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ARTICLE

Six-transmembrane epithelial antigen of the prostate 1 protects against increased oxidative stress via a nuclear erythroid 2-related factor pathway in colorectal cancer

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Abstract

The over-expression of six-transmembrane epithelial antigen of the prostate 1 (STEAP1) underlies the pathogenesis of a large subset of human cancers. Expressed on the cancer cell surface, STEAP1 is an attractive target for antibody-based therapy or immunotherapy. However, its role in modulating the pathophysiology of colorectal cancer (CRC) remains relatively unexplored. In this study, we first demonstrated that the *STEAP1* transcript level was significantly higher in CRC tissues than in normal colonic tissues. Of note, *STEAP1* expression negatively correlated with overall survival as determined from a publicly accessible gene expression profile data set. A loss-of-function approach in cultured CRC cell lines revealed that *STEAP1* silencing suppressed cell growth and increased reactive oxygen species (ROS) production, followed by apoptosis, through an intrinsic pathway. Mechanistically, the inhibition of STEAP1 was associated with decreased expression of antioxidant molecules regulated by the transcription factor, nuclear erythroid 2-related factor (NRF2), in CRC cells. Taken together, we identified high *STEAP1* transcript levels leading to reduced ROS production that prevented apoptosis via the NRF2 pathway in CRC cells as a pathological mechanism in CRC. This study highlights the STEAP1–NRF2 axis as a therapeutic target for CRC and its manipulation as a novel strategy to conquer CRC.

Introduction

Colorectal cancer (CRC) makes up the fourth largest contribution to cancer-related mortality worldwide, accounting for around 1.3 million new patients and 700,000 deaths each year [1]. Although several combination chemotherapy regimens with cytotoxic drugs and molecular-targeted agents have led to improved overall survival of patients with CRC, a low 5-year survival rate for metastatic CRC highlights the need to develop novel treatment strategies [2, 3]. Reactive oxygen species (ROS), a group of quite reactive ions and molecules, are byproducts of a wide variety of cellular metabolic reactions [4]. ROS act as oncogenic signal transducers at low to moderate levels to activate cell proliferation, migration/invasion, and angiogenesis [5]. Conversely, a highly elevated ROS level can induce several types of cell death, such as apoptosis, autophagy, and necrosis [6]. We previously reported that manipulating ROS production may be used as a therapeutic intervention for multiple myeloma [7, 8]. As such, disrupting the equilibrium of cellular oxidative stress is an attractive tool for cancer treatment in CRC [9]. Therefore, understanding how CRC cells regulate conditions for ROS is critical to developing novel strategies to combat CRC. Six-transmembrane epithelial antigen of the prostate 1 (STEAP1), identified in prostate cancer (PC) cells as a cell surface protein that is suggested to work as an intracellular transporter, is over-expressed in a subset of human cancers, including CRC [10–12]. Regarding PC, STEAP1 may be useful as a negative prognostic marker, and shows oncogenic properties such as the ability to stimulate cell growth [13–15]. In

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cases of Ewing sarcoma (EWS), STEAP1 augmented cell proliferation and invasion and was accompanied by increased ROS levels, although how STEAP1 induced ROS production has not been elucidated [16]. The transcription factor, nuclear erythroid 2-related factor (NRF2), is a pivotal regulator of antioxidant responses in both normal and cancerous cells [17]. Growing evidence suggests that NRF2 has two contradictory effects in cancer [18]. Basically, NRF2 is regarded as a tumor suppressor since it can protect cells from multiple insults, including oxidative stress, and has cancer chemopreventive capabilities [19]. The other function is as an oncogene because NRF2 is activated and over-expressed in cancer and promotes cancer cell survival [20]. The expression and activity of NRF2 is tightly regulated by numerous molecules, including kelch-like ECH-associated protein 1 (KEAP1), at transcriptional and post-transcriptional levels [21]. However, a relationship between NRF2 and STEAP1 has not been described, and the biological functions of STEAP1 in cancer remain undefined. Here, we characterized the biological impact of STEAP1 in CRC pathogenesis. We showed that *STEAP1* transcript levels were over-expressed in CRC compared to normal cells using three public independent data sets. Over-expression of *STEAP1* was associated with poor prognosis in patients with CRC. The knock-down of *STEAP1* induced apoptosis by targeting *NRF2* suppression, followed by the inhibition *NRF2*-targeted genes that activated antioxidant reactions, leading to the overproduction of intracellular ROS. Our data provides a rationale for a new treatment strategy inhibiting the STEAP1–NRF2 pathway in CRC.

Materials and methods

Databases and gene expression data analysis

Gene expression levels of *STEAP1* in normal and cancerous tissues were evaluated using the publicly accessible data set (GSE25070, GSE21510, and GSE37364) from the Gene Expression Omnibus. The correlation between *STEAP1* messenger RNA (mRNA) levels and clinical outcome was investigated using the publicly accessible data set (GSE17538) from the Gene Expression Omnibus. We used receiver operating characteristic (ROC) curve to determine the cutoff value. In total, 232 patients were categorized as having high or low levels of *STEAP1*, and Kaplan–Meier analysis of overall survival comparing the two groups was performed. Pearson's correlation coefficient between STEAP1 and NRF2 in samples of patients with CRC was analyzed using publicly accessible gene expression profiling data, GSE25070 and GSE21510.

Cell lines and culture conditions

All CRC cell lines were purchased from the American Type Culture Collection (Manassas, VA, USA). DLD-1, HCT-116, and SW480 cell lines were cultured in RPMI 1640 (Sigma–Aldrich, St. Louis, MO, USA) containing 10% fetal bovine serum (FBS), 2 μM L-glutamine and 1% penicillin–streptomycin. CCD 841 CoTr, Lovo, and LS180 cell lines were cultured in Dulbecco's Modified Eagle Medium (Sigma–Aldrich) containing 10% fetal bovine serum (FBS), 2 μM L-glutamine and 1% penicillin–streptomycin. Cell lines were authenticated by short tandem repeat DNA profiling in 2018.

