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Signal transducer and activator of transcription 3 and 5 regulate system Xc- and redox balance in human breast cancer cells

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Abstract System Xc- is a cystine/glutamate antiporter that contributes to the maintenance of cellular redox balance. The human xCT (*SLC7A11*) gene encodes the functional subunit of system Xc-. Transcription factors regulating antioxidant defense mechanisms including system Xc- are of therapeutic interest, especially given that aggressive breast cancer cells exhibit increased system Xc- function. This investigation provides evidence that xCT expression is regulated by STAT3 and/or STAT5A, functionally affecting the antiporter in human breast cancer cells. Computationally analyzing two kilobase pairs of the xCT promoter/5′ flanking region identified a distal gamma-activated site (GAS) motif, with truncations significantly increasing luciferase reporter activity. Similar transcriptional increases were obtained after treating cells transiently transfected with the full-length xCT promoter construct with STAT3/5 pharmacological inhibitors. Knock-down of STAT3 or STAT5A with siRNAs produced similar results. However, GAS site mutation significantly reduced xCT transcriptional activity, suggesting that STATs may interact with other transcription factors at more proximal promoter sites. STAT3 and STAT5A were bound to the xCT promoter in MDA-MB-231 cells, and binding was disrupted by pre-treatment with STAT inhibitors. Pharmacologically suppressing STAT3/5 activation significantly increased xCT mRNA and protein levels, as well as cystine uptake, glutamate release, and total levels of intracellular glutathione. Our data suggest that STAT proteins negatively regulate basal xCT expression. Blocking STAT3/5-mediated signaling induces an adaptive, compensatory mechanism to protect breast cancer cells from stress, including reactive oxygen species, by up-regulating xCT expression and the function of system Xc-. We propose that targeting system Xc- together with STAT3/5 inhibitors may heighten therapeutic anti-cancer effects.

Keywords System Xc- · xCT · SLC7A11 · STAT3 · STAT5 · Oxidative stress

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Introduction

Cancer cells adapt to high levels of oxidative stress to survive and proliferate by countering the accumulation of damage-inducing molecules, including reactive oxygen species (ROS), by up-regulating neutralizing enzymes and increasing the production of intracellular antioxidant molecules. One of the main antioxidant mechanisms by which many cancer cells preserve redox balance is through glutathione (GSH). GSH synthesis requires intracellular cysteine, and levels of this amino acid are maintained through the activity of the cell surface transport system Xc-, a cystine/glutamate antiporter that exports glutamate and imports cystine, which is then reduced to cysteine (reviewed in [1]). Under normal physiological conditions, the xCT (*SLC7A11*) gene, which encodes the functional light chain subunit of system Xc-, is most prominently expressed in the central nervous and immune systems, as well as the eye (reviewed in [1]). A deficiency in xCT sensitizes cancer cells to oxidative stress-induced damage [2, 3], also inhibiting their growth [4] and

metastatic potential [5]. Assessing relative levels of xCT expression may therefore provide a mean to predict chemosensitivity and drug resistance [6]. Given the promising synergistic results achieved by treating glioma cells with sulfasalazine, a functional inhibitor of system Xc-, and celastrol, which chemically blocks the effects of heat shock protein 90 (HSP90) [7], combinatorial approaches may prove to elicit the most effective anti-cancer effects.

xCT is linked by a disulfide bridge to the glycoprotein 4F2 heavy chain (4F2hc, CD98) at the extracellular membrane [8], and heterodimers of xCT-4F2hc also complex with the cell adhesion molecule CD44v in cancer cells [2]. As 4F2hc promiscuously interacts with the light chain of several other amino acid transporters, levels of xCT are thought to more prominently influence system Xc- activity [8]. This notion has been experimentally supported by over-expressing xCT in diverse cell types, resulting in significant increases in system Xc- function (reviewed in [1]). The expression of xCT is up-regulated in cultured cells (references in [1]) and various pathological states including cancer (reviewed in [4]). Inflammation, mitochondrial dysfunction, endoplasmic reticulum stress, and excitotoxicity also alter xCT levels in different cell populations [1]. Diverse stimuli that cell-specifically alter the activity of system Xc- or change the expression of xCT include amino acid availability [9, 10], electrophilic agents [9], oxygen [11], bacterial lipopolysaccharide (LPS) [12], tumor necrosis factor alpha (TNF-α) [12], interleukin-1 beta (IL-1β) [13], erythropoietin (EPO) [14], fibroblast growth factor 2 (FGF-2) [15], insulin-like growth factor 1 (IGF-1) [16], iron [17], aspirin [18], and ethanol [19]. In addition, the microRNA miR-26b targets xCT mRNA [20], and xCT trafficking to the extracellular membrane also plays a role in its function.

Several of the mechanisms underlying xCT expression have been examined at the level of transcription. The activity of system xC- may be induced by cystine deprivation [9]. However, functional up-regulation and increased xCT expression also occur upon withdrawal of other amino acids from culture media [10]. A common pathway that underlies amino acid deprivation is the phosphorylation of eukaryotic initiation factor 2 alpha (eIF2α), which inhibits ubiquitous cap-dependent translation while potentiating the translation of specific transcripts, including activating transcription factor 4 (ATF-4) (reviewed in [21]). ATF-4 is able to heterodimerize with CCAAT/enhancer-binding protein (C/EBP) and activator protein 1 (AP-1) family members, binding to amino acid response elements (AAREs) in target gene promoters. The proximal 5′ flanking region of the murine xCT gene has been shown to contain two closely spaced AAREs, with ATF-4 physically binding to the more 5′ site, although both cooperatively mediate xCT transcriptional activity in response to amino acid deprivation [10]. The human xCT promoter has also been confirmed to contain these ATF-4 binding sites [22].

One of the key transcriptional regulators of oxidative stress responses is nuclear factor E2-related factor 2 (NRF2) [23]. Its activity may also be controlled by signaling pathways such as MAPK and PI3K that target NRF2 or its repressor, Kelch-like ECH-associated protein 1 (KEAP1) [24]. The functional up-regulation of system Xc- by electrophilic agents and ROS is dependent on NRF2 in cultured murine macrophages [25]. A proximal electrophile response element (EpRE)/antioxidant response element (ARE) present in the promoter of the murine xCT gene mediates diethylmaleate (DEM)-induced transcriptional activation of xCT in hamster kidney cells, a response that is lost in NRF2-mutant fibroblasts relative to wild-type counterparts [26]. Furthermore, in murine hepatocytes, NRF2 together with NRF1 regulates the stress-inducible expression of xCT [27]. In this scenario, NRF1 acts as a basal transcriptional repressor, suppressing xCT transactivation until cells are exposed to electrophilic/oxidative stress, at which point it is displaced, concurrent with recruitment of NRF2 to the ARE [27]. NRF2 also up-regulates xCT in human bladder carcinoma cells by binding to the ARE [22]. However, LPS-stimulated activity of system Xc- is retained in murine $NRF2^{-/-}$ cells, indicating that other mechanisms are also at play [25].

LPS and TNF-α are inflammatory agents that stimulate system Xc- in vitro [12]. LPS binds to toll-like receptor 4 (TLR-4), thereby activating multiple signaling pathways, including those related to nuclear factor kappa B (NF-κB), which plays a central role in regulating the expression of inflammation-mediated target genes (reviewed in [28]). A putative binding site for NF-κB is present in the 5′ flanking region of the murine xCT gene, although concentrations of LPS too low to sufficiently stimulate signaling through NF-κB nevertheless robustly up-regulate xCT expression in macrophages [29]. This would suggest that as-yet-to-be defined mechanisms underlie the LPS-mediated changes in xCT expression. Similarly, how the pro-inflammatory cytokine TNF-α, which activates numerous intracellular signaling cascades including NF-κB (reviewed in [30]), induces system Xc- remains to be determined. Blocking FGF-2 signaling through FGF receptor 1 (FGFR-1) by inducing either the MEK/ERK or PI3K pathways partially reduces FGF-2-mediated cystine uptake in mixed glial and neuronal cultures [15]. High AP-1 activity due to redox stimulation has been shown to correlate with high xCT expression [31], and AP-1, being one of the most downstream components of the MEK/ERK signaling cascade, provides a potential link between FGF-2 and system Xc-. Another mechanism that is at play during stress responses involves octamer-binding transcription factor 1 (OCT-1). In murine hepatocytes, ethanol inhibits the binding of

OCT-1 to a site in the murine xCT promoter, thereby up-regulating xCT expression [19].

