

CST1 stabilizes GPX4 to suppress ferroptosis and promote bladder cancer progression

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Abstract

Background

Cystatin SN (CST1), a cysteine protease inhibitor, participates in various cancers, yet its function and mechanism in bladder cancer (BCa) are unclear.

Methods

Public databases, BCa cells and clinical samples were used to analyze CST expression profiles. Functional experiments detected cell proliferation, migration and invasion. Intracellular iron, ROS, MDA, GSH and mitochondrial morphology reflected ferroptosis. Co-IP assays verified the intracellular interaction between CST1 and GPX4, and ubiquitination assays clarified GPX4 stabilization via suppressed proteasomal degradation. Xenograft and rescue assays validated the CST1-GPX4 axis.

Results

The expression of CST1 is markedly higher in bladder cancer cells than in normal urothelial cells. CST1 knockdown inhibits malignant phenotypes and induces ferroptosis. CST1 binds GPX4 to block its ubiquitination and post-translationally elevate GPX4 protein without altering its transcription. GPX4 overexpression rescues CST1 silencing-induced ferroptosis and growth defects. In vivo CST1 facilitates tumor growth and GPX4 upregulation.

Conclusion

CST1 interacts with GPX4 to inhibit its ubiquitin-dependent degradation, suppress ferroptosis and promote BCa progression, and may serve as a potential therapeutic target for bladder cancer.

1 Introduction

Bladder cancer (BCa) is one of the most common malignancies of the urinary system. According to global cancer statistics, BCa ranks the sixth most common malignancy in men, with over 570,000 new cases and more than 210,000 deaths annually [1]. BCa is classified by depth of invasion into non-muscle-invasive bladder cancer (NMIBC) and muscle-invasive bladder cancer (MIBC), the latter of which is highly aggressive with strong metastatic potential and extremely poor prognosis [2]. Despite continuous advances in surgery, chemotherapy, and immunotherapy, the 5-year survival rate of patients with metastatic BCa remains very low [3]. Therefore, elucidating the molecular mechanisms underlying BCa progression and identifying novel therapeutic targets is of substantial clinical significance.

The Cystatin superfamily comprises a group of proteins widely distributed in human body fluids and tissues that function as inhibitors of cysteine proteases [4]. Cystatin SN (CST1), encoded by the CST1 gene, is a secreted polypeptide belonging to the type 2 subfamily of Cystatins [5]. CST1 protein is predominantly expressed in the submandibular gland, gallbladder, and uterus, but is also highly expressed in various malignant tumors [6]. Recent studies have demonstrated that CST1 overexpression is involved in the proliferation, invasion, and metastasis of multiple cancers, including lung cancer, breast cancer, and colorectal cancer [7–9]. Moreover, CST1 has been shown to reduce auranofin-induced cell death by inhibiting intracellular reactive oxygen species (ROS) production in colorectal cancer cells [10]. In gastric cancer, CST1 has been reported to promote malignant progression by regulating the transcription factor HOXC10 and the Wnt signaling pathway [11–13]. However, the expression and biological function of CST1 in BCa remain poorly characterized.

Ferroptosis is a distinct iron-dependent form of regulated cell death driven by lipid peroxidation, characterized by elevated intracellular Fe^{2+} levels, increased reactive oxygen species (ROS) production, accumulation of the lipid peroxidation product malondialdehyde (MDA), glutathione (GSH) depletion, and mitochondria with reduced volume and increased membrane density [14, 15]. In recent years, the critical role of ferroptosis in tumor progression has been increasingly recognized. For instance, lymph protects metastasizing melanoma cells from ferroptosis to promote metastasis [16]; additionally, induction of ferroptosis suppresses brain metastasis in a syngeneic mouse model of spontaneous HER2-positive breast cancer [17].

GPX4 (glutathione peroxidase 4) is the core regulator of ferroptosis, functioning to maintain intracellular redox homeostasis and protect cells from membrane lipid peroxidation-induced death by suppressing lipid peroxidation [18]. GPX4 is highly expressed in various tumors and is closely associated with tumor progression [19, 20]. Lu et al. demonstrated that KLF2 suppresses the migration and invasion of clear cell renal cell carcinoma by regulating ferroptosis through GPX4 [21]. However, the role of the GPX4-ferroptosis axis in BCa progression remains to be further elucidated.

The ubiquitin-proteasome system (UPS) is the principal pathway for selective protein degradation in eukaryotic cells and is essential for maintaining protein homeostasis [22]. Ubiquitination is a highly conserved post-translational modification process, in which ubiquitin molecules are covalently conjugated to lysine residues of substrate proteins through a sequential enzymatic cascade involving ubiquitin-activating enzymes (E1), ubiquitin-conjugating enzymes (E2), and ubiquitin ligases (E3), thereby targeting the substrate proteins for recognition and degradation by the 26S proteasome [23]. Deubiquitinases (DUBs) specifically recognize and remove ubiquitin chains from substrate proteins, reversing ubiquitin modifications, and play critical roles in maintaining protein stability and regulating signal transduction [24]. In recent years, aberrant alterations of the ubiquitination-deubiquitination regulatory network in tumor initiation and progression have drawn widespread attention. Studies have shown that dysregulated ubiquitination of various tumor-associated proteins is involved in key biological processes including cell proliferation, apoptosis, invasion, metastasis, and ferroptosis [25]. Notably, as the core negative regulator of ferroptosis, GPX4 protein stability is tightly governed by ubiquitination

modifications [18]. Studies have demonstrated that multiple E3 ubiquitin ligases and deubiquitinases (such as OTUB1) can influence ferroptosis execution by modulating GPX4 ubiquitination levels [26]. However, the specific mechanisms by which ubiquitination regulates GPX4 protein stability and ferroptosis to influence tumor progression in BCa remain to be thoroughly elucidated.

In this study, we investigated the molecular mechanism of CST1 in BCa and how it regulates GPX4 to suppress ferroptosis in BCa cells, thereby promoting BCa initiation and progression.

2 Results

2.1 CST1 is highly expressed in BCa

To investigate CST1 expression at the pan-cancer level, we first performed pan-cancer analysis using the TIMER3.0 database. The results showed that CST1 expression was significantly higher in multiple tumor tissues compared with normal tissues, including BCa (BLCA) (Fig. 1A), suggesting that CST1 may play an oncogenic role in various malignancies.

To further clarify the expression and clinical significance of CST1 in BCa, we analyzed TCGA database. The results showed that CST1 expression was markedly elevated in tumor tissues of most BCa patients compared with normal bladder tissues (Fig. 1B).

Based on the bioinformatics analysis, we further validated CST1 expression at the protein level in BCa cell lines. Western blot results showed that CST1 protein expression was significantly higher in T24, 5637, UM-UC-3, and J82 BCa cell lines compared with the human normal bladder epithelial cell line SV-HUC-1 (Fig. 1C, D).

To verify CST1 expression in clinical specimens, we collected 10 paired BCa and adjacent normal tissues for western blot analysis. The results showed that CST1 protein levels in BCa tissues were significantly higher than those in paired adjacent normal tissues (Fig. 1E, F). Immunohistochemistry (IHC) staining further confirmed that CST1 was highly expressed in BCa tissues (Fig. 1G).

Collectively, these results demonstrate that CST1 is generally upregulated in BCa cell lines and clinical tissues, implying that CST1 may participate in the initiation and progression of bladder cancer.

2.2 CST1 promotes proliferation, invasion, and migration of BCa cells

To investigate the impact of CST1 on the malignant biological behavior of BCa cells, we selected T24 and 5637 cells, which express high levels of CST1, to establish CST1 stable knockdown cell lines. Western blot confirmed the knockdown efficiency (Fig. 2A, B). We evaluated the effect of CST1 on BCa cell proliferation using EdU and colony formation assays. EdU results showed that the percentage of EdU-positive cells was significantly lower in the CST1 knockdown group than in the control group ($P < 0.01$) (Fig. 2C, D). Colony formation assay results were consistent with the EdU findings (Fig. 2E, F),

demonstrating that CST1 significantly promotes BCa cell proliferation. Subsequently, Transwell invasion assays confirmed that the number of invaded cells in the CST1 knockdown group was significantly reduced compared with the control group ($P < 0.001$) (Fig. 2G, H). Wound healing assay results showed that the wound closure ability of T24 and 5637 cells was significantly impaired upon CST1 knockdown ($P < 0.001$) (Fig. 2I, J). Taken together, these results demonstrate that CST1 significantly promotes the proliferation, invasion, and migration of BCa cells, indicating that CST1 plays an important tumor-promoting role in the malignant progression of BCa.

