Cystine/glutamate antiporter xCT (SLC7A11) facilitates oncogenic RAS transformation by preserving intracellular redox balance

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The *RAS* family of proto-oncogenes are among the most commonly mutated genes in human cancers and predict poor clinical outcome. Several mechanisms underlying oncogenic RAS transformation are well documented, including constitutive signaling through the RAF-MEK-ERK proproliferative pathway as well as the PI3K-AKT prosurvival pathway. Notably, control of redox balance has also been proposed to contribute to RAS transformation. However, how homeostasis between reactive oxygen species (ROS) and antioxidants, which have opposing effects in the cell, ultimately influence RAS-mediated transformation and tumor progression is still a matter of debate and the mechanisms involved have not been fully elucidated. Here, we show that oncogenic KRAS protects fibroblasts from oxidative stress by enhancing intracellular GSH levels. Using a whole transcriptome approach, we discovered that this is attributable to transcriptional up-regulation of *xCT*, the gene encoding the cystine/glutamate antiporter. This is in line with the function of xCT, which mediates the uptake of cystine, a precursor for GSH biosynthesis. Moreover, our results reveal that the ETS-1 transcription factor downstream of the RAS-RAF-MEK-ERK signaling cascade directly transactivates the *xCT* promoter in synergy with the ATF4 endoplasmic reticulum stress-associated transcription factor. Strikingly, xCT was found to be essential for oncogenic KRAS-mediated transformation in vitro and in vivo by mitigating oxidative stress, as knockdown of *xCT* strongly impaired growth of tumor xenografts established from KRAS-transformed cells. Overall, this study uncovers a mechanism by which oncogenic RAS preserves intracellular redox balance and identifies an unexpected role for xCT in supporting RAS-induced transformation and tumorigenicity.

RAS | oncogene | xCT | antioxidants

The human *RAS* family of proto-oncogenes is comprised of *HRAS*, *KRAS*, and *NRAS* (1), which are among the most mutated genes in human cancers (2). *RAS* encodes a GTPase that relays signals from growth factor receptors to downstream signaling cascades. Mutations in RAS favor GTP binding, resulting in a constitutively active form of the protein, sufficient to transform cells and induce tumorigenesis in vivo (3). A number of mechanisms underlying RAS transformation have been proposed. These encompass constitutive induction of the proproliferative RAF-MEK-ERK pathway (4) and the pleiotropic PI3K-AKT pathway to prevent apoptosis (5). Other proposed mechanisms include increases of extracellular proteases as well as increased calcium signaling (6). Notably, the control of redox balance has also been suggested to support RAS transformation. Initially, reactive oxygen species (ROS) were believed to contribute to RAS transformation as oncogenic *Ras* expression was shown to up-regulate the NADPH oxidase system, causing increased superoxide production (7–9). It was also reported that mitochondrial ROS are required for *K-Ras*^{G12D}-induced tumorigenicity (10). More recently, several studies have demonstrated that on the contrary, activation of antioxidant pathways is necessary to support Ras transformation. Indeed, *K-Ras*^{G12D} induces the expression of the transcription factor Nrf2, the master regulator of intracellular antioxidant response, to support *K-Ras*^{G12D}–driven tumor development (11). In addition, oncogenic RAS cells undergo oncogene-directed metabolic reprogramming, in which glucose and glutamine are rechannelled to maintain cellular redox balance, ultimately contributing to tumor progression (12, 13). Thus, the role of ROS versus antioxidants in RAS transformation is still a matter of debate, and the mechanisms involved are not yet fully resolved.

A critical modulator of intracellular redox balance is the system x_c^- transporter, which mediates the exchange of intracellular glutamate for extracellular cystine, an essential precursor for GSH synthesis. This complex consists of xCT, a light-chain subunit that confers cystine transport function (14), and the

Significance

***RAS* genes are among the most mutated proto-oncogenes in human cancer. The mechanisms supporting RAS transformation are not fully understood, particularly regarding the relative contributions of oxidant versus antioxidant pathways. Here, we report that the cystine/glutamate transporter xCT is essential for RAS-induced tumorigenicity by enhancing antioxidant glutathione synthesis. Our findings uncover that RAS controls *xCT* transcription by downstream activation of ETS-1 to synergize with ATF4. This has clinical relevance since *xCT* expression is upregulated in human cancers exhibiting an activated RAS pathway. Therefore, oncogenic RAS transformation is supported by induction of an antioxidant program, highlighting xCT as a potential vulnerability for therapeutic targeting.**

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CD98 heavy-chain subunit, which localizes system x_c^- to the plasma membrane. *XCT* expression is induced in response to ROS-inducing agents such as hydrogen peroxide and sodium arsenite, leading to enhanced GSH production (15). This is attributed to *cis*-acting transcriptional regulatory elements present in the *xCT* promoter, including an antioxidant response element (ARE) principally recognized by NRF2 (16), and the amino acid response element (AARE), which is bound by ATF4 (17), a major player in the integrated stress response and oxidative stress response. Expression of *xCT* is deregulated in multiple cancers. Indeed, overexpression of *xCT* has been reported in nonsmall cell lung cancer, breast cancer, and liver cancer and is associated with poor outcomes (18–21). Furthermore, xCT has been implicated in promoting tumorigenesis through its antioxidant function, which supports breast cancer cell proliferation (19), matrix invasion of glioma (22), and in vivo tumor growth of gastric cancer (23). These studies and others also demonstrated that genetic or pharmacological inhibition of xCT, such as with sulfasalazine and erastin, hold promise as a therapeutic strategy in these model systems (24–26). Significantly, it was recently revealed that xCT is involved in tumor initiation as it is directly repressed by p53 or BAP1 as a means to exert tumor suppression (27, 28). Nonetheless, whether xCT directly contributes to oncogene-driven tumorigenesis is unknown.

Here, we report that oncogenic KRAS protects fibroblasts against oxidative stress by stimulating *xCT* transcription to enhance GSH levels. Our results reveal that this is mediated downstream of the Ras-Raf-Mek-Erk pathway by the Ets-1 transcription factor which, in synergy with Atf4, directly transactivates the *xCT* promoter. Finally, our data highlight that xCT mediates oncogenic KRAS-mediated tumorigenesis in vitro and in vivo by maintaining the redox balance. Together, our study provides a mechanism contributing to RAS transformation and link the control of xCT expression to the RAS pathway.

Results

Oncogenic Transformation with KRAS Protects Fibroblasts from Oxidative Stress. To define the impact of oncogenic RAS signaling on the cellular response to oxidative stress and the mechanisms involved, we initially used 3T3 fibroblasts transformed by activated $KRAS^{G12V}$ (3T3 $KRAS^{V12}$) or the ETV6-NTRK3 (EN) chimeric tyrosine kinase (3T3 EN) (29). As both oncoproteins constitutively activate Ras-Erk and PI3K-Akt signaling, we used both $KRAS^{V12}$ and EN-transformed cell lines to avoid cell line-specific effects (30). Expression of oncogenic KRAS and EN were confirmed in the transformed fibroblasts (Fig. 1*A*).

To assess the impact of oncogenic KRAS and EN transformation on the susceptibility of fibroblasts to exogenous oxidative stress, we subjected the cells to increasing concentrations of hydrogen peroxide (H_2O_2) and quantified cell death. While H_2O_2 treatment induced massive cell death in nontransformed control cells (3T3 MSCV), especially at higher concentrations, 3T3 $KRAS^{V12}$ and 3T3 EN cells were relatively protected (Fig. 1*B*), indicating that oncogenic transformation by KRAS and EN provide protection against oxidative stress. Similar effects were observed for mutant KRAS using a second oxidative stress inducer, diethyl maleate (DEM) (*SI Appendix*, Fig. S1*A*). These differences in cell death were linked to amounts of intracellular ROS; indeed, while 3T3 MSCV cells displayed higher levels of intracellular ROS under ambient conditions and showed rapid accumulation of ROS following H_2O_2 treatment (Fig. 1*C*) or DEM treatment (*SI Appendix*, Fig. S1*B*), 3T3 $KRAS^{V12}$ cells maintained lower levels of intracellular ROS throughout these conditions. This is in line with previous work demonstrating that $KRas^{G12D}$ expression actively suppresses ROS due to basal activation of the Nrf2 detoxification program (11). Similar effects were also seen with 3T3 EN cells (*SI Appendix*, Fig. S1*C*). This was further validated by analyzing levels of protein oxidation, which indicated that following exposure to