Interestingly, LPS [32], IL-1β [32], TNF-α [33], EPO [34], and FGF [35] are all able to induce intracellular signaling mediated by signal transducer and activator of transcription (STAT) proteins. STATs have emerged as potential targets for the development of novel anti-cancer therapies. In particular, inhibitors of STAT3/5 may become clinically relevant for treating brain and breast cancers, as well as leukemia [36, 37]. There is a potential for STATs to interact with multiple transcription factors, including AP-1 [38], NF-κB [39], and OCT-1 [40]. STAT proteins may also regulate the expression of other transcription factors, creating feedback loops. In addition, significant cross-talk exists between STAT3 and STAT5 and changes in oxidative metabolism, with ROS regulating the activity of STATs and STATs modifying ROS production in both normal and cancer cells (reviewed in [41]). A project referred to as the NRF2-ome, which was designed to identify proteins and regulatory networks associated with NRF2, presented the possibility that NRF2 and the JAK/STAT pathway are connected through STAT1 and STAT3, with STAT3 predicted to physically interact with NRF2 [42]. Interestingly, microarray studies have provided potential links between STATs and xCT expression [43, 44]. The current investigation therefore examined whether STAT proteins, in particular STAT3 and STAT5A, influence the expression of xCT or the function of system Xc- in human breast cancer cells. We aim to develop a framework for understanding the complex mechanisms at play, given significant cross-talk that culminates in STAT activation in breast cancer cells.

Materials and methods

Reagents and antibodies

Antibodies against total STAT3 and STAT5, phospho-STAT3, actin, and calnexin were obtained from Cell Signaling Technologies, Inc. The xCT antibody was from Novus Biologicals, and anti-rabbit IgG horseradish peroxidise secondary antibody was from Cell Signaling Technologies, Inc. (E)-3(6-bromopyridin-2-yl)-2-cyano-N-((S0-1-phenylethyl)acrylamide) (WP1066; Sigma), STAT5 Inhibitor (Calbiochem), the MEK1/2 inhibitor PD098059 (NEB), and BP-1-102, BP-4-018, SF-1-066, and SH-4-54, novel STAT3/5 inhibitors synthesized in the laboratory of Dr. Gunning [37], were reconstituted in DMSO. WP1066 is an analogue of AG490 which potently inhibits JAK2 phosphorylation, also degrading JAK2 protein, thereby dose- and time-dependently blocking downstream activation of the STAT3 and STAT5 as well as the PI3K pathways (see the supplier's specification sheet). Individual aliquots of each compound were stored at −20 °C, and cells were treated with vehicle or an appropriate working concentration of inhibitor for various times at 37 °C in 5 % CO_2. Individual aliquots of recombinant human IL-6 (R&D Systems) were prepared at a concentration of 100 μg/mL by reconstituting the lyophilate in 1× PBS supplemented with 0.1 % BSA, and stored at −20 °C. The final working concentrations were as follows: WP1066 at 5 μM, STAT5 Inhibitor at 100 μM, PD098059 at 20 μM, SH-4-54 at 6.5 μM, and IL-6 at 25 ng/mL. N-acteyl-cysteine (NAC; Sigma) was used at a final concentration of 5 mM, and antimycin A (Sigma) at 50 μM.

Cell lines and culture

All human cell lines were used in accordance with institutional biosafety guidelines. MDA-MB-231, MCF-7, and T47D human breast cancer cells lines were cultured according to the specifications delineated by ATCC for no more than 20 total passages. Cells were plated into 6-well tissue culture-treated plates at 2.5×10^5 cells/well 24 h prior to manipulation to allow optimal attachment, and media were changed prior to treatments.

Cloning of the full-length human xCT promoter region

Cloning of the 2607 bp full-length xCT promoter region, including the 5′-UTR preceding the ATG translational start codon (+1 to +278 bp) and the promoter/5′ flanking region (−1 to −2329 bp) was carried out by PCR amplification using MDA-MB-231 cell-derived genomic DNA as template and the primers xCT-FOR (5′-TTATGGTACCGAGGAAGCTAGGACTATTTCT-3′) and xCT-REV (5′-ATAACTCGAGAGTAGGGACACACGGGGGA-3′), with added KpnI or XhoI site, respectively, underlined. Platinum *Pfx* DNA polymerase and 2× PCR_x Enhancer Solution (Invitrogen) were used in an optimized PCR reaction, the product was extracted using phenol/chloroform, and the KpnI/XhoI digested fragment was cloned into the pGL3-Basic vector (Promega). Construction of the xCT truncation reporter constructs was achieved in a similar manner using the following primers containing KpnI sites paired with the xCT-REV primer above: 5′-TTATGGTACCGATCATCCTCACCATACTTG-3′(−1269/+278), 5′-TTATGGTACCTGACAATGAAGGATATGTATC-3′(−759/+278), 5′-TTATGGTACCGAATTTACTACTTCTGGATTG-3′(−246/+278), and 5′-TTATGGTACCATGAGGAAGCTGAGCTGG-3′(−18/+278). All products were confirmed by gel electrophoresis and bi-directional sequencing.

Site-directed mutagenesis

Mutation of the GAS site within the distal xCT promoter region at −2321 (5′-TGCTTAGAA-3′) was achieved using the QuikChange Site-Directed Mutagenesis kit (Stratagene) and complementary mutant oligonucleotides corresponding to GASmut-Sense (5′-TTCTACAGATTTTGGGGTGAGTTTAAGAGGTACACTGGTGCCC-3′), with the antisense oligo being exactly reverse complementary. The mutated site is underlined. The mutation was confirmed by bi-directional sequencing.

Cloning of STAT3 and STAT5A over-expression vectors

Sequence-verified cDNAs for STAT3 (Clone ID 3347434) and STAT5A (CloneID 5138686) purchased from the Mammalian Gene Collection (GE Healthcare Dharmacon, Inc.) were amplified by PCR, introducing 5′ HindIII and 3′ XhoI sites to facilitate subcloning into the pcDNA3.1 mammalian expression vector (Invitrogen). The following primers were used, with the start and stop codons indicated in bold and restriction enzyme sites underlined: STAT3-FOR (5′-ATTATCTCGAG**ATG**GCCCAATGGAATCAG-3′), STAT3-REV (5′-ATAATAAGCTTTCACATGGGGGAGGTAGC-3′), and STAT5A-FOR (5′-ATTATCTCGAG**ATG**GCGGGCTGGATCCAG-3′), STAT5A-REV (5′-ATAATAAGCTT**TCA**TGAGAGGGAGCCTCT-3′), resulting in products of 2309 and 2384 bp, respectively.

Luciferase reporter assays

Cells were transfected using Lipofectamine 3000 (Invitrogen), and luciferase activity of cell lysates was determined as described previously [45]. All Firefly data were normalized to Renilla luciferase, or Renilla luciferase over total protein, relative to untreated pGL3-Basic.

siRNA transfections

A negative control (Ctrl_Control_1) and experimentally verified siRNAs specifically targeting STAT3 (Hs_STAT3_7) or STAT5A (Hs_STAT5A_2) (Qiagen) were transiently transfected into cells using Hiperfect reagent (Qiagen). Cells were plated at low density (1.25×10^5 cells/well) into 6-well plates 3 h prior to transfection as described previously [45].

Quantitative real-time PCR

cDNA was isolated from treated cells, and quantitative real-time PCR was carried out using primers SLC7A11-FOR (5′-CCTCTATTCGGACCCATTTAGT-3′) and SLCA11-REV (5′-CTGGGTTTCTTGTCCCATATAA-3′) to amplify a 99 bp fragment specific to the human xCT gene, which was verified by sequencing. Housekeeping primers to amplify the RNA polymerase II gene have been described previously [45]. The $2^{-[\Delta][\Delta]C_t}$ method was used to calculate relative mRNA levels, and the results are presented as the fold changes of untreated controls.

Immunoblotting

Total cell lysates were prepared, 50 μg of protein was subjected to SDS-PAGE electrophoresis on 10 % polyacrylamide gels, and immunoblotting was carried out as described previously [45]. Following signal detection, PVDF membranes were stripped and re-probed with primary anti-Actin antibody and anti-mouse IgG-HRP.

Chromatin immunoprecipitations

ChIP assays were carried out using the ChIP-IT Express Enzymatic kit (Active Motif) and chromatin isolated from MDA-MB-231 cells as described previously [45]. Immunoprecipitations included either normal rabbit IgG, STAT3, or STAT5A antibody (sc-2027, sc-7179X, or sc-1081X, respectively; Santa Cruz Biotechnology). Enriched DNA and input were analyzed by quantitative real-time PCR with primers spanning the putative GAS site (GAS-FOR: 5′-TTTACAAGCTTAGAACTGGCAAT-3′ and GAS-REV: 5′-GGCCACCACTCAGAATGTTT-3′), as well as primers specific for a region of the xCT promoter that does not contain a putative STAT binding site (NS-FOR: 5′-TTGAGCAACAAGCTCCTCCT-3′ and NS-REV: 5′-CAAACCAGCTCAGCTTCCTC-3′). The specificity of each product was verified by agarose gel electrophoresis, with bands migrating at 154 and 178 bp, respectively. Fold enrichment relative to IgG was calculated for immunoprecipitated samples using standard methods.