Inhibition of *STEAP1* expression by small-interfering RNA

Non-targeting control small-interfering (siRNA; Control; #4390843, Thermo Fisher Scientific, Waltham, MA, USA) and two independent siRNAs targeting human *STEAP1* (siSTEAP1; MQ-003713-01 and MQ-003713-02, Dharmacon, Lafayette, CO, USA) were transfected into cell lines using Lipofectamine RNAiMAX (Thermo Fisher Scientific) according to the manufacturer's protocol.

Quantitative reverse transcription-PCR

RNA was extracted with TRIzol Reagent (Thermo Fisher Scientific) according to the manufacturer's protocol. Total RNA (1 μg) was reverse-transcribed using a SuperScript VILO cDNA synthesis kit (Thermo Fisher Scientific), and quantitative reverse transcription-PCR (qRT-PCR) was performed with an Applied Biosystems 7300 Real-time PCR system (Applied Biosystems, Foster City, CA, USA). The analysis of target genes was conducted in quadruplicate using a POWER SYBR Green Master Mix (Thermo Fisher Scientific) as previously described [22]. Transcripts levels were normalized to β-actin expression. PCR primer sets for targeted genes are listed in Supplementary Table.

Western blot

Cells were solubilized in radioimmunoprecipitation assay (RIPA) lysis buffer (50 mM Tris-HCI, pH 7.5, 1% NP-40, 0.5% Na-deoxycholate, 1mM EDTA, 150mM NaCl, 1mM EGTA, and protease inhibitor cocktail), and then centrifuged at $12{,}000 \times g$ for 10 min. The supernatants were collected, and protein concentrations were determined using a bicinchoninic acid (BCA) Protein Assay Kit (Thermo Fisher Scientific). Equal amounts of protein were run on 4–20% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) gels and transferred to polyvinylidene fluoride (PVDF) membranes (Millipore Corp.,

Billerica, MA, USA). The blots were probed using the following primary antibodies: anti-STEAP1 (sc25514; Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-NRF2 (ab62352; abcam, Cambridge, MA, USA), and anti-actin-horse radish peroxidase (HRP; sc1615; Santa Cruz Biotechnology).

Evaluation of cell growth

Cells were seeded at densities of 2×10^3 (DLD-1), 1×10^4 (SW480), and 5×10^3 (LS180) cells/well into 96-well plates. Non-targeting siRNA and two independent siRNAs targeting human *STEAP1* were transfected 24 h later. Cell viability was assessed at 0, 24, 48, and 72 h using a WST-1 assay (Premix WST-1 Cell Proliferation Assay; Takara Bio, Otsu, Japan) and an Infinite M1000 PRO microplate reader (Tecan Japan, Kawasaki, Japan). A growth curve was constructed by plotting absorbance against time.

Flow cytometry analysis

Cells were seeded at densities of 1.5×10^5 (DLD-1), 6×10^5 (SW480), and 3×10^5 (LS180) cells/well into 6-well plates and cultured for 24 h. Then, non-targeting siRNA and a siRNA targeting human *STEAP1* were transfected into cells, and these then incubated for 72 h. *STEAP1*-silenced cells were pretreated with or without 10mM N-acetyl-cysteine (NAC) for 12 h.

Apoptosis was quantified using an Annexin V/7-AAD staining kit (BD Biosciences, San Jose, CA, USA) according to the manufacturer's protocol, followed by analysis on a BD FACS Canto II instrument (BD Biosciences, Tokyo, Japan) with FACSDiva software (BD Biosciences) as previously described [23].

Levels of caspases 8 and 9 were indirectly evaluated using a Caspase8 Assay Kit, Green Fluorescence, CaspaLux8-L1D2 (OncoImmunin Inc, Gaithersburg, MD, USA) and a Caspase9 Assay Kit, Green Fluorescence, CaspaLux9-M1D2 (OncoImmunin) in accordance with the manufacturer's protocol, followed by flow cytometry.

Measurements of cytosolic ROS were performed with CellROX Deep Red (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. Briefly, cells were stained for 30 min at 37 °C. After staining, cells were washed and suspended in phosphate-buffered saline. For the quantification of ROS, cells were analyzed with a BD FACS Canto II instrument (BD Biosciences).

PCR array

For PCR array, total RNA was reverse-transcribed using a RT² First Strand Kit (QIAGEN, Germantown, MD, USA). PCR array was performed using a Signal Transduction Pathway Finder RT² Profiler PCR array (PAHS-014Z; QIAGEN) according to the manufacturer's protocol.

Immunofluorescence

Cells were seeded at densities of 1.5×10^5 (DLD-1) and 6×10^5 (SW480) cells/well into 6-well plates with coverslips on the bottom. Non-targeting siRNA and a siRNA targeting human *STEAP1* were transfected 24 h later. The culture medium was removed 72 h after transfection and cells were fixed with 4% formaldehyde. Immunofluorescence analysis was performed using an anti-NRF2 primary antibody as above, followed by anti-rabbit IgG(H+L), F(ab')2 Fragment (Alexa Fluor® 488 Conjugate; Cell Signaling Technology, Danvers, MA, USA). Slides were mounted in ProLong Gold Antifade Reagent with DAPI (Cell Signaling Technology). The subcellular localization of NRF2 was observed using a Biozero BZ-8000 fluorescence microscope (KEYENCE Laboratories, Osaka, Japan).

Statistical analysis

The significance of differences was determined by Student *t*-test or Mann–Whitney *U*-test. Statistical significance was defined as $P < 0.05$. All statistical analyses were performed using EZR software [24].