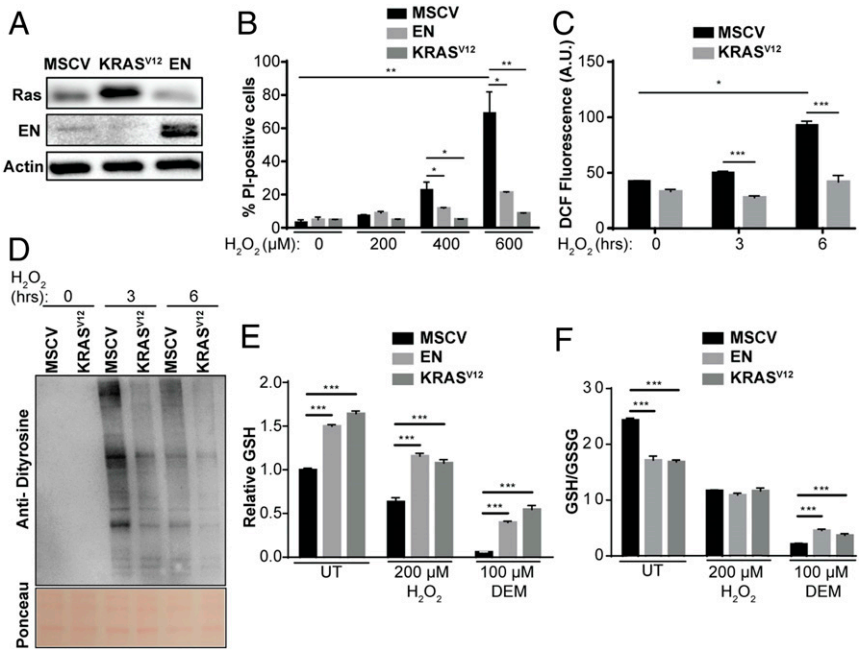

Fig. 1. Oncogenic KRAS protects fibroblasts against exogenous oxidative stress. (*A*) Western blot analysis of lysates obtained from 3T3 $KRAS^{V12}$, EN, and MSCV cells. Actin was used as a loading control. (*B*) 3T3 $KRAS^{V12}$, EN and MSCV cells were treated with indicated concentrations of H_2O_2 for 16 h, and cell death was determined by propidium iodide (PI) staining and flow cytometry ($n = 3$). (*C*) ROS levels in 3T3 $KRAS^{V12}$ and MSCV cells treated with 200 μM H_2O_2 for the indicated times were determined by 6-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate, acetyl ester (CM-H_2DCFDA) staining and flow cytometry ($n = 3$). (*D*) 3T3 $KRAS^{V12}$ and MSCV cells were treated with 200 μM H_2O_2 for the indicated times and dityrosine levels were determined using Western blot. Ponceau was used as a loading control ($n = 3$). (*E*) 3T3 $KRAS^{V12}$, EN, and MSCV cells were treated with 200 μM H_2O_2 or 100 μM DEM for 6 h, and reduced GSH levels were determined using GSH-Glo assay (Promega) ($n = 3$). (*F*) 3T3 $KRAS^{V12}$, EN, and MSCV cells were treated with 200 μM H_2O_2 or 100 μM DEM for 6 h, and GSH/GSSG ratio was determined using GSH/GSSG-Glo assay (Promega) ($n = 3$). Where shown, data are reported as means ± SD with indicated significance (*$P < 0.05$, **$P < 0.01$, and ***$P < 0.005$).

H_2O_2, 3T3 MSCV cells showed higher levels of protein oxidation relative to 3T3 $KRAS^{V12}$ cells, as measured by dityrosine levels (Fig. 1*D*). Moreover, together with reduced ROS, 3T3 $KRAS^{V12}$ and EN cells consistently displayed higher reduced glutathione (GSH) levels (Fig. 1*E*) and higher total glutathione (GSH+GSSG) levels (*SI Appendix*, Fig. S1*D*) relative to 3T3 MSCV cells, both under ambient conditions and in response to oxidative stress by H_2O_2 or DEM treatment. Interestingly, while 3T3 $KRAS^{V12}$ and EN cells exhibited a lower GSH/GSSG ratio relative to 3T3 MSCV cells under ambient conditions, these cells had similar GSH/GSSG ratios as 3T3 MSCV cells following H_2O_2 treatment (Fig. 1*F*). This suggests that in our cellular model, protection of cells against oxidative stress following oncogenic transformation with KRAS or EN may be attributed to enhanced GSH biosynthesis rather than increased GSH-GSSG recycling. Supportive of this, 3T3 $KRAS^{V12}$ and EN cells showed higher total GSH and GSH/GSSG ratios compared with 3T3 MSCV cells when treated with DEM, which conjugates and, therefore, depletes GSH availability (Fig. 1*F* and *SI Appendix*, Fig. S1*D*). Together, these results suggest that oncogenic transformation with KRAS or EN protects fibroblasts against exogenous oxidative stress by preserving the redox balance and enhancing intracellular GSH capacity.

Oncogenic RAS Leads to Enhanced Induction of *xCT*. To uncover the mechanisms underlying $KRAS^{V12}$- or EN-mediated oxidative stress resistance, we performed whole-transcriptome microarray analysis of 3T3 $KRAS^{V12}$, EN, and MSCV cells under basal conditions (UT) and H_2O_2 treatment to identify differentially regulated transcripts potentially involved in the response to oxidative stress. When 3T3 $KRAS^{V12}$ and 3T3 EN cells were individually analyzed following H_2O_2 treatment, we found overlap of a core of 90 probesets (corresponding to 67 unique genes) that were significantly up-regulated >twofold in both cell lines relative to 3T3 MSCV cells. Gene ontology (GO) overrepresentation analysis of these 67 genes showed significant enrichment for functional categories relating to cellular stress response and cell death, including the GO categories "cellular response to hypoxia," "endoplasmic reticulum unfolded protein response," and "apoptotic process" (Fig. 2*A*, *Left*). These categories comprised many up-regulated genes of the integrated stress response