Glutamic acid assay

Cells were treated with various compounds and cultured for 48 h. Media were collected, and cells were lysed to extract total protein. The level of glutamic acid in the extracellular media was determined using the Amplex Red Glutamic Acid/Glutamate Oxidase Assay Kit according to the manufacturer's protocol, with modifications (Invitrogen). The fluorescent readings obtained using a CytoFluor Series 4000 plate reader (Perseptive Biosystems), with excitation and emission wavelengths set at 530 and 590 nm, respectively, were normalized to total protein and presented as fold changes relative to the DMSO control group.

Radiolabeled C^{14}-cystine uptake

The uptake of radiolabeled cystine was assessed as described previously [46], with modifications. Various inhibitors were added to the complete culture media for 48 h. Cells were washed with HEPES-buffered saline solution (HBSS) and incubated for 20 min at 37 °C with ^{14}C-Cystine (0.5 μCi/mL; Perkin Elmer). Cells were then washed 3 times with ice-cold HBSS and lysed in 220 μl of 0.1 N NaOH/0.1 % Triton X-100 [46] for 20 min. A 100 μl aliquot was added to 1 mL of scintillation fluid and placed in a Beckman LS 6000 counter, with 3 min reads for each sample. A fraction of the second aliquot was used to measure total protein. Background was subtracted from each sample, and values were normalized to ^{14}C-cystine uptake obtained for DMSO-treated controls relative to total protein.

Measurements of total intracellular glutathione

Total intracellular glutathione levels were assessed using the protocol detailed as part of the glutathione assay kit (Sigma), and data were normalized to cell counts for each treatment.

ROS assays

The DCFDA assay was used to measure intracellular levels of ROS (including hydroxyl, peroxyl, and other ROS activity within a cell) using CM-H_2DCFDA (Molecular Probes). Briefly, cells were plated into 6-well plates, loaded with the DCFDA reagent (25 μM final concentration in 1× PBS) for 30 min at 37 °C, washed with 1× PBS, and incubated in media without phenol red containing the various treatments for up to 48 h. Fluorescent endpoint reads were determined on a CytoFluor Series 4000 plate reader (Perseptive Biosystems), with an excitation wavelength at 485 nm and an emission wavelength at 535 nm. Each experiment contained a control group of unlabeled cells to verify staining. Results were normalized to cell numbers obtained by crystal violet staining.

Statistical analyses

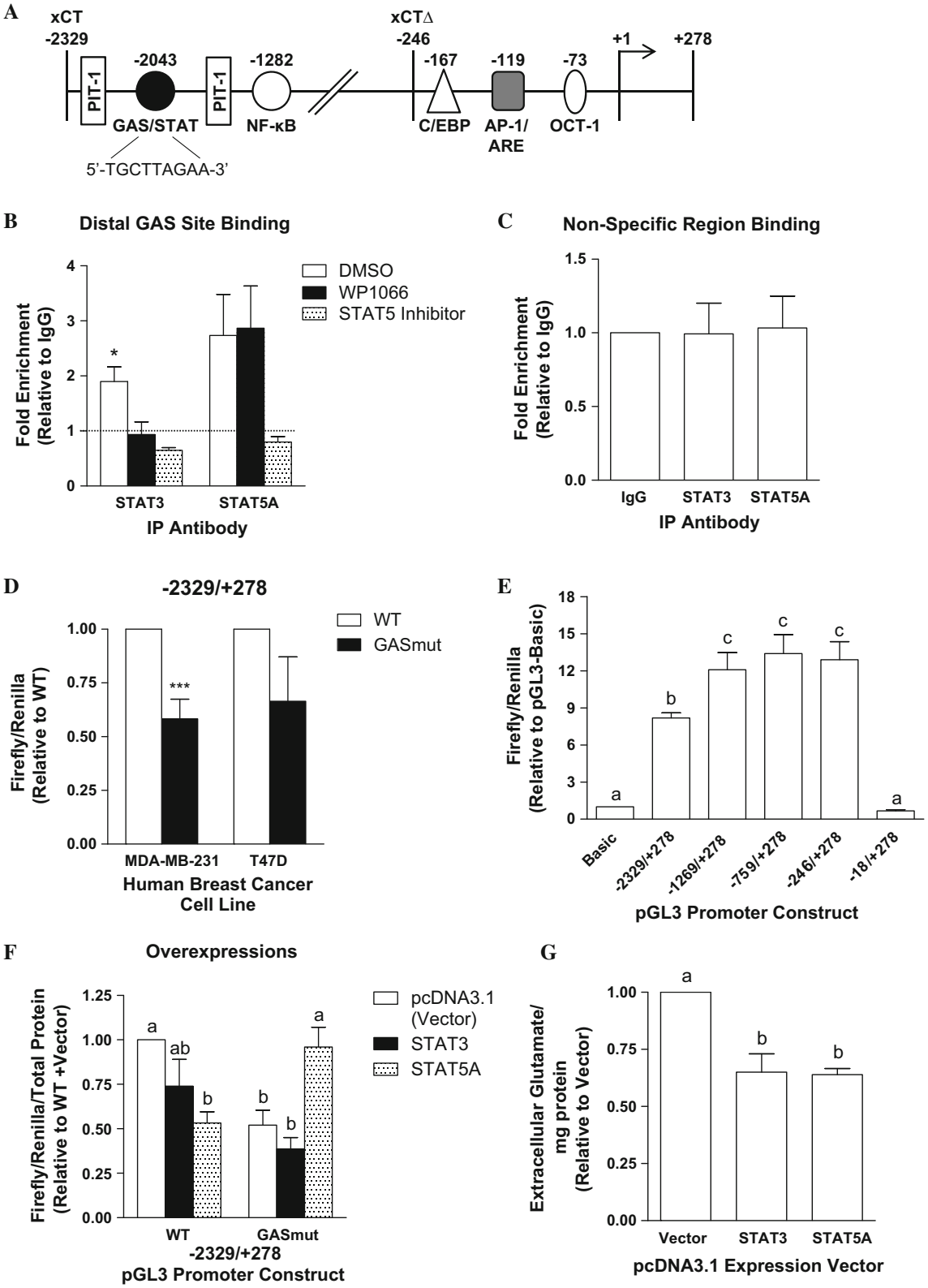
Results represent the mean ± SEM of at least three independent replicates. GraphPad Prism software was used to analyze statistical differences between treatment groups by either *t* test (denoted by stars) or 1-way ANOVA with a Tukey's post-test (denoted by different letters). Results were considered significant at $P < 0.05$. The results of immunoblotting depict a representative image of three independent experiments.

Results

The distal human xCT promoter region contains a putative GAS site to which STAT3 and STAT5A bind

Using NSITE software (Softberry Inc.) paired with a TRANSFAC data base search, computational analysis of the human xCT promoter and 5′ flanking region from −2329 to +278 bp confirmed the location of DNA binding elements previously reported to be present in the human or murine promoters, including several AP-4 sites within the proximal promoter corresponding to characterized AAREs [10, 22], an EpRE/ARE overlapping with a putative AP-1 site [22, 29] at −119/−120, and an NF-κB site [29] at −1282 (Fig. 1a). Several other computationally identified sites were of potential interest, including a highly conserved CCAAT–enhancer binding protein (C/EBP) site at −167 (5′-AGCAA-3′) and two highly conserved POU domain class 1 transcription factor 1 (PIT-1) binding sites within the human xCT 5′ flanking region at −2130 and −2477 (5′-ATTCA-3′) (Fig. 1a). The PIT-1 transcription factor, which binds at PIT-1 sites, is in the same protein family as OCT-1, and an OCT-1 site that acts as a silencer element has previously been characterized in the murine xCT promoter, localizing between −2029 and −2040 upstream of the transcriptional start site [19]. Another interesting hit was a putative gamma-activated site (GAS) at −2321 (5′- TgCTTAGAA-3′) (Fig. 1a). Upon phosphorylation/activation, dimerization, and nuclear translocation, STAT proteins transcriptionally regulate diverse target genes by binding within promoter regions containing GAS motifs. STAT family members preferentially bind to TTC(N_n)GAA, which represents a consensus GAS sequence with $n = 3$ conferring optimal binding of STAT3 and STAT5 [47, 48]. The computationally identified site within the human xCT promoter differs from this motif by one base pair. In addition, STAT proteins may also bind to considerably less well-defined DNA recognition sequences in a promoter-dependent manner, requiring only the recognition sequence TT(N5)AA [49], with a multitude of these sequences present in the 2.6 kilobase pair human xCT promoter region analyzed in the current investigation.