2.3 CST1 interacts with GPX4 and enhances its protein stability by inhibiting ubiquitination-mediated degradation

Liu et al. demonstrated that CST1 interacts with the core ferroptosis regulator GPX4 in gastric cancer cells, protecting them from ferroptosis and thereby promoting gastric cancer progression and metastasis [26]. To explore the molecular mechanism by which CST1 drives malignant progression of BCa, we hypothesized that CST1 exerts its oncogenic function through a similar pathway in BCa. We first performed endogenous Co-IP in T24 and 5637 cells (Fig. 3A), which confirmed the endogenous interaction between CST1 and GPX4 in BCa cells. Subsequently, we constructed His-tagged GPX4 (His-GPX4) and Flag-tagged CST1 (Flag-CST1) overexpression plasmids and conducted two-way Co-IP experiments in HEK293T cells (Fig. 3B), further verifying the intracellular interaction between CST1 and GPX4.

Next, we investigated whether CST1 regulates GPX4 expression. Western blot analysis showed that GPX4 protein levels were markedly reduced upon CST1 knockdown (Fig. 3C, D). To determine whether CST1 regulates GPX4 at the transcriptional or post-translational level, we performed RT-qPCR. The results revealed no significant change in GPX4 mRNA levels following CST1 knockdown (Fig. 3E), suggesting that CST1 regulates GPX4 at the post-translational level, potentially affecting GPX4 protein degradation.

To examine whether CST1 regulates GPX4 degradation through the ubiquitin-proteasome pathway, we treated control and CST1-knockdown cells with the 26S proteasome inhibitor MG132. The results showed that MG132 treatment markedly restored GPX4 protein levels in CST1-knockdown cells (Fig. 3F), demonstrating that CST1 inhibits the proteasomal degradation of GPX4.

To validate the above hypothesis, we performed a cycloheximide (CHX, 100 $\mu\text{g/mL}$) protein half-life assay to assess the effect of CST1 on GPX4 protein stability. Western blot results showed that compared with the control group, GPX4 protein degradation was significantly accelerated and its half-life was markedly shortened upon CST1 knockdown (Fig. 3G), indicating that CST1 enhances GPX4 protein stability.

Finally, to directly verify whether CST1 regulates GPX4 through modulating its ubiquitination, we performed ubiquitination assays. Cells transfected with HA-Ub and His-GPX4 were co-transfected with

either empty vector or Flag-CST1, with MG132 treatment as an intervention control. The results showed that MG132 treatment significantly accumulated GPX4 ubiquitination bands, whereas concurrent overexpression of CST1 markedly attenuated GPX4 ubiquitination levels (Fig. 3H). Collectively, these results demonstrate that CST1 inhibits GPX4 ubiquitination, thereby reducing its proteasomal degradation and enhancing GPX4 protein stability.

2.4 CST1 suppresses ferroptosis in BCa cells

Given that we had confirmed an interaction between CST1 and GPX4 in Section 2.3, and GPX4 is a canonical regulator of the ferroptosis pathway, we hypothesized that CST1 may influence ferroptosis in BCa cells through GPX4. To test this hypothesis and exclude the involvement of other programmed cell death modalities, we first performed intervention experiments using different cell death inhibitors. CCK-8 results showed that in the shNC control group, treatment with the ferroptosis inducer Erastin significantly reduced cell viability ($P < 0.01$), an effect that was significantly reversed by co-treatment with the ferroptosis-specific inhibitor Ferrostatin-1 (Fer-1) ($P < 0.01$), whereas the apoptosis inhibitor Z-VAD-FMK and the autophagy inhibitor 3-MA showed no significant rescue effect. In the shCST1 knockdown group, Erastin-induced reduction of cell viability was further exacerbated compared with the shNC group ($P < 0.001$), and co-treatment with Fer-1 still significantly restored cell viability ($P < 0.01$), whereas the protective effects of Z-VAD-FMK and 3-MA were limited (Fig. 4A). These results demonstrate that CST1 knockdown sensitizes BCa cells to ferroptosis rather than apoptosis or autophagy.

To comprehensively validate the regulatory role of CST1 in ferroptosis, we examined key biochemical markers of ferroptosis. Measurement of intracellular Fe^{2+} levels using a colorimetric assay kit showed that Fe^{2+} levels were significantly elevated in 5637 and T24 cells upon CST1 knockdown ($P < 0.01$) (Fig. 4B). Flow cytometry analysis of intracellular ROS levels revealed that ROS levels in the shCST1 group were significantly higher than those in the shNC control group (Fig. 4C, D). We further employed the BODIPY lipid peroxidation fluorescent probe to detect lipid peroxide levels following CST1 knockdown. In the shNC control group, cells exhibited weak green oxidized BODIPY fluorescence and strong red reduced BODIPY fluorescence, whereas in the CST1 knockdown group, the green fluorescence signal was markedly enhanced, indicating substantial accumulation of intracellular lipid peroxides (Fig. 4E). MDA content measurement showed that the level of MDA, a terminal product of lipid peroxidation, was significantly increased upon CST1 knockdown (Fig. 4F). GSH assay results demonstrated that intracellular GSH content was significantly decreased following CST1 knockdown ($P < 0.01$) (Fig. 4G).

In addition, we examined mitochondrial ultrastructure by transmission electron microscopy (TEM). The results showed that mitochondria in the shNC control group displayed normal morphology with clearly visible cristae, whereas mitochondria in the shCST1 group were markedly shrunken with increased membrane density and reduced or absent cristae, exhibiting the characteristic mitochondrial morphological features of ferroptosis (Fig. 4H).

Collectively, these results demonstrate that CST1 knockdown elevates intracellular Fe^{2+} and ROS levels, increases lipid peroxidation product accumulation, reduces GSH content, and abolishes the protection of mitochondria from ferroptosis-associated morphological damage, thereby promoting ferroptosis in BCa cells.

2.5 Rescue experiments confirm that GPX4 is a key downstream effector of CST1 in promoting malignant phenotypes of BCa

To determine whether the promotion of malignant phenotypes and regulation of ferroptosis by CST1 are dependent on GPX4, we performed rescue experiments by overexpressing GPX4 in CST1 stable knockdown T24 and 5637 (shCST1) cells. Western blot confirmed the efficiency of CST1 knockdown and GPX4 overexpression (Fig. 5A, B).

We first evaluated the rescue effect of GPX4 overexpression on the malignant biological behavior of BCa cells. EdU assay results showed that the percentage of EdU-positive T24 cells was significantly reduced upon CST1 knockdown ($P < 0.01$), whereas overexpression of GPX4 partially restored cell proliferation ($P < 0.01$) (Fig. 5C, D). Wound healing assay results demonstrated that the migration rate of T24 cells was significantly slowed following CST1 knockdown ($P < 0.001$), and overexpression of GPX4 partially rescued the migration ability ($P < 0.01$) (Fig. 5E, F).

Subsequently, we examined the rescue effect of GPX4 overexpression on ferroptosis. Flow cytometry analysis of intracellular ROS levels showed that ROS were significantly elevated in T24 cells upon CST1 knockdown ($P < 0.01$), whereas overexpression of GPX4 partially reversed this effect ($P < 0.01$) (Fig. 5G, H). We further measured intracellular Fe^{2+} , MDA, and GSH levels following GPX4 overexpression (Fig. 5I, J, K). All results consistently demonstrated that overexpression of GPX4 partially reversed the promoting effect of CST1 knockdown on ferroptosis in BCa cells, indicating that GPX4 is a key downstream molecule through which CST1 regulates ferroptosis.

2.6 CST1 accelerates bladder cancer growth in vivo

To validate the tumor-promoting function of CST1 in vivo, we established a nude mouse xenograft tumor model. The results showed that tumor volume, weight, and growth rate in the CST1 knockdown group were significantly lower than those in the control group, whereas tumor volume, weight, and growth rate in the CST1 overexpression group were significantly greater than those in the control group (Fig. 6A, B, C). IHC analysis revealed that Ki-67 expression was reduced in the knockdown group and elevated in the overexpression group (Fig. 6D), indicating that CST1 promotes BCa growth in vivo.

2.7 CST1 stabilizes GPX4 to suppress ferroptosis and promote BCa progression

Our mechanistic findings confirm an interaction between CST1 and GPX4 in BCa cells. This association inhibits GPX4 ubiquitination, blocks its proteasomal degradation, and leads to a significant increase in

intracellular GPX4 protein levels. The accumulated GPX4 suppresses ferroptosis in BCa cells. Inhibition of ferroptosis further promotes multiple malignant phenotypes of BCa, including accelerated cell proliferation, enhanced migration and invasion, and increased tumor growth in vivo. The xenograft tumor model also confirmed that CST1 overexpression significantly increased tumor volume and weight in nude mice, whereas CST1 knockdown exerted the opposite effect.