Results

STEAP1 is over-expressed and associated with poor prognosis in CRC

To explore the hypothesis that STEAP1 may be related to CRC pathogenesis, we first investigated *STEAP1* expression in patients with CRC using publicly available gene expression profiling data. *STEAP1* transcript levels were significantly higher in CRC tissues than in normal colonic tissues (Fig. 1a, b). Moreover, *STEAP1* expression in colorectal adenoma was higher than in normal colonic tissues, and lower than in CRC tissues (Fig. 1c). Although statistical differences between these groups were non-existent, this data indicates that *STEAP1* expression seems to be increasingly elevated in the process of an adenoma–carcinoma sequence [25]. We also evaluated STEAP1 expression in CRC cell lines. *STEAP1* mRNA expression was significantly higher in several common CRC cell lines compared to the normal colonic epithelial cell line, CCD 841 CoTr, as determined by qRT-PCR studies (Fig. 1d). Similarly, western blotting showed that STEAP1 protein levels in CRC cell lines were significantly higher than in CCD 841 CoTr cells (Fig. 1e). Thus, our data implied that STEAP1 may have oncogenic functions, as also reported in

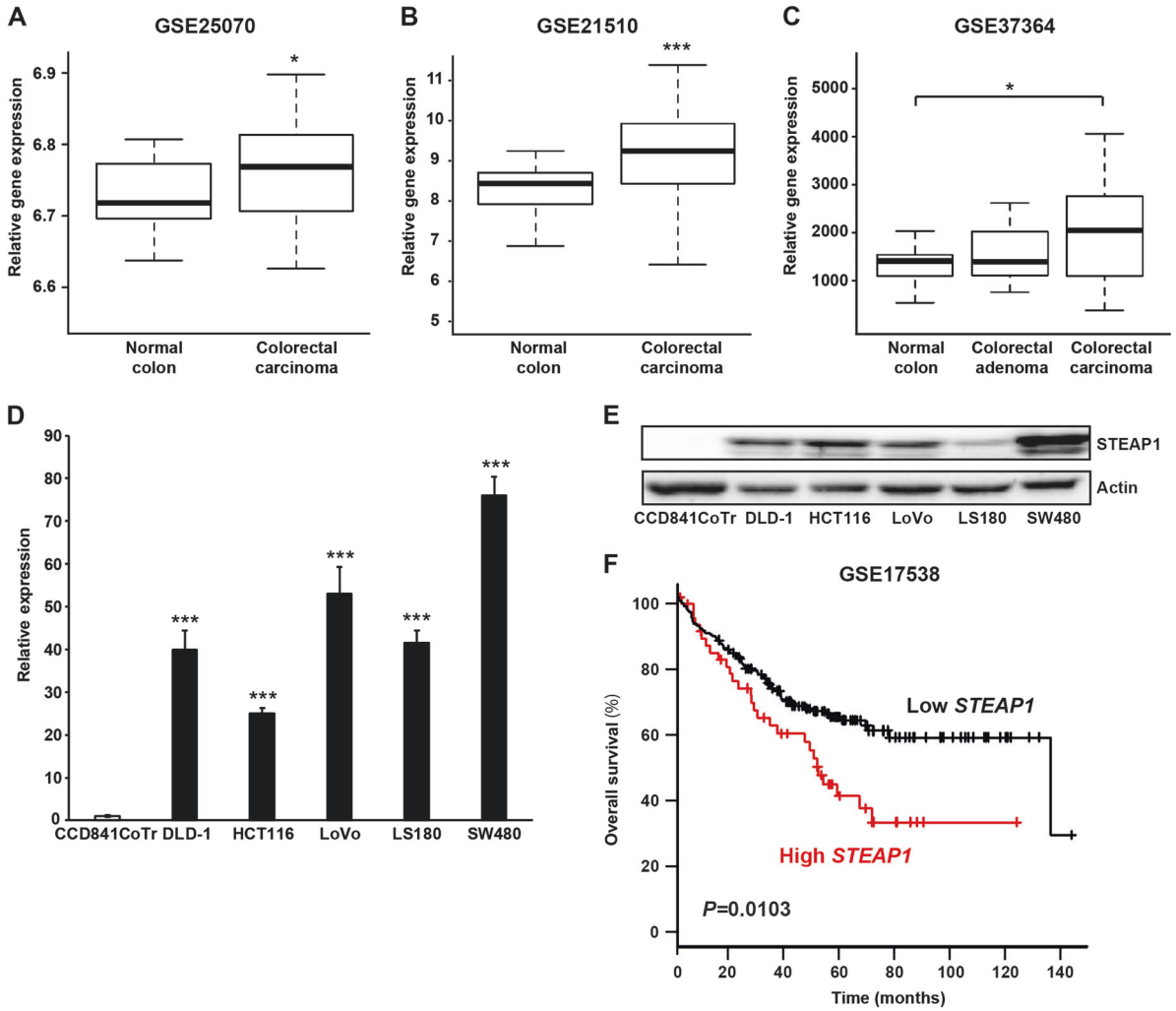


Fig. 1 STEAP1 expression is upregulated and associated with poor overall survival in CRC. **a**, **b**, **c** Publicly accessible gene expression profiling data GSE25070 (**a**), GSE21510 (**b**), and GSE37364 (**c**) were analyzed to evaluate six-transmembrane epithelial antigen of the prostate 1 (*STEAP1*) expression in patients with CRC. $*P < 0.05$, $***P < 0.001$. **d** Expression of *STEAP1* mRNA in colorectal carcinoma (CRC) cell lines was evaluated by quantitative reverse transcription (qRT)-PCR. Data are the mean of quadruplicate measurements. Error bars represent the standard deviation. $***P < 0.001$. **e** Whole-cell lysates from colorectal cancer cell lines were subjected to immunoblotting using anti-STEAP1 and actin-horse radish peroxidase (HRP) antibodies. **f** Correlation between *STEAP1* expression and overall survival in samples of patients with CRC was analyzed using publicly accessible gene expression profiling data, GSE17538

PC cells [15]. To investigate the impact of *STEAP1* expression on survival in patients with CRC, we analyzed publicly gene expression profiling data. *STEAP1* transcript levels were correlated with overall survival, with a statistically significant negative correlation between *STEAP1* levels and overall survival (Fig. 1f).