To establish whether STAT3 and/or STAT5A are able to bind to the putative GAS site, chromatin derived from MDA-MB-231 cells was subjected to ChIP coupled with quantitative real-time PCR. Both STAT3 and STAT5A were basally bound to the distal xCT promoter region containing the GAS site, indicated by a 1.9- and 2.7-fold enrichment relative to non-specific IgG ($P = 0.03$ and $P = 0.07$, respectively; Fig. 1b). The binding of STAT3 was significantly reduced after pretreating cells with either


A
xCT
-2329
-2043
-1282
xCTΔ
-246
-167
-119
-73
+1
+278
PIT-1
PIT-1
GAS/STAT
NF-κB
C/EBP
AP-1/
ARE
OCT-1
5'-TGCTTAGAA-3'
B
Distal GAS Site Binding
DMSO
WP1066
STAT5 Inhibitor
Fold Enrichment
(Relative to IgG)
STAT3
STAT5A
IP Antibody
C
Non-Specific Region Binding
Fold Enrichment
(Relative to IgG)
IgG
STAT3
STAT5A
IP Antibody
D
-2329/+278
WT
GASmut
Firefly/Renilla
(Relative to WT)
MDA-MB-231
T47D
Human Breast Cancer
Cell Line
E
Firefly/Renilla
(Relative to pGL3-Basic)
Basic
-2329/+278
-1269/+278
-759/+278
-246/+278
-18/+278
pGL3 Promoter Construct
F
Overexpressions
pcDNA3.1
(Vector)
STAT3
STAT5A
Firefly/Renilla/Total Protein
(Relative to WT +Vector)
WT
GASmut
-2329/+278
pGL3 Promoter Construct
G
Extracellular Glutamate/
mg protein
(Relative to Vector)
Vector
STAT3
STAT5A
pcDNA3.1 Expression Vector

◀**Fig. 1** A putative GAS site within the distal human xCT promoter is bound by STAT3 and STAT5A. **a** A diagram representing −2329 to +278 bp of the human xCT promoter region, with the computationally identified GAS/STAT binding site at −2321. The xCTΔ-246 truncation (−246/+278) and the location of putative PIT-1, NF-κB, C/EBP, AP-1/ARE, and OCT-1 sites are also shown. (BC) ChIP-qPCR with primers designed to specifically amplify a region spanning the GAS site or a non-specific region of the xCT promoter from input, STAT3 and STAT5A antibody, or normal rabbit IgG-immunoprecipitated chromatin from differentially treated MDA-MB-231 cells. **b** Basal STAT3/5A binding occurred within the region containing the GAS site, which was blocked by treating cells with STAT inhibitors. **c** Neither STAT protein was bound to a non-specific region. **d** GAS site mutation (GASmut) decreased xCT promoter activity in MDA-MB-231 and T47D cells. **e** Truncation of the xCT promoter indicated the presence of a silencer element in the region from −2329 to −1270. The region between −19 and −246 is required for basal transcriptional activation of the xCT promoter in breast cancer cells. **f** Transient over-expression of STAT3 and STAT5A repressed wild-type full-length xCT promoter activity, while over-expression of STAT5A in combination with the full-length GASmut promoter returned transcriptional activity to baseline. **g** Over-expression of STAT3 and STAT5A significantly reduced glutamate release from MDA-MB-231 cells. Data represent the mean of three independent experiments (±SEM) calculated relative to appropriate controls, with a *star* (*) indicating statistically significant changes using a *t* test and different *letters* denoting significant differences between groups determined by one-way ANOVA with a Tukey test ($P < 0.05$)

WP1066 or STAT5 Inhibitor prior to isolating chromatin, while STAT5A was displaced only following pretreatment with STAT5 Inhibitor (Fig. 1b). Neither STAT protein was basally bound to a region of the xCT promoter that did not contain a putative GAS site (Fig. 1c).

Mutating the GAS site within the context of the full-length promoter significantly reduced basal transcriptional activity in MDA-MB-231 cells, with similar results obtained in T47D cells (Fig. 1d), suggesting that STAT proteins could be important in modulating the expression of xCT in human breast cancer cells. Truncating the distal region containing the GAS, PIT-1, and NF-κB sites significantly increased basal human xCT promoter activity by 1.5-fold ($P < 0.001$), indicating the likely presence of a silencer element within −2329 to −1270 (Fig. 1e). Further truncations revealed that the region encompassed by xCTΔ-246 (−246/+278) is required for basal transcription (Fig. 1e). Given that STAT proteins may bind to DNA directly or by interacting with AP-1 [38], NF-κB [39], and other transcription factors such as OCT-1, the contribution of the GAS site to basal xCT promoter activity was assessed further.

Transient over-expression of STAT3 or STAT5A in MDA-MB-231 cells decreased basal xCT transcriptional activity by 25 and 50 %, respectively (Fig. 1f). Interestingly, upon mutation of the GAS site, STAT5A no longer functioned repressively, with its over-expression significantly increasing transcriptional activity by 2-fold relative to vector only, suggesting that this transcription factor could potentially be interacting with (an) other site(s) within the human xCT promoter region when the GAS site is unavailable (Fig. 1f). In contrast, STAT3 did not alter promoter activity upon GAS site mutation (Fig. 1f). Over-expressing STAT3 or STAT5A in MDA-MB-231 cells significantly decreased glutamate release by 35 % (Fig. 1g), linking transcriptional changes with system Xc- function.

Blocking STAT proteins increases xCT transcriptional activity

To directly assess the contribution of STAT proteins in regulating human xCT promoter activity, an siRNA approach was employed. Transient, specific knock-down of each target in MDA-MB-231 cells had been previously validated at the protein level compared to a NS siRNA [45]. Knock-down of STAT3 or STAT5A relative to the NS control resulted in significant up-regulation of full-length xCT promoter activity by 1.9- and 1.6-fold, respectively (Fig. 2a). However, neither siRNA significantly affected basal transcriptional activity in cells transfected with the truncated −246/+278 promoter construct (Fig. 2a). The transcriptional changes in cells transfected with STAT3 or STAT5A siRNA were reflected at the protein level, with an increase in xCT expression (Fig. 2b; 55 kDa variant). Chemically blocking STAT protein activation with WP1066 or STAT5 Inhibitor resulted in significant 2-fold increases in xCT promoter activity (Fig. 2c, d). These responses were lost when the promoter was truncated to −18/+278 (Fig. 2c, d). In contrast to results obtained with STAT3 and STAT5A siRNAs, the region from −246/+278 conferred significant up-regulation of transcriptional activity when the STAT3/5 pathway was chemically blocked, suggesting that sites within this particular promoter region promote off-target responsiveness to the inhibitors. This particular region contains several putative binding sites, including C/EBP, AP-1/ARE, and OCT-1, that could mediate off-target effects. Several novel small-molecule chemical inhibitors developed to more specifically target phospho-STAT3/5 were also assessed, including BP-1-102, BP-4-018, SF-1-066, and SH-4-54, with 5 μM of the latter significantly increasing full-length xCT promoter activity by approximately 2-fold (Fig. 2e). SH-4-54 was previously shown to strongly bind to STAT3, effectively suppressing its phosphorylation and downstream transcriptional targets [37]. As SH-4-54 produced the most pronounced transcriptional effects at the xCT locus in human breast cancer cells without significantly reducing cell number, this compound was utilized for all further analyses. PD098059, which effectively blocks signaling through ERK1/2, produced an inverse effect on xCT promoter activity (Fig. 2f).