3 Discussion

In this study, we systematically investigated the biological function and molecular mechanism of CST1 in BCa progression. Through bioinformatics analysis and clinical specimen validation, we revealed for the first time that CST1 is highly expressed in BCa tissues and multiple BCa cell lines. Functional experiments demonstrated that CST1 significantly promotes the proliferation, migration, and invasion of BCa cells. Mechanistically, we confirmed an interaction between CST1 and GPX4 in bladder cancer cells. This interaction suppresses GPX4 ubiquitination and proteasomal degradation and enhances GPX4 protein stability. It consequently lowers intracellular Fe^{2+} and ROS levels, attenuates lipid peroxidation product accumulation, maintains GSH content, preserves mitochondrial morphology, suppresses ferroptosis, and ultimately promotes the malignant progression of BCa in a GPX4-dependent manner. This study establishes, for the first time, a complete functional framework for the CST1-GPX4-ferroptosis regulatory axis in BCa.

CST1 belongs to the type 2 Cystatin superfamily, which comprises 10 members, all located at chromosome 20p11.21[27]. Recent studies have demonstrated that type 2 Cystatin family members play important regulatory roles in multiple malignancies; CST2 and CST4 have been identified as independent prognostic markers and therapeutic targets in gastric cancer [28]. CST1 has been reported to exert oncogenic functions in several tumor types. For example, in hepatocellular carcinoma, CST1 promotes malignant progression through the PI3K/AKT pathway [29]. However, the expression and function of CST1 in BCa had not been systematically characterized prior to this study. In the present study, pan-cancer analysis using the TIMER3.0 database revealed that CST1 expression is elevated in multiple tumors, including BCa. TCGA database analysis further verified that CST1 expression was markedly elevated in bladder tumor tissues compared with normal bladder tissues. We found that CST1 protein expression was significantly elevated in highly invasive BCa cell lines, including T24, 5637, UM-UC-3, and J82, but was low in the normal urothelial cell line SV-HUC-1, indicating that CST1 expression levels are closely correlated with the malignancy of BCa cells. Western blot and IHC analyses further confirmed the high expression of CST1 in BCa tissues at the protein level. In terms of functional characterization, we systematically evaluated the effects of CST1 on the malignant biological behavior of BCa cells through EdU, colony formation, wound healing, and Transwell invasion assays. The results demonstrated that CST1 knockdown significantly suppressed the proliferation, colony formation, migration, and invasion of BCa cells. These findings are consistent with those reported by Chen et al. [11] in gastric cancer, suggesting that the function of CST1 in promoting tumor migration and invasion may be a common feature across different tumor types.

Ferroptosis, first designated in 2012, is a distinct iron-dependent form of regulated cell death driven by lipid peroxidation, with core features including intracellular Fe^{2+} accumulation, ROS burst, lipid peroxide (e.g., MDA) accumulation, GSH depletion, and characteristic mitochondrial morphological alterations. The dual role of ferroptosis in tumor initiation and progression has drawn increasing attention: on the one hand, induction of ferroptosis can suppress tumor growth and metastasis; on the other hand, tumor cells can evade ferroptosis by upregulating antioxidant defense systems, thereby gaining a survival advantage [30, 31]. In this study, we provided multi-layered experimental evidence demonstrating that CST1 suppresses ferroptosis in BCa cells. First, the programmed cell death inhibitor intervention experiment represents one of the important innovations of this study. Erastin is a classic ferroptosis inducer that blocks cystine uptake by inhibiting xCT (SLC7A11), thereby depleting intracellular GSH, inactivating GPX4, and triggering lipid peroxidation [32]. Our results showed that Erastin-induced cell death was effectively reversed by the ferroptosis-specific inhibitor Fer-1, whereas the apoptosis inhibitor Z-VAD-FMK and the autophagy inhibitor 3-MA showed no significant rescue effect, clearly ruling out the involvement of apoptosis and autophagy and confirming that CST1 deficiency sensitizes BCa cells to ferroptosis. Second, we comprehensively examined four classic biochemical markers of ferroptosis. The results demonstrated that CST1 knockdown led to elevated intracellular Fe^{2+} and ROS levels, increased MDA content, and decreased GSH content, presenting typical biochemical hallmarks of ferroptosis. The integrated analysis of these multiple indicators provides compelling evidence that CST1 regulates ferroptosis in BCa cells.

GPX4 is the core negative regulator of the ferroptosis network, utilizing GSH as a cofactor to reduce toxic lipid peroxides to non-toxic lipid alcohols, thereby protecting cells from ferroptosis. The regulation of GPX4 protein through ubiquitination modification is a frontier focus in ferroptosis research. In 2024, Li et al. [33] discovered that the deubiquitinase USP8 directly removes K48-linked ubiquitin chains from GPX4, preventing its proteasomal degradation, thereby suppressing ferroptosis and influencing the efficacy of cancer immunotherapy. Chu et al. [34] reported that OTUD5 inhibits ferroptosis in renal ischemia-reperfusion injury through deubiquitination of GPX4. Moreover, the natural product Plumbagin promotes GPX4 ubiquitination and degradation by inhibiting the deubiquitinase USP31 [35]. In gastric cancer, Li et al. [26] found that CST1 stabilizes GPX4 protein by removing ubiquitin chains from GPX4 through OTUB1, thereby inhibiting ferroptosis and promoting gastric cancer metastasis. The present study confirms the inhibitory effect of CST1 on GPX4 ubiquitination in BCa, extending this regulatory mechanism to urological tumors.

This study has several limitations that should be acknowledged. (1) The upstream regulatory mechanism underlying the aberrantly high expression of CST1 in BCa remains unclear. Recent studies have shown that transcription factors such as SPI1 can drive CST1 overexpression in esophageal squamous cell carcinoma [36]; which transcription factors or epigenetic modifications drive CST1 upregulation in BCa warrants further investigation. (2) Although this study demonstrated that CST1 inhibits GPX4 ubiquitination, the specific deubiquitinase involved remains to be identified. Given that multiple DUBs, including USP8, OTUD5, and OTUB1, have been shown to regulate GPX4 deubiquitination, whether CST1 cooperates with a specific DUB to stabilize GPX4 protein needs to be identified through

systematic approaches such as DUB library screening. (3) This study only employed a subcutaneous xenograft tumor model to assess the effect of CST1 on tumor growth; the impact of CST1 on BCa distant metastasis requires more comprehensive evaluation using a tail vein injection metastasis model or an orthotopic bladder transplantation model. (4) Large-scale, multi-center clinical cohort validation is lacking to confirm the clinical therapeutic value of CST1 for bladder cancer. (5) Whether the function of the CST1-GPX4-ferroptosis axis differs across different molecular subtypes of BCa also merits further investigation.

In summary, this study reveals for the first time the oncogenic function and molecular mechanism of CST1 in BCa: CST1 binds to GPX4, inhibits its ubiquitination-proteasomal degradation, and enhances GPX4 stability, thereby reducing intracellular Fe^{2+} and ROS levels, attenuating lipid peroxidation product accumulation, maintaining GSH content, protecting mitochondria from ferroptosis-associated morphological damage, and suppressing ferroptosis, which in turn promotes BCa cell proliferation, migration, invasion, and in vivo tumor growth. CST1 may serve as a promising therapeutic target, and targeting the CST1–GPX4–ferroptosis axis could offer a novel strategy for precision treatment of bladder cancer.

4 Materials and methods

4.1 Cell lines and cell culture

The immortalized human uroepithelial cell line SV-HUC-1 (CL-0222) and BCa cell lines 5637 (CL-0002), T24 (CL-0227), J82 (CL-0125), and UM-UC-3 (CL-0463) were obtained from Procell Life Science & Technology Co., Ltd. (Wuhan, China). SV-HUC-1 cells were cultured in Ham's F-12K medium (Servicebio, G4560-500ML, Wuhan, China), 5637 and T24 cells in RPMI-1640 medium (Servicebio, G4515-500ML), and J82 and UM-UC-3 cells in MEM medium (Servicebio, G4532-500ML). Each medium was supplemented with 10% fetal bovine serum (Servicebio, G8003-100ML, Wuhan, China) and 1% penicillin-streptomycin-amphotericin B solution (Biosharp, BL142A, China). All cell lines were cultured at 37°C in a humidified atmosphere with 5% CO_2 . All experiments were performed using cells in the logarithmic growth phase, and cell morphology and growth status were regularly monitored to ensure experimental consistency.

4.2 Clinical sample collection

Tumor tissues and paired adjacent normal tissues from 10 BCa patients were provided by Renmin Hospital of Wuhan University. All specimens were pathologically confirmed. Informed consent was obtained from all patients, and the study was approved by the Ethics Committee of Renmin Hospital of Wuhan University (WDRY2025-K321). Samples were immediately cryopreserved after collection.

4.3 Experimental animals

BALB/c nude mice (6–8 weeks old) were purchased from the Laboratory Animal Center of Renmin Hospital of Wuhan University. Nude mice, which lack T cells, are suitable for tumor xenograft models.