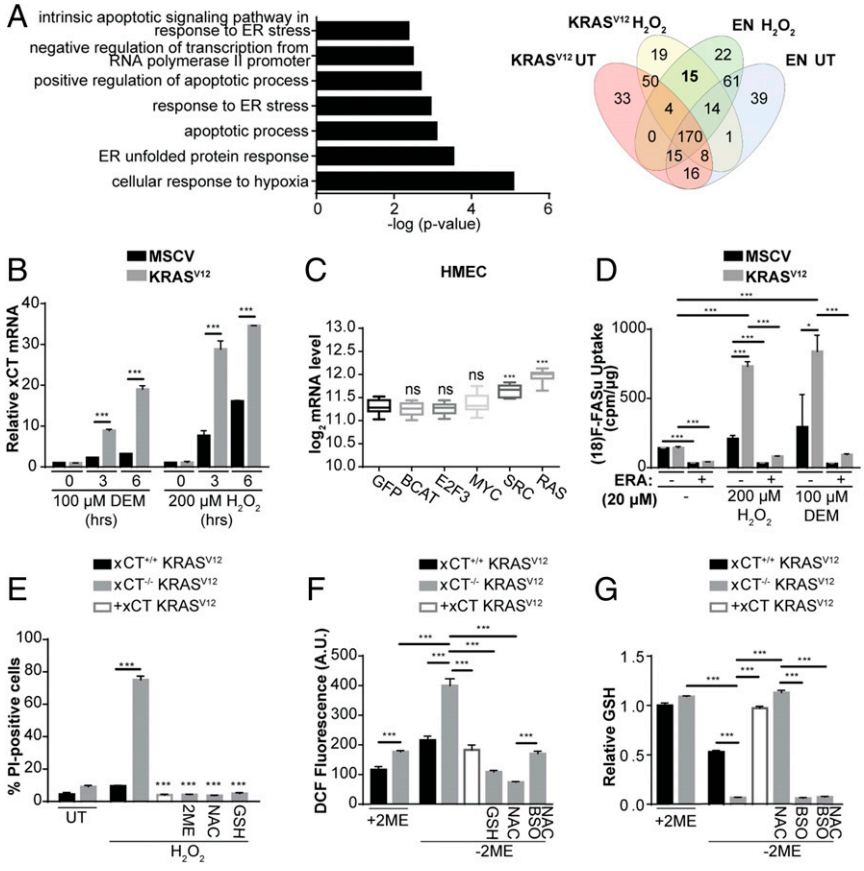

Fig. 2. Oncogenic KRAS enhances *xCT* induction. (*A*, *Left*) GO biological process categories overrepresented in 67 genes up-regulated in 3T3 $KRAS^{V12}$ and 3T3 EN cells following 200 μM H_2O_2 for 3 h, for all categories with FDR < 0.10. The *x* axis represents the negative log of the significant score *P* values generated from DAVID. (*A*, *Right*) Venn diagram depicting the overlap of up-regulated genes in 3T3 $KRAS^{V12}$ versus 3T3 MSCV and 3T3 EN cells versus MSCV under 200 μM H_2O_2 for 3 h and basal condition. (*B*) 3T3 $KRAS^{V12}$ and MSCV cells were treated with 100 μM DEM or 200 μM H_2O_2 for the indicated times, and *xCT* mRNA levels was determined using qRT-PCR. ($n = 3$). (*C*) Expression levels of *xCT* in human mammary epithelial cells (HMEC) ectopically expressing indicated oncogenes or control GFP. Expression levels are displayed as log2 of mRNA. (*D*) xCT activity in 3T3 $KRAS^{V12}$ and MSCV cells treated with 200 μM H_2O_2 or 100 μM DEM for 6 h, with or without 20 μM erastin was determined by FASu uptake. FASu radioactivity was normalized to protein concentration ($n = 3$). (*E*) $xCT^{+/+}$ $KRAS^{V12}$ and $xCT^{-/-}$ $KRAS^{V12}$ cells were removed from regular media containing 2ME and treated with 200 μM H_2O_2 for 16 h; $xCT^{-/-}$ $KRAS^{V12}$ was rescued with 50 μM 2ME, 5 mM NAC, 5 mM GSH, or reexpression of xCT (+xCT); and cell death was determined by propidium iodide (PI) staining and flow cytometry ($n = 3$). (*F*) ROS levels in $xCT^{+/+}$ $KRAS^{V12}$ and $xCT^{-/-}$ $KRAS^{V12}$ cells removed from regular media containing 2ME and rescued with 5 mM GSH, 5 mM NAC, or 5 mM NAC and 100 μM BSO, or reexpression of xCT (+xCT) were determined by 6-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate, acetyl ester (CM-H_2DCFDA) staining and flow cytometry ($n = 3$). (*G*) $xCT^{+/+}$ $KRAS^{V12}$ and $xCT^{-/-}$ $KRAS^{V12}$ cells were removed from regular media containing 2ME and rescued with 5 mM NAC or 5 mM NAC and 100 μM BSO, or reexpression of xCT and reduced GSH levels were determined using GSH-Glo assay (Promega) ($n = 3$). Where shown, data are reported as means ± SD with indicated significance (*$P < 0.05$, **$P < 0.01$, and ***$P < 0.005$).

pathway such as *Ppp1r15a*, *Atf3*, *Trib3*, *Chac1*, and *Ddit3* as well as oxidative stress response genes such as *Hmox1*, *Plk3*, and *Trp53inp1* (Dataset S1).

To better characterize individual genes uniquely up-regulated by $KRAS^{V12}$ and EN in response to oxidative stress, we subdivided genes to identify those that were differentially up-regulated by $KRAS^{V12}$ and EN transformation relative to control cells only under H_2O_2 treatment, and not under basal conditions. From this analysis, we identified only 15 genes with overlap across both cell lines (Fig. 2*A*, *Right*). Among these, solute carrier family 7 member 11 (*Slc7a11*; also commonly referred to as *xCT*) was of interest due to its known role in redox regulation through GSH biosynthesis. Differential expression of *xCT* mRNA was validated by qRT-PCR, confirming that while *xCT* expression was induced in all cell lines in response to DEM or H_2O_2, it was fourfold and twofold higher in 3T3 $KRAS^{V12}$ compared with 3T3 MSCV cells at 6 h treatment with DEM and H_2O_2, respectively (Fig. 2*B*). To corroborate these findings in another cell type, we analyzed levels of *xCT* expression in a publicly available gene expression dataset of human mammary epithelial cells (HMEC) ectopically expressing either activated HRAS or a panel of oncogenes [β-*catenin* (*BCAT*), *E2F3*, *MYC*, or *SRC*] (31). In line with our findings, we observed that *xCT* is significantly up-regulated in $HRAS^{V12}$ and *SRC*-transformed cells, but not in other oncogene expressing cells, compared with control GFP HMECs (Fig. 2*C*). Furthermore, *xCT* induction is not RAS isoform specific in 3T3 cells, as $HRAS^{V12}$ transformation also resulted in enhanced induction of *xCT* in response to H_2O_2 (*SI Appendix*, Fig. S2*A*). To further corroborate the link between RAS signaling and xCT expression, we used *Nras*/*Hras* double knockout MEFs with 4-OHT–inducible knockout of endogenous *Kras* (32). As shown in *SI Appendix*, Fig. S2*B*, in the presence of 4-OHT, the conditional *Kras* alleles become fully excised rendering the cells "Rasless." This complete knockout of *Kras* led to a dose-dependent decrease in xCT expression, further linking xCT induction to RAS signaling (*SI Appendix*, Fig. S2*B*). In contrast, levels of GPX4, another oxidative stress response protein, were unchanged. Together, these data support the notion that oncogenic RAS pathway activation leads to induction of *xCT* expression.

We next investigated the impact of oncogenic KRAS on xCT activity to determine whether increased *xCT* mRNA levels led to enhanced protein activity. We opted for this approach rather than Western blotting since it has been documented that at present, all commercially available antibodies display nonspecific immunoreactivity when used to detect xCT protein in mouse cell lines and tissues (33). We therefore performed in vitro uptake assays of the xCT-specific PET tracer ^{18}F-5-fluoroaminosuberic acid (FASu), which was developed as a diagnostic tracer of oxidative stress via system x_c^- activity (34, 35). In sharp contrast to nontransformed 3T3 cells, which showed no significant increase of FASu uptake following H_2O_2 and DEM exposure, 3T3 $KRAS^{V12}$ cells consistently exhibited a five- to sixfold induction of system x_c^- activity in response to these compounds (Fig. 2*D*), in keeping with higher levels of *xCT* mRNA detected in transformed cells. FASu uptake was effectively blocked with a known xCT inhibitor, erastin, confirming the specificity of FASu for xCT. Moreover, increased FASu uptake observed in $KRAS^{V12}$ transformed cells was not linked to altered expression of the heavy-chain subunit of system x_c^-, i.e., CD98, as expression of the latter was similar in transformed versus nontransformed cells under basal and H_2O_2 treatment conditions (*SI Appendix*, Fig. S2*C*). This points to a major role for xCT in $KRAS^{V12}$-mediated induction of system x_c^- activity. Together, these results indicate that oncogenic $KRAS^{V12}$ leads to enhanced levels of *xCT* expression and activity in response to exogenous oxidative stress.

xCT Mediates Oncogenic KRAS-Induced Resistance to Oxidative Stress by Preserving Redox Balance. Given that xCT activity is essential to support GSH biosynthesis pathway and therefore to maintain redox balance, we next asked whether xCT promotes the oxidative stress resistance conferred by oncogenic KRAS (15). To address this, we acquired mouse embryonic fibroblasts (MEFs) derived from wild-type ($xCT^{+/+}$) or $xCT^{-/-}$ mice into which we stably expressed K-Ras^{V12} (36). $xCT^{-/-}$ cells are routinely cultured in the reducing agent 2-mercaptoethanol (2ME), allowing these cells to circumvent the block in cysteine uptake by instead importing its reduced form, cysteine, via neutral amino acid transporters (36). Strikingly, *xCT* knockout cells transformed with K-Ras^{V12} showed marked cell death following exposure to H_2O_2 compared with wild-type cells (Fig. 2*E*). Similarly, knocking down *xCT* with two nonoverlapping siRNAs in 3T3 $KRAS^{V12}$ cells increased cell death induced by H_2O_2 (*SI Appendix*, Fig. S2 *D* and *E*). Susceptibility to oxidative stress was reversed in MEFs and 3T3 models either by supplementation with the antioxidants GSH and NAC or with 2ME (specifically in $xCT^{-/-}$ $KRAS^{V12}$) (Fig. 2*E* and *SI Appendix*, Fig. S2*E*). Further, ectopic overexpression of xCT in 3T3 MSCV cells phenocopied effects of oncogenic KRAS on susceptibility to H_2O_2 by protecting cells against this treatment (*SI Appendix*, Fig. S2*F*). Together, these data clearly indicate that xCT mediates oncogenic KRAS-induced cytoprotection against oxidative stress. This function is linked to antioxidant activity of xCT. 2ME depletion led to a significant increase of ROS in $xCT^{-/-}$ $KRAS^{V12}$ relative to $xCT^{+/+}$ $KRAS^{V12}$ cells, but this was reversed by reexpressing xCT (+xCT $KRAS^{V12}$) or by supplementation with 2ME, GSH, or NAC, but less so under cotreatment with NAC and 1-buthionine-S,R-sulfoximine (BSO), a glutamate-cysteine ligase (GCL) inhibitor (Fig. 2*F*). This suggests that xCT promotes KRAS-mediated oxidative stress resistance by providing cystine intermediates for the synthesis of GSH via GCL. Similarly, knockdown of *xCT* in 3T3 $KRAS^{V12}$ cells was accompanied by an increase in ROS levels under both basal and H_2O_2 treatment conditions, which was also reversed by GSH or NAC, but not NAC and BSO together (*SI Appendix*, Fig. S2*G*). Consistent with this finding, knockout (Fig. 2*G*) or knockdown (*SI Appendix*, Fig. S2*H*) of *xCT* led to significant decreases in GSH levels under basal and oxidative stress conditions (2ME for MEFs and H_2O_2 treatment for 3T3), which could be reversed by either 2ME (specifically in $xCT^{-/-}$ $KRAS^{V12}$) or NAC, but not NAC with BSO. Taken together, these data provide compelling evidence that xCT mediates resistance to oxidative stress conferred by oncogenic KRAS by providing cystine for GSH synthesis.