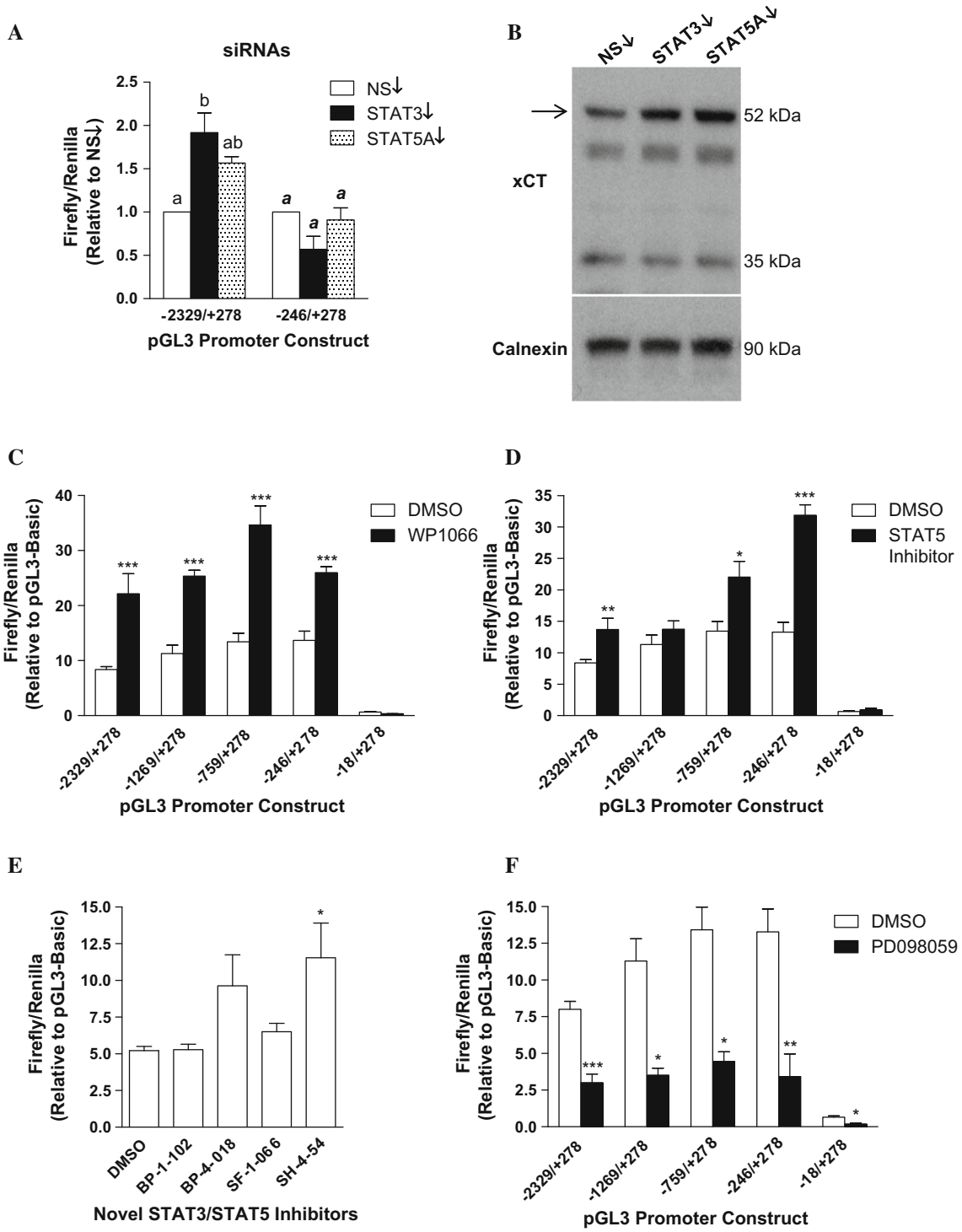


Fig. 2 Suppressing STAT3 and STAT5A increases xCT transcriptional activity. **a** Knock-down of STAT3 (S3↓) or STAT5A (S5A↓) relative to a non-specific (NS↓) siRNA significantly increased full-length promoter activity but failed to alter transcriptional activity conferred by the −246/+278 truncated region. **b** siRNA-mediated knock-down of STAT3 and STAT5A also increased xCT expression at the protein level. Pharmacologically blocking STAT3/5 activation with **c** WP1066 or **d** STAT5 Inhibitor significantly up-regulated xCT transcriptional activity in MDA-MB-231 cells. **e** Treatment with one of the novel STAT3/5 pathway blockers, SH-4-54, also significantly activated the xCT promoter, while **f** PD098059 produced the opposite effect. Data represent the mean of three independent experiments (±SEM) calculated relative to appropriate controls, with a *star* (*) indicating statistically significant changes using a *t* test and different *letters* denoting significant differences between groups determined by one-way ANOVA with a Tukey test ($P < 0.05$)

Mutating the distal GAS site within context of the xCT full-length promoter completely and significantly abolished the transcriptional up-regulation conferred by WP1066 in both MDA-MB-231 (Fig. 3a) and T47D (Fig. 3b) cells. Mutating the GAS site reduced SH-4-54-mediated transcriptional up-regulation in MDA-MB-231 cells by 1.6-fold (Fig. 3a) while completely blocking the effect of SH-4-54 in T47D cells (Fig. 3b). In MDA-MB-231 cells, the stimulatory effects of both inhibitors on wild-type promoter activity were lower than the fold changes obtained in T47D cells. Although PD098059 alone reduced promoter activity by 3-fold relative to the DMSO-treated control (Fig. 3c), treating MDA-MB-231 cells transfected with the full-length wild-type promoter construct with WP1066 or SH0545 in combination with PD098059 did not alter the stimulatory effect of either of the two STAT inhibitors (Fig. 3c). This suggests that their action on the xCT promoter is likely independent of the MAPK pathway.

Treatment with STAT inhibitors increases xCT expression and up-regulates the function of system Xc-

Significant increases in xCT expression at the mRNA level were observed in MDA-MB-231, T47D, and MCF-7 human breast cancer cells following a 6 h treatment with WP1066 or SH-4-54 (Fig. 4a). Of note, the effect of both inhibitors on xCT mRNA levels was more dramatic in T47D and MCF-7 cells compared to MDA-MB-231 cells. Increases were sustained in MDA-MB-231 cells after 24 h (up to 48 h, results not shown), with STAT5 Inhibitor also significantly up-regulating mRNA levels (Fig. 4b). Furthermore, expression of xCT at the protein level in MDA-MB-231 cells was increased by treatment with WP1066 and STAT5 Inhibitor (Fig. 4c), as well as SH-4-54, which produced maximal effects at 5 μM (Fig. 4d). WP1066 and SH-4-54 also significantly reduced levels of phospho-

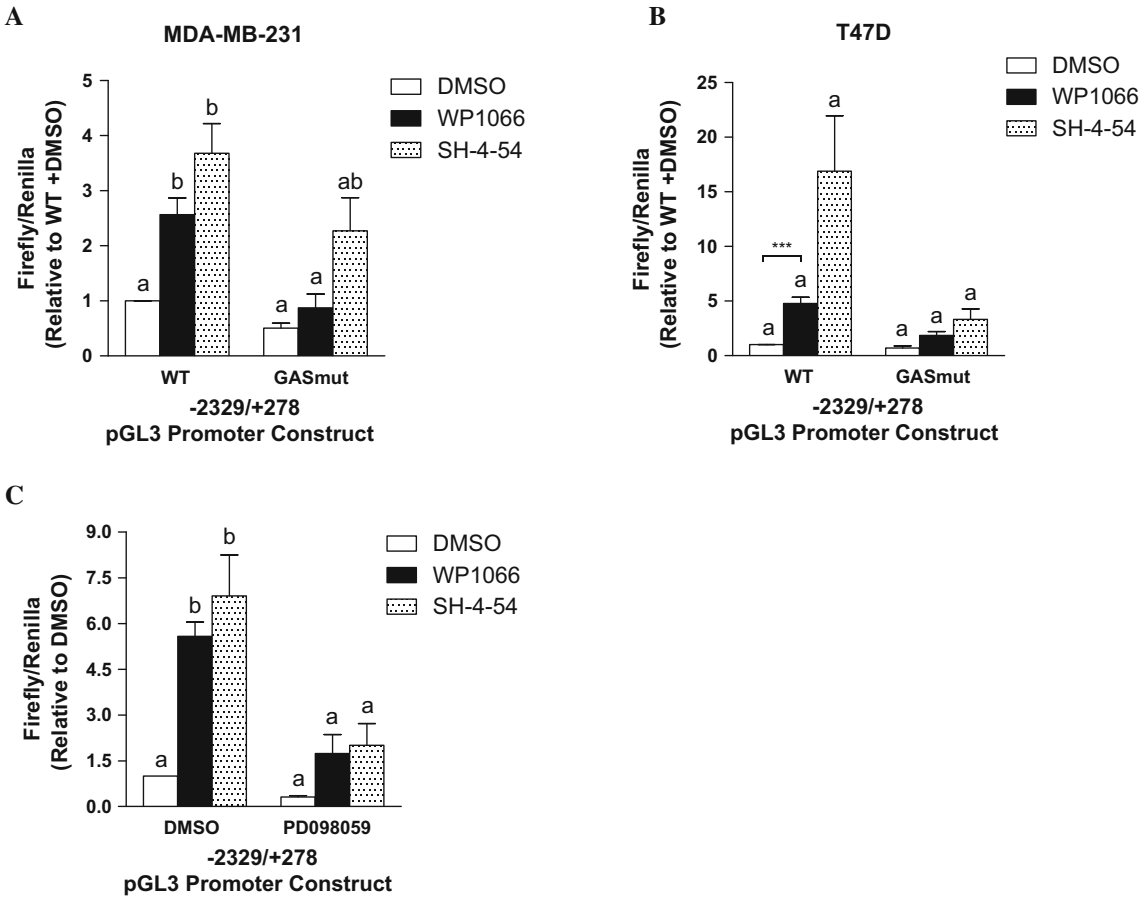
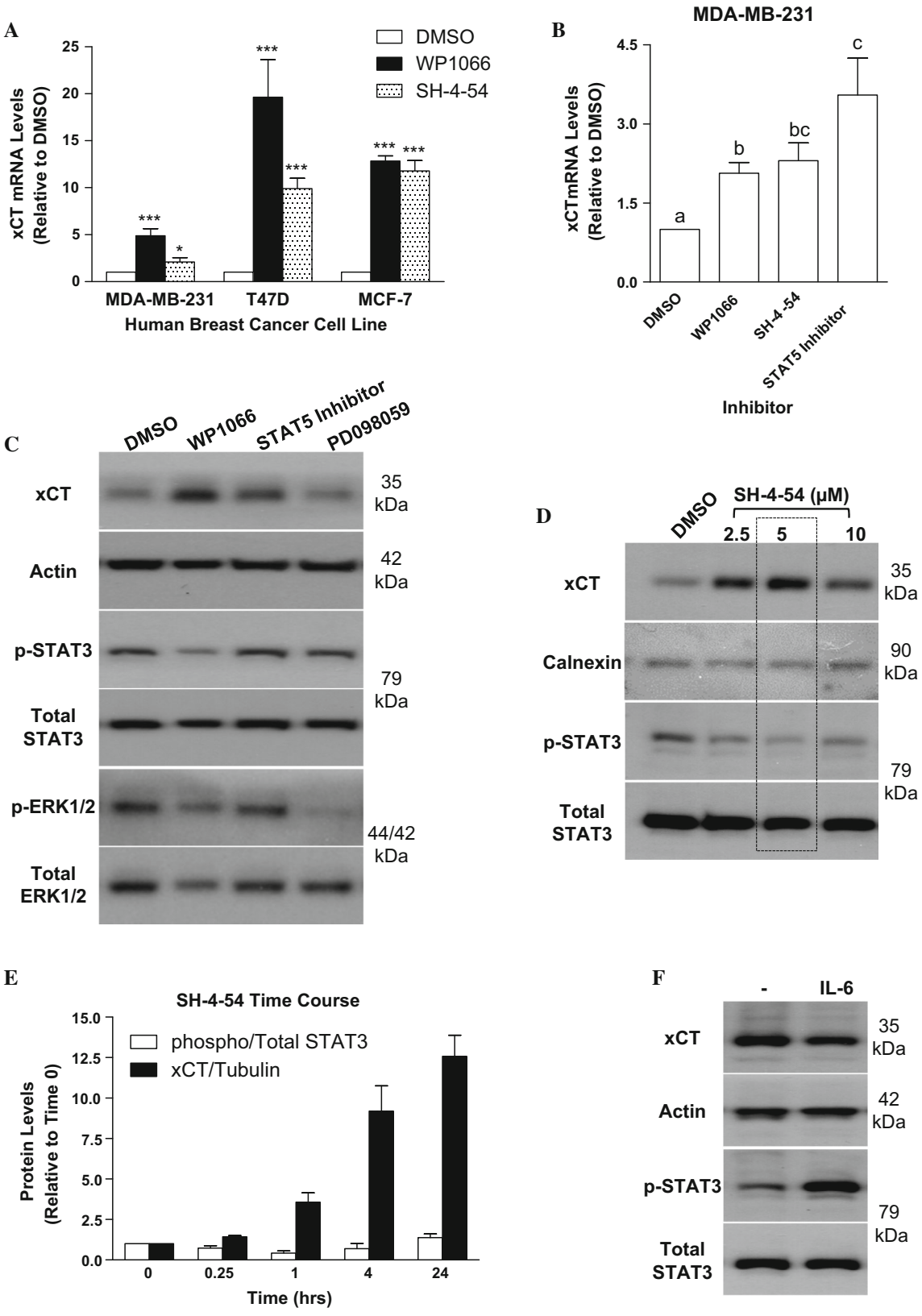