STEAP1 silencing inhibits proliferation of CRC cell lines

To examine the impact of STEAP1 on CRC, we conducted siRNA knock-down of *STEAP1* in three CRC cell lines, DLD-1 (mutant p53), SW480 (mutant p53), and LS180 (wild-type p53). Knock-down efficiency was examined by qRT-PCR and western blot. *STEAP1* expression in all cell lines was significantly downregulated by two independent siRNAs (Fig. 2a, b, d, e, g, h). Next, we tested the influence of *STEAP1* silencing on the proliferation of CRC cell lines using WST-1 assays. *STEAP1* silencing significantly reduced the proliferation of CRC cell lines for both siRNA transfectants (Fig. 2c, f, i). Thus, in accordance with loss-of-function analysis, STEAP1 activated proliferation in CRC cells regardless of their p53 status.

Knock-down of *STEAP1* evokes intrinsic apoptosis in CRC cell lines

To clarify the mechanism of cytotoxicity induced by *STEAP1* silencing in CRC cell lines, we performed flow cytometry analysis. Annexin V/7-AAD staining showed an

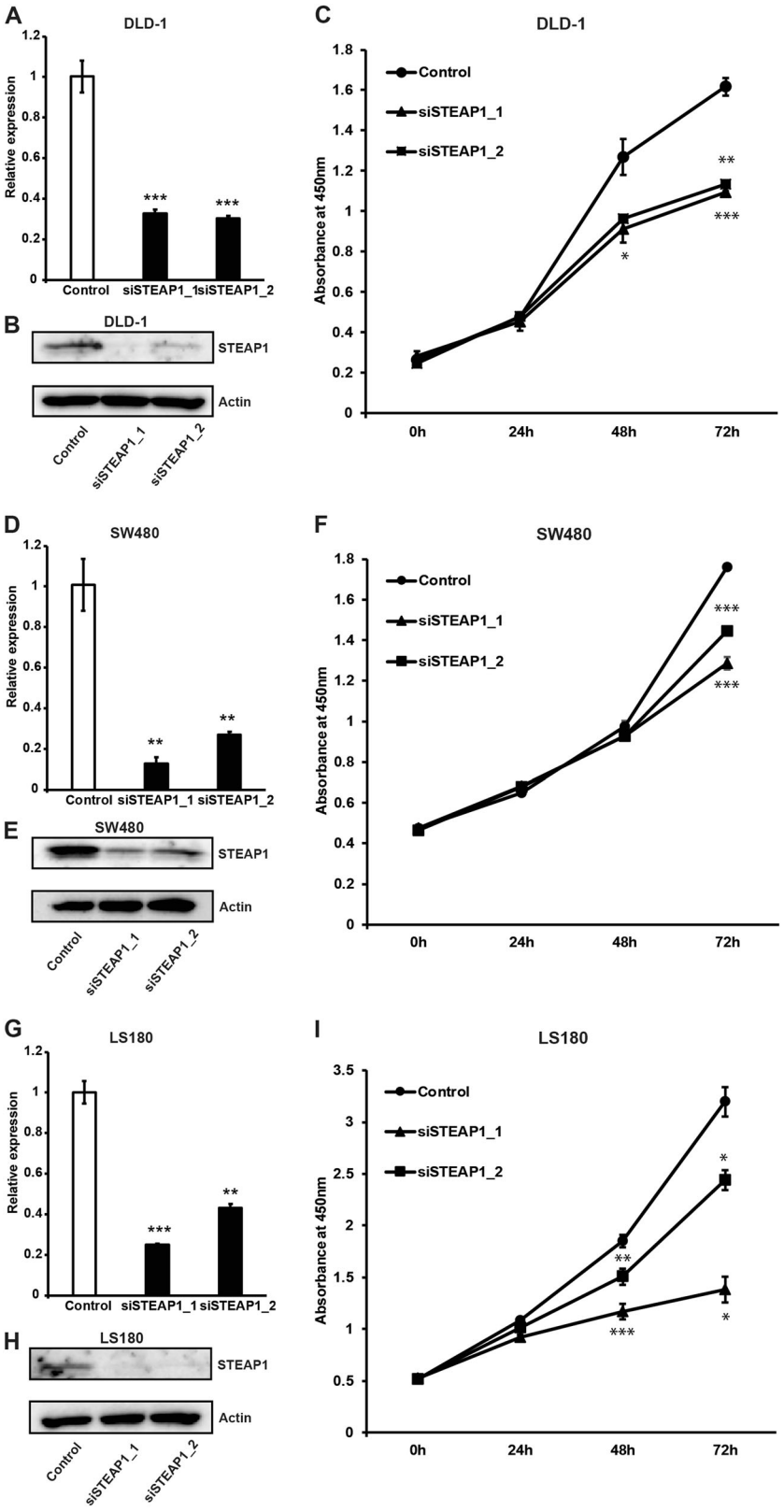
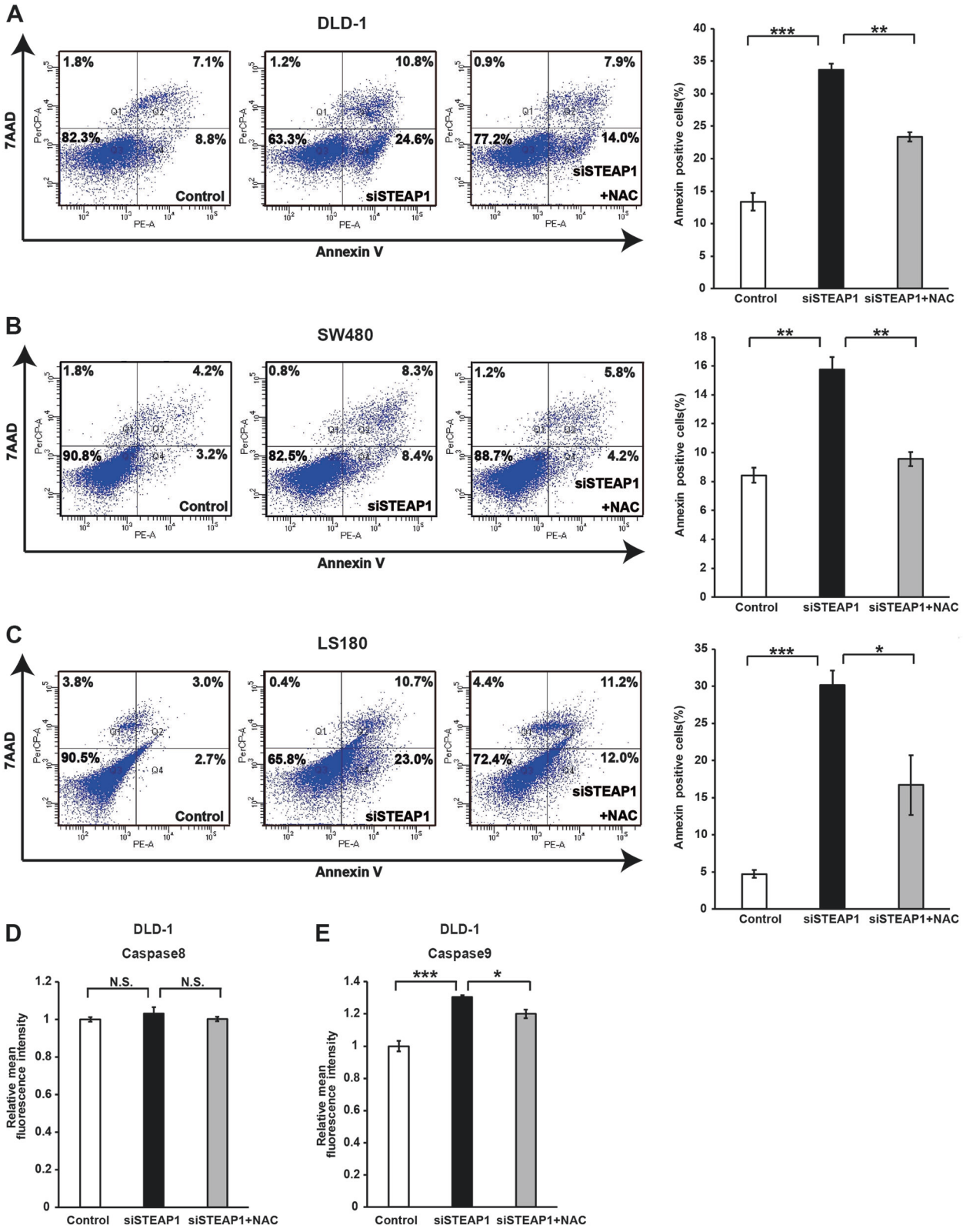