Mice were housed under specific pathogen-free (SPF) conditions at the Animal Center of Renmin Hospital of Wuhan University, with a controlled temperature of $22 \pm 2^{\circ}\text{C}$, relative humidity of 40%–60%, and a 12 h light/dark cycle. Sterile food and water were provided, and the health status of the mice was monitored regularly. The animal study protocol was approved by the Institutional Animal Care and Use Committee of WHUPENN LIFE SCIENCE Co., Ltd. (LBSM2026037) and was conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

4.4 Protein extraction and western blot analysis

Cells were seeded in 100 mm culture dishes (Servicebio, CCD-100H) and cultured until reaching over 90% confluency. Cells were washed three times with pre-cooled phosphate-buffered saline (PBS) and lysed in RIPA buffer (Servicebio, G2002-100ML) supplemented with 1 mM PMSF (Servicebio, G2008-1ML) on ice for 30 min. After sonication, the samples were centrifuged at 12,000 rpm for 15 min at 4°C . The supernatant was collected and mixed with 5× SDS-PAGE sample loading buffer (EpiZyme, LT101S, Shanghai, China) at a 4:1 (v/v) ratio. The mixture was boiled at 100°C for 10 min and centrifuged at 12,000 rpm for 3 min at 4°C . Prepared samples were stored at -20°C .

Proteins were separated by 10% SDS-PAGE gel (EpiZyme, PG212) and transferred onto nitrocellulose membranes (Vazyme, E803-01, China). Membranes were blocked with 5% nonfat milk in TBST for 1 h at room temperature, followed by overnight incubation at 4°C with primary antibodies. Primary antibodies and their dilutions were as follows: anti-CST1 (Huabio, ER63197, China, 1:2000 dilution), anti-GPX4 (Proteintech, 30388-1-AP, Wuhan, China, 1:1000 dilution), anti-Flag tag (Proteintech, 20543-1-AP, 1:50,000 dilution), anti-His tag (Proteintech, 10001-0-AP, 1:1000 dilution), anti- β -actin (Proteintech, 20536-1-AP, 1:10,000 dilution). After three washes with TBST, membranes were incubated with HRP-conjugated secondary antibody (Proteintech, SA00001-2, Wuhan, China, 1:1000 dilution) for 1 h at room temperature. Following three additional TBST washes, membranes were visualized using ECL FemtoLight Substrate (EpiZyme, SQ201).

4.5 EdU assay

Cells were seeded onto sterile coverslips placed in 24-well plates and cultured until reaching approximately 70% confluency. A 20 μM EdU working solution (Servicebio, G1603) was prepared. Half of the culture medium in each well was removed and replaced with an equal volume of pre-warmed EdU working solution, followed by incubation in a cell culture incubator for 2 h. After washing with PBS, cells were fixed with 4% paraformaldehyde (PFA) for 15 min at room temperature and permeabilized with permeabilization buffer (Servicebio, G1204-100ML) for 15 min at room temperature. The Click reaction cocktail was prepared, added to the samples, and incubated for 30 min at room temperature in the dark. Following additional washes, the coverslips were mounted onto slides using an antifade mounting medium containing DAPI (Servicebio, G1407-25ML). Samples were observed and photographed under a fluorescence microscope (Olympus, Japan).

4.6 Transwell invasion assay

The upper chamber of each Transwell insert (Corning, 8 µm pore size) was pre-coated with 50 µL of diluted Matrigel (1:8 dilution in serum-free medium, Corning). Cells were serum-starved in serum-free medium for 12 h, then harvested, resuspended in serum-free medium, and counted. A 200 µL aliquot of cell suspension (containing 20,000–50,000 cells) was seeded into the upper chamber, while the lower chamber was filled with 600 µL of complete medium containing 10% FBS. After incubation for 24 h, the inserts were rinsed with PBS, fixed with 4% PFA (Servicebio, G1101-500ML), and stained with 0.1% crystal violet (Servicebio, G1014-50ML). Non-invading cells on the upper membrane surface were removed with a cotton-tipped swab. Invaded cells were counted in three randomly selected fields under a microscope.

4.7 Wound healing assay

Cells were seeded in 6-well plates and cultured until reaching over 90% confluency. A straight scratch was created by manually dragging a sterile 100 µL pipette tip perpendicularly across the center of each well. After washing with PBS, the medium was replaced with serum-free medium. The scratch area was photographed at 0 h and 24 h using an inverted microscope. Wound width was measured using ImageJ software to calculate the cell migration rate.

4.8 Colony formation assay

Cells in the logarithmic growth phase were trypsinized to prepare single-cell suspensions and counted. Cells were seeded into 6-well plates at a density of 500 cells per well and cultured at 37°C in a 5% CO₂ incubator, with the medium refreshed as needed. After 7–14 days of culture, the medium was discarded. Cells were washed with PBS, fixed with 4% PFA (Servicebio, G1101-500ML) for 15 min at room temperature, and stained with 0.1% crystal violet (Servicebio, G1014-50ML) for 10 min. Colonies containing ≥ 50 cells were counted manually under a microscope. Experiments were performed with multiple replicate wells and independently repeated three times.

4.9 CCK-8 cell viability assay

Cells were collected, counted, and seeded into 96-well plates at a density of 2,000 cells per well for proliferation assays or 5,000 cells per well for cytotoxicity assays, and incubated at 37°C in a 5% CO₂ incubator for 24–72 h. Then 10 µL of CCK-8 solution (C0038, Beyotime Biotechnology, Shanghai, China) was added to each well and incubated for an additional 1 h. Absorbance at 450 nm was measured using a microplate reader (Molecular Devices, USA), and cell viability was calculated.

4.10 Co-immunoprecipitation (Co-IP)

Cells were seeded in 100 mm culture dishes (Servicebio, CCD-100H) and cultured until reaching over 90% confluency. Cells were washed twice with pre-cooled PBS and lysed on ice for 10 min in IP lysis buffer (EpiZyme, PC105) supplemented with 1 mM PMSF. The lysates were centrifuged at 14,000 rpm for 10 min at 4°C, and the supernatant was transferred to a new centrifuge tube. A 500 µL aliquot of total protein lysate was incubated with 5 µg of specific primary antibody or control IgG (Proteintech, 30000-0-AP) overnight on a rotary mixer at 4°C. Meanwhile, 25 µL of Protein A/G magnetic beads (EpiZyme,

YJ003) were transferred to a new tube and washed twice with IP lysis buffer. The antigen-antibody complexes were then added to the prepared beads and incubated for an additional 4 h at 4°C with rotation. After incubation, the bead-bound complexes were collected using a magnetic rack and washed three times with IP lysis buffer. The washed beads were resuspended in 1× SDS-PAGE sample loading buffer and heated at 100°C for 10 min. The beads were separated magnetically, and the supernatant was subjected to SDS-PAGE followed by western blot analysis.

4.11 Real-time quantitative polymerase chain reaction (RT-qPCR)

Total RNA was extracted from cultured cells using TRIzol Reagent (Invitrogen, 15596018CN, Carlsbad, USA). RNA concentration and purity were assessed with a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, USA). First-strand cDNA was synthesized from 1 µg of qualified RNA using a reverse transcription kit with gDNA remover (Servicebio, G3333-50), employing random hexamer primers and oligo(dT) primers. RT-qPCR was performed on a CFX96 Real-Time PCR System (Bio-Rad, Hercules, USA) using 2× SYBR Green qPCR Master Mix (Servicebio, G3326-01). Each 10 µL reaction contained 5 µL of 2× Master Mix, 0.5 µL of each primer, and 4 µL of diluted cDNA. The cycling conditions were 95°C for 30 s, followed by 40 cycles of 95°C for 15 s and 60°C for 30 s, with a melting curve analysis performed after amplification to verify product specificity. β -actin was used as the internal reference gene, and data were analyzed using the $2^{-\Delta\Delta C_t}$ method. Primer sequences are listed below.

CST1 (Forward) 5'-ACACGACGGCTTCGCTC-3'

(Reverse) 5'-TGCGTTTAAGCACTTCGCAA-3'

GPX4 (Forward) 5'-ACAAGAACGGCTGCGTGGTGAA-3'

(Reverse) 5'-GCCACACACTTGTGGAGCTAGA-3'

β -actin (Forward) 5'-GGCTGTATTCCCCTCCATCG-3'

(Reverse) 5'-CCAGTTGGTAACAATGCCATGT-3'

4.12 Lipid peroxidation assay

Malondialdehyde (MDA) assay was used to evaluate cellular lipid peroxidation levels. Cells were collected and lysed in RIPA buffer. Then 100 µL of cell lysate was mixed thoroughly with 200 µL of MDA detection working solution (Beyotime, S0131S). The mixture was placed in light-protected sealed tubes, heated at 100°C in a water bath for 15 min, cooled naturally, and centrifuged at 1,000 g for 10 min at room temperature. A 200 µL aliquot of the supernatant was transferred to a 96-well plate, and the absorbance at 532 nm was measured using a microplate reader (Molecular Devices, USA). MDA content was calculated based on a standard curve prepared using MDA standards. Additionally, BODIPY 581/591 C11 staining was used to detect lipid peroxidation. Cells were washed twice with PBS, incubated with 2

μM BODIPY 581/591 C11 staining solution (Beyotime, S0043S) for 20 min at 37°C in the dark, washed twice again with PBS, and observed under a fluorescence microscope (Olympus, Japan).