xCT Expression and Activity Are Induced by Mutant KRAS in Human Cancer Cells. To assess regulation of xCT by oncogenic KRAS in human cancer cells, we analyzed various cell lines originating from mutant KRAS-driven human cancers, such as lung adenocarcinoma (LUAD), colorectal adenocarcinoma (COAD), and pancreatic ductal adenocarcinoma (PDAC). We surveyed xCT expression in mutant KRAS versus wild-type KRAS cancer cells from LUAD, COAD, and PDAC. Normal epithelial cells HPL1D and HPDE6 from lung and pancreas, respectively, were included as controls. We found that xCT expression is consistently higher in mutant KRAS cancer cell lines compared with wild-type KRAS cell lines originating from the same tumor types and in normal epithelial cells (Fig. 3*A*), pointing to a correlation between KRAS activation and xCT expression in human cancer cells. To test this, we performed mutant KRAS knockdown, which markedly decreased xCT expression at the mRNA level in the COAD cell line SW620 (Fig. 3*B*), as well as at the protein level in SW620 and in the LUAD cell line H460 (Fig. 3*C*). Consistent with this, knockdown of mutant KRAS led to reduction of xCT activity (FASu uptake; Fig. 3*D*) and reduced GSH (Fig. 3*E*) in SW620 cells under basal and H_2O_2 treatment conditions. The decrease in reduced GSH levels following mutant KRAS knockdown under basal conditions was partially rescued by NAC, thereby phenocopying the effect of *xCT*

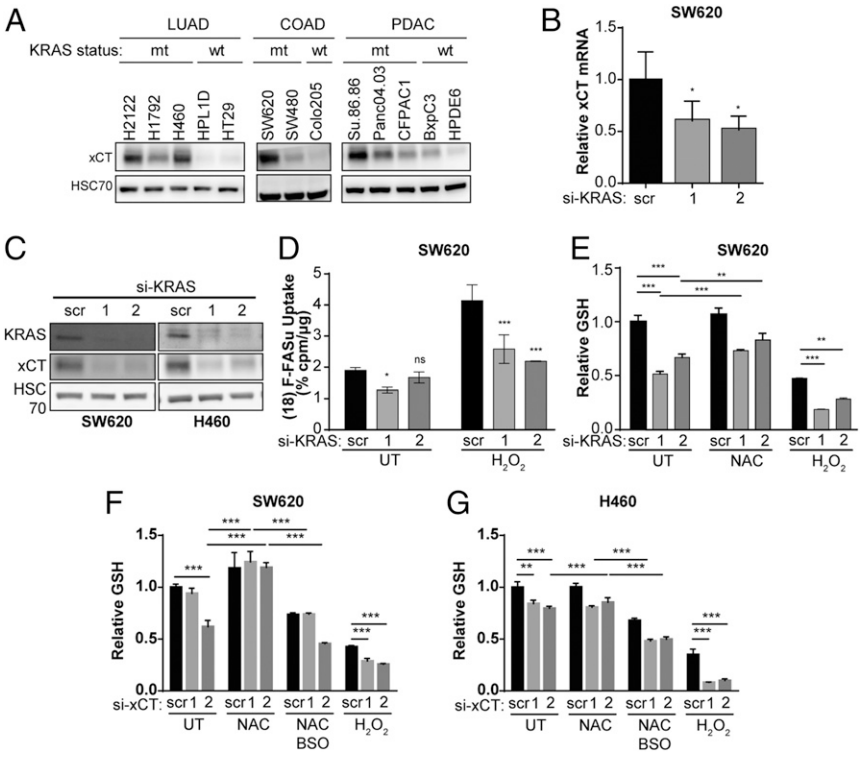

Fig. 3. Mutant KRAS regulates xCT expression and activity in human cancer cell lines. (*A*) Western blot analysis of xCT levels from lysates of lung adenocarcinoma (LUAD), colorectal adenocarcinoma (COAD), and pancreatic ductal adenocarcinoma (PDAC) cell lines with corresponding normal epithelial cells (HPL1D and HPDE6). HSC70 was used as a loading control. (*B*) SW620 cells were transfected with siRNA targeting *KRAS* or scrambled control for 72 h, and *xCT* mRNA levels were quantified using qRT-PCR ($n = 3$). (*C*) SW620 and H460 cells were transfected with siRNA targeting *KRAS* or scrambled control for 72 h, and xCT expression levels were determined using Western blot analysis. HSC70 was used as a loading control ($n = 3$). (*D*) xCT activity in SW620 cells transfected with siRNA targeting *KRAS* or scrambled control for 72 h and treated with 200 μM H_2O_2 for the indicated times, was determined by FASu uptake. (*E*) SW620 cells were transfected with siRNA targeting *KRAS* or scrambled control for 48 h, treated with 5 mM NAC or 100 μM H_2O_2 for 16 h, following which reduced GSH levels were determined using GSH-Glo assay (Promega) ($n = 3$). SW620 cells (*F*) and H460 cells (*G*) were transfected with siRNA targeting *xCT* or scrambled control for 48 h, treated with 5 mM NAC or 100 μM H_2O_2 for 16 h, following which reduced GSH levels were determined using GSH-Glo assay (Promega) ($n = 3$). Where shown, data are reported as means ± SD with indicated significance (*$P < 0.05$, **$P < 0.01$, and ***$P < 0.005$).

knockdown on reduced GSH levels as observed in 3T3 $KRAS^{V12}$. Consistent with 3T3 $KRAS^{V12}$, knockdown of *xCT* in SW620 cells (Fig. 3*F* and *SI Appendix*, Fig. S2 *I* and *J*) and H460 cells (Fig. 3*G*) led to significant decreases in GSH levels under basal and oxidative stress conditions, which could be reversed by either NAC, but not NAC in the presence of BSO, indicating that these cell lines are also dependent on *xCT* for GSH synthesis via GCL. Moreover, siRNA-mediated knockdown of *xCT* led to increased ROS levels under both basal and oxidative stress conditions (*SI Appendix*, Fig. S2*K*). Together, these results indicate that oncogenic KRAS promotes xCT expression and activity in human cancer cells to control the redox balance.

ETS-1 Mediates the Induction of *xCT* by Oncogenic KRAS via Synergistic Cooperation with ATF4. To elucidate how oncogenic KRAS regulates *xCT* expression, we analyzed transcription factor networks by performing gene set enrichment analysis (GSEA) of mRNA expression data obtained in 3T3 $KRAS^{V12}$ versus 3T3 MSCV cells. This revealed significant enrichment of genes controlled by the transcription factor Ets-1 in oncogenic KRAS cells (*SI Appendix*, Fig. S3*A*). Ets-1 is a known substrate of ERK, downstream of the Ras-Raf-Mek pathway, but its effects on *xCT* transcription have not been reported. Confirming a role for the Ras-Raf-Mek pathway in *xCT* regulation, pharmacological inhibition of Mek (by PD184352), but not of Akt (Akt Inhibitor VIII), attenuated *xCT* expression in 3T3 $KRAS^{V12}$ cells under both basal and H_2O_2 treatment conditions (*SI Appendix*, Fig. S3 *B* and *C*). This suggests that Ets-1 mediates $KRAS^{V12}$ induction of *xCT* downstream of the Ras-Raf-Mek-Erk pathway. Indeed, knockdown of *Ets-1* with two nonoverlapping siRNAs in 3T3 $KRAS^{V12}$ cells (*SI Appendix*, Fig. S3*D*) significantly restricted *xCT* induction in response to H_2O_2 treatment versus control siRNAs (Fig. 4*A*). Accordingly, *Ets-1* knockdown increased ROS levels in $KRAS^{V12}$-transformed cells under basal conditions, which was rescued by *xCT* overexpression or NAC supplementation, and dramatically increased ROS levels under H_2O_2 treatment (*SI Appendix*, Fig. S3*E*). Moreover, blocking *Ets-1* expression increased sensitivity of 3T3 $KRAS^{V12}$ cells to H_2O_2 (*SI Appendix*, Fig. S3*F*). Therefore, Ets-1 confers $KRAS^{V12}$ cytoprotection against oxidative stress, potentially by controlling *xCT* expression. In human cancer cells harboring KRAS mutations, *ETS-1* knockdown similarly decreased xCT expression at both the mRNA and protein level compared with siRNA controls (Fig. 4 *B* and *C*). To corroborate this, we ectopically expressed *ETS-1* in the DLD-1 colon cancer cell line devoid of ETS-1 expression, which was sufficient to up-regulate xCT expression at both the mRNA and protein level under H_2O_2 treatment (Fig. 4*D*). Together, these highlight a role for ETS-1 in mediating $KRAS^{V12}$ induction of *xCT* expression.