Fig. 2 *STEAP1* silencing leads to cell growth inhibition in CRC cell lines. **a**, **b**, **d**, **e**, **g, h** Three different colorectal carcinoma (CRC) cell lines (DLD-1; **a**, **b**, SW480; **d**, **e**, LS180; **g**, **h**) were transfected with non-targeting siRNA (Control) or two independent siRNAs targeting six-transmembrane epithelial antigen of the prostate 1 (*STEAP1*; siSTEAP1_1, siSTEAP1_2). Knock-down efficiency was evaluated by quantitative reverse transcription (qRT)-PCR (**a**, **d**, **g**) and western blot (**b**, **e**, **h**). Data of qRT-PCR experiments are the mean of quadruplicate measurements. Error bars represent the standard deviation (SD). $^{**}P < 0.01$, $^{***}P < 0.001$. **c**, **f**, **i** Cell growth of three different CRC cell lines (DLD-1; **c**, SW480; **f**, LS180; **i**) was evaluated using a WST-1 assay at 0, 24, 48, and 96h after siRNA transfection. Data are the mean of triplicate measurements. Error bars represent the SD. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$

increased percentage of apoptosis in *STEAP1*-silenced CRC cell lines derived from DLD-1 (Fig. 3a), SW480 (Fig. 3b), and LS180 (Fig. 3c). A previous report suggested that STEAP1 accelerated tumor invasiveness via the over-production of ROS in EWS [16]. To assess the contribution of ROS in apoptosis, we investigated whether treatment



with the ROS scavenger, N-acetyl-cysteine (NAC), might affect apoptosis. Interestingly, NAC supplementation markedly decreased the percentage of apoptotic cells in *STEAP1*-silenced CRC cell lines. These data strongly indicated that the apoptosis that followed the knock-down of *STEAP1* was caused by ROS elevation. We next

◀ **Fig. 3** Knock-down of *STEAP1* induces intrinsic apoptosis in CRC cell lines. **a**, **b, c** DLD-1 (**a**), SW480 (**b**), and LS180 (**c**) cells were transfected with non-targeting siRNA (Control) or six-transmembrane epithelial antigen of the prostate 1 (*STEAP1*) targeting siRNA (siSTEAP1) in the absence or presence of N-acetyl-cysteine (NAC) pretreatment. Apoptosis was measured by flow cytometry using Annexin V/7-AAD staining. Data are the mean of triplicate measurements. Error bars represent the standard deviation (SD). $*P < 0.05$, $**P < 0.01$, $***P < 0.001$. **d**, **e** DLD-1 cells were transfected with non-targeting siRNA (Control) or *STEAP1*-targeting siRNA (siSTEAP1) in the absence or presence of NAC pretreatment. Caspase 8 (**d**) and Caspase 9 (**e**) activities were evaluated by flow cytometry. Data are the mean of triplicate measurements. Error bars represent the SD. N.S.; not significant. $*P < 0.05$. $***P < 0.001$

examined whether this apoptosis was induced via an intrinsic or extrinsic pathway using flow cytometry. The activity of Caspase 8 in *STEAP1*-silenced DLD-1 cells was equivalent to that observed in control cells (Fig. 3d). By contrast, the activity of Caspase 9 was significantly upregulated in *STEAP1*-silenced DLD-1 cells (Fig. 3e). Furthermore, NAC treatment significantly suppressed Caspase 9 activities induced by *STEAP1* knock-down. Collectively, apoptosis induced by *STEAP1* silencing was stimulated in an ROS-dependent manner and was mediated via an intrinsic pathway.