Fig. 3 The GAS site influences the effect of WP1066 and SH-4-54. Mutation of the GAS site in **a** MDA-MB231 cells or **b** T47D cells completely abolished the stimulatory effect of WP1066 on xCT promoter activity, while the effect of SH-4-54 was partially blocked in MDA-MB-231 cells and completely abrogated in T47D cells. **c** Co-treatment with PD098059 did not significantly block the effect of both drugs on the full-length xCT promoter in transiently transfected MDA-MB-231 cells. Data represent the mean of three independent experiments (±SEM) calculated relative to appropriate controls, with a *star* (*) indicating statistically significant changes using a *t* test and different *letters* denoting significant differences between groups determined by one-way ANOVA with a Tukey test ($P < 0.05$)


A
DMSO
WP1066
SH-4-54
xCT mRNA Levels (Relative to DMSO)
MDA-MB-231
T47D
MCF-7
Human Breast Cancer Cell Line
B
MDA-MB-231
xCTmRNA Levels (Relative to DMSO)
DMSO
WP1066
SH-4-54
STAT5 Inhibitor
Inhibitor
C
DMSO
WP1066
STAT5 Inhibitor
PD098059
xCT
Actin
p-STAT3
Total STAT3
p-ERK1/2
Total ERK1/2
35 kDa
42 kDa
79 kDa
44/42 kDa
D
DMSO
SH-4-54 (μM)
2.5
5
10
xCT
Calnexin
p-STAT3
Total STAT3
35 kDa
90 kDa
79 kDa
E
SH-4-54 Time Course
phospho/Total STAT3
xCT/Tubulin
Protein Levels (Relative to Time 0)
Time (hrs)
F
-
IL-6
xCT
Actin
p-STAT3
Total STAT3
35 kDa
42 kDa
79 kDa

◀**Fig. 4** Treatment of MDA-MB-231 cells with STAT inhibitors up-regulates xCT expression, also selectively increasing the function of system Xc-. **a** Pharmacologically blocking STAT signaling with WP1066 and SH-4-54 for 6 h significantly increased levels of xCT mRNA in MDA-MB-231, MCF-7, and T47D cells, **b** a response that was sustained in MD-MB-231 cells after a 24 h treatment, with STAT5 Inhibitor also significantly up-regulating xCT mRNA levels. **c** WP1066 and STAT5 Inhibitor, as well as **d** SH-4-54, increased xCT protein levels, with WP1066 reducing phospho-STAT3. PD098059 did not alter levels of xCT at the protein level, but phospho-ERK1/2 was reduced. **e** Densitometric analysis of a time course with SH-4-54 confirmed a significant block of STAT3 phosphorylation over 24 h with a concomitant increase in levels of xCT protein levels, whereas **f** recombinant human IL-6, which up-regulated phospho-STAT3, produced the inverse effect. Data represent the mean of three independent experiments (±SEM) calculated relative to appropriate controls, with a *star* (*) indicating statistically significant changes using a *t* test and different *letters* denoting significant differences between groups determined by one-way ANOVA with a Tukey test ($P < 0.05$)

STAT3 (Fig. 4c, d). Similar results were obtained with WP1066 in MCF-7 cells (results not shown). Treatment with PD098059 blocked phospho-ERK1/2 in MDA-MB-231 cells without altering levels of xCT protein (Fig. 4c). A time course with SH-4-54 at 5 μM, analyzed by densitometric analysis, confirmed that xCT expression increased with inhibition of phospho-STAT3 (Fig. 4e). To assess whether an extracellular stimulus known to induce STAT3 phosphorylation could alter xCT expression, MDA-MB-231 cells were treated with IL-6, which down-regulated xCT protein levels concomitant with a marked increase in phospho-STAT3 (Fig. 4f).

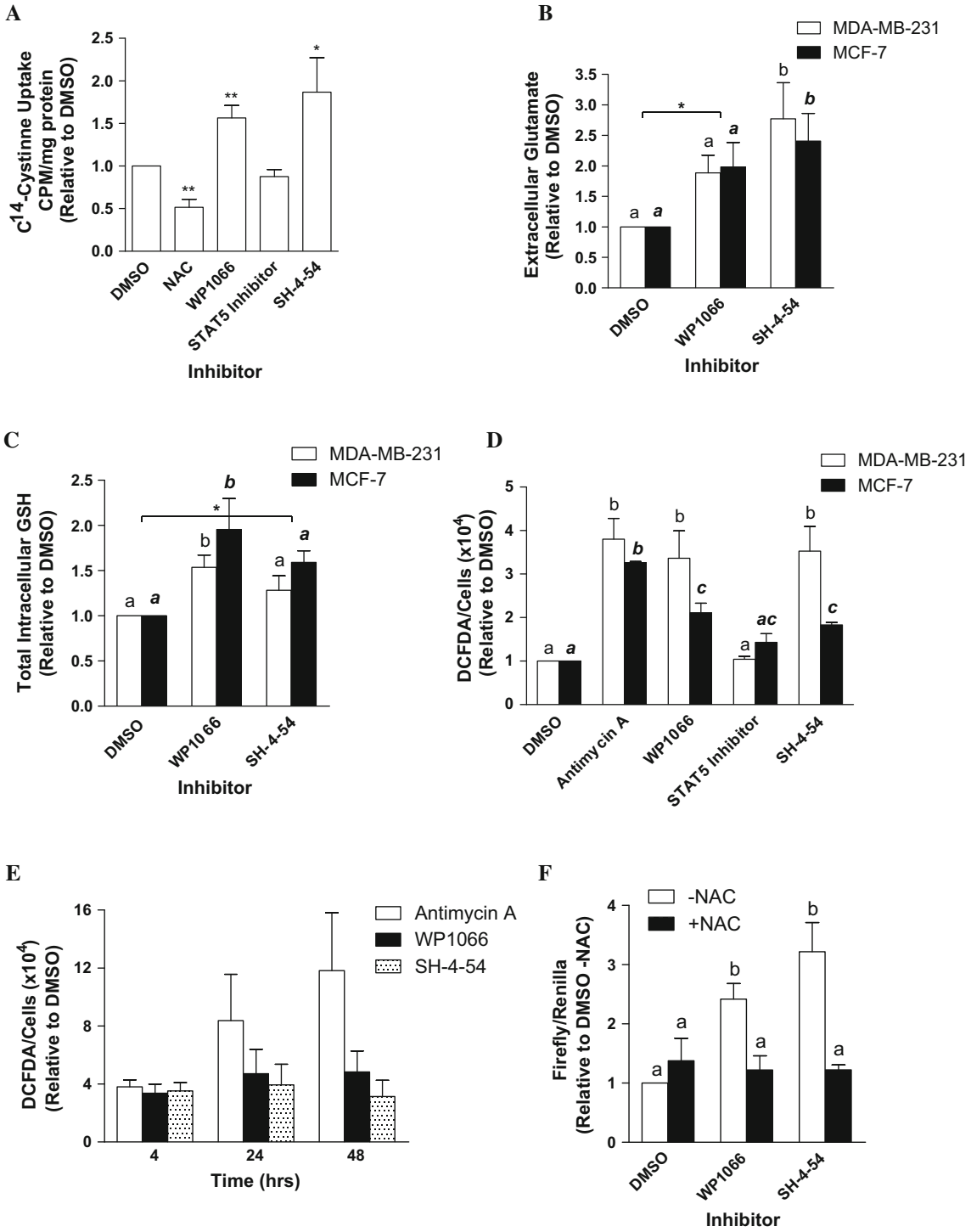
In addition to affecting xCT expression, system Xc- was functionally up-regulated in MDA-MB-231 cells. While exposure to NAC, which may act as a source of cysteine, decreased the radiolabeled uptake of cystine by 50 %, both WP1066 and SH-4-54 significantly increased cystine uptake by 1.6- and 1.9-fold, respectively (Fig. 5a). Treatment with STAT5 Inhibitor did not correlate with an increase in cystine uptake (Fig. 5a). Therefore, its effect on glutamate release and production of total levels of intracellular GSH were not evaluated. Glutamate release increased by approximately 2-fold in response to WP1066 and SH-4-54 in both MDA-MB-231 and MCF-7 cells (Fig. 5b). In addition, both inhibitors also enhanced intracellular GSH production in both cell types (Fig. 5c).

