranco, Glutathione levels and variability in breast tumors and normal tissue, Cancer 72 (1993) 783–787.
[61] S.J. Berger, D. Gosky, E. Zborowska, J.K. Willson, N.A. Berger, Sensitive enzymatic cycling assay for glutathione: measurements of glutathione content and its modulation by buthionine sulfoximine in vivo and in vitro in human colon cancer, Cancer Research 54 (1994) 4077–4083.
[62] F. Joncourt, A.E. Oberli-Schrammli, M. Stadler, K. Buser, L. Franscini, M.F. Fey, T. Cerny, Patterns of drug resistance parameters in adult leukemia, Leukocyte Lymphoma 17 (1995) 101–109. http://dx.doi.org/10.3109/10428199509051709.
[63] J.A. Cook, H.I. Pass, S.N. Iype, N. Friedman, W. DeGraff, A. Russo, J.B. Mitchell, Cellular glutathione and thiol measurements from surgically resected human lung tumor and normal lung tissue, Cancer Research 51 (1991) 4287–4294.
[64] R.A. Britten, J.A. Green, H.M. Warenius, Cellular glutathione (GSH) and glutathione S-transferase (GST) activity in human ovarian tumor biopsies following exposure to alkylating agents, International Journal of Radiation Oncology, Biology, Physics 24 (1992) 527–531.
[65] K. Zhang, M. Chew, E.B. Yang, K.P. Wong, P. Mack, Modulation of cisplatin cytotoxicity and cisplatin-induced DNA cross-links in HepG2 cells by regulation of glutathione-related mechanisms, Molecular Pharmacology 59 (2001) 837–843.
[66] G. Loschen, A. Azzi, C. Richter, L. Flohe, Superoxide radicals as precursors of mitochondrial hydrogen peroxide, FEBS Letters 42 (1974) 68–72.
[67] T.P. Szatrowski, C.F. Nathan, Production of large amounts of hydrogen peroxide by human tumor cells, Cancer Research 51 (1991) 794–798.
[68] A. Kobayashi, M.I. Kang, Y. Watai, K.I. Tong, T. Shibata, K. Uchida, M. Yamamoto, Oxidative and electrophilic stresses activate Nrf2 through inhibition of ubiquitination activity of Keap1, Molecular and Cellular Biology 26 (2006) 221–229. http://dx.doi.org/10.1128/MCB.26.1.221-229.2006.
[69] A. Takada, S. Bannai, Transport of cystine in isolated rat hepatocytes in primary culture, Journal of Biological Chemistry 259 (1984) 2441–2445.
[70] C. Zhuang, Z. Miao, C. Sheng, W. Zhang, Updated Research and applications of small molecule inhibitors of Keap1-Nrf2 protein–protein interaction: a review, Current Medicinal Chemistry (2014).