Intracellular ROS level is related to STEAP1 expression in CRC cell lines

As shown in Fig. 3a–c, the apoptosis evoked by *STEAP1* silencing seems to be associated with ROS. We, therefore, measured intracellular ROS levels using a flow cytometer to evaluate whether *STEAP1* inhibition stimulated ROS generation in CRC cell lines. Increased generation of intracellular ROS was observed in *STEAP1*-silenced DLD-1 (Fig. 4a), SW480 (Fig. 4b), and LS180 (Fig. 4c) cell lines. As expected, NAC treatment canceled any increment of ROS induced by the silencing of *STEAP1*.

STEAP1 regulates the NRF2-related oxidative stress response in CRC

To understand the mechanisms involved in increased intracellular ROS induced by *STEAP1* silencing, we analyzed components of several signal transduction pathways by PCR array. We found that heme oxygenase 1 (*HMOX1*), NAD(P)H quinone dehydrogenase 1 (*NQO1*), and thioredoxin reductase 1 (*TXNRD1*) transcript expression was significantly downregulated by *STEAP1* silencing (Fig. 5a). These genes reportedly give rise to NRF2-mediated antioxidant molecules [26]. The mRNA expression levels of these molecules after *STEAP1* silencing were measured by qRT–PCR. Downregulation of these molecules was observed when DLD-1 and SW480 cells were transfected with two independent siRNAs targeting *STEAP1* compared to non-targeting siRNA (Fig. 5b, c). Based on these data, we hypothesized the existence of a relationship between *STEAP1* and *NRF2* expression. Therefore, we evaluated their expression using publicly accessible gene expression profiling data, GSE25070 and GSE21510. As expected, Pearson's correlation coefficient analysis revealed a significant positive relationship between *STEAP1* and *NRF2* expression in both data sets (Fig. 5d, e). Subsequently, the expression of *NRF2* after *STEAP1* silencing in DLD-1 and SW480 cells was evaluated by qRT-PCR and western blot. Downregulation of NRF2 was observed in cells when transfected with two independent siRNAs targeting *STEAP1*

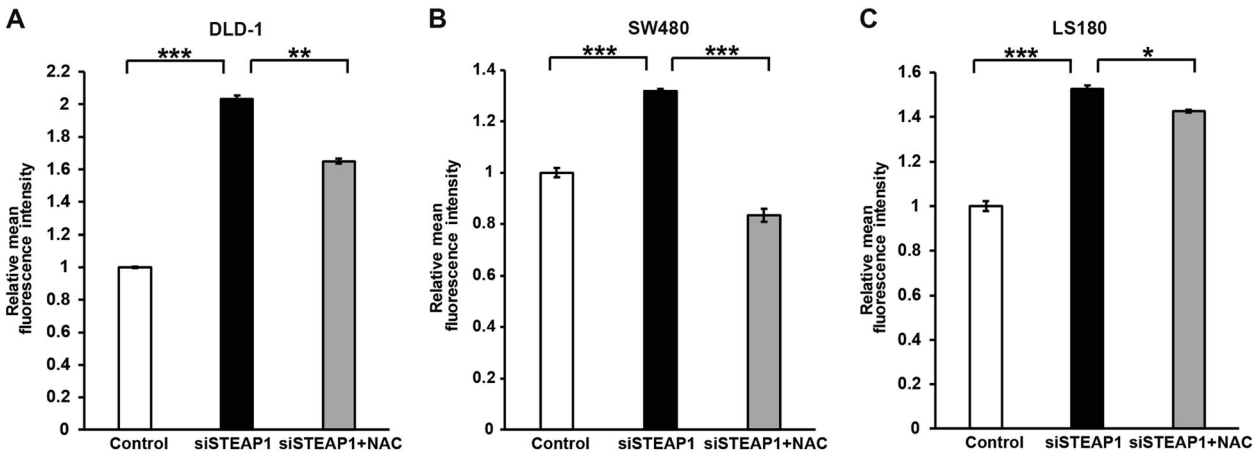
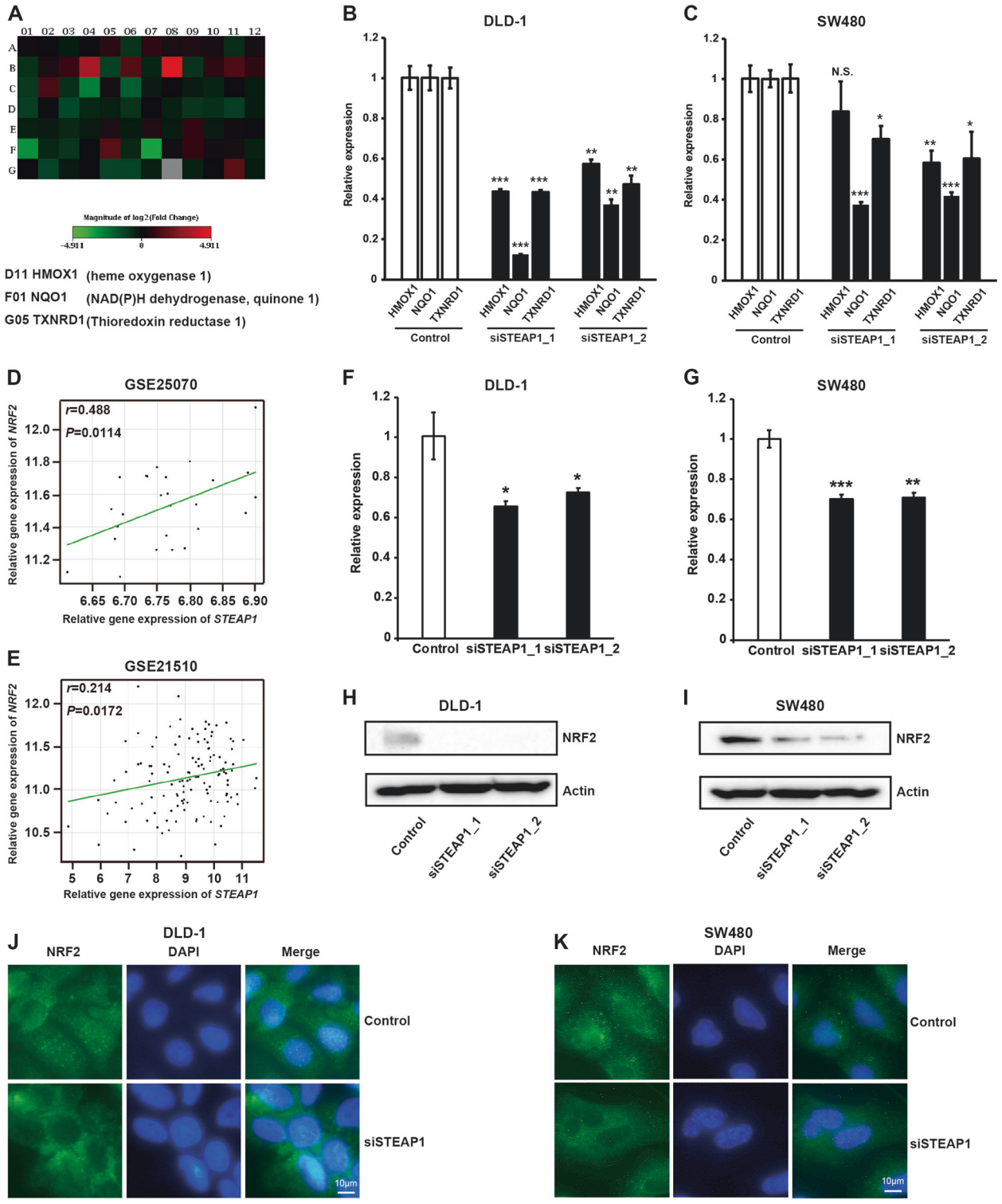