The transcriptional responses induced by WP1066 and SH-4-54 arise, at least in part, due to ROS production

As WP1066, STAT5 Inhibitor, and SH-4-54 all significantly up-regulated xCT promoter activity within the truncated −246/+278 reporter construct, which encompasses a region that contains an ARE likely to mediate oxidative stress responses, the effect of these drugs on ROS production was examined in MDA-MB-231 cells. Using antimycin A as a positive control to induce oxidative stress, both WP1066 and SH-4-54 significantly increased levels of intracellular ROS in MDA-MB-231 cells by approximately 3-fold after a 4 h treatment relative to DMSO (Fig. 5d). Whereas the production of ROS continued to increase over 48 h in cells treated with antimycin A, levels of ROS were stabilized by 24 h in those treated with WP1066 or SH-4-54 (Fig. 5e) consistent with up-regulated xCT expression and system Xc- function. Co-treating cells with NAC abolished the stimulatory effects elicited by WP1066 and SH-4-54 on xCT promoter activity (Fig. 5f). The STAT5 Inhibitor did not significantly up-regulate ROS levels (Fig. 5d), suggesting an alternate mechanism of action for this particular compound.

Discussion

System Xc- is an important adaptive mechanism utilized by cancer cells to manage altered metabolism and effectively maintain redox balance (Fig. 6a), ensuring their long-term survival. While diverse extracellular stimuli affect the expression of xCT and/or the function of system Xc-, the current investigation has initiated an exploration of how STAT3 and STAT5 proteins may regulate xCT transcriptional activity in human breast cancer cells. This approach is particularly relevant given that STAT3/5 are key regulators that control the expression of multiple genes required for cancer cell survival and proliferation, potentially integrating signals from diverse extracellular stimuli including hormones, growth factors, cytokines, and ROS (Fig. 6b). The response of cancer cells to ROS production is complex, as numerous signaling pathways may be affected by this process [50, 51]. Chronic extra- and intracellular exposure to oxidative stress may induce some cancer cells to undergo growth arrest (cellular senescence) or apoptosis [2]. Other types of cancer cells adapt by re-establishing redox balance [52], promoting survival under unfavorable conditions, also contributing to further genomic instability [53], metastasis [54], and multi-drug resistance [55]. In the current investigation, chemically targeting STAT3/5 activation with either the known JAK2/STAT3/5 pathway inhibitor WP1066 or the novel STAT3/5 small molecule inhibitor SH-4-54, which has been shown to specifically block the SH2 domain with minimal off-target effects [37], significantly up-regulated ROS production in MDA-MB-231 cells. However, unlike treatment with antimycin A, which directly affects the mitochondrial electron transport chain, thereby driving continuous increases in ROS over 48 h, the production of ROS leveled


A
C^{14}-Cystinne Uptake CPM/mg protein (Relative to DMSO)
DMSO
NAC
WP1066
STAT5 Inhibitor
SH-4-54
Inhibitor
B
MDA-MB-231
MCF-7
Extracellular Glutamate (Relative to DMSO)
DMSO
WP1066
SH-4-54
Inhibitor
C
MDA-MB-231
MCF-7
Total Intracellular GSH (Relative to DMSO)
DMSO
WP1066
SH-4-54
Inhibitor
D
MDA-MB-231
MCF-7
DCFDA/Cells (x10^4) (Relative to DMSO)
DMSO
Antimycin A
WP1066
STAT5 Inhibitor
SH-4-54
E
Antimycin A
WP1066
SH-4-54
DCFDA/Cells (x10^4) (Relative to DMSO)
Time (hrs)
F
-NAC
+NAC
Firefly/Renilla (Relative to DMSO -NAC)
DMSO
WP1066
SH-4-54
Inhibitor

◀**Fig. 5** Chemically blocking STAT activation increases the function of system Xc- to counter levels of intracellular ROS. **a** In MDA-MB-231 cells, WP1066 and SH-4-54 significantly increased cystine uptake, while STAT5 Inhibitor did not affect this process. In both MDA-MB-231 and MCF-7 cells, **b** accumulation of extracellular glutamate and **c** levels of total intracellular GSH were increased by WP1066 and SH-4-54 treatment. **d** A 4 h treatment with WP1066, SH-4-54, or Antimycin A significantly up-regulated the level of intracellular ROS relative to DMSO-treated controls in MDA-MB-231 and MCF-7 cells, whereas STAT5 Inhibitor did not significantly alter endogenous ROS levels. **e** A time course revealed that ROS were maximally increased within 4 h of WP1066 and SH-4-54 treatment, with Antimycin A producing further increases in ROS over a 48 h period. **f** Co-treating cells transiently transfected with the full-length xCT promoter construct with NAC completely abolished the stimulatory transcriptional effects of WP1066 and SH-4-54. Data represent the mean of three independent experiments (±SEM) calculated relative to appropriate controls, with a *star* (*) indicating statistically significant changes using a *t* test and different *letters* denoting significant differences between groups determined by one-way ANOVA with a Tukey test ($P < 0.05$)

off at 4 h following treatment with the STAT inhibitors. This particular response is likely due to compensatory up-regulation of xCT expression and a concurrent increase in the function of system xC-, culminating in increased GSH production in response to the STAT inhibitors. Both STAT3 and STAT5 are intricately and complexly involved in oxidative metabolism in cancer cells (reviewed in [41]), with conflicting literature regarding their regulation by ROS as well as their influence on ROS production. Growth factors and cytokines may induce the production of ROS [56, 57], with ROS inactivating tyrosine phosphatases [58], thereby potentially up-regulating the phosphorylation and activation of JAK kinases, STAT3, and STAT5, as well as other signaling molecules [59]. Conversely, it has been shown that oxidative stress and ROS production may interfere with the JAK/STAT pathway [60, 61].