4.13 Reactive oxygen species (ROS) assay

Intracellular reactive oxygen species (ROS) levels were detected using the DCFH-DA fluorescent probe. Cells were washed twice with PBS and incubated with 10 μM DCFH-DA staining solution (Beyotime, S0033S) at 37°C for 20 min in the dark. After incubation, cells were washed three times with PBS, and fluorescence was detected using a flow cytometer (Agilent, NovoCyteD2060R, Hangzhou, China). A minimum of 10,000 events were acquired per sample.

4.14 Transmission electron microscopy (TEM)

Cells were collected and fixed with 2.5% glutaraldehyde (Servicebio, G1102-1.5ML) for 24 h at 4°C. After fixation, the samples were washed three times with 0.1 M phosphate buffer (PB, pH 7.4), each for 15 min, followed by post-fixation with 1% osmium tetroxide for 2 h at room temperature in the dark. The samples were then washed again with PB (three times, 15 min each), dehydrated through a graded ethanol series, and embedded in epoxy resin. Ultrathin sections (60–80 nm) were cut, stained with 2% uranyl acetate and 2.6% lead citrate, and observed under a Hitachi HT7800 transmission electron microscope (Tokyo, Japan).

4.15 Reduced glutathione (GSH) assay

Reduced (GSH) and oxidized (GSSG) glutathione were assessed using the GSH and GSSG assay kit (Beyotime, S0053). Cells were washed with PBS, centrifuged, and resuspended in protein removal reagent M. The suspension was subjected to two freeze-thaw cycles (liquid nitrogen/37°C water bath), incubated on ice for 5 min, and centrifuged at 10,000 g for 10 min at 4°C. For total glutathione measurement, the supernatant was mixed with 150 μL of total glutathione detection buffer, incubated at room temperature for 5 min, followed by addition of 50 μL of 0.5 mg/mL NADPH. Absorbance at 412 nm was measured every 5 min over a continuous 25 min period. For GSSG measurement, the supernatant was first incubated with the GSH scavenger system at 25°C for 1 h to remove GSH from the sample, and the remaining assay procedure was identical to that of total glutathione. Concentrations of total glutathione and GSSG were calculated from a standard curve, and the intracellular GSH content was ultimately derived.

4.16 Ferrous iron (Fe²⁺) assay

Intracellular Fe²⁺ levels were quantitatively detected using a colorimetric ferrous iron assay kit (Beyotime, S1068S). Cells were washed with PBS, centrifuged, and resuspended in BeyoLysis™ H buffer for metabolic assays. The suspension was homogenized at 4°C, lysed on ice for 10 min, and then centrifuged. A 100 μM standard working solution was prepared by diluting 4 μL of 50 mM ferrous iron standard in 1.996 mL of assay buffer. The supernatant or the standard solution was mixed with 5 μL of decontamination solution and adjusted to a final volume of 190 μL with assay buffer. The mixture was gently vortexed and incubated at 37°C for 30 min in the dark. Absorbance at 593 nm was measured

using a microplate reader. The Fe^{2+} concentration in each sample was calculated from a standard curve and normalized to the total protein content.

4.17 Nude mouse xenograft tumor model

The experiment was divided into three groups: the knockdown group (injected with T24 cells stably expressing shCST1), the overexpression group (injected with T24 cells stably overexpressing CST1), and the control group (injected with T24 cells transfected with empty vector). Each group contained three nude mice. Each mouse was subcutaneously inoculated with 5×10^6 cells into the right axilla. Tumor length and width were measured every 5 days, and tumor volume was calculated as $V = 0.5 \times \text{length} \times \text{width}^2$. After 30 days, the mice were euthanized using cervical dislocation, and the tumor tissues were excised, weighed, and subjected to pathological and molecular biological analyses. At the experimental endpoint, all mice were deeply anesthetized with inhaled isoflurane prior to cervical dislocation for humane euthanasia.

4.18 Statistical analysis

Statistical analyses were performed using SPSS and GraphPad Prism. Quantitative data were presented as mean $\pm$ standard deviation (SD). Comparisons between two groups were performed using independent samples t-test, and comparisons among multiple groups were performed using one-way analysis of variance (ANOVA). $P < 0.05$ was considered statistically significant.

Declarations

Ethics approval and consent to participate:

The study was conducted in accordance with the Declaration of Helsinki. The research involving human clinical specimens was approved by the Ethics Committee of Renmin Hospital of Wuhan University (approval number: WDRY2025-K321). The animal study protocol was approved by the Institutional Animal Care and Use Committee of WHUPENN LIFE SCIENCE Co., Ltd. (approval number: LBSM2026037) and was performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. At the endpoint of all in vivo xenograft experiments, mice received isoflurane inhalation anesthesia before cervical dislocation for humane euthanasia. All subjects participating in the study provided written informed consent. All human specimen experiments and animal operations strictly complied with relevant institutional, national ethical guidelines and regulatory standards.

Competing interests:

The authors declare no competing interests.

Fundings:

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Author Contribution

Zhengjing Zhang: Conceptualization, Data curation, Formal analysis, Writing – original draft. Xinmiao Ni: Conceptualization, Data curation, Formal analysis, Writing – original draft. Antong Guo: Conceptualization, Data curation, Formal analysis, Writing – original draft. Lei Wang: Data curation, Formal analysis. Qianxue Lu: Data curation, Formal analysis. Sijin Dong: Data curation, Formal analysis. Xiuheng Liu: Conceptualization, Data curation, Formal analysis, Supervision, Writing – review & editing. Zhiyuan Chen: Conceptualization, Data curation, Formal analysis, Supervision, Writing – review & editing.

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Data Availability

Data availability: The data that support the findings of this study are available from the corresponding authors upon reasonable request.