Fig. 4 ROS production is increased by STEAP1 inhibition. **a**, **b**, **c** DLD-1 (**a**), SW480 (**b**), and LS180 (**c**) cells were transfected with non-targeting siRNA (Control) or six-transmembrane epithelial antigen of the prostate 1 (*STEAP1*) targeting siRNA (siSTEAP1) in the absence or presence of N-acetyl-cysteine (NAC) pretreatment. Cytosolic reactive oxygen species (ROS) levels were measured by flow cytometry using CellROX Deep Red. Data are the mean of triplicate measurements. Error bars represent the standard deviation. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$



compared to non-targeting siRNA (Fig. 5f–i). Furthermore, we assessed the cellular localization of NRF2 by immunocytochemistry when DLD-1 and SW480 cells were transfected with *STEAP1*-targeting or non-targeting siRNA because NRF2 works as a transcription factor to regulate downstream-targeted genes [20]. The nuclear translocation of NRF2 was inhibited by *STEAP1* silencing (Fig. 5j, k). Taken together, our data suggest that CRC cells avoid ROS-dependent apoptosis using a STEAP1–NRF2 pathway (Supplementary Fig.).

◀ **Fig. 5** Inhibition of STEAP1 suppresses downstream-targeted genes of NRF2 in CRC cell lines. **a** Heat map representation of the 89 genes listed in rows (**a**–**g**) and in columns [1–12] for a DLD-1 signature validated by PCR array. Heme oxygenase 1 (*HMOX1*), NAD(P)H quinone dehydrogenase 1 (*NQO1*), and thioredoxin reductase 1 (*TXNRD1*) are downregulated genes related to a kelch-like ECH-associated protein 1 (KEAP1)–nuclear erythroid 2-related factor (NRF2) pathway. **b**, **c** DLD-1 (**b**) and SW480 (**c**) cells were transfected with non-targeting siRNA (Control) or two independent siRNAs targeting six-transmembrane epithelial antigen of the prostate 1 (*STEAP1;* siSTEAP1_1, siSTEAP1_2). Expression of *HMOX1*, *NQO1*, and *TXNRD1* was evaluated by quantitative reverse transcription (qRT)-PCR. Data are the mean of quadruplicate measurements. Error bars represent the standard deviation (SD). N.S.; not significant. $*P < 0.05$. $**P < 0.01$, $***P < 0.001$. **d**, **e** Correlation between *STEAP1* and *NRF2* in samples of patients with CRC was analyzed using publicly accessible gene expression profiling data, GSE25070 (**d**) and GSE21510 (**e**). Pearson's correlation coefficient, $r = 0.488$; $P = 0.0114$ (**d**) and $r = 0.214$; $P = 0.0172$ (**e**). **f**–**i** Two different CRC cell lines (DLD-1: **f**, **h**; SW480: **g**, **i**). Cells were transfected with non-targeting siRNA (Control) or two independent siRNAs targeting *STEAP1* (siSTEAP1_1, siSTEAP1_2). The expression of *NRF2* was evaluated by qRT-PCR (**f**, **g**) and western blot (**h**, **i**). Data are the mean of quadruplicate measurements. Error bars represent the SD. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$. **j, k** DLD-1 (**j**) and SW480 (**k**) cells were transfected with non-targeting siRNA (Control) or *STEAP1*-targeting siRNA (siSTEAP1). Cellular localization of NRF2 (green) was assessed using fluorescence microscopy. DAPI: blue nuclear stain. Scale bar, 10 μm