Next, to demonstrate whether ETS-1 controls the activity of *xCT* promoter, we performed luciferase transactivation assays in HEK293 cells. Notably, one potential ETS-1 binding site (E1BS), 5′-TGAGGAAGCT-3′ containing the consensus GGAA/T core motif at position −15 of the human *xCT* promoter, was detected in silico (*SI Appendix*, Fig. S3*G*). Indeed, exogenous ETS-1 activates a luciferase reporter containing the *xCT* promoter in a concentration-dependent manner (Fig. 4*E*). Furthermore, chromatin immunoprecipitation (ChIP) of 3T3 $KRAS^{V12}$ cells revealed that endogenous Ets-1 occupies the promoter region of the *xCT* gene under both basal and H_2O_2 treatment conditions (Fig. 4*F*). This strongly supports *xCT* as a target gene of Ets-1. However, we noticed that the levels of total and phosphorylated Ets-1 (p-Ets-1, the active form of Ets-1; ref. 37), are unchanged following H_2O_2 exposure in 3T3 $KRAS^{V12}$ cells (*SI Appendix*, Fig. S3*H*). This implies that while Ets-1 can activate *xCT* transcription downstream of KRAS signaling, there may be other mechanisms to explain how *xCT* expression is enhanced in response to oxidative stress. Therefore, to uncover other transcription factors potentially involved in *xCT* regulation specifically under oxidative stress, we employed GSEA in H_2O_2-treated versus untreated 3T3 $KRAS^{V12}$ cells. This revealed enrichment of genes regulated by Atf4 under H_2O_2 treatment (*SI Appendix*, Fig. S4*A*) and conversely no enrichment of genes regulated

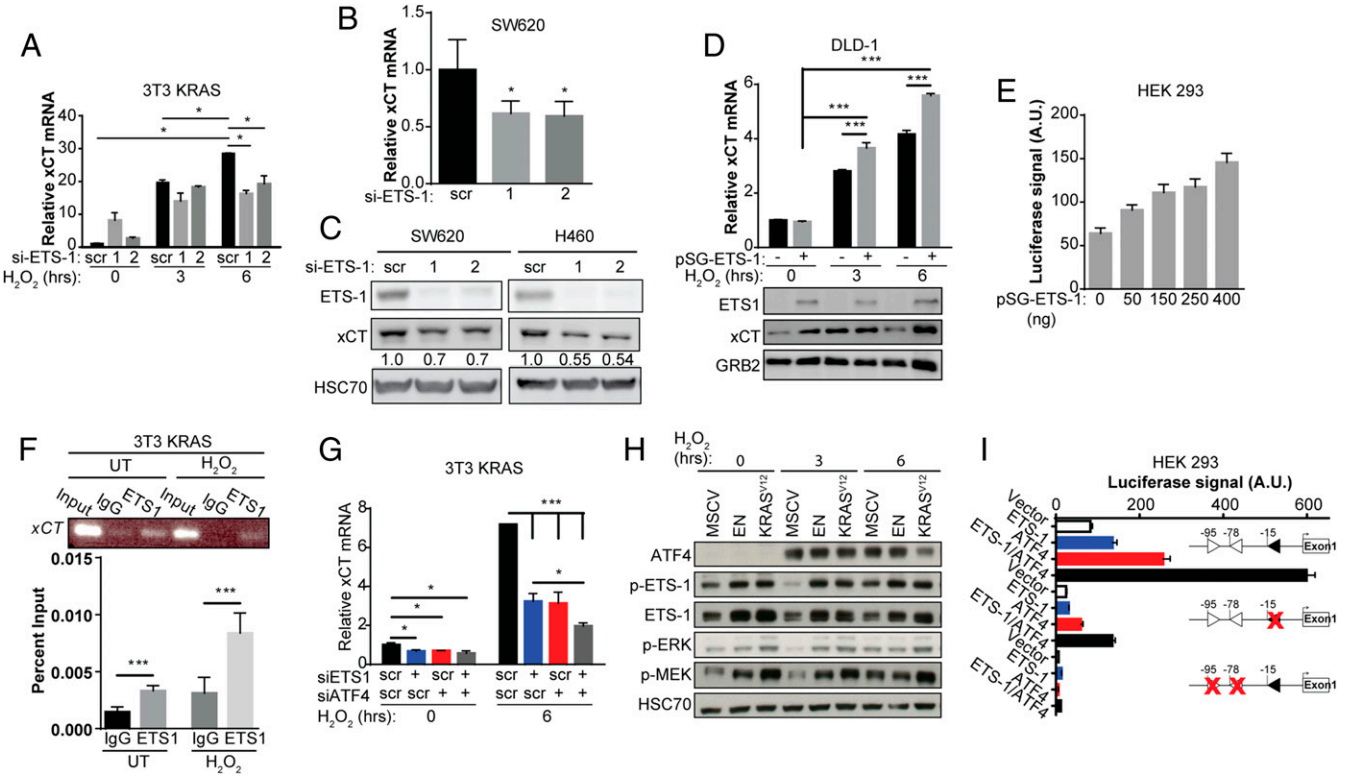

Fig. 4. ETS-1 directly transactivates *xCT* and synergizes with ATF4. (*A*) 3T3 KRAS^{V12} cells were transfected with siRNA targeting *Ets-1* or scrambled control for 72 h, treated with 200 μM H_2O_2 for the indicated times, and *xCT* mRNA levels were determined by qRT-PCR ($n = 3$). (*B*) SW620 cells were transfected with siRNA targeting *ETS-1* for 72 h and *xCT* mRNA levels were determined by qRT-PCR ($n = 3$). (*C*) SW620 and H460 cells were transfected with siRNA targeting ETS-1 or scrambled control for 72 h, and xCT levels were determined by Western blot analysis. HSC70 was used as a loading control ($n = 3$). (*D*) DLD-1 cells were transfected with *ETS-1* expression construct or control for 72 h and treated with 200 μM H_2O_2 for the indicated times and *xCT* mRNA, and protein levels were determined by qRT-PCR and Western blot, respectively ($n = 3$). (*E*) HEK293 cells were transfected with luciferase construct containing *xCT* promoter and indicated concentrations of *ETS-1* expression construct for 72 h, and luciferase activity was determined by Dual-Luciferase Reporter Assay System (Promega) ($n = 3$). (*F*) 3T3 KRAS^{V12} cells were treated with 200 μM H_2O_2 for 6 h, and ChIP assay was carried out. qRT-PCR was used to determined percent enrichment of ETS-1 binding over input ($n = 3$). (*G*) 3T3 KRAS^{V12} cells were transfected with siRNA targeting *Ets-1*, *Atf4*, or both for 72 h, and *xCT* mRNA levels were determined by qRT-PCR ($n = 3$). (*H*) Western blot analysis showing ATF4, phospho-ETS-1, ETS-1 levels, phospho-ERK, and phospho-MEK in 3T3 KRAS^{V12}, EN, and MSCV cells treated with 200 μM H_2O_2 for the indicated times. HSC70 was used as a loading control ($n = 3$). (*I*) HEK293 cells were transfected with *ETS-1*, *ATF4* expression construct, or both, as well as luciferase construct containing wild-type *xCT* promoter, *xCT* promoter with E1BS mutation, or *xCT* promoter with AARE mutations for 72 h. Luciferase activity was determined by the Dual-Luciferase Reporter Assay System (Promega) ($n = 3$). Where shown, data are reported as means ± SD with indicated significance (*$P < 0.05$, **$P < 0.01$, and ***$P < 0.005$).

by Ets-1. This is consistent with the established role of Atf4 as a transcriptional regulator of *xCT* expression in response to oxidative stress (17). Indeed, knockdown of *Atf4* markedly suppressed *xCT* induction (*SI Appendix*, Fig. S4*B*) and xCT protein expression (*SI Appendix*, Fig. S4*C*) in response to H_2O_2 in 3T3 KRAS^{V12} and H460 cells, respectively. Notably, knocking down *Ets-1* together with *Atf4* achieved a stronger suppression of *xCT* induction under oxidative stress conditions than targeting *Ets-1* or *Atf4* alone (Fig. 4*G*), suggesting an interplay between these two transcription factors in regulating *xCT* expression. While total and phosphorylated Ets-1 are up-regulated in KRAS^{V12}-transformed cells compared with nontransformed cells but unaffected by H_2O_2 treatment, Atf4 expression is conversely strongly induced by H_2O_2 without any differences between MSCV and KRAS^{V12} cells (Fig. 4*H*). These results suggest that both transcription factors may cooperate to regulate *xCT* expression in response to both oncogenic KRAS and oxidative stress, with Ets-1 being a component of RAS signaling and Atf4 being independently regulated by oxidative stress.