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Figures

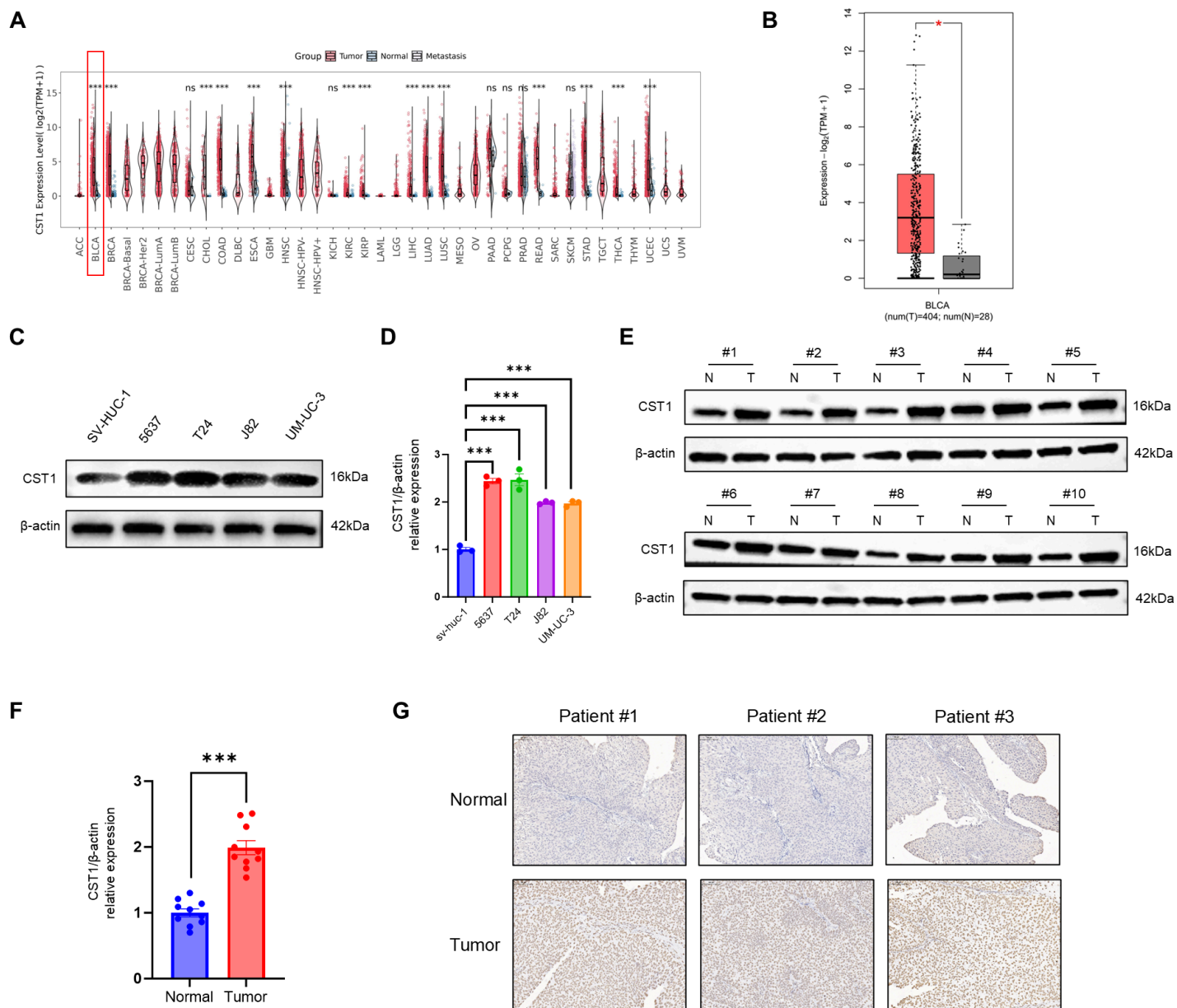


Figure 1

CST1 is highly expressed in BCa A. Differential expression of CST1 in various tumor and adjacent normal tissues using TIMER3.0. B. CST1 expression in BCa versus normal bladder tissues in TCGA cohort. C, D. Western blot analysis and quantification of CST1 in SV-HUC-1 and BCa cell lines (T24, 5637, UM-UC-3, J82). E, F. Western blot analysis and quantification of CST1 in 10 paired BCa and adjacent normal tissues. G. Representative IHC images of CST1 in BCa and adjacent normal tissues. ** $P < 0.01$, *** $P < 0.001$. Full-length uncropped original blots have been uploaded as supplementary materials for review.

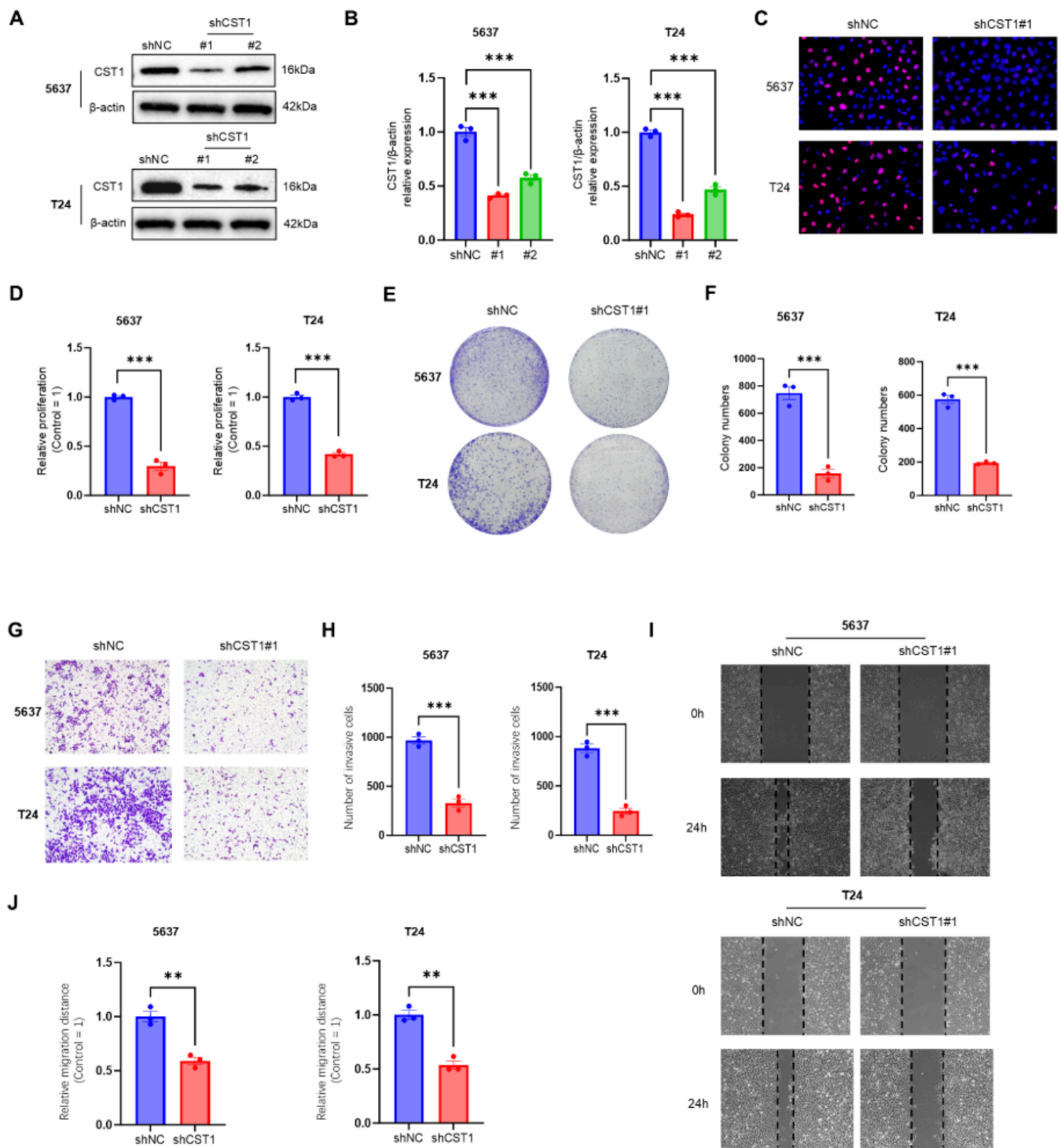


Figure 2

CST1 promotes proliferation, invasion, and migration of BCa cells. A, B. Western blot analysis of CST1 knockdown efficiency in T24 and 5637 cells. C, D. EdU assay and quantification of cell proliferation in CST1 knockdown T24 and 5637 cells. E, F. Colony formation assay and quantification in CST1 knockdown T24 and 5637 cells. G, H. Transwell invasion assay and quantification in CST1 knockdown T24 and 5637 cells. I, J. Wound healing assay and quantification in CST1 knockdown T24 and 5637 cells.