Discussion

In the current study, we first demonstrated that *STEAP1* transcript levels were over-expressed in gene expression profiling of patients with CRC. These results prompted us to investigate the biological significance of STEAP1 in CRC cells using loss-of-function analysis. Our results from *STEAP1* silencing-induced apoptosis implied that STEAP1 has oncogenic properties in CRC. In fact, the high expression of *STEAP1* was associated with poor overall survival [12]. Most recently, the knock-down of *STEAP1* suppressed cell growth in PC cells, which are well documented regarding STEAP1, accompanied by apoptosis [15]. However, the mechanisms of apoptosis induced by *STEAP1* knock-down in PC cells were not elucidated. We found that apoptosis induced by *STEAP1* silencing in CRC cells was evoked through increased intracellular ROS levels. In contrast, a previous report indicated that the knock-down of STEAP1 reduced ROS production and redox reactions in EWS cells [16]. Although these findings seem inconsistent, numerous studies have shown that ROS have dual effects in cancer cells, like a double-edged sword. Namely, ROS promotes both cell death, including apoptosis, as well as tumor growth and invasiveness [4]. The fate of cells in response to ROS may be determined by cellular ROS levels, which differ among the cancer cell types. As mentioned above, disrupting the equilibrium of oxidative stress is an attractive tool for cancer treatment because ROS contribute to tumor aggressiveness while a high amount of ROS generation can induce apoptosis [9]. Similarly, we found that the overproduction of ROS triggered apoptosis in CRC cells. However, a high level of intracellular ROS is more harmful for normal compared to CRC cells [27]. In the context of translating ROS overproduction to CRC therapeutics, the targeting of cancer cells is required to prevent damaging normal cells. In this regard, STEAP1 would be a suitable target for CRC treatment to stimulate ROS production and induce cancer cell death because STEAP1 expression is limited in normal tissues [10]. Importantly, we have identified NRF2 as a downstream-targeted gene of STEAP1 using PCR array, microarray data sets, qRT-PCR and immunoblot for the first time (Fig. 5). NRF2 is a master regulator that represses oxidative stress; thus down-regulating NRF2 leads to increasing intracellular ROS [28]. In the context of our experimental setting, NRF2 inhibitors may be effective for CRC treatment. However, NRF2 inhibitors have not been introduced as a therapeutics in the clinic as yet since NRF2 has a dual function in both oncogenic and tumor suppressive activities [18]. Therefore, our results may prove valuable in developing a STEAP1-driven CRC treatment to manipulate intracellular NRF2 expression and therefore ROS production. Collectively, the current study demonstrated that *STEAP1* was over-expressed in CRC cells compared to normal colonic mucosa and adenoma. *STEAP1* silencing induced apoptosis in CRC cells through an overload of ROS due to the suppression of NRF2-induced antioxidant activities. Our results highlight the STEAP1–NRF2 axis as a therapeutic target for CRC and its manipulation as a novel strategy to combat CRC.

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Compliance with ethical standards

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

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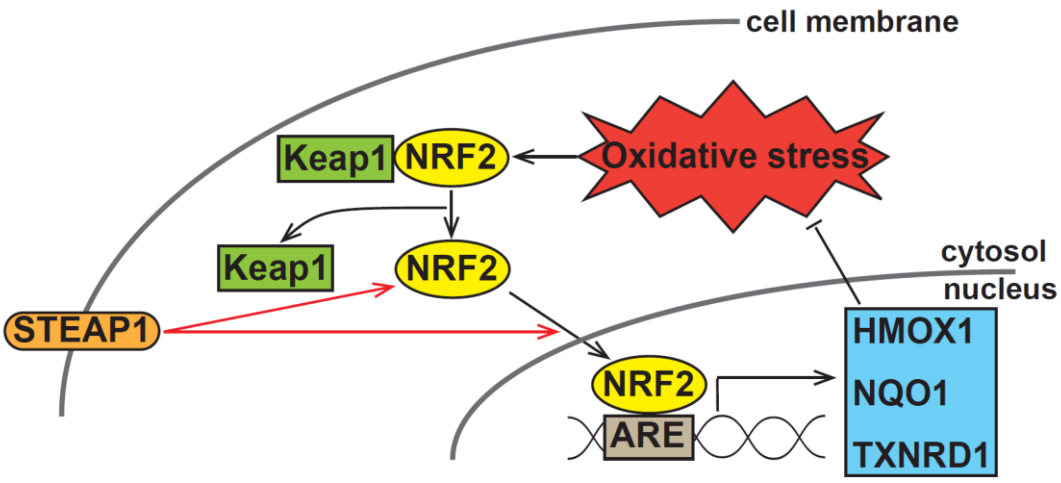
Supplementary Table. Primer sets used for qRT-PCR

Gene	Forward Primer (5' to 3')	Reverse Primer (5' to 3')
β-actin	5’-GGCATCCTCACCCTGAAGTA-3’	5’-GAAGGTGTGGTGCCAGATTT-3’
STEAP1	5’-CCCTTCTACTGGGCACAATACA-3’	5’-GCATGGCAGGAATAGTATGCTTT-3’
HMOX1	5'-TCTCCGATGGGTCCTTACACTC-3'	5'-GGCATAAAGCCCTACAGCAACT-3'
NQO1	5'-ATGGTCGGCAGAAGAGC-3'	5'-GGAAATGATGGGATTGAAGT-3'
TXNRD1	5’-CCACTGGTGAAAGACCACGTT-3’	5’-AGGAGAAAAGATCATCACTGCTGAT-3’
NRF2	5’-CACATCCAGTCAGAAACCAGTGG-3’	5’- GGAATGTCTGCGCCAAAAGCTG -3’

STEAP1, six-transmembrane epithelial antigen of the prostate 1; HMOX1, heme oxygenase 1; NQO1, NAD(P)H quinone dehydrogenase 1; TXNRD1, thioredoxin reductase 1; NRF2, nuclear erythroid 2-related factor.



Supplementary Figure. Schematic representation of STEAP1–NRF2 pathway

Under normal conditions, nuclear erythroid 2-related factor (NRF2) is located in the cytoplasm, adjacent to kelch-like ECH-associated protein 1 (KEAP1). Oxidative stress causes the dissociation of NRF2 from KEAP1. NRF2 enters the nucleus and activates several antioxidant genes to mitigate oxidative stress. Our data suggests that Six-transmembrane epithelial antigen of the prostate 1 (STEAP1) plays an important role in up-regulating this pathway. HMOX1, heme oxygenase 1; NQO1, NAD(P)H quinone dehydrogenase 1; TXNRD1, thioredoxin reductase 1, ARE, antioxidant response element.