To determine potential cooperativity between Ets-1 and Atf4 in regulating *xCT* promoter activity, we constructed a series of *xCT* promoter constructs containing mutations in the putative E1BS at position −15, or in the known ATF4 binding sites (AARE) at position −95 and −78. Ectopically expressed ETS-1 and ATF4 were able to synergistically activate the wild-type *xCT* promoter compared with the activity induced by each alone (Fig. 4*I*; see top bars). However, when either the E1BS alone or the Atf4 AAREs alone were mutated, activation of the luciferase reporter was strongly decreased (Fig. 4*I*). Moreover, association between endogenous Ets-1 and Atf4 in a putative protein–protein complex within 3T3 KRAS^{V12} cells was confirmed by coimmunoprecipitation experiments, and this association was enhanced under oxidative stress (*SI Appendix*, Fig. S4*D*). Taken together, these results provide strong evidence that ETS-1 and ATF4 synergistically transactivate the *xCT* promoter downstream of oncogenic RAS signaling and in response to oxidative stress, possibly as a cotranscriptional activating complex.

The master regulator of the oxidative stress response, Nrf2, has also previously been shown as being induced by oncogenic KRAS (11) and to control *xCT* promoter activity in response to electrophilic agents in cooperation with Atf4 (38). We therefore investigated the role of Nrf2 in Ets-1 regulation of *xCT* transcription downstream of oncogenic KRAS. We found that, at least in 3T3 fibroblasts, while Nrf2 was increased following ectopic expression of oncogenic KRAS (or EN), its levels remain unchanged following exposure to H_2O_2 (*SI Appendix*, Fig. S4*E*).

Furthermore, siRNA-mediated knockdown of Ets-1 (upper blots) or Atf4 (lower blots) failed to alter expression of Nrf2 (*SI Appendix*, Fig. S4*F*). Conversely, siRNA-mediated *Nrf2* knockdown did not alter Ets-1 or Atf4 expression, nor did ATF4 overexpression alter ETS-1 levels (*SI Appendix*, Fig. S4*G*). This indicates that Ets-1 and Nrf2 are independently regulated downstream of the Ras-Raf-Mek pathway and, therefore, likely comprise two distinct pathways controlling *xCT* promoter activity. As expected, *Nrf2* knockdown resulted in decreased *xCT* transcript levels, which interestingly could be at least partially rescued by Atf4 overexpression (*SI Appendix*, Fig. S4*G*). These data suggest that while Nrf2 does not regulate Ets-1 or Atf4 to control *xCT* transcription, it may potentially coregulate xCT promoter together with these transcription factors. Indeed, luciferase transactivation assays reveal that NRF2 exerts an additive effect with ETS1 or ATF4 or with ETS-1 and ATF4 on *xCT* promoter activity (*SI Appendix*, Fig. S4*H*). However, NRF2 was unable to act in synergy with these transcription factors, contrasting with the effect observed between ETS1 and ATF4. Together, our studies provide evidence that the Ras-Raf-Mek pathway controls the *xCT* promoter through Ets-1 in synergy with Atf4, but independently of the Nrf2 pathway.

xCT Supports Oncogenic KRAS Transformation and Tumorigenicity in Vivo. We next asked whether xCT contributes to KRAS oncogenic transformation and tumorigenicity. We first performed soft agar colony formation assays and found that siRNA-mediated *xCT* silencing results in ∼70% inhibition of colony formation in 3T3 KRAS^{V12} cells (Fig. 5*A*). Similarly, *xCT* knockout cells transformed with K-Ras^{V12} showed markedly reduced colony formation compared with wild-type cells transformed with K-Ras^{V12} (*SI Appendix*, Fig. S5*A*). These results were recapitulated in the human cancer cells H460 (Fig. 5*B*), SW620, and SW480 (*SI Appendix*, Fig. S5 *B* and *C*, respectively) harboring KRAS mutations. Moreover, pharmacological inhibition of xCT using the inhibitor erastin significantly impaired the ability of 3T3 KRAS^{V12} and H460 cells to form colonies (*SI Appendix*, Fig. S5 *D* and *E*). These results strongly indicate that *xCT* is essential for KRAS-mediated oncogenic transformation and maintenance of tumorigenicity in vitro. To explore the potential mechanism involved, we assessed whether xCT reduces oxidative stress, proposed to be critical for oncogenic transformation (39). Notably, addition of NAC partially restored the ability of *xCT*-deficient and xCT-targeted cells to form colonies in soft agar (Fig. 5 *A* and *B* and *SI Appendix*, Fig. S5 *A*–*C*), consistent with an important role for xCT in supporting oncogenic KRAS-mediated transformation through its antioxidant function. To confirm that ETS-1 and ATF4 also contribute to oncogenic KRAS transformation by regulating *xCT* transcription, we carried out siRNA-mediated silencing of *ETS-1* and *ATF4* alone, or in combination. We found these to result in more than 70% inhibition of colony formation of 3T3 KRAS^{V12} cells, compared with siRNA controls, which could be partially rescued with xCT overexpression or supplementation with NAC (*SI Appendix*, Fig. S5*F*). This highlights that ETS-1 and ATF4, by regulating *xCT* transcription, can mediate oncogenic KRAS transformation.

To determine the in vivo relevance of these findings, 3T3 KRAS^{V12} cells stably expressing scr or two individual shRNAs targeting *xCT* were s.c. implanted in *nu*/*nu* immunocompromised mice. Tumor xenografts established from 3T3 KRAS^{V12} cells expressing *xCT*-specific shRNA were severely impaired in their growth compared with tumors established from control scr cells (Fig. 5*C*). This data were accompanied by dramatically improved survival of mice bearing tumors with *xCT* silencing compared with control tumors (Fig. 5*D*). These highlight that xCT is required for KRAS-mediated tumorigenesis in vivo. To determine whether the impairment of KRAS-driven tumor growth induced by xCT deficiency is associated with oxidative stress, we measured GSH levels and found that tissues from *xCT*-knockdown tumors exhibited significantly reduced GSH levels compared with control tumors (Fig. 5*E*). Further, immunoblots performed on *xCT* knockdown tumor tissues demonstrated an increase in levels of the oxidized protein marker, Dityrosine, relative to controls, providing evidence for increased oxidative stress levels in these tumors (Fig. 5*F*). These data support a model whereby

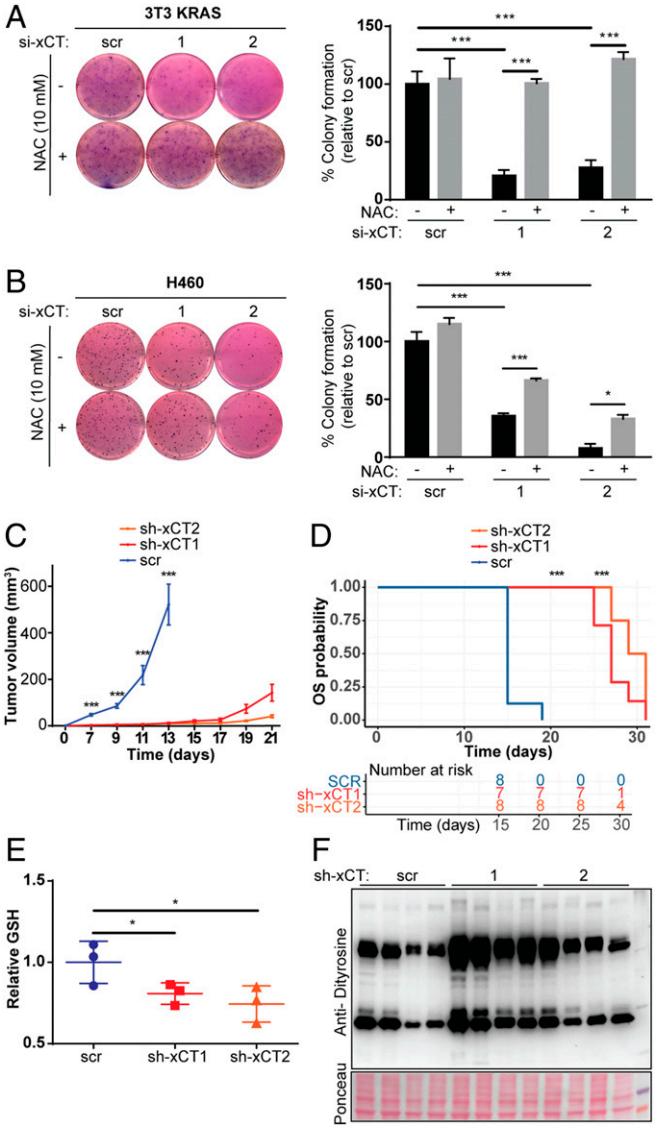

Fig. 5. xCT is required for oncogenic KRAS-mediated transformation and tumorigenicity. (*A*) 3T3 KRAS^{V12} cells were transfected with siRNA targeting *xCT* or scrambled control for 24 h and seeded into soft agar with or without 10 mM NAC for 3 wk, following which colony formation was determined by MTT staining ($n = 3$). (*B*) H460 cells were transfected with siRNA targeting *xCT* or scrambled control for 24 h and seeded into soft agar with or without 10 mM NAC for 3 wk, following which colony formation was determined by MTT staining ($n = 3$). (*C*) 3T3 KRAS^{V12} cells were transfected with shRNA targeting *xCT* or scrambled control and were s.c. implanted in *nu*/*nu* immunocompromised mice. Tumor volume was determined every 2 d ($n = 8$). (*D*) Survival of mice bearing tumors from 3T3 KRAS^{V12} cells expressing shRNA targeting *xCT* or scrambled control ($n = 8$). (*E*) Quantification of reduced GSH levels of tumor lysate from 3T3 KRAS^{V12} cells expressing shRNA targeting *xCT* or scrambled control using GSH-Glo assay (Promega) ($n = 8$). (*F*) Western blot analysis of dityrosine levels of tumor lysate from 3T3 KRAS^{V12} cells expressing shRNA targeting *xCT* or scrambled control. Where shown, data are reported as means ± SD with indicated significance (*$P < 0.05$, **$P < 0.01$, and ***$P < 0.005$).