In the current investigation, the GAS site located in the distal human xCT promoter was shown, under basal conditions, to be bound by STAT3 and/or STAT5A in MDA-MB-231 cells, with binding of these proteins specifically blocked by treatment with various STAT inhibitors. While blocking STAT3 and STAT5A chemically or with siRNAs up-regulated basal xCT transcriptional activity, over-expression of STAT3 and STAT5A or mutation of the GAS site in conjunction with inhibitor treatments produced the inverse effect. This finding suggests that binding of STATs to the GAS site is repressive but also dependent on the action of other factors. Blocking STAT3 or STAT5A via siRNAs confirms the repressive role of these factors on xCT transcriptional activity, while chemical inhibition also induces off-target effects, via, for example, the production of ROS, which required separate examination to tease out the direct effect of STAT3/5 inhibition. Several potential binding sites for STAT3/5 interacting factors are present in the proximal human xCT promoter region, including C/EBP, OCT-1, and AP-1/ARE. Interestingly, it has been shown that AP-1 interacts with STAT3 to induce target gene expression [38], AP-1 activity is increased in neurons in response to up-regulated ROS production [62], and its activity due to redox stimulation has been linked to high xCT expression [31]. In human breast cancer cells, a reciprocal relationship between STAT5 and AP-1 has been described, with activated STAT5 inhibiting AP-1 activity while the over-expression of STAT3 enhanced AP-1 responses [63]. However, treating MDA-MB-231 human breast cancer cells with PD098059, a MEK1/2 inhibitor that blocks ERK1/2 phosphorylation, which is linked with FOS/JUN and AP-1 activation, in combination with WP1066 or SH-4-54, did not alter the stimulatory effect of the latter two drugs. The proximal AP-1/ARE site may nevertheless be integral to the transcriptional responses described in the current investigation, given that NAC completely abolished the effects mediated by WP1066 and SH-4-54. These compounds increased cellular ROS levels, and ROS up-regulate the nuclear translocation of NRF2, another FOS/JUN family member (reviewed in [64]), which has been shown to bind to the ARE in the proximal xCT promoter region [26]. Blocking ROS production with NAC would thereby counteract up-regulated transcriptional activity through either AP-1 or NRF2 in a manner independent from MAPK signaling. It will be of future interest to establish whether the STAT3/5 inhibitors influence AP-1 or NRF2 binding at the xCT locus, and whether this interaction is influenced by STAT occupancy at the distal GAS site. NAC may also act as a source of intracellular cysteine [65], thereby potentially reducing the levels of ROS directly generated in response to treatment with the STAT inhibitors. In addition, NAC alters signaling by producing cytokine effects [66], with a study demonstrating that NAC stimulates the JAK/STAT3 pathway [67]. This latter effect could also potentially explain its opposing action to the STAT inhibitors on xCT transcriptional activation in breast cancer cells.

Another finding from the current investigation is that estrogen receptor (ER)-negative MDA-MB-231 cells, in which STAT3 has been shown to be constitutively active [68], supported a less dramatic increase in xCT transcriptional activity and mRNA levels in response to WP1066 or SH-4-54 treatment than T47D and MCF-7 ER-positive cells. A study by Yamashita and Iwase [69] reported that in these latter two breast cancer cell lines, which exhibit constitutively activated STAT5, targeting STAT5 represents a potential means to suppress ER activity. Interestingly, we computationally identified a putative estrogen response element (ERE) in the xCT promoter region (data

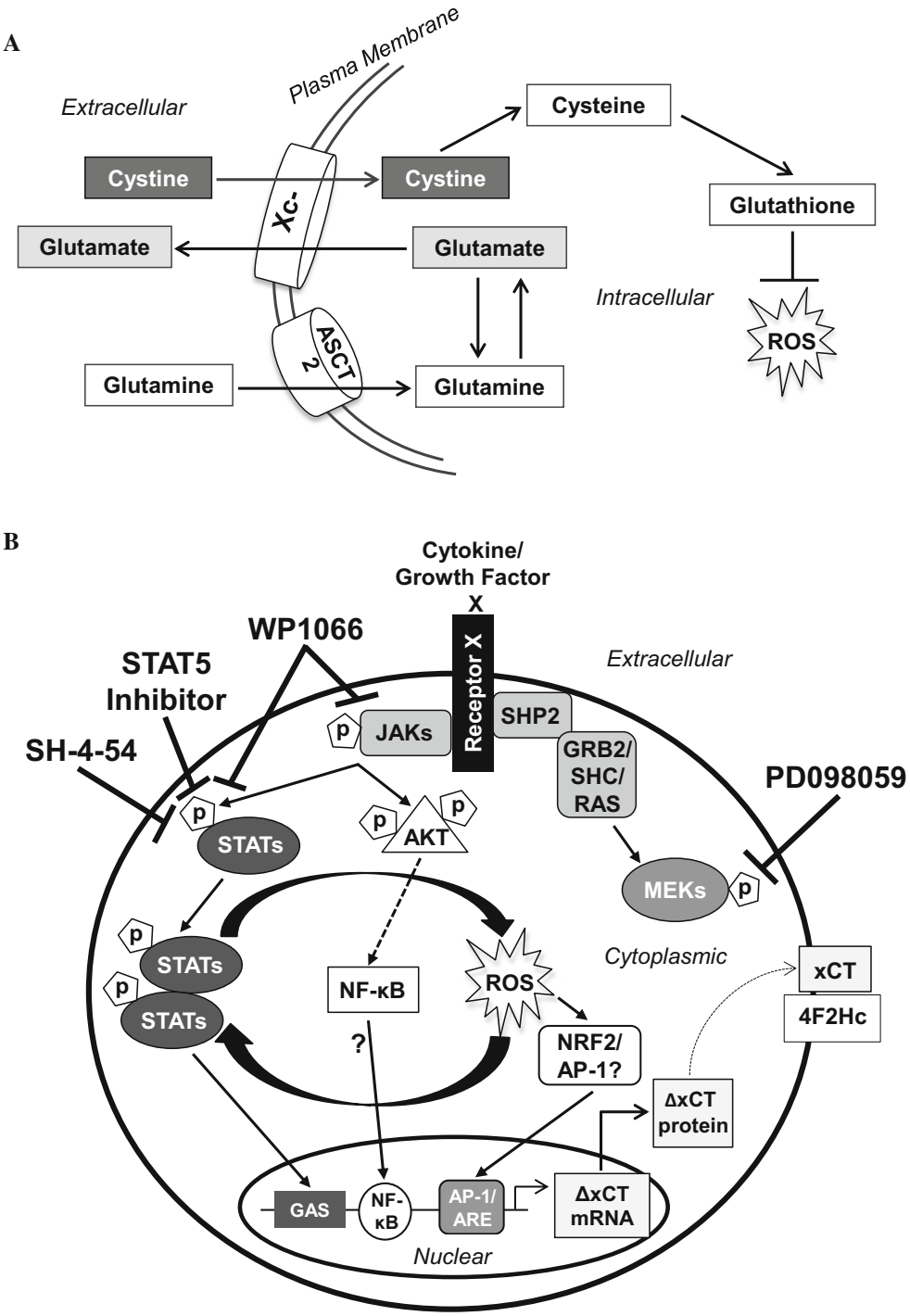


Fig. 6 The cellular redox cycle and potential pathways affecting system Xc-. **a** Cancer cells adapt to high levels of oxidative stress by countering the accumulation of ROS with an increased production of intracellular antioxidant molecules such as GSH. In response to increased glutamine metabolism, system Xc- exports glutamate while importing cystine, thereby mediating levels of cysteine required for GSH synthesis and the maintenance of cellular redox balance. **b** Transcription factors that regulate system Xc- may be of therapeutic interest, especially given that aggressive breast cancer cells have been shown to exhibit increased expression of xCT and function of system Xc-. STAT proteins have emerged as potential targets for the development of novel anti-cancer therapies. In particular, inhibitors of STAT3/5 may become clinically relevant. Upon phosphorylation, dimerization, and nuclear translocation, STATs transcriptionally regulate diverse target genes by binding to promoter regions containing GAS motifs. Diverse cross-talk/interactions may occur to regulate xCT expression in response to either extracellular stimuli or metabolic adaptations

not shown), as well as two highly conserved PIT-1 binding sites that flank the GAS/STAT site. It has been shown that several Pit-1 binding sites are required to facilitate estrogen responsiveness of the rat prolactin gene, and that there is a DNA-dependent interaction between Pit-1 and ER [70]. In this context, it remains to be determined whether targeting STAT5 with the inhibitors used in the present investigation influences the ER, which, based on our results, appears to be acting in a repressive manner at the xCT locus in ER-positive breast cancer cells. It will also be interesting to mechanistically evaluate a potential relationship between ER and PIT-1 in regulating xCT expression.

Clearly, STAT3 and STAT5 contribute to the regulation of xCT expression and the function of system Xc- in breast cancer cells. However, cancer cells may survive drug treatments aimed at blocking STAT proteins, potentially

contributing to drug resistance through system Xc-. Abrogating this type of resistance mechanism by modulating the cellular redox balance could have significant clinical implications, and targeting STATs in combination with system Xc- using specific antiporter inhibitors such as sulfasalazine may be a more effective strategy to target adaptive cancer cells. Indeed, in a murine sarcoma model, pairing the use of the cyclooxygenase inhibitor ibuprofen with pharmacological inhibition of system Xc- via sulfasalazine synergistically improved antitumor efficacy [71]. In an extension of the current investigation, stable breast cancer cells selected through long-term treatment with WP1066 and SH-4-54 will be assessed for changes in xCT expression and the function of system Xc-. Furthermore, given the potential role of system Xc- on responses to chemo- or radiotherapy, it will be of considerable future interest to examine whether the use of STAT3/5 inhibitors such as WP1066 and SH-4-54 result in up-regulation of xCT in vivo, thereby conferring resistance to commonly used anti-cancer agents.

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Conflict of interest The authors declare that they have no conflict of interest.

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