****P<0.01, ***P<0.001.** Full-length uncropped original blots have been uploaded as supplementary materials for review.

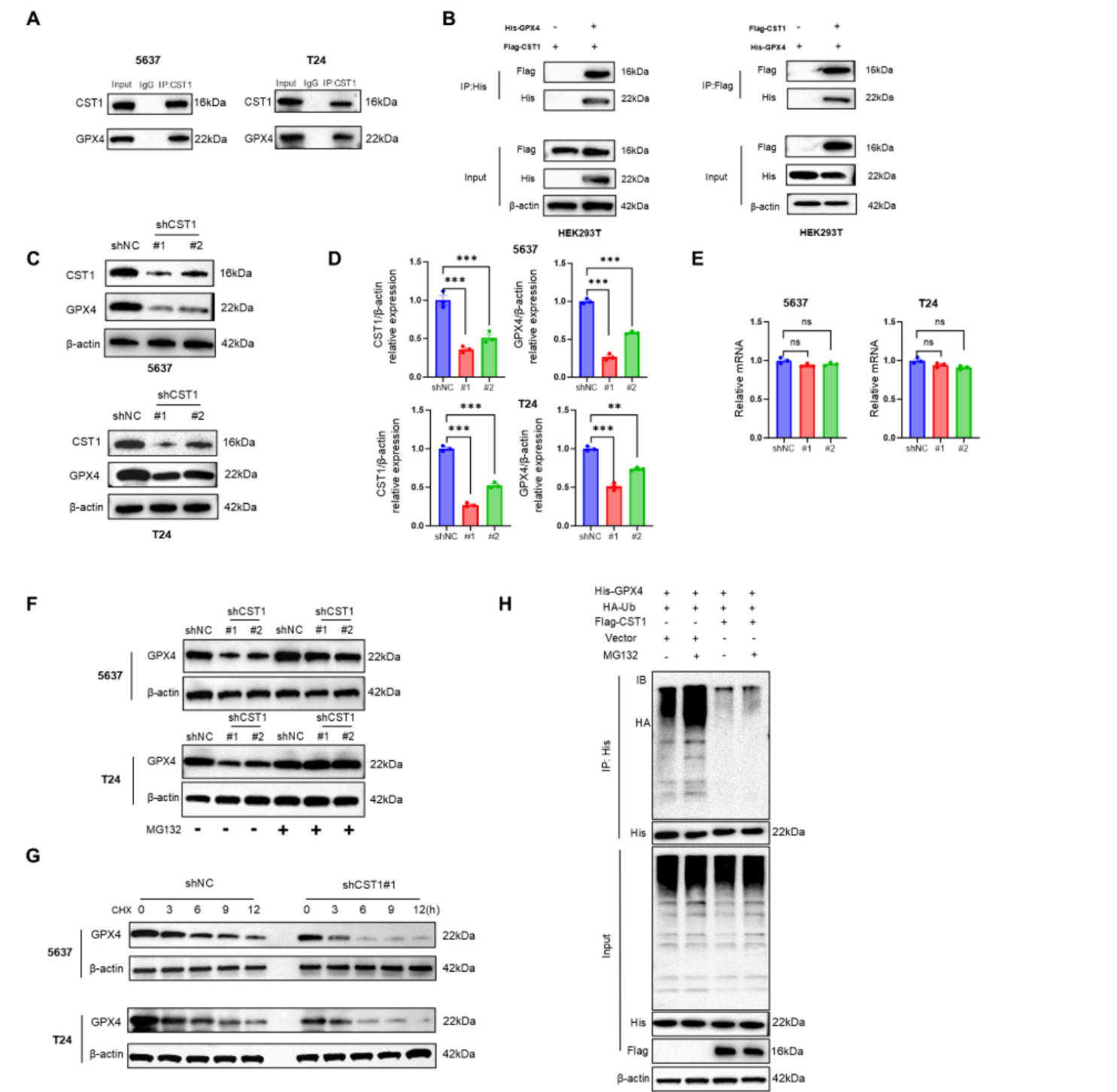


Figure 3

CST1 interacts with GPX4 and enhances its protein stability by inhibiting ubiquitination-mediated degradation. A. Endogenous Co-IP analysis of CST1-GPX4 interaction in T24 and 5637 cells. B.

Exogenous Co-IP analysis of Flag-CST1 and His-GPX4 interaction in HEK293T cells. C, D. Western blot analysis and quantification of GPX4 protein levels following CST1 knockdown. E. RT-qPCR analysis of GPX4 mRNA levels following CST1 knockdown (ns, not significant). F. Western blot analysis and quantification of GPX4 protein levels with or without MG132 treatment in CST1 knockdown cells. G. CHX chase assay of GPX4 protein half-life in control and CST1 knockdown cells. H. Ubiquitination assay of GPX4 in cells transfected with HA-Ub and His-GPX4, with or without Flag-CST1 overexpression and MG132 treatment. **P<0.01, ***P<0.001. Full-length uncropped original blots have been uploaded as supplementary materials for review.

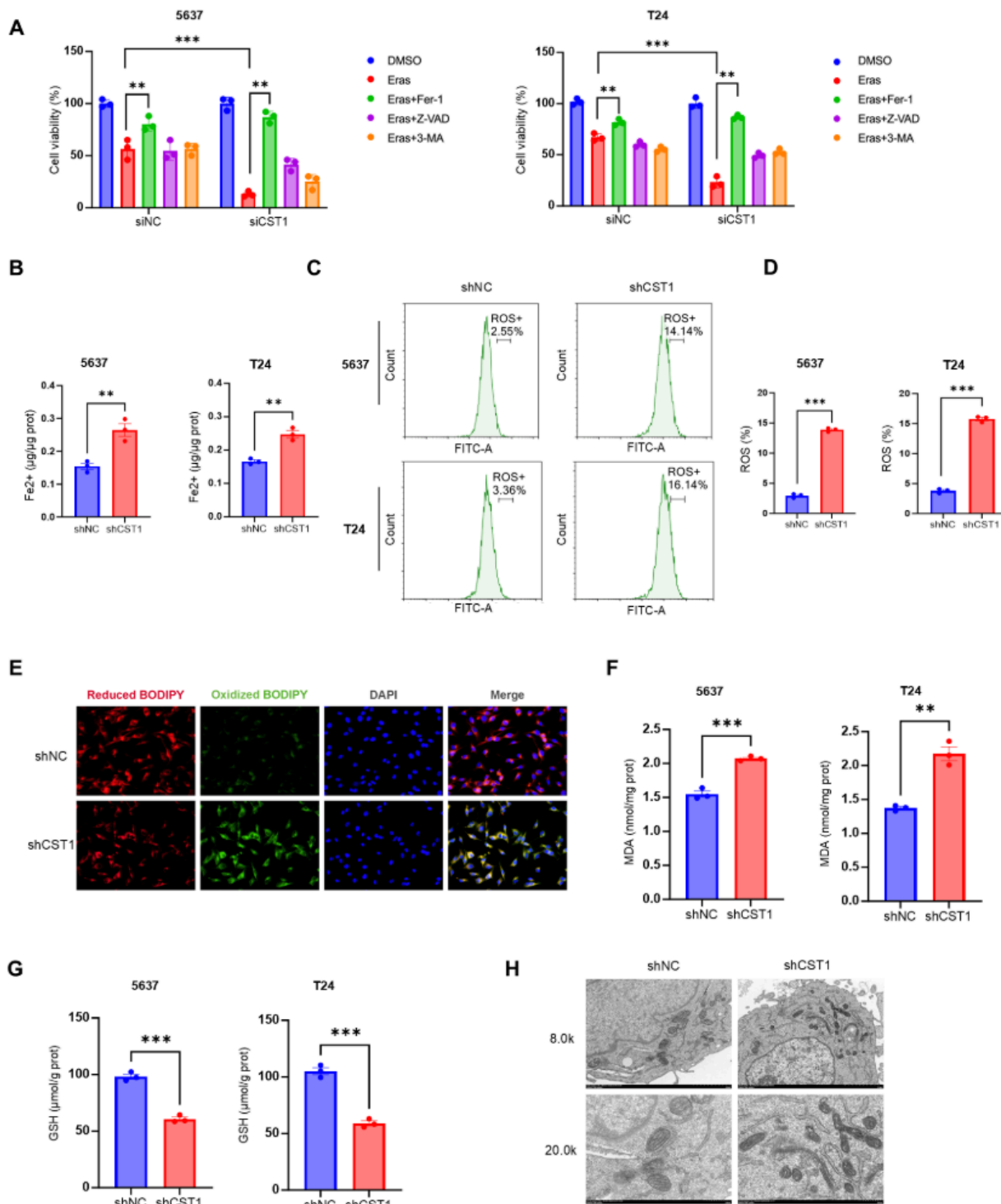


Figure 4

CST1 suppresses ferroptosis in BCa cells. A. CCK-8 assay of cell viability in CST1 knockdown cells treated with Erastin alone or in combination with Ferrostatin-1, Z-VAD-FMK, or 3-MA. B. Measurement of intracellular Fe^{2+} levels in CST1 knockdown 5637 and T24 cells. C, D. Flow cytometry analysis and quantification of intracellular ROS levels in CST1 knockdown BCa cells. E. Representative images of intracellular lipid peroxidation detected by BODIPY 581/591 C11 staining. F. MDA content in CST1

knockdown BCa cells. G. GSH content in CST1 knockdown BCa cells. H. Representative TEM images of mitochondrial morphology in control and CST1 knockdown T24 cells. **P<0.01, ***P<0.001.