xCT is critical for supporting KRAS oncogenic transformation and tumorigenicity in vitro and in vivo by increasing antioxidant capacity and mitigating oxidative stress.

High *xCT* Expression Predicts Poor Outcome in Mutant KRAS-Driven Human Tumors. We next investigated the relevance of these findings in preclinical and clinical models of RAS-driven cancers. We first interrogated mRNA expression data from genetically engineered mouse models and found that lung tumor specimens from transgenic mice expressing $Kras^{G12D}$ mutant display higher levels of *xCT* mRNA compared with those from oncogenic *c-Myc*–expressing mice or from normal lung tissue (Fig. 6*A*). Additionally, elevated levels of *xCT* mRNA were also found in lung tumor specimens from transgenic mice expressing either *EGFR* in-frame exon 19 deletion mutant or the $EGFR^{L858R}$ mutant, providing evidence that constitutively active RAS signaling in addition to expression of mutant RAS proteins are associated with *xCT* induction.

These findings were confirmed in clinical cancer specimens by analyzing publicly available gene expression data from lung and colon cancer and glioma patients. This revealed that *xCT* expression is up-regulated in patient tumors that are positive for *KRAS* mutations (Fig. 6 *B* and *C*). Interestingly, *xCT* expression is also up-regulated in glioma tumors that are positive for *EGFR* amplification, further highlighting that constitutive RAS signaling is associated with *xCT* induction (Fig. 6*D*). Furthermore, hypergeometric analyses of gene expression datasets from TCGA LUAD, LUSC, and COAD cohorts revealed that the top 1% genes positively coexpressed with *xCT* are those that are significantly enriched in association with constitutively active MEK (*SI Appendix*, Fig. S6*A*). Similar analyses of these gene expression datasets also showed that the top 5% genes positively coexpressed with *xCT* are significantly enriched for genes controlled by the ETS-1 transcription factor (*SI Appendix*, Fig. S6*B*). Altogether, these results suggest that *xCT* is elevated downstream of overactive RAS-RAF-MEK-ERK-ETS-1 signaling axis in human tumors harboring *KRAS* mutations.

Finally, we assessed the prognostic value of *xCT* expression in colon cancer and glioma patients. We observed that high *xCT* levels correlates with poorer outcomes across all colon cancer subgroups (Fig. 6*E*) and in glioma patients (Fig. 6*F*). This is in line with previous reports in triple-negative breast cancer (20) as well as nonsmall cell lung cancer (18), and it suggests that *xCT* predicts poor prognosis in tumor types associated with increased RAS pathway activation. In summary, these data strongly suggest that *xCT* expression has prognostic value in *KRAS* mutant-expressing tumors, further reinforcing the link between oncogenic KRAS and *xCT* expression.

Discussion

Oncogenic RAS is well known to exert cytoprotective effects in tumor cells under diverse stress-inducing conditions (40–44). Here, we demonstrate that oncogenic KRAS directly protects cells against oxidative stress by stimulating *xCT* transcription to enhance intracellular GSH levels. This is in agreement with the role of xCT as an integral player in the cellular response to oxidative stress. Indeed, xCT is reported to enhance the antioxidant capacity of cancer cells as a means to support tumorigenicity and chemoresistance (25, 26, 45). We show using two models of oncogenic RAS signaling, namely $KRAS^{V12}$ and EN-transformed cell lines, that oncogenic RAS activation promotes the transcription of *xCT* in response to oxidative stress by a synergistic cooperation between ETS-1 and ATF4. This is in keeping with previous reports that ATF4 is a transcriptional regulator of *xCT* expression in response to oxidative stress (17). While ATF4 and several other transcription factors including NRF2 and P53 are reported to control *xCT* expression, ETS-1 downstream of the RAS pathway is a previously unappreciated regulator of *xCT*. Moreover, our data suggests that while ATF4 is necessary to induce *xCT*, it is insufficient to elicit full induction of the latter, and that ETS-1 is required for a more complete response to oxidative stress. ETS-1 is known to synergize with other transcription factors to activate downstream targets (46), but to our knowledge, synergy between ETS-1 and ATF4 on promoters of a target gene has not

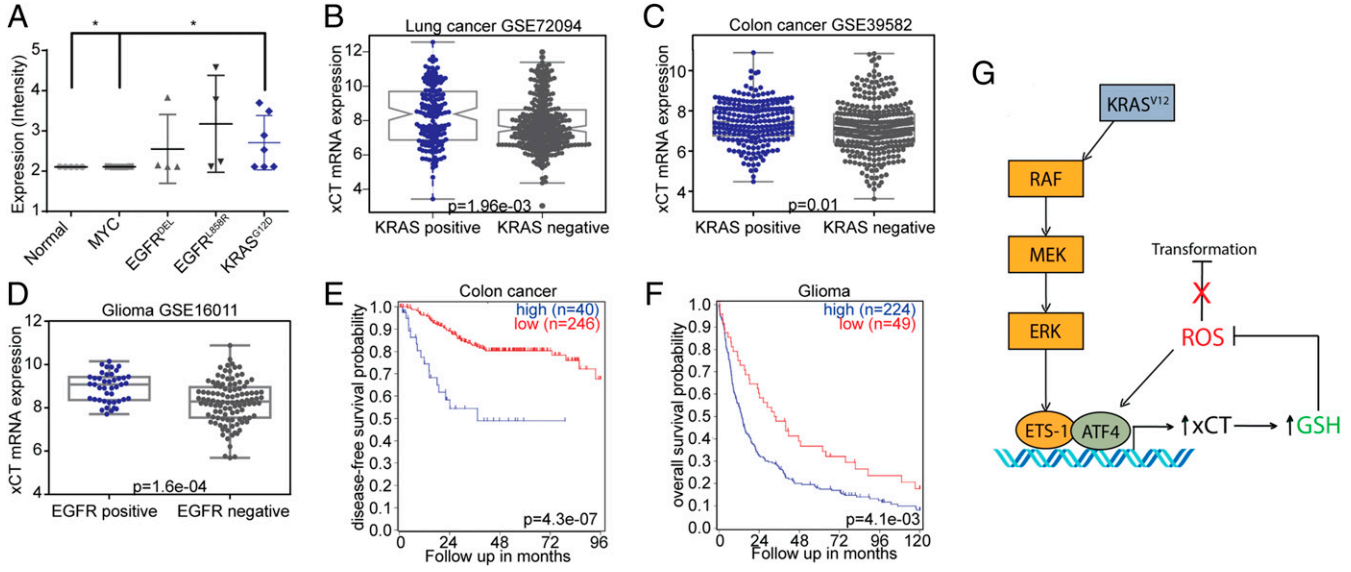

Fig. 6. Clinical relevance of *xCT* expression in mutant KRAS-driven tumors. (*A*) *xCT* expression in lung tumor specimens of transgenic mice expressing $Kras^{G12D}$, c-*Myc*, *EGFR* in-frame exon 19 deletion mutant or $EGFR^{L858R}$ mutant, and normal lung tissue. Gene expression data showing *xCT* levels in lung cancer (*B*), colon cancer (*C*), and glioma (*D*) patient cohorts with positive or negative status for *KRAS* mutation. Survival analysis by Kaplan–Meyer plotting based on *xCT* expression in colon cancer (*E*) and glioma (*F*) patients. (*G*) Schematic diagram depicting oncogenic RAS activation promoting the transcription of *xCT* in response to oxidative stress by a synergistic cooperation between ETS-1 downstream of RAS-ERK signaling and ATF4. The induction of *xCT* leads to enhanced GSH biosynthesis, which is suggested to mitigate oxidative stress arising during transformation/tumorigenicity. Where shown, data are reported as means ± SD with indicated significance (*$P < 0.05$, **$P < 0.01$, and ***$P < 0.005$).

been documented. Given the evidence presented for a physical association between endogenous ETS-1 and ATF4, and for the binding of ETS-1 to the *xCT* promoter, both of which being enhanced under oxidative stress (*SI Appendix*, Fig. S4 *D* and *F*, respectively), we further speculate that this synergy may involve the direct recruitment of ETS-1 to the *xCT* promoter by ATF4, or that both transcription factors are necessary to form a functional transcriptional complex under oxidative stress. These findings represent an unanticipated mechanism to link RAS signaling to the cellular oxidative stress response. Interestingly, while oncogenic KRAS-transformed 3T3 fibroblasts show enhanced induction of *xCT* in response to oxidative stress, RAS-transformed HMECs have elevated *xCT* expression even in the basal state (Fig. 2*C*). This may be explained by the observation that while RAS-transformed HMECs exhibit an increase in *ATF4* expression under ambient conditions, in comparison with control HMECs (*SI Appendix*, Fig. S6*C*), this is not the case for 3T3 fibroblasts. Thus, the regulation of *xCT* by the RAS-ETS1/ATF4 cascade is observable across multiple cellular models of RAS transformation.

In ovarian carcinoma cells, *ETS-1* is a target gene of NRF2 and is up-regulated under oxidative stress (47). In contrast, we did not find evidence to support that ETS-1 expression levels are increased in response to oxidative stress or that it is downstream of NRF2 regulation in RAS-transformed 3T3 fibroblasts, which may be attributed to cell line-specific differences. Similarly, although a recent study in non-small cell lung cancer found that NRF2 up-regulates ATF4 transcription downstream of the KRAS-PI3K signaling axis in response to nutrient deprivation (42), we did not find a similar link in our study. Such regulation again may be lung tissue specific, but it may also be explained by differences in the time points investigated or the type of stress stimuli involved, as the regulation of ATF4 by NRF2 was determined at 72 h following glutamine deprivation. However, given that *xCT* is a target gene of NRF2, and *NRF2* itself is transcriptionally up-regulated downstream of RAS (11), RAS-ETS1/ATF4 and RAS-ERK-NRF2 axes may form two independent signaling cascades to ultimately achieve the same output. While seemingly redundant, it is conceivable that RAS utilizes both pathways to mitigate oxidative stress and maximize tumor fitness. Two observations lend support to this idea; first, NRF2 has an additive effect on induction of *xCT* by ETS-1 and ATF4, suggesting that both the RAS-ETS-1/ATF4 and RAS-ERK-NRF2 pathways are complementary. Second, ETS-1 is activated by RAS at the posttranslational level, as a known substrate of ERK, while NRF2 is induced at the transcriptional level, providing separate mechanisms to ensure acute and sustained responses to oxidative stress through up-regulation of *xCT*. The cross-talk between these pathways and how they influence RAS-mediated antioxidant response warrants further investigation.

Our data demonstrates that *xCT* transcription is directly controlled by oncogenic RAS signaling through ETS-1, itself encoded by a proto-oncogene (48). The only other reported oncogenic mechanism controlling *xCT* expression involves oncogenic PI3K, as it was shown that this pathway suppresses *xCT* transcription and activity (49). Notably, in addition to oncogenic $KRAS^{V12}$, overexpression of $HRAS^{V12}$ or *ETV6-NTRK3* also induced *xCT* expression. In this regard, *xCT* overexpression may be generalizable to RAS pathway-driven cancers. This may include cancers that do not harbor *RAS* mutations but exhibit constitutive RAS signaling, such as lung cancer with *EGFR* mutations, glioblastoma with *NF1* mutations or *EGFR* amplification, and colon cancer and melanoma with *BRAF* mutations. Many of these tumors are notable for being highly refractory to standard chemotherapeutic agents (50–52), and therefore targeting xCT with pharmacological inhibitors represents an attractive therapeutic strategy to potentially sensitize RAS-driven cancers to chemotherapy. Indeed, our data lends support to this notion as preclinical models of *EGFR*-mutant lung cancer and clinical models of *EGFR*-amplified glioma were associated with enhanced *xCT* overexpression (Fig. 6 *A*–*D*). In contrast, there was no evidence that up-regulation of *xCT* was driven by other oncogenes such as β-*catenin*, *E2F3*, or *MYC*, highlighting the specificity for oncogenic *RAS* signaling. These findings thus enhance our general understanding of how tumors up-regulate *xCT*, which is clinically relevant since it is overexpressed in multiple cancers and is a marker of poor prognosis (18–21).

The role of ROS versus antioxidants in RAS transformation is controversial, and the mechanisms involved have not been completely elucidated. We provide further evidence that antioxidants indeed contribute to RAS transformation, as xCT facilitates oncogenic KRAS-mediated tumorigenesis in vitro and in vivo by maintaining the redox balance. Previously proposed mechanisms of antioxidant-mediated RAS transformation include up-regulation of NRF2, the master regulator of intracellular antioxidant response, and rechannelling of glucose and glutamine into GSH and NADPH-generating metabolic pathways (11–13). Furthermore, broad evidence now shows that cancer cells that are able to initiate intrinsic antioxidant responses are selected for during tumor progression to cope with oxidative stress arising in response to oncogene activation, accumulation of genetic instability, aberrant metabolism, and mitochondrial dysfunction (53). For example, it has been observed that a subset of cancer stem cells in human and mouse mammary tumors contain lower levels of ROS and display less DNA damage relative to their nontumorigenic counterparts, due to increased GSH biosynthesis genes such as *GCLM* and *GSS* (54). In addition, GSH pathways are critical for tumor initiation, as genetic loss of *Gclm* resulted in the inability of mice to form mammary tumors (55). More recently, Truitt et al. (39) demonstrated that cancer cells hijack an eIF4E-dependent translation program that is selectively enriched for mRNAs involved in antioxidant responses to support cell survival and fuel oncogenic transformation. Therefore, while cancer cells rely on ROS induction to promote protumorigenic processes such as proliferation, they are also critically dependent on adaptive or intrinsic ROS-scavenging pathways to restrict oxidative damage that can ultimately impede tumor progression. This dependence on ROS-clearing mechanisms thus reveals a vulnerability that represents a tractable therapeutic strategy, such as the use of high-dose vitamin C to exacerbate oxidative stress by depleting GSH, thereby selectively killing *KRAS* and *BRAF*-mutated colorectal cancer cells (56). Such a strategy may also extend to targeting other steps of cancer progression, such as metastasis, as a clear role for antioxidants in promoting tumor metastasis has been shown (57–59).

In summary, our work reveals that oncogenic RAS protects tumor cells from oxidative stress by enhancing GSH via *xCT* up-regulation. We present evidence that ETS-1 synergizes with ATF4 to directly transactivate the *xCT* promoter downstream of the RAS-ERK pathway in response to oxidative stress (Fig. 6*G*). As such, xCT supports oncogenic KRAS-mediated transformation by maintaining the redox balance, presenting a candidate therapeutic target for this subset of therapy-resistant tumors.

Materials and Methods

Cell Culture. NIH 3T3 cells stably expressing EN or $KRAS^{V12}$ were as described (60). $xCT^{-/-}$ (KO) and $xCT^{+/+}$ (WT) MEFs were a kind gift from Hideyo Sato (Niigata University, Niigata, Japan) and routinely cultured in DMEM supplemented with 10% FBS (36). $xCT^{-/-}$ MEFs were also supplemented with 50 μM 2-mercaptoethanol (Sigma-Aldrich). H460 and SW620 cell lines were purchased from American Type Culture Collection (Rockville, MD) and maintained in RPMI 1640 media supplemented with 10% FBS. *Nras*/*Hras* double knockout MEFs with 4-OHT–inducible knockout of endogenous *Kras* were kindly provided by Mariano Barbacid, National Centre for Cancer Research, Madrid (32).

Tumorigenicity Assay. Aliquots of 0.5×10^6 cells were resuspended in 200 μL of PBS and injected s.c. into the flanks of 5- to 6-wk-old female Nu/Nu

immunodeficient mice using standard procedures. Starting from day 13 after injection, mice euthanasia was required when tumors exceeded humane practice guidelines (500 mm^3). Mice were evaluated for tumor growth every 2 d until the experimental endpoints. Tumors were measured with a caliper, and volumes were estimated using the following formula: tumor length × (tumor width)^2 × π/6 mm^3. All animal experiments underwent ethical approval from the Animal Care Committee of the University of British Columbia (A16-0050; A16-0050-A001). Full material and methods are available in *SI Appendix, Materials and Methods*.

Gene Expression of Clinical Cancer Specimens. Analyses of gene expression data from human subjects did not require approval from the institutional review board (IRB), as the data were all obtained from publicly available databases in which each study had utilized strict human subjects protection guidelines, informed consent, and respective IRB review of protocols.

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