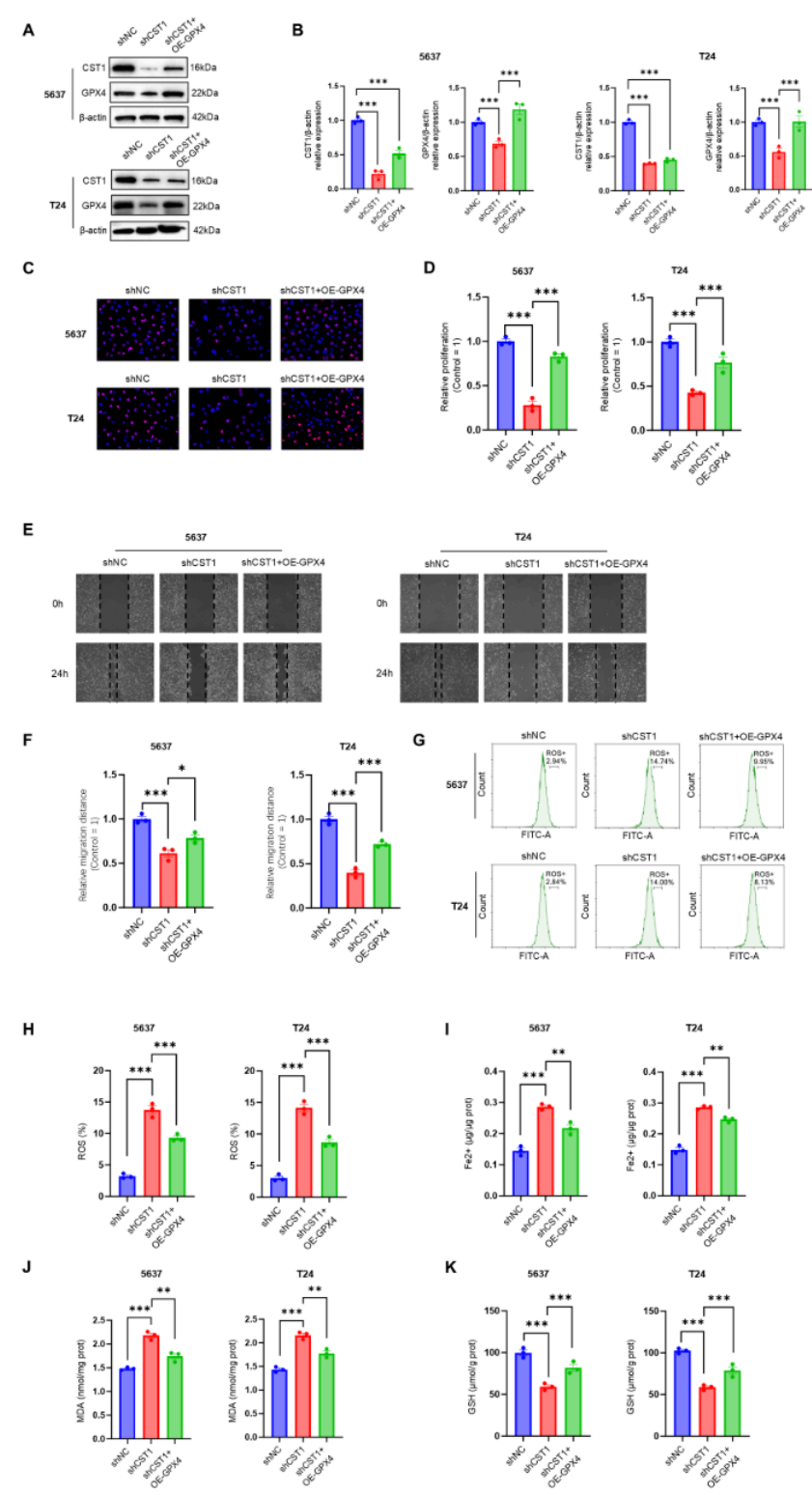


Figure 5

Rescue experiments confirm that GPX4 is a key downstream effector of CST1 in promoting malignant phenotypes of BCa. A, B. Western blot analysis confirming CST1 knockdown and GPX4 overexpression

efficiency. C, D. EdU assay and quantification showing rescue of cell proliferation by GPX4 overexpression in CST1 knockdown T24 cells. E, F. Wound healing assay and quantification showing rescue of cell migration by GPX4 overexpression in CST1 knockdown T24 cells. G, H. Flow cytometry analysis and quantification of ROS levels showing rescue by GPX4 overexpression in CST1 knockdown T24 cells. I. Measurement of intracellular Fe^{2+} levels. J. MDA content. K. GSH content. $**P<0.01$, $***P<0.001$. Full-length uncropped original blots have been uploaded as supplementary materials for review.

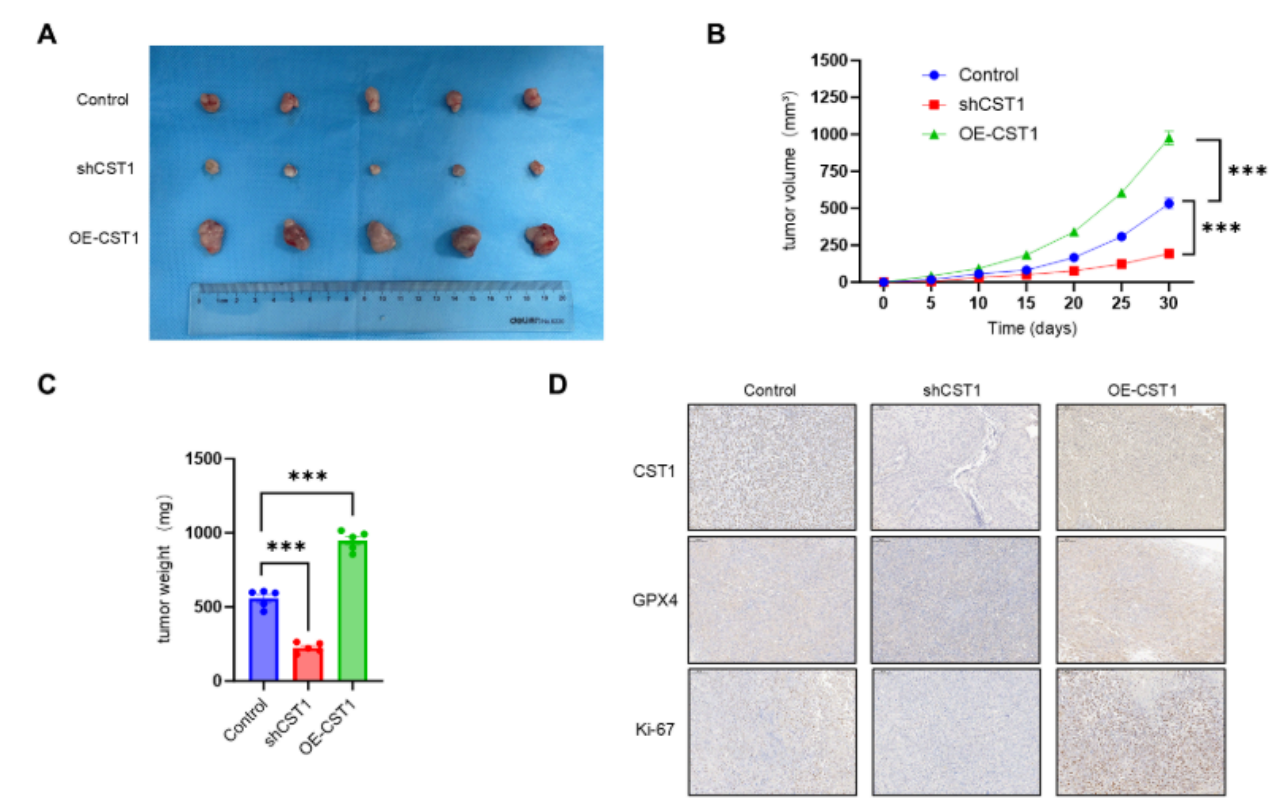


Figure 6

CST1 accelerates bladder cancer growth in vivo. A. Representative images of xenograft tumors from control, CST1 knockdown, and CST1 overexpression groups. B. Tumor weight in each group. C. Tumor volume growth curves in each group. D. Representative IHC images of Ki-67 in xenograft tumor tissues from each group. $**P<0.01$, $***P<0.001$.

A

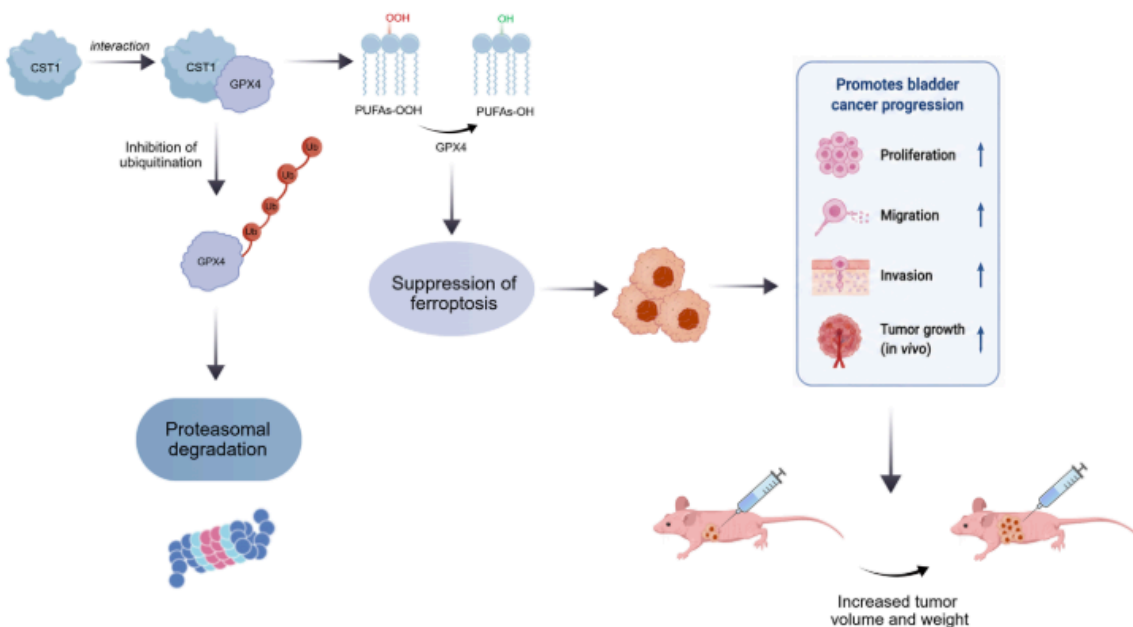


Figure 7

Schematic diagram of the proposed mechanism. CST1 interacts with GPX4, inhibits its ubiquitination-proteasomal degradation, and enhances GPX4 protein stability, thereby suppressing ferroptosis and promoting BCa cell proliferation, migration, invasion, and in vivo tumor growth.

Supplementary